Discovery of dormancy associated antigens of Mycobacterium tuberculosis : novel targets for the development of post-exposure or therapeutic tuberculosis vaccines

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Voor de wetenschap

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Introduction

Tuberculosis: preface

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB, also known as the "white plague"), was identified by Robert Koch in 1882. *M. tuberculosis*' highly complex interactions with the human host have been studied intensely ever since. Despite these efforts many critical gaps in our knowledge remain, precluding successful control of the TB pandemic. *M. tuberculosis* is one of the world's most successful and sophisticated pathogens, as it causes persistent infection in what is estimated to be over 2 billion people, yet largely without causing clinical symptoms (asymptomatic or "latent" infection") (1-3).
Human beings represent the only known natural reservoir of the bacillus. Following primary infection, only 5-10% of those infected will ever develop active TB disease, mostly within two years and commonly presenting as pulmonary TB in the adult. Eight to 10 million individuals newly develop active TB each year, and 2-3 million die from the disease. The remaining 90-95% of infected cases develops latent *M. tuberculosis* infection, which can be maintained for the lifetime of the person unless the immunologic balance between pathogen and host is perturbed; this can trigger reactivation of *M. tuberculosis* and result in progression to active TB. The enormous reservoir of latently infected individuals represents the main source of new TB cases (2), although it has become clear that new TB cases can also arise due to exogenous re-infection with *M. tuberculosis* in areas with high TB endemicity (4,5). The best-known factor driving progression of latent towards active infection is human immunodeficiency virus (HIV) co-infection: this increases the proportion of TB disease reactivation from 5-10% in a lifetime to 5-10% per life-year (6). Due to the expanding HIV/AIDS pandemic the number of TB reactivation related casualties is growing. Other host and environmental factors involved in compromising the host's ability to control *M. tuberculosis* infection include malnutrition, ageing, stress (7), type-2 diabetes (8), the use of immunosuppressive agents (such as corticosteroids and anti-TNF monoclonal antibodies) and likely genetically controlled host factors. On the pathogen's side, the bacterial factors essential to waking up this sleeping "giant" and their precise interplay with host molecules remain to be identified.

Anti-TB strategies: past and present
The most efficient and cost-effective intervention strategy against any infectious disease is vaccination. Since the 1920's *Mycobacterium bovis* bacille Calmette-Guérin (BCG) strains have been widely used as prophylactic TB vaccines. BCG vaccines are live attenuated bacterial strains that share a high degree of genetic and genomic homology (>95 %) with *M. tuberculosis* (9). Despite its use in massive vaccination programmes, BCG's benefits and efficacy remain subjects of debate. BCG has proven to be highly efficacious against severe TB in children, including TB meningitis and miliary TB. In addition, BCG protects adults against leprosy, which is caused by the related species *Mycobacterium leprae* (10-12). Unfortunately, this does not translate to sufficient protection of adults against the main and contagious form of TB, which is

pulmonary TB in the adult. Several randomised controlled trials and observational studies in adults have revealed rather discrepant results for TB, reporting protective efficacies ranging from 0 to 80% (10). Many explanations have been put forward to account for this conundrum, including differences in trial methodologies, host population genetics, regional differences in *M. tuberculosis* strains and differences in the BCG vaccine strains used. It is currently believed that heterologous immunity induced by environmental mycobacteria plays a significant role in blocking or masking BCG's protective efficacy (13, 14). BCG also clearly fails to prevent TB reactivation from latent infection. Moreover, BCG revaccination offers no additive protection against TB (15). These are the primary reasons that BCG has had little impact on the global prevalence and epidemiology of TB.

Developing better TB vaccines that can complement or replace BCG constitute a major global research area. Basically, there are three main strategies:

1) *TB subunit vaccines*, based upon immunodominant mycobacterial antigens delivered in selected platforms, including non-replicating viruses (MVA, Adenoviruses) or recombinant proteins admixed with potent adjuvants;
2) live vaccines based upon *genetically improved BCG*; and
3) live vaccines based upon highly *attenuated live M. tuberculosis.*

The subunit based approaches aim to induce high levels of cell mediated immunity and memory to a single or restricted number of mycobacterial antigens, preferably those that are immunodominant and essential for mycobacterial virulence. The concept behind the use of live mycobacterial vaccines is the induction of a broader cellular immune response against a plethora of antigens which may help generating optimal protective immunity. These different approaches have been discussed in detail recently (16-18).

Almost all new generation TB vaccines that are currently in clinical development have been designed as pre-exposure vaccines. Prophylactic subunit vaccines are generally considered to be particularly effective in boosting immunity induced by prior BCG vaccination, whereas live mycobacterial vaccines aim to replace BCG by more efficient vaccine strains. Whilst these vaccines aim to increase host resistance *prior* to infection, they are unlikely to be effective as post-exposure or therapeutic vaccines in latently infected individuals (19, 20). This is underlined by the aforementioned inefficacy of BCG revaccination in affording added protection compared to single BCG administration. It is clear that post-exposure or "therapeutic" vaccines that control, or even better, eradicate dormant persistent bacteria may be important tools to help protecting against TB reactivation and thereby enhance TB control (21).

Stage variation during M. tuberculosis infection

TB-vaccine discovery approaches rest on the assumption that the vaccine antigens administered are expressed by infected host cells, where they are recognized by T cells that execute the desired effector response, either assisting phagocytes in controlling or eliminating live bacteria, or -alternatively- by cytolysis of infected cells. Despite significant advances, relatively little is known about the *M. tuberculosis* antigen repertoire that is truly expressed on the surface of infected cells, and which is considered relevant to human T cells. This is particularly evident for molecules encoded by recently discovered "groups" of *M. tuberculosis* genes expressed during

specific phases -or stages- of *M. tuberculosis* infection. Proteins that are strongly expressed during the early phase of infection can be highly efficacious vaccine targets in models of acute infection. Traditionally, TB vaccine discovery has focused mostly on such antigens, several of which have shown strong protective efficacy in animal models, including the well known early stage antigens (e.g. early secreted antigenic target-6 (ESAT-6), TB10.4, Ag85A and Ag85B).

However, one caveat of this approach is that antigens that are highly expressed during early infection -or under laboratory conditions of log phase growth- may not necessarily induce optimal immunity and concomitant protection during the later stages of infection, since they might not be expressed optimally during later stages of *M. tuberculosis* infection. Adequate control of late stage infection may require different subsets of T cell clones, including T cells specific for antigens expressed during late stage TB infection. Several factors may contribute to the lack of "early" antigens in inducing protection against late stage TB infection, including exhaustion of relevant T cell memory populations, insufficient expression of early antigens on infected cells, and immune regulatory effects affecting the T cells specific for early immuno-dominant antigens.

Another important issue is that neither natural infection nor BCG vaccination may be able to achieve optimal activation of such T cells specific for late stage antigens. Detailed analysis of the *M. tuberculosis* genes that are switched on selectively (or at least predominantly) during late stage infection may help to identify novel antigens to activate T cells with the potential to control such stages of infection. Targeting and redirecting the human immune response to these antigens may thus help preventing TB reactivation.

One of the best controlled laboratory models to study adaptive responses of *M. tuberculosis* during non-proliferating conditions is the "Wayne" model (22). This model is based on the gradual depletion of oxygen from *M. tuberculosis* cultures, which triggers *M. tuberculosis* to enter a state of non replicating persistence (NRP) (3). Two different NRP stages can be discriminated: the microaerobic (oxygen levels approaching 1% saturation) NRP1 stage, which is characterized by a slow rate of increase in turbidity without corresponding increase in CFU, an arrest of DNA synthesis and to a large extent also reduced general mRNA synthesis (3,22). Expression of genes encoding early stage proteins such as ESAT-6 and Ag85B is repressed during this stationary phase (23). During NRP1 the bacilli start producing large amounts of glycine dehydrogenase, which continues during further NRP1. Additionally, steady ATP concentrations are observed. However, as soon as oxygen saturation levels decrease below 0.06% saturation, *M. tuberculosis* enters the NRP2 stage. NPR2 is an anaerobic stage with no further increase in turbidity, no DNA synthesis and most likely also no mRNA synthesis (22). Furthermore, in contrast to NRP1, decreased levels of glycine dehydrogenase and a drop in ATP concentration can be observed. During these NRP stages bacteria are typically resistant to the bactericidal actions of drugs that target actively replicating bacilli (3).

The use of these models combined with genome wide transcriptome profiling has led to the identification of *M. tuberculosis* genes that are particularly expressed during conditions thought to replicate bacterial dormancy. Voskuil *et al.* initially studied *M. tuberculosis* during *in vitro* conditions of nitric oxide (NO) and hypoxia, and identified

a regulon called the DosR or *devR* (dormancy) regulon, that consists of 48 genes which are coordinately up-regulated in *M. tuberculosis* under these conditions (24). Soon afterwards, several studies showed expression and up-regulation of many of the DosR/*devR* regulon genes by *M. tuberculosis* in the NRP stages of the Wayne model (25, 26). Work of Sherman et al. has shown before that *M. tuberculosis*' response to hypoxia was characterized by significant alterations in the expression of approximately 100 genes. The expression of over 40 genes, mainly including members of the DosR regulon, was induced whereas that of most other genes was significantly repressed (27). These studies thus suggest that *M. tuberculosis* is capable of showing phase specific gene expression. This may provide not only new insights into its intriguing infection biology, but also provide new directions for antigen discovery and TB vaccine and drug design. In this introduction several aspects of host–pathogen interactions will be discussed followed by the scope and outline of this thesis.

Infection and disease

Tuberculosis infection is acquired through inhalation of aerosolized infectious particles containing *M. tuberculosis*, which can reach the alveoli in the distal airways (28). The bacteria are generally taken up by alveolar macrophages where they persist and replicate slowly.
The presence of *M. tuberculosis* will trigger both the innate (see below) and the adaptive immune responses, which include helper ($CD4^+$) T cells, cytotoxic ($CD8^+$) T cells, γδ T cells and production of cytokines as interferon γ (IFNγ) and tumor necrosis factor α (TNFα), both vital in immunity to TB. As a result, the infected individual will typically convert to a positive tuberculin skin test (TST), which is based on acquired specific immunity (delayed-type hypersensitivity) to *M. tuberculosis*. Despite the essential role of IFNγ in host resistance to *M. tuberculosis*, evidence is accumulating that IFNγ in itself is not a sufficient correlate of protective immunity to *M. tuberculosis* (29). Recently, it has become evident that so-called multifunctional T cells i.e. simultaneously producing IFNγ, TNFα and IL-2, define a correlate of vaccine-mediated protection against *Leishmania major* (30) and *M. tuberculosis* (31). Additionally, IP-10 possibly in combination with IL-2 have been put forward as new biomarkers for TB e.g. in the diagnosis of latent TB (32). Synergistic interactions between different pathways, such as evident for IFNγ, TNFα and IL-2, may mask the essential role of single individual components in vaccine induced immunity. This illustrates the necessity of exploring and determining more complex, multi-factorial biomarker profiles in protection of TB.

Diagnosis of M. tuberculosis infection
The typical symptoms of pulmonary TB are productive prolonged cough, chest pain, fever, easy fatigability, night sweating and weight loss. TB can only be confirmed by either radiographic examination and/or diagnostic microbiology. Latent TB is more difficult to diagnose. From the late 1930's, detection of latent TB infection has classically relied on the TST, which is performed by intradermal administration of purified protein derivative (PPD or tuberculin) of *M. tuberculosis*. One of the major

shortcomings of the TST is its limited specificity due to cross-reactivity with environmental mycobacteria as well as with BCG.
To overcome this problem, there currently are two commercial first generation assays available aiming at improved detection of latent tuberculosis infection. Both the QuantiFERON©-TB Gold assay and T-SPOT.TB test are based on detecting IFNγ responses to the *M. tuberculosis* complex specific antigens ESAT-6 and culture filtrate protein 10 (CFP-10) and are being promoted as new diagnostic tests in TB (33). At this stage, however, insufficient formation is available to evaluate the precise potential contribution of these tests to TB diagnosis, despite their enhanced specificity. This particularly holds for cases in areas with high TB endemicity. In areas with low endemicity, these assays may detect recently but not so much more distantly exposed individuals.

Treatment of TB
TB treatment requires long-term treatment with a combination of drugs, as recommended by the World Health Organisation (WHO). Directly observed treatment short course (DOTS) is the internationally recommended tuberculosis treatment building on five key elements: political commitment, case detection, standardised observed therapy, effective drug supply and monitoring and evaluation (34). Despite the enormous impact of multiple drug therapy (MDT) on TB prevalence, limited drug availability and compliance problems have allowed drug resistance to arise in TB. Numerous multi-drug resistant (MDR) and also extensively drug resistant (XDR) (35) *M. tuberculosis* strains are rapidly arising which in some cases are virtually untreatable with current drugs. If these escape variants will turn out to be infection- and transmission competent, which is supported by current data, this will pose serious challenges to the future control of TB and may bring us back to the pre-antibiotic era. The scarcity of new drugable targets for intracellular pathogens such as mycobacteria and *Salmonella* (36) underlines the need for completely new approaches in drug discovery for the control of intracellular bacterial diseases.

Genetic predisposition of TB disease
It has long been recognized that both environmental and genetic factors contribute to the susceptibility to TB in humans. The host genes involved are probably numerous, and almost certainly involve complex gene-gene interactions. Thus, synergistic interactions between different genes and downstream pathways are likely to exist, which may mask the contribution of single individual components. Despite the limitations posed by these problems on TB-susceptibility gene discovery searches, consistent associations have been reported for several genes with host susceptibility to TB. These include natural resistance associated macrophage protein-1 or *NRAMP1* (37-39), the vitamin-D receptor (*VDR*) gene and MHC genes, particularly *HLADRB1* (40).
Deficiencies in signalling pathways of adaptive and innate immunity can also result in remarkably selective susceptibility to mycobacteria. Patients suffering from severe infections due to otherwise poorly pathogenic mycobacteria (e.g. non tuberculous mycobacteria or BCG) have been diagnosed with molecular defects in essential proteins of the type 1 cytokine axis (IL-12/23p40, IL-12/23Rβ1, IFN-γR1, IFN-γR2,

Stat-1, Tyk2) (41,42). In line with this, Sahiratmadja et al. showed that some polymorphisms in IL-12/IFNγ pathway genes were associated with susceptibility to pulmonary tuberculosis in Indonesia (43).
Also deficiencies in innate cell signalling through Toll like receptor (TLR-see below) pathways seem involved in TB susceptibility. The adaptor protein Mal, which I encoded by *TIRAP*, is involved in the downstream signalling of TLR2 and TLR4 and variants in this gene have been associated with TB in one study (44); however, a larger study was unable to confirm this (45). Additionally, a genetic association study performed in Indonesia and Russia showed that polymorphisms in the TLR8 pathway are implied in the increased susceptibility to adult pulmonary TB (46).
Interestingly, human IL-1 receptor-associated kinase 4 (IRAK4) deficiency, leading to impaired TLR/IL-1 receptor dependent signalling, does not enhance susceptibility to mycobacteria but rather to pyogenic bacteria (47,48).
Several other genes have been associated to the predisposition to TB disease. Pan *et al.* identified the *Ipr1* (intracellular pathogen resistance 1) locus as a major new TB susceptibility locus in mice (49). Subsequent studies investigated the closest human homologue of *Ipr1* namely SP110 and its role in genetic susceptibility in TB. Indeed, polymorphisms in *SP110* were associated with susceptibility to TB in humans (50). Work of Baghdadi *et al.* revealed the existence of a major tuberculosis susceptibility locus (8q12-q13) containing an autosomal dominant susceptibility gene (51).
Since susceptibility associated gene polymorphisms are often different between different studies, it is likely that multiple genes and genetic polymorphisms co-ordinately determine host susceptibility to TB. High density DNA typing technologies combined with large scale population studies will shed new light on the genetic elements and the downstream pathways that regulate host susceptibility to TB. Most importantly, however, most genetic factors identified to date have significant roles in controlling innate and adaptive components of the cell mediated immune response in general, and to intracellular pathogens in particular (reviewed by (41,47)). Thus, genetic factors that control the balance of the cell mediated immune response are likely to impact on TB resistance and susceptibility.

Face to face: host responses to *M. tuberculosis*

Innate immunity to M. tuberculosis
Macrophages and dendritic cells (DCs) are the first host cells targeted by invading *M. tuberculosis* bacteria. They are the key mediators of innate immunity to *M. tuberculosis*, and recognize pathogen components through highly conserved pattern recognition receptors (PRRs), including members of the Toll-like receptor family (TLR). Each of the ten human TLR family members recognizes a distinct class of conserved microbial molecules. For example, TLR2, in conjunction with TLR1 or TLR6, recognizes microbial lipo-proteins (LP), lipo-mannans (LM) and lipo-arabinomannans (LAM), all of which are characteristic components of mycobacteria. TLR activation induces the: 1) release of cytokines (e.g. IL-12) and chemokines; 2) differentiation of DCs; 3) regulation of phagocytosis; and 4) triggering of anti-microbial activities (52, 53).

The role of TLRs in anti-mycobacterial activity has been linked to vitamin D: Vitamin D mediated antimicrobial activity to *M. tuberculosisis* was found to be dependent on the antimicrobial peptide cathelicidin (LL37) which needs to be activated by TLR2/1 signalling (54).
Other relevant PRRs include mannose receptors (MR), intracellular NOD/NLR receptors (55) and C-type lectins like dendritic cell-specific ICAM grabbing nonintegrin (DC-SIGN). It has been reported that *M. tuberculosis* is able to prevent DC maturation by the binding of mannosylated LAM (Man-LAM) (as well as other components) to DC-SIGN, thereby inducing the production of IL-10 (56) which suppresses immunity and T cell activation. In addition, macrophages express Fcγ receptors and complement receptors (CR) which promote recognition of opsonised bacteria (57, 58).
In the course of infection, increasing numbers of macrophages and resident DCs are recruited to the site of infection. Mature DCs re-locate to the lymph nodes where they produce inflammatory cytokines and prime $CD4^+$ and $CD8^+$ T-cells against mycobacteria (7, 59).

The T cell response to M. tuberculosis
M. tuberculosis primarily resides in vacuoles within macrophages, and presents antigens via the endocytic system in the context of MHC class II molecules to $CD4^+$ T cells. Ample evidence points to the indispensable role of $CD4^+$ lymphocytes in protective immunity to *M. tuberculosis*. In humans, loss of $CD4^+$ T cells due to progressing HIV disease greatly increases the chance of TB reactivation and TB re-infection (6). Mice deficient in $CD4^+$ T cells have impaired ability to control infection and eventually die from TB (60). In animal models, adoptive transfer of $CD4^+$ T cells taken at the height of the primary immune response to *M. tuberculosis* conferred protection against *M. tuberculosis* in T cell deficient mice (61).
$CD4^+$ T cells comprise several subclasses, among which T helper 1 (Th1) cells are the most abundant and prominent. Th1 cells are characterized by the production of IFNγ and TNF (62-64); other $CD4^+$ T cell subsets encompass Th2 cells, T-regulatory cells (Tregs) and Th17 cells. Th2 cells produce IL-4, IL-10 and TGFβ and influence immunity to *M. tuberculosis* possibly by antagonizing Th1 cells through IL-4 (65). Tregs comprise multiple subsets and are important in controlling immunity and inflammation. In *M. tuberculosis* infection Tregs were shown to be associated with active TB disease (66) but they are also induced efficiently by BCG (67) and *M. leprae* (68). Recently, a newly detected subset of $CD4^+$ T cells, Th17 cells, was described. These cells are characterized by the production of the pro-inflammatory cytokine IL-17 and IL-22. In mice, Th-17 cells are associated with inflammatory diseases such as rheumatoid arthritis, collagen induced arthritis and experimental autoimmune encephalitis (69). Khader *et al*. showed that IL-23 is required for the establishment of IL-17 producing $CD4^+$ T cells in the lungs of mice infected with *M. tuberculosis*. It is thought that Th17 cells may contribute to the secondary immune (recall) response by promoting the expression of chemokine receptors in the lung and the recruitment of IFNγ producing T cells (70, 71). Also the attraction of neutrophils which can kill *M. tuberculosis,* may contribute to IL-17 dependent protection. As yet, the exact roles and

modes of action of these latter three populations of $CD4^+$ T cells in human TB remain to be established.

An involvement of $CD8^+$ T cells in the containment of *M. tuberculosis* has been suggested by the rapid migration of such cells to the sites of infection (72) and their presence in granulomas (73). Mycobacteria specific $CD8^+$ T cells can produce IFNγ, but are mostly recognized for their cytotoxic function (74). $CD8^+$ T cells are typically activated by antigens derived from the cytoplasm, which in the form of peptides have been translocated to the ER and complexed to MHC class I molecules. For long, it has been unclear how antigens from the phagosome in which *M. tuberculosis* resides, could access the MHC class I route. Two pathways have now been identified by which this may take place (75): first, mycobacteria are able to induce apoptosis in infected macrophages, thus initiating release of apoptotic vesicles; these can then be taken up by DCs, processed and shuttled into the canonical MHC class I presentation pathway, a process commonly referred to as "cross-priming". A second, although as yet controversial mechanism, involves the possible recruitment of ER membranes to the phagocytic cup. This would allow access of the MHC class I processing machinery to intracellular bacteria, providing a source of peptides for coupling to MHC class I molecules.

Of interest, $CD8^+$ T cells have been shown to preferentially recognize heavily infected cells whereas $CD4^+$ T cells do not shown make this distinction (76). It has also been suggested that $CD8^+$ T cells control later stage *M. tuberculosis* infection whereas $CD4^+$ T cells are important during early stages (77). Furthermore, since *M. tuberculosis* can also infect non phagocytic cells such as type II alveolar epithelial cells (78), which are typically devoid of MHC class II expression but do express MHC class I molecules, $CD8^+$ T cells may be endowed with the unique capacity to recognise and eliminate this unique subclass of infected cells. Thus, a growing body of evidence emphasizes an important and perhaps unique role of $CD8^+$ T cells in different stages of adaptive immunity in *M. tuberculosis* infection (79).

$TCR\alpha\beta^+$ $CD8^+$ T cells and $CD4^+$ T cells recognize antigens in the context of MHC class I and II respectively, yet bacterial glycolipid antigens are typically presented to T cells by CD1 molecules that are abundantly expressed on DCs (80). There are four human isoforms of CD1: CD1a, b, c, and d which can be segregated into two groups. The group 1 or CD1a, b and c isoforms present lipid antigens to T cells, whereas group 2 CD1 molecules are composed of CD1d and the murine orthologue CD1, and present lipid antigens to NK T cells (81, 82). Recently, diacylated sulfoglycolipids were identified as highly potent non protein mycobacterial antigens that are presented in the context of CD1 restricted T cells (83). $\gamma\delta^+$ T cells recognize phosphate-group containing non-protein antigens in the absence of an antigen presenting molecule (16). These latter two populations are commonly referred to as unconventional T cells and make up a smaller fraction of circulating T cells. Unconventional T cells however may contribute to antibacterial immunity, particularly in the early phase of infection.

Upon activation, both $CD4^+$ and $CD8^+$ T cells produce several effector molecules. As mentioned, IFNγ synergizes with TNFα in activating *M. tuberculosis* infected macrophages and subsequent killing of *M. tuberculosis* (84).

The importance of IFNγ in controlling mycobacterial infections including *M. tuberculosis* is emphasized by the extreme susceptibility of individuals genetically and

functionally deficient in the IL-12/IL-23/IFNγ axis. The vital role of TNFα in the control of (latent) TB infection was recently emphasized again by the use of TNF blockers in chronic inflammatory diseases like rheumatoid arthritis and Crohn's disease: neutralization of TNF by anti-TNF antibodies led to disproportionally frequent reactivation of latent *M. tuberculosis* infection and disseminating TB in treated individuals (85, 86). In line with this, *M. tuberculosis* infected mice neutralized for TNFα, or deficient in TNF receptor signaling, also rapidly died with markedly higher bacterial burden (87). As mentioned before, it is clear that the role of synergistic interactions between different cytokine pathways must be explored as well.

Activated T cells are able to exert direct cytolytic and microbical effector functions by the production of perforin, granzymes and granulysin (88, 89). These molecules are secreted from granules and are released in the immunological synapse between effector T cell and infected target cell. Perforin forms pores in the target cell membrane, causing lysis by the influx of water and ions. Moreover, perforin mediates translocation of granzymes, which enter the nucleus and induce apoptosis (90). Granulysin directly induces apoptosis (91); when accessing bacteria, it can alter the membrane integrity. It is thought that perforin helps granulysin to access phagosomal bacteria such as *M. tuberculosis*, thereby eventually reducing mycobacterial viability (92). An additional mechanism to induce target-cell apoptosis occurs by the ligation of target-cell expressed Fas (CD95) to FasL (CD95L) on the effector T cell (88, 93). Apoptosis is an important host defence mechanism against intracellular bacteria, and virulent *M. tuberculosis* is able to resist apoptosis induction in human cells (94-96).

The granuloma: M. tuberculosis' hideout

In most cases the cell mediated response is able to contain *M. tuberculosis* in well-organised granulomas, in which it can persist for decades without causing any symptoms. It is possible that in these lesions truly dormant bacilli are present that persist in a metabolically reduced or inactive state (80). Alternatively, (a subpopulation of) persisting bacteria might continue to replicate slowly. Regardless, in both cases latent infection arises, representing a balance between host defense and bacterial dormancy/slow replicating persistence, which can be maintained for many decades.

Granuloma formation is a hallmark of latent *M. tuberculosis* infection, and provides a microenvironment in which interactions between T cells, macrophages and cytokines are facilitated and bacteria are sequestered from spreading further into the host. Disruption of these organized structures is a typical feature of TB reactivation.

Due to chronic stimulation, macrophages in these lesions eventually fuse to form giant cells that are characteristic for granulomas. The granuloma is a highly dynamic structure in which $CD4^+$ and $CD8^+$ T cells occupy discrete and different sites that are most likely associated with their functions in different stages of infection and disease (97, 98). Distinct temporal accumulations of immune cells including T cells, macrophages, neutrophils, B cells and DCs in the lungs of mice during progression of acute to chronic state of infection have been observed; macrophages and neutrophils were predominantly present during the acute phase of infection whereas numbers of $CD3^+$ T cells rapidly expanded in the lungs during the course of infection (2-4 weeks

post-infection). Simultaneously, total numbers of macrophages increased as well. Neutrophil numbers decreased towards the chronic phase of infection and eventually $CD3^+$ T cells and macrophages were shown to characterize the chronic tuberculous lung (97).
Granulomas can be formed anywhere in the lung, yet 90% of the post-primary TB cases occurs in the upper lobes (99). Previously it was reported that in fewer than 10% of the tuberculous lesions viable bacteria could be detected whereas in 50% of normal lung tissue these bacteria could be recovered (100). In concordance, *M. tuberculosis* DNA was detected in the upper lobes of normal lung tissue during latency (101). Together, these studies suggest that *M. tuberculosis* may also persist in human lung tissue outside the classic tuberculous granulomas but this important issue needs substantial further investigation.
Two members of the TNF superfamily (now comprising a total number of 19 ligands and 29 receptors (102) are particularly involved in controlling granuloma formation: TNFα and Lymphotoxin α (LTα). Not only macrophages but also $CD4^+$ Th1 and Th17 cells produce these molecules. LTα binds to the TNF receptor molecule TNFR1. Mice lacking LTα also exhibit a distinct susceptibility to TB infection despite normal TNFα levels (103).
TNF family members are also involved in regulating chemokine and chemokine receptor expression, which are responsible for the recruitment of inflammatory cells. Elevated levels of the chemokines MCP-1, MIP1α/β, RANTES, IL-8 and IP-10 in bronchial alveolar lavage of TB patients compared to healthy controls, are consistent with a role for these molecules in the immunopathology in TB disease (104). Also adequate IL-12/IL-23/IFNγ signalling is required for both the control of *M. tuberculosis* infections and for development of mature granulomas, since subjects genetically deficient in this axis often fail to develop mature granulomas (41, 42). Likewise, mice deficient in IFNγ signalling fail to form granulomas following *M. tuberculosis* infection (105).
The containment of the bacteria and the local intra-granuloma interactions between the host and *M. tuberculosis* eventually determines the outcome of disease.

Face to face: *M. tuberculosis'* evasive manoeuvres from immune killing inside the hostile macrophage environment.

The alveolar macrophage's phagosome is the favoured milieu for *M. tuberculosis*' replication and persistence, and perhaps also for the survival of dormant TB bacilli. Intracellular bacilli are usually degraded in highly bactericidal acidic phagolysosomes. However, *M. tuberculosis* has adopted powerful strategies to resist, or escape from, these otherwise microbicidal pathways.

Escape from Reactive Nitrogen Intermediates (RNI)
Activation of macrophages by IFNγ and TNF induces the activity of nitric oxide synthase 2 (NOS2). NOS2 catalyzes the conversion of L-arginine into NO and related nitrogen intermediates (RNI) (106) which can act as potent cytotoxic agents. In vitro studies with mouse derived macrophages have shown that the L-arginine-dependent production of RNI is the principal effector mechanism in activated murine

macrophages, responsible for inhibiting virulent *M. tuberculosis* (107). Not surprisingly, therefore, *M. tuberculosis* appears to have armed itself against such oxidative and nitrosative host responses through the presence of *noxR1* (108), *noxR3* (109) and *ahpC* (110) genes, which all have been shown to aid in resistance to RNIs.

Escape of M. tuberculosis from phago-lysosomal fusion
After uptake of intracellular bacteria by professional phagocytes into classical phagosomes, the latter typically fuse with lysosomes to form phagolysosomes. These compartments are highly acidic, deprived of nutrients ("starvation" stress), hypoxic and rich in hydrolytic enzymes that degrade and kill bacteria (111). Already in the early 1970's it was recognized that in order to evade killing, *M. tuberculosis* arrested phagosome maturation by preventing fusion with lysosomes (112). Proteins and molecular mechanisms associated with this phagosomal maturation block include LAMP1, GTPases of the Rab family and the calcium binding protein calmodulin, coronin-1 (or TACO) (113, 114). More recently, using chemical genetics, a network of human kinases was demonstrated to be essential in phagosome maturation. Functional disruption of kinases in the Akt1 network by RNAi or chemical inhibitors led to enhanced killing of intracellular bacteria, including *M. tuberculosis* (36). In addition, interactions of mycobacteria (mostly BCG) with cholesterol have been shown to allow entry of mycobacteria into macrophages (115). These pathways have recently been reviewed in detail (113, 116, 117).
A recent provocative study has suggested that *M. tuberculosis* and *M. leprae* -but not BCG- can escape from the phagosome, at least upon longer-term culture in human cells: *M. tuberculosis* containing phagosomes fused with lysosomes, but *M. tuberculosis* subsequently translocated from the phagolysosomes into the cytosol. This process was dependent on a bacillary secretion system involving CFP-10 (118). The subject of possible translocation of *M. tuberculosis* remains a matter of debate, and further studies are needed to dissect this process further.

The quest for novel targets for anti-tuberculosis intervention

The availability of several complete *M. tuberculosis* genome sequences (119), (www.TBDB.org) combined with the possibility to study genome wide expression profiles under controlled *M. tuberculosis* infection-phase simulating conditions will allow insight into the blueprint of microbial persistence in humans, and help identifying novel targets for intervention in (latent) TB.
Several studies have applied DNA microarray techniques to study the transcriptome of *M. tuberculosis* in models that are representative of distinct phases of *M. tuberculosis* infection. In a recent study, Murphy and Brown described the results of a meta-analysis on different gene expression profiles that had been specifically studied in models simulating bacterial dormancy. Their analysis set out to identify novel TB drug targets against dormant phase *M. tuberculosis* infections based on the expression data combined with genome-wide insertional mutagenesis as indicative for gene indispensability. Novel anti-TB targets which could thus be identified included regulatory genes (*devR*/*devS*, *relA*, *mprAB*) and several enzymes involved in bacterial metabolism (120).

Using micro-array based approaches, genes expressed by *M. tuberculosis* during oxygen deprivation have been identified, as discussed in more detail below. Besides oxygen deprivation, low pH- and ROI/RNI- exposure, intracellular mycobacteria are also exposed to nutrient starvation (*e.g.* Fe, carbon). It is interesting that *M. tuberculosis* adapts to these specific limitations by uniquely alterations in its gene expression patterns (121). Transcriptional profiling also allows the gathering of detailed information in direct cross-talk between *M. tuberculosis* and infected human DCs and macrophages (122). Nevertheless, the bacterial factors that alter the balance from NRP to reactivation and proliferation still remain unknown. Recently, a family of *M. tuberculosis* growth factors (resuscitation promotion factors, Rpfs) was identified, which appeared to be required for the virulence and resuscitation of dormant bacteria (123,124). Deletions of *rpf* genes resulted in defective growth of the bacteria in vivo, defective resuscitation in vitro (125) and delayed reactivation from chronic TB in vivo (126). In addition, Rpf appear to be associated in normal cell division in *M. smegmatis* (127). Whether this can be extrapolated to *M. tuberculosis* remains to be seen. The mechanisms involved in control and expression of Rpfs in TB are largely unclear but a growing body of evidence points towards a role for Rpfs in bacterial virulence, persistence, immune modulation and reactivation (124,128).

Obviously, molecules involved in *M. tuberculosis*' remarkable and abundant lipid metabolism are highly attractive antibiotic targets. These include methyltransferases, cyclopropane synthases (involved in the synthesis of mycobacterium specific mycolic acids) (129,130) as well as lipolytic molecules (degradation of lipids) (131). One of the most effective anti-TB drugs, isoniazid (INH), indeed targets cell wall biosynthesis. Very recently, a publication appeared discussing the role of other potential genes in controlling the response to hypoxia in *M. tuberculosis*, next to the DosR regulon (132). Genes of the enduring hypoxic response (EHR, encompassing 230 genes which also include members of the DosR regulon) were significantly induced at four and seven days of hypoxia in a simple in vitro tube system, but not at initial time points. The EHR encompasses several transcriptional regulators that could control the program of bacteriostasis.

Thus, many of the above mentioned gene families offer interesting potential targets for anti-mycobacterial drug development.

The M. tuberculosis DosR regulon: stage or phase specific gene expression

M. tuberculosis has an unusual ability to survive in the human host for many decades. Tuberculous lesions are essentially avascular and deprived of oxygen and nutrients (3). Given the fact that *M. tuberculosis* needs oxygen and is able to persist despite these low oxygen levels in lesions, *M. tuberculosis* must be able to adapt to gradual oxygen depletion. Studies have demonstrated that *M. tuberculosis '* adaptation to hypoxic shift down is characterized by nitrate reduction, altered metabolism and chromosomal and structural changes of the non-replicating bacteria (133-135). The *M. tuberculosis* DosR regulon was found to play a critical role in preparing *M. tuberculosis* for this metabolic shift-down, as an essential step towards bacterial dormancy (24, 136).

During hypoxia, *M. tuberculosis* was first found to massively up-regulate expression of the 16kDa (α-crystallin (acr), Rv2031c, HspX) protein (137). These initial observations led to the search for similarly up-regulated genes using whole genome microarray approaches. Voskuil et al. showed that low concentrations of NO induced expression of a 48-gene (dormancy or latency) regulon, including HspX, in *M. tuberculosis,* which was coined "the DosR regulon" (24). The induction of all 48 DosR regulon genes occurred swiftly after only 5 minutes of NO exposure or hypoxia, and appeared to be under the control of the Rv3133c (*dosR/devR*) regulator gene. Another newly identified stimulus capable of inducing the *M. tuberculosis* DosR regulon is host-generated carbon-monoxide (CO) (138-140). The importance of the DosR regulon was further underscored by altered *M. tuberculosis* survival rates in in vitro hypoxia models, in which survival of wild type *M. tuberculosis* was superior to that of *M. tuberculosis* DosR deletion mutants (24).

In vivo regulation of the DosR regulon was studied by transcriptional profiling of *M. tuberculosis* in infected murine lung tissue. Five dormancy genes were selected for closer study based on their strong expression in IFNγ activated macrophages. All five genes were highly expressed in mouse lungs 21 days post-infection and remained abundantly expressed there after (136). The same study showed that most dormancy regulon genes were also strongly induced by *M. tuberculosis* in IFNγ activated wild type macrophages but *not* in IFNγ activated macrophages from NOS2 deficient mice. In accordance with this, expression of three *M. tuberculosis* dormancy genes (HspX, Rv2623, and Rv2626c) was delayed in the lungs of IFNγ KO compared to wild type mice. Together, these studies show that host (type-1) immunity is essential in inducing the expression of bacterial transcription patterns characteristic of NRP (141). Indeed, immune-compromised humans and animals succumb rapidly from TB-infection, probably without a phase of latency, suggesting that also the human immune response is involved in initiating TB-latency. Without such immune pressure, *M. tuberculosis* may not be activated to up-regulate its DosR/Rv3133c regulon, and thus fails to transit from replicating to non-replicating persistence.

Many of the DosR genes are conserved hypothetical proteins (CHP) or hypothetical proteins (HP) with as yet still unknown products and functions. Only a few genes have been designated a function such as probable phosphofructokinases, possible transmembrane proteins or probable ferredoxin A proteins (Table 1).

Previous work in BCG had already demonstrated that Rv3133c was up-regulated in a similar dormancy like response (142). Besides Rv3133c, two other DosR regulon

proteins (Rv2623 and Rv2626c) also displayed increased expression during conditions simulating dormancy in BCG. The response regulator Rv3133c was named DosR for 'dormancy survival regulator' since disruption of the gene disabled BCG in its adaptation to hypoxia, and caused loss of induction of the three dormancy proteins HspX, Rv2623 and Rv2626c (143).

Bacteria typically use two-component systems in their adaptation to the environment. Rv3133c is part of a two-component system: it is a transcription factor that mediates the hypoxic response of *M. tuberculosis* and its metabolic shift down to NRP (144).

Two component systems usually consist of a response regulator (here Rv3133c) and a histidine sensor kinase, which in this case turned out to be the adjacent gene, Rv3132c. Signal transduction through two component systems is attained by transient phosphorylation of both components (145). Rv3133c and Rv3132c are co-transcribed and conserved in *M. tuberculosis* and BCG (146), and Rv3132c is able to phosphorylate Rv3133c in vitro. A third gene, Rv2027c (*dosT*), which also encodes a sensor kinase, can also phosphorylate the Rv3133c/Rv3132c two component system (145, 147).

Rv2027c bears strong homology with Rv3132c. Sousa et al. (148) have shown that Rv2027c and Rv3132c are oxygen-switched kinases, which under normal oxygen levels are saturated with O_2, but during hypoxia exist in a deoxy-state, by which they become enzymatically active (148). Kumar *et al*. confirmed that Rv2027c is a hypoxia sensor, but found that Rv3132c was a redox sensor that does not need O_2 binding for its activation, but rather uses oxidation of its heme iron for inducing autokinase activity (149). These results imply that activation of both hypoxia- and redox- sensor kinases is involved in phosphorylation of Rv3133c, but that these sensors are differentially induced in persistent bacilli. Recent studies have revealed more insight into the mechanisms behind Rv3133c/DosR (DevR) signaling (150,151).

Conflicting reports have been published concerning the *dosR-dosS* two component system in *M. tuberculosis*' virulence. Deletion of Rv3133c was shown to have an increased (152) or neutral effect (132) on *M. tuberculosis*' virulence when assessing mice infected with either wild type *M. tuberculosis* or a *dosR* deletion mutant.

However, three other studies concluded otherwise: guinea pigs infected with a Rv3133c disrupted *M. tuberculosis* mutant had decreased bacterial loads and significantly decreased lesions in lung, liver and spleen (153). A second study applying a Δ*dosR-S M. tuberculosis* mutant in three different animal models reported similar growth defect and defective survival (154). Thirdly, a possible role for DosR as a virulence factor was also suggested when analyzing virulent W/Beijing lineages of *M. tuberculosis,* which display enhanced epidemic spread. These strains had constitutive up-regulation of DosR regulon genes compared to non-Beijing strains (155), and accumulated high levels of triacylglycerides, likely due to the 10-fold up-regulation of the DosR regulon gene Rv3130c, which encodes a triacylglycerides synthase (*tsg1*). Indeed, Rv3130c deficient *M. tuberculosis* strains fail to accumulate triacylglycerides under hypoxic conditions (156).

Table 1. List of the 48 DosR regulon encoded genes of *Mycobacterium tuberculosis* [a,b]

Rv number	Gene name	Size (aa)	Gene Product	References
Rv0079		273	HP	
Rv0080		152	CHP	
Rv0081		114	transcriptional regulatory protein (ArsR family)	
Rv0569		88	CHP	(164)
Rv0570	*ndrZ*	692	ribonucleoside-diphosphate reductase (large subunit) NrdZ (ribonucleotide reductase)	
Rv0571c		443	CHP	
Rv0572c		113	HP	
Rv0573c		463	CHP	
Rv0574c		380	CHP	
Rv1733c		210	conserved transmembrane protein	(165-167)
Rv1734c		80	CHP	
Rv1735c		165	hypothetical membrane protein	
Rv1736c	*narX*	652	fused nitrate reductase NarX	(168,169,170-172)
Rv1737c	*narK2*	395	nitrate/nitrite transporter NarK2	(141,168,171-174)
Rv1738		94	CHP	(150,165-167,174)
Rv1812c		400	probable dehydrogenase	175
Rv1813c		143	CHP	176
Rv1996		317	CHP	
Rv1997	*ctpF*	905	metal cation transporter P-type ATPase CtpF	
Rv1998		258	CHP	
Rv2003c		285	CHP	
Rv2004c		498	CHP	177
Rv2005c		295	CHP	178
Rv2006	*otsB1*	1327	trehalose-6-phosphate phosphatase OtsB1	179
Rv2007c	*fdxA*	114	ferredoxin FdxA	
Rv2028c		279	CHP	
Rv2029c	*pfkB*	339	phosphofructokinase PfkB (phosphohexokinase)	(165-167)
Rv2030c		681	CHP	
Rv2031c	*hspX, acr*	140	heat shock protein HspX (a-crystallin homologue) (14kDa antigen)	(141,161,162,174,180,181)
Rv2032	*acg*	331	CHP Acg	(166, 182,183)
Rv2623	*TB31.7*	297	CHP TB31.7	(141,142,164,174,183)
Rv2624c		272	CHP	
Rv2625c		393	conserved transmembrane alanine and leucine rich protein	
Rv2626c		143	CHP	(141,142, 164,166,175,184)
Rv2627c		413	CHP	(165-167,175)
Rv2628		120	HP	(165-167,175)
Rv2629		374	CHP	(178, 185-187)
Rv2630		179	HP	
Rv2631		432	CHP	
Rv3126c		104	HP	
Rv3127		344	CHP	
Rv3128c		337	CHP	
Rv3129		110	CHP	
Rv3130c	*tgs1*	463	Triacylglycerol synthase (diacylglycerol acyltransferase)	(155,156,188)
Rv3131		332	CHP	
Rv3132c	*devS, dosS*	578	two component sensor histidine kinase devS/dosS	(27,139,145,149,154,189,190)
Rv3133c	*devR,DosR*	217	two component transcriptional regulatory protein DevR/DosR probably LuxR/UhpA-family	(27,144,152,191) (24,143,145,154,155,189,192)
Rv3134c		268	CHP	(27,145,151,154,189,190)

[a] Abbreviations: HP, hypothetical protein; CHP, conserved hypothetical protein.
[b] 48 Dormancy genes of *Mycobacterium tuberculosis* as described by Voskuil et al., 2003
Annotations are from: http://genolist.pasteur.fr/TubercuList/, TB database (www.TBDB.org) and Murphy DJ and Brown JR (2007) supplemental files:
supplemental file 2; http://www.biomedcentral.com/content/supplementary/1471-2334-7-84-s2.xls and supplemental file 4; http://www.biomedcentral.com/content/supplementary/1471-2334-7-84-s4.doc.

The DosR regulon: novel phase specific *M. tuberculosis* antigens and source of novel anti-tuberculosis vaccine targets?

An important question remains: are *M. tuberculosis* DosR regulon genes, which all have been found to be regulated in vitro or in animal models of TB, in fact expressed during natural *M. tuberculosis* infection in humans? HspX (a-crystallin, *acr*), the archetypal DosR regulon gene, is a small heat-shock protein that has been studied most intensely. Cellular immune responses to HspX have been observed in latently infected individuals while antibodies to this antigen were predominantly found in individuals with active TB disease (157). This antigen is a major target for the human immune system and is recognized well by $CD4^+$ (157-159) and $CD8\ T^+$ cells (159, 160). HspX is required for mycobacterial persistence within the macrophage and is dominantly expressed in bacterial stationary phase and under reduced oxygen levels (161).

Clues about its role in host pathogenesis and mycobacterial persistence came from studies in which the *acr* gene was deleted from *M. tuberculosis*; in vivo bacterial growth was increased in (resting and activated) macrophages from Balb/c mice infected with a Δ*acr* mutant (162). Similarly, infection of C57/B6 mice with an Δ*acr* mutant of *M. tuberculosis* demonstrated a 1-2 log higher bacillary load in the lungs in comparison with mice infected with the parental *M. tuberculosis* H37Rv strain. Thus, disruption of *acr* results in increased bacterial replication and lung pathology (163).
These studies have shown a significant and prominent role for this DosR regulated gene HspX in mycobacterial persistence. Human immune responses to other DosR regulon encoded genes than HspX had not been studied before. Here, we extend the studies on human immunity to *M. tuberculosis* DosR regulated genes with findings concerning HspX and other members of this late stage specific regulon.

Scope and Outline of this thesis

The work described in this thesis focuses on the search for and immunologic evaluation of novel vaccine targets for the development of post-exposure- or therapeutic TB vaccines which can control TB infection, stop TB reactivation or even better, eradicate *M. tuberculosis*. Such vaccines can however be given also in a prophylactic setting. Both people harbouring latent *M. tuberculosis* infection and un-infected individuals would benefit from such vaccines, since most TB cases arise from the immense reservoir of individuals carrying latent TB infection (>2 billion individuals are thought to carry latent TB).
The recently identified 48-gene *M. tuberculosis* DosR regulon is expressed by tubercle bacilli during in vitro exposure to hypoxia, low dose nitric oxide or carbon monoxide, conditions thought to be encountered by *M. tuberculosis* in vivo when persisting in immuno-competent hosts. Genes expressed by persisting bacilli represent attractive targets for (post-exposure or therapeutic) vaccination against TB. In this thesis DosR regulon encoded *M. tuberculosis* antigens and the immune response they induce in humans have been studied.
Chapter 2 describes T cell responses to the first set of 25 *M. tuberculosis* DosR encoded antigens in humans. To this end recombinant DosR proteins were produced and immune responses to these antigens were studied using $CD4^+$ T cell lines and PBMC from TB patients, TST converters and healthy, *M. tuberculosis*-un-infected controls. Studying these different study groups provided new insights into relevant immune recognition profiles and antigens among this set of genes which are strongly up-regulated during intracellular infection.
Since protective cellular immunity to *M. tuberculosis* thrives on both $CD4^+$ and $CD8^+$ T cell mediated responses, $CD4^+$ and $CD8^+$ T cell responses to a number of (dominant) *M. tuberculosis* DosR encoded antigens were studied in mycobacteria primed individuals in **Chapter 3**. Furthermore, HLA class I and II restricted peptide-epitopes were identified. These results further increased our understanding of the human immune response to *M. tuberculosis* phase specific antigens.
The only currently available anti-TB vaccine is the widely used BCG vaccine. However, BCG is effective mainly in preventing severe pediatric forms of TB, but is insufficient in preventing pulmonary TB in adults, the major and contagious form of TB disease. Relatively little is known about the immune response profiles following BCG vaccination in relation to protection against TB, let alone the involvement of *M. tuberculosis* DosR genes in protective anti-mycobacterial responses. In **Chapters 4 and 5** we studied induction of immune responses to *M. tuberculosis* DosR antigens following BCG vaccination. **Chapter 4** describes the immune response profiles to a series of DosR antigens responses, including HspX, in a longitudinal BCG vaccination study (human and mice) and in a cross-sectional BCG vaccination analysis (human). Moreover, transcriptional profiles of different BCG strains were studied, combined with an in silico analysis of *M. tuberculosis* DosR homologs in BCG. As mentioned before, HspX has already been studied in anti-mycobacterial immunity. In **Chapter 5,** findings regarding HspX are extended with results from assessment of responses to HspX following BCG vaccination (in mice) and responses to HspX in PBMC from TB

patient, TST converters, BCG vaccinated individuals and healthy, *M. tuberculosis*-uninfected controls.
Unexpectedly, responses to *M. tuberculosis* and *M. tuberculosis* DosR antigens were observed also in *M. tuberculosis* uninfected/ non-exposed, non-BCG vaccinated healthy controls, suggesting prior exposure to environmental non-tuberculous mycobacteria (NTM). NTM exposure has long been suspected to modulate human responses to *M. tuberculosis* infection and BCG vaccination. In **Chapter 6** therefore the hypothesis was tested that NTM infection/exposure induces cross-reactive immunity to *M. tuberculosis* DosR antigens. For this purpose, *M. tuberculosis* DosR antigen specific T-cell responses were studied in PBMC of NTM infected or exposed individuals in combination with in silico analysis to determine the presence of *M. tuberculosis* DosR-regulon gene homologs among environmental mycobacteria and non-mycobacteria.
DNA vaccines against tuberculosis have been proposed as a result of their ability to induce strong cellular immunity, which is needed for control of TB. The study described in **Chapter 7** evaluates the immunogenicity of a DNA vaccine encoding the *M. tuberculosis* DosR antigen Rv1733c and explores different vaccine regimes including, DNA-prime/protein-boost immunization, route of administration and co-formulation with nanoparticles in mice. Following vaccination, mouse splenocytes were assessed for immune parameters such as cell proliferation and IFNγ production and immunogenicity of the different vaccine regimen were compared.
The main findings of these studies are summarized and discussed in **Chapter 8**.

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Chapter 2

Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of *Mycobacterium tuberculosis*

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Abstract

The dormancy (DosR) regulon of *Mycobacterium tuberculosis* is expressed in vitro during hypoxia and low-dose nitric oxide stimulation. Tubercle bacilli are thought to encounter these conditions in humans during latent infection. In this study, immune responses were evaluated to the 25 most strongly induced DosR-regulon-encoded proteins, referred to as latency antigens. Proliferation assays were performed using *M. tuberculosis*-specific T-cell lines and peripheral blood mononuclear cells (PBMC) from tuberculosis (TB) patients, tuberculin skin test positive (TST+) individuals and uninfected controls. All 25 latency antigens were able to induce production of interferon-gamma (IFN-γ) by T-cell lines. Eighteen latency antigens were also recognized by PBMC of *M. tuberculosis*-infected individuals, which indicates expression of the DosR-regulon during natural infection. Differential analysis showed that TST+ individuals recognized more latency antigens and with a stronger cumulative IFN-γ response than TB patients, while the opposite profile was found for culture filtrate protein-10. In particular Rv1733c, Rv2029c, Rv2627c and Rv2628 induced strong IFN-γ responses in TST+ individuals, with 61%, 61%, 52% and 35% responders, respectively. In conclusion, several new *M. tuberculosis* antigens were identified within the DosR-regulon. Particularly strong IFN-γ responses to latency antigens were observed in latently infected individuals, suggesting that immune responses against these antigens may contribute to controlling latent *M. tuberculosis* infection.

Introduction

Tuberculosis (TB) is a major threat to global health with an estimate of four persons dying of TB every minute. Surveys with tuberculin skin tests (TST) suggest that one-third of the world's population is latently infected with *Mycobacterium tuberculosis*. This enormous reservoir of latent TB, from which most cases of active disease arise, embodies a major obstacle in achieving worldwide control of TB [1]. In particular when taken into account the currently ongoing AIDS pandemic, as in HIV-1 infected individuals risk of progression from a latent infection to active TB increases significantly, up to 8-10% per year [1,2]. The available vaccine against TB, *M. bovis* bacillus Calmette-Guérin (BCG), only provides significant protection against severe TB disease in young infants and establishment of *M. tuberculosis* infection [3], but affords limited and highly variable protection against pulmonary TB in adults, which is mostly due to reactivation of latent *M. tuberculosis* [4,5].

Both $CD4^+$ and $CD8^+$ T-cells and the cytokines IFN-γ and TNF-α play an important role in the immune response against mycobacteria and in controlling latent infection [6,7]. During latent infection, tubercle bacilli are typically contained within immune-mediated granulomas [8]. However, before the era of chemotherapy, it has been shown that live tubercle bacilli can also be present in macroscopically normal lung tissue [9]. This finding was recently confirmed by in-situ PCR [10], but these tubercle bacilli could not be visualized using Ziehl-Neelsen staining. It has been reported that this is caused by distinct cell-wall alterations of tubercle bacilli during persistence [11].

It is generally thought that persisting tubercle bacilli are subjected to nutrient and oxygen deprivation, microbicidal molecules such as reactive oxygen intermediates and nitrogen intermediates, and immune-mediated killing (reviewed in [12,13]). However, little is known about antigen-specific human T-cell responses against persisting mycobacteria, particularly in the context of latent infection and protection against TB disease. Previous studies of tubercle bacilli cultured under low oxygen report that HspX, also known as 16-kDa alpha-crystallin homologue (Acr) or Rv2031c, is highly expressed during these conditions [14,15]. Cellular immune responses to HspX have been observed in latently infected individuals, while antibodies to this antigen were predominantly found in persons with active TB disease [16]. More recently, HspX was found to be part of the so-called dormancy (DosR) regulon of *M. tuberculosis* comprising 48 genes [15,17-20]. Expression of the DosR regulon is observed as part of the adaptive response of *M. tuberculosis* to hypoxia, including gradual oxygen depletion, which results in distinct stages of non-replicating persistence in the so-called Wayne model [21], and to low-dose nitric oxide stimulation [19]. These in vitro culture models represent proxies of conditions that tubercle bacilli are thought to encounter in vivo during persistence in immune competent hosts [13,22]. This is supported by observations that genes of the DosR regulon are upregulated in IFN-γ activated *M. tuberculosis*-infected murine macrophages and in lungs of chronically infected mice [20,22,23]. In humans, increased levels of mRNA of HspX were found in lungs of patients with chronic active TB [23].

The functions of most DosR regulon encoded proteins are unknown [14,24]. At present it is also not known to which extent DosR regulon encoded proteins are expressed during the different stages of natural *M. tuberculosis* infection in human, nor is it known whether these proteins are targeted as part of the protective immune response to *M. tuberculosis*. To start addressing these questions we have selected 25 of the most strongly expressed proteins of the DosR regulon, further referred to as latency antigens, and evaluated IFN-γ responses and proliferation of human T-cells in TB patients, TST positive (TST+) individuals and *M. tuberculosis*-uninfected controls.

Materials and Methods

Study subjects

Our primary goal was to assess the potential immunogenicity of novel candidate *M. tuberculosis* latency antigens. Therefore, we selected a wide spectrum of *M. tuberculosis* infected individuals, as is described in detail below. In total, three populations were studied: TB patients, TST^+ healthy individuals and *M. tuberculosis* uninfected healthy individuals.

The group of TB patients (n=20) consisted of 11 persons with TB during treatment (mean duration of treatment: 10 weeks, range 2-25) and 9 with cured TB who were treated between 4-63 years before blood sampling (mean interval 29 years). Eleven patients had pulmonary and 9 had extra-pulmonary TB. The mean age was 46 years (range 17-75 years), 14 were male. Eight TB patients were of Dutch origin, 9 of African and 3 of Asian origin. TB patients were either HIV-1 seronegative or had no risk factors for exposure to HIV.

All 23 TST+ healthy individuals were of Dutch origin and were not BCG vaccinated. They had a documented TST result of ≥ 10 mm induration, mostly (n=14) after contact with a case of smear-positive pulmonary TB. The mean age was 37 years (range 21–63), 14 were male. From 12 persons, blood was drawn within 6 months after TST conversion, of whom only 5 were treated with isoniazid. In the remaining 11 TST+ individuals, the mean interval between conversion and blood sampling was 5.4 years (range 2-8). Only 2 of these remote TST converters had received isoniazid. Up to the time of this writing, none of the TST+ individuals had developed active TB after a mean period of 5.5 years (range 2.5-11) since TST conversion. These individuals are regarded as latently infected persons who most likely have acquired natural immunity against development of TB disease.

As a control group, 21 uninfected healthy individuals were studied; none of whom were BCG vaccinated or had had any known exposure to TB. They were either TST negative (n=18; in the others no TST was done) and/or did not respond to *M. tuberculosis*-specific proteins ESAT-6 and CFP-10 in an IFN-γ ELISPOT (known from a previous project [25]). All controls were of Dutch origin with an average age of 30 years (range 22-44), 7 were male.

Blood samples were collected after written informed consent was obtained. PBMC were isolated and stored in liquid nitrogen as previously described [26]. The study

protocol (P207/99) was approved by the institutional review board of the Leiden University Medical Center.

M. tuberculosis antigens and peptides

M. tuberculosis H37Rv was grown for 24 hours in tubes with tightly screwed caps, harvested and lysed as previously described [15]. The culture filtrate of this low oxygen culture was concentrated with a centriprep-concentrator and protein concentration was determined by the BCA test (Pierce, Rockford, Illinois). The lysate from *M. tuberculosis* cultured under standard aerated conditions was provided by the RIVM (Bilthoven, the Netherlands).

DosR regulon encoded genes were selected on basis of their RNA expression level in microarray experiments [19], as most of these 48 genes are hypothetical open reading frames with unknown biological function. The 25 most strongly induced genes were selected and sequences were obtained from http://genolist.pasteur.fr/TubercuList (Table 1). Genes were cloned and proteins were overexpressed in *Escherichia coli* and purified, as previously described [27]. Size and purity were checked by gel electrophoresis and Western blotting with anti-His antibodies. Residual endotoxin levels were determined with a Limulus Amebocyte Lysate assay (Cambrex) and were found to be below 50 IU/mg recombinant protein. Protein batches were tested for non-specific T-cell stimulation in lymphocyte stimulation assays using PBMC of BCG-unvaccinated, TST-negative healthy donors.

Synthetic peptides from culture filtrate protein 10 (CFP-10) were produced as previously described [26].

T-cell lines

Eight long-term T-cell lines were generated with either lysate (n=4) or culture filtrate (n=4) of *M. tuberculosis* grown under low oxygen conditions, using PBMC obtained from two cured TB patients and two TST+ individuals known to respond to HspX. In addition, 4 T-cell lines were made by stimulating PBMC from three TB patients and one TST+ individual with lysate from *M. tuberculosis* cultured under standard aerated conditions. T-cell lines were generated as previously described [26].

Proliferation assays and IFN-γ detection

T-cells ($1.5x10^4$/well) were cultured with autologous or HLA-DR matched irradiated PBMC ($5x10^4$/well) for 3 days and PBMC ($1.5x10^5$/well) were cultured for 6 days in the presence or absence of antigen, as previously described [26]. All latency antigens (Table I) were tested at a final concentration of 0.33 μM (average concentration 9.3 μg/ml, range 3.1-24) in order to allow for direct comparison of immunogenicity between proteins which vary considerably in size (9-74 kDa). Standard *M. tuberculosis* lysate, hypoxic lysate and culture filtrate were used at 1 μg/ml and the peptide-pool of CFP-10 at 1 μg/ml/peptide. The same antigens and identical batches were used throughout all experiments. Due to a shortage of the Rv1733c batch the number of study subjects that could be tested for this antigen was limited to 17 controls, 18 TST+ persons and 16 TB patients. After culture, supernatants were collected for the detection of IFN-γ and proliferation of T-cells was measured by [^{3}H]-thymidine incorporation as described elsewhere [26]. The concentration of IFN-γ was measured

by ELISA (U-CyTech, Utrecht, The Netherlands; detection limit of 20 pg/ml). The mean value of unstimulated cultures was subtracted from the mean value of stimulated cultures.

Statistical analysis

For comparison of the proportion of responders in each study group, the chi-square test was used. For all study subjects who were able to respond to an antigen, levels of IFN-γ production were evaluated non-parametrically using Kruskal-Wallis and Mann-Whitney tests for comparison of study groups. The Wilcoxon-signed-rank test was performed to compare the strength of the response between two antigens for each individual. In controls responses to latency antigens were correlated to the response to *M. tuberculosis* lysate using a Spearman rank correlation. A regression analysis was done to compare the cumulative IFN-γ response to latency antigens between groups, while controlling for the effect of age, general responsiveness of T-cells and time between initial infection and blood sampling.

Results

Human T-cell lines recognize latency antigens

Long-term T-cell lines were generated against either lysate (n=4) or culture filtrate (n=4) of *M. tuberculosis* grown under low oxygen conditions or standard aerated conditions (n=4). All lines responded strongly to the preparations of *M. tuberculosis* grown under hypoxic conditions. Readouts included T-cell proliferation (*data not shown*) and IFN-γ production. Figure 1a shows the individual IFN-γ responses to 25 latency antigens of all generated *M. tuberculosis*-specific T-cell lines. Both T-cell lines generated from cured TB patients as well as from TST+ individuals were able to recognize a large number of latency antigens.

In order to ensure the selection of all proteins with possible immunogenic capacity for further evaluation (see below), we used a relatively low level of IFN-γ as cut-off value (50 pg/ml above background) and calculated the number of T-cell lines that responded to each latency antigen (Figure 1b). This figure shows that all 25 latency antigens were recognized by at least 1 out of 12 T-cell lines tested and 21 antigens by at least 4 T-cell lines. Latency antigen Rv2031c (HspX), and Rv2032 (Acg) were most frequently recognized by 75% of tested T-cell lines (median IFN-γ levels among responding lines: 507 and 129 pg/ml respectively). The observed responses to latency antigens by T-cell lines generated with the standard-aerated *M. tuberculosis* lysate are explained by the finding of HspX in this preparation (Western blot analysis; *data not shown*), indicating the expression of the DosR regulon. Most latency antigens were recognized by T-cell lines raised with hypoxic-lysate as well as with hypoxic culture filtrate, suggesting that some latency antigens might also be found extra-cellularly. This is in line with previous findings showing that Rv0569, Rv2623 and Rv2626c proteins were present in culture filtrate of *M. tuberculosis* grown under hypoxic conditions [15]. In conclusion, using human T-cell lines specific for *M. tuberculosis*, we show that all 25 novel mycobacterial latency antigens are potentially able to elicit cellular immune responses in *M. tuberculosis*-infected individuals.

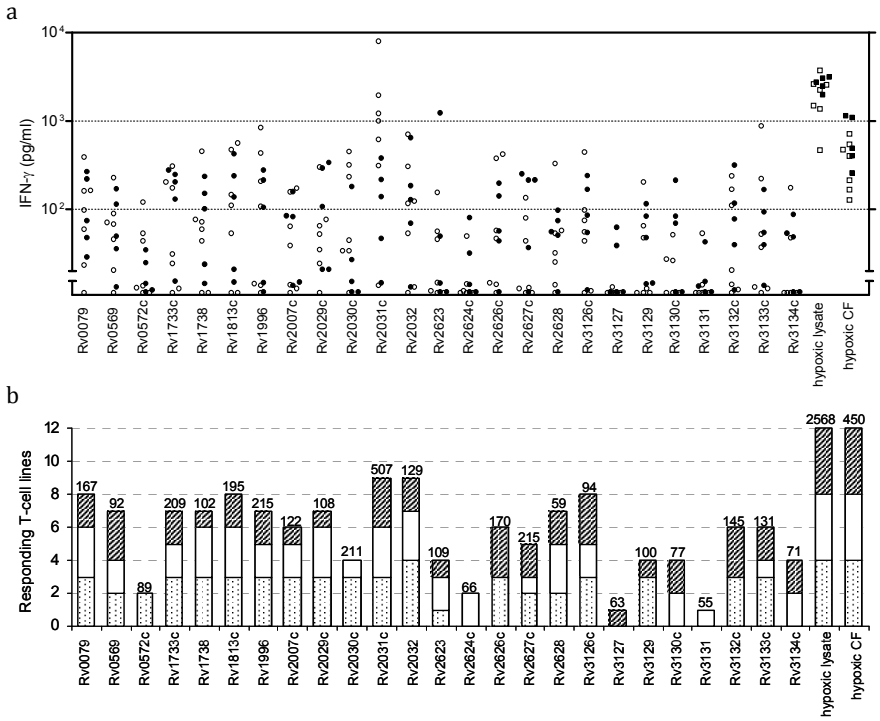

Figure 1. Long-term T-cell lines (n=12) responding to 25 M. tuberculosis latency antigens, hypoxic-M. tuberculosis lysate and culture filtrate (CF)*. Hypoxic lysate and CF were prepared from M. tuberculosis H37Rv which was grown for 24 hours in tubes with tightly screwed caps. 8 lines were generated by stimulating PBMC obtained from TST converters (n=2) or cured TB patients (n=2) with either hypoxic-CF (n=4, cross-hatched bar) or hypoxic-lysate (n=4, white bar). Additionally, PBMC from one TST converter and 3 cured TB patients were stimulated with lysate of M. tuberculosis, which was grown under standard, aerated conditions (n=4, dotted bar).* ***(a)*** *IFN-γ responses of all 12 M. tuberculosis-specific T-cell lines. Dots indicate the IFN-γ response minus the IFN-γ value in the unstimulated cultures. Lines were either generated from PBMC of TST converters (closed circles (•)) or cured TB patients (open circles (o)).* ***(b)*** *Bars indicate the number of lines responding to a latency antigen with an IFN-γ production of at least 50 pg/ml. The number at the top of each bar indicates the median IFN-γ production of these responding lines.*

Recognition of latency antigens by PBMC of *M. tuberculosis* infected individuals

Subsequently, direct peripheral blood ex-vivo stimulation assays were performed using PBMC from 20 TB patients, 23 TST+ individuals and 21 uninfected controls. In order to identify those proteins with a strong capacity to induce IFN-γ production, we calculated for each antigen the proportion of individuals who responded to the antigen with an IFN-γ production level exceeding 100 pg/ml (Table 1). This analysis revealed that 18 of the 25 latency antigens were recognized by at least 10% of either TB patients or TST+ individuals (Table 1). Of these, Rv1733c, Rv2029c and Rv2627c, were even recognized by the majority of TST+ individuals. The remaining 7 antigens tested (Rv0572c, Rv2007c, Rv2623, Rv2624c, Rv3127, Rv3131, Rv3134c) were not or very poorly recognized by PBMC from *M. tuberculosis*-infected individuals. These

Table 1. Immunogenicity of latency antigens[a]

Latency antigen	Gene name	Product	Response (%) [b] HC (n=21)	TB (n=20)	TST+ (n=23)
Rv0079		HP	14	10	26
Rv0569		CHP	-	5	22
Rv0572c		HP	10	5	-
Rv1733c		possible transmembrane protein	41	50	61
Rv1738		CHP	-	11	13
Rv1813c		CHP	14	15	17
Rv1996		CHP	14	11	4
Rv2007c	*fdxA*	probable ferredoxin FDXA	-	-	9
Rv2029c	*pfkB*	probable phosphofructokinase PfkB	29	25	61*
Rv2030c		CHP	14	15	26
Rv2031c	*hspX*	heat shock protein HspX	5	20	-*
Rv2032	*acg*	CHP Acg	14	20	30
Rv2623	*TB31.7*	CHP TB31.7	-	-	-
Rv2624c		CHP	-	-	-
Rv2626c		CHP	14	10	30
Rv2627c		CHP	38	30	52
Rv2628		HP	10	16	35
Rv3126c		HP	19	10	30
Rv3127		CHP	-	-	4
Rv3129		CHP	19	21	35
Rv3130c		CHP	-	-	13
Rv3131		CHP	-	-	4
Rv3132c	*devS*	2-component sensor histidine kinase	24	15	17
Rv3133c	*dosR*	2-component transcriptional regulatory protein	14	32	30
Rv3134c		CHP	-	-	4

[a] Abbreviations: HC, healthy controls; TB, TB patients; TST+, tuberculin skin test positive individuals;
HP, hypothetical protein; CHP, conserved hypothetical protein. Annotations are from http://genolist.pasteur.fr/TubercuList/.

[b] Percentage of individuals with an IFNγ response of ≥100 pg/ml.
-, in none of the study subjects an IFNγ response of ≥100 pg/ml was observed.

*P < 0.05, $\chi 2$ test comparing TB patients with TST+ individuals.

antigens were also the weakest inducers of IFN-γ production among the above tested T-cell lines. Similar results were obtained when proliferation was analysed (*data not shown*).

When comparing the proportion of strong responders among study groups, most latency antigens were recognized by a larger proportion of TST+ individuals as compared to TB patients and controls (Table 1); this trend was only statistically significant for Rv2029c when comparing TST+ persons with TB patients (P=0.02).

HspX was relatively poorly recognized, and more frequently by TB patients than by TST+ individuals (P=0.02) (Table 1). Although no further statistical analysis was possible because of small numbers, it was interesting to note that TST+ individuals who showed at least some response to HspX (IFN-γ of 20-100 pg/ml) all had had recent TST conversion (<6 month).

Recognition of latency antigens by uninfected controls

Somewhat unexpectedly, 16 of the 25 latency antigens were also recognized by T-cells from a minority of *M. tuberculosis*-uninfected controls (Table 1). Controls were BCG-unvaccinated, were TST-negative and did not recognize CFP-10 and ESAT-6 (known from a previous project [28]). Therefore, we hypothesized that observed responses to

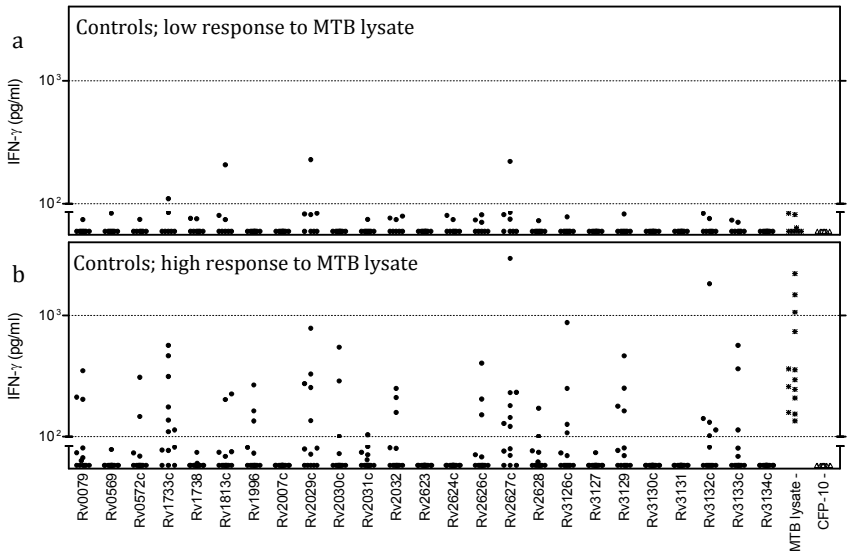

Figure 2. IFNγ responses of M. tuberculosis uninfected healthy controls to 25 latency antigens, M. tuberculosis (MTB) lysate and CFP-10. (a) *Controls with a low IFNγ response (< 100 pg/ml) to standard M. tuberculosis lysate (n = 8)* ***(b)*** *Controls with a strong IFN-γ response (> 100 pg/ml) to standard M. tuberculosis lysate (n = 13). None of the controls responded to the M. tuberculosis specific antigen CFP-10. MTB lysate, M. tuberculosis lysate.*

latency antigens in controls were not caused by infection with *M. tuberculosis* complex species, but more likely by previous exposure to other mycobacteria. Indeed, thirteen 62%) of 21 *M. tuberculosis*-uninfected controls appeared to strongly respond to *M. tuberculosis* lysate (IFN-γ >100 pg/ml; median level 591 pg/ml IFN-γ), which is most likely due to exposure to non-tuberculous mycobacteria. Significant responses to latency antigens were almost exclusively observed in this group of control subjects (Figure 2b), while in the remaining 8 control subjects with low responses to mycobacterial lysate only sporadically low responses to latency antigens were observed (Figure 2a). Responses to latency antigens indeed correlated significantly to the response to *M. tuberculosis* lysate (Spearman R=0.56, P =0.02).

IFN-γ responses to frequently recognized latency antigens and CFP-10

Rv1733c, Rv2029c, Rv2627c and Rv2628 were particularly broadly recognized, predominantly by TST+ individuals. IFN-γ responses to those 4 antigens were analysed in more detail as shown in Figure 3. For comparison of the magnitude of the IFN-γ response between groups, only those individuals were analysed who were able to recognize the antigen with an IFN-γ response above the detection level of the assay. Median IFN-γ production among responders in the group of TST+ individuals in response to Rv1733c, Rv2029c, Rv2627c and Rv2628 was 564, 382, 195 and 160 pg/ml, respectively, while in the group of TB patients lower IFN-γ responses were found, with medians of 160, 137, 194 and 79 pg/ml, respectively (Figure 3). This difference was statistically significant for Rv1733c (P=0.008). In order to compare responses to latency antigens with responses to a *M. tuberculosis*-specific antigen

outside the DosR regulon, PBMC of the same individuals were also stimulated with a peptide pool of CFP-10, an antigen that is secreted by tubercle bacilli during active replication. In contrast to latency antigens, CFP-10 induced significantly stronger IFN-γ responses among TB patients as compared to TST+ individuals, with a median among responders of 242 and 59 pg/ml, respectively (P = 0.042) (Figure 3).

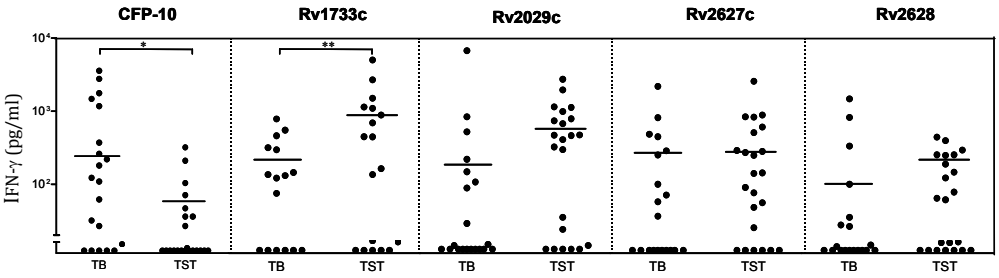

Figure 3. IFN-γ response profiles to the four best recognized M. tuberculosis latency antigens and the M. tuberculosis-specific secreted antigen CFP-10. *IFN-γ production by PBMC from TB patients (TB) and TST positive individuals (TST) in response to Rv1733c, Rv2029c, Rv2627c Rv2628 and CFP-10 (peptide pool) is shown. Horizontal lines indicate the median IFN-γ production among persons who are able to respond to the antigen. Responses were compared using a Mann-Whitney U test; *, P < .05; ** P <.01*

To analyze this difference in antigen recognition pattern between TB patients and latently infected individuals further, we compared the strength of the immune response to the 4 best recognized latency antigens versus CFP-10 within each individual, using the Wilcoxon signed rank test. In TST+ individuals IFN-γ responses to Rv1733c, Rv2029c, Rv2627c and Rv2628 were higher than to CFP-10 (P<0.01, P<0.01, P<0.01 and P=0.07, respectively). The opposite antigen recognition profile was found for TB patients, irrespective of whether they had active or cured disease: there was a stronger IFN-γ response to CFP-10 than to Rv1733c, Rv2029c, Rv2627c and Rv2628 (P=0.19, P=0.01, P=0.04 and P<0.01, respectively). Subsequently, for each individual the IFN-γ response to CFP-10 was subtracted from the response to Rv1733c such that a positive value would indicate that the response to Rv1733c is stronger while a negative value would indicate a stronger response to CFP-10. The same was done for Rv2029c, Rv2627c and Rv2628. This complementary analysis revealed that in TST+ individuals the mean difference in IFN-γ response between CFP-10 and Rv1733c, Rv2029c, Rv2627c and Rv2628 was 451, 308, 184 and 44 pg/ml respectively, while in TB patients negative values were obtained (-373, -365, -458 and -536 pg/ml respectively (P<0.01, P=0.04, P=0.01 and P=0.02).

Cumulative IFN- y response to antigens of the dormancy regulon

As each individual was found to be able to recognize a different set of latency antigens, we calculated the cumulative IFN-γ response to all 25 latency antigens to capture the overall immune response to the tested set of DosR regulon encoded antigens within each person. This analysis showed that a significantly higher cumulative IFN-γ

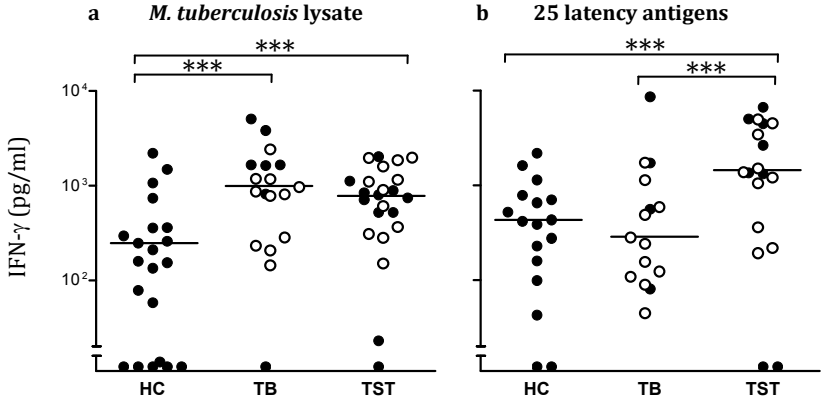

Figure 4. IFN-γ response profiles to M. tuberculosis lysate and the group of 25 latency antigens. *IFN-γ production by PBMC from uninfected healthy controls (HC), TB patients (TB) and TST positive individuals (TST) in response to* ***(a)*** *M. tuberculosis lysate and* ***(b)*** *the cumulative IFN-γ production to 25 latency antigens. TB patients during treatment are indicated with open circles (o) and cured TB patients with closed circles (•). TST+ persons with recent exposure are indicated with open circles (o) and TST+ with a more remote exposure with (•). Horizontal lines indicate the median IFN-γ production among responders, and responses were compared using a Mann-Whitney U test; ***, P< 0.01.*

response to the antigens encoded by the DosR regulon was seen in the group of TST+ individuals compared to TB patients (median IFN-γ of 1453 and 263 pg/ml respectively, *P*=.003) and to controls (median IFN-γ of 434 pg/ml, *P*=.003)(Figure 4). In contrast, for whole *M. tuberculosis*-lysate no significant differences were observed between TB patients and TST+ individuals but their responses were significantly higher when compared to healthy controls (*P*<0.01) (Figure 4). Figure 4 visualizes responses in TB patients both during active disease and following cure, but subgroups were too small for separate statistical analysis. Subgroup analysis was also done for the group of TST converters which was split up into those with recent exposure to *M. tuberculosis* and those who had been exposed more than 2 years prior to the time of blood sampling. Subsequently, a regression analysis was done in which cumulative IFN-γ responses to latency antigens were compared between the two groups, while controlling for the effect of age, general responsiveness of T-cells (by adjusting for the response to *M. tuberculosis*-lysate and PHA) and possible waning of immunity (by adjusting for the time between initial infection with *M. tuberculosis* and the time of blood sampling). This analysis showed that, also after adjusting for all of the above-mentioned variables, a significantly higher cumulative IFN-γ response to the antigens of the DosR regulon was observed in TST+ individuals compared to TB patients.

Finally, response profiles also differed between the two groups of *M. tuberculosis*-infected individuals with regard to the absolute number of latency antigens that were recognized: TST+ persons recognized significantly more latency antigens (mean of 5.4) than TB patients (mean of 2.4) (*P*=0.04).

Discussion

The present study provides the first description of human T-cell responses to a large series of proteins encoded by the recently described dormancy (DosR) regulon of *M. tuberculosis.* All 25 tested latency antigens were able to induce human T-cell responses, using *M. tuberculosis*-specific T-cell lines. Eighteen of these latency antigens were recognized by PBMC from *M. tuberculosis*-infected individuals, indirectly confirm that DosR regulon encoded proteins are expressed during natural *M. tuberculosis* infection in humans. Our results indicate than TST+ individuals tend to recognize more latency antigens, and have a stronger cumulative IFN-γ response when compared to individuals who developed TB. In contrast, IFN-γ responses to CFP-10, a *M. tuberculosis*-specific antigen secreted during active replication, were significantly stronger in TB patients.

Out of the 18 latency antigens that were recognized by PBMC of *M. tuberculosis*-infected individuals only HspX had been previously identified as a *M. tuberculosis* antigen [16]. Of these newly identified antigens, Rv1733c, Rv2029c, Rv2627c and Rv2628 were most frequently recognized, by 61%, 61%, 52% and 35% of TST+ individuals, respectively. These antigens were found to induce relatively strong IFN-γ responses when compared to the well-known immunodominant secreted antigen CFP-10.

Positive responses to latency antigens in TB patients were not entirely unexpected as most patients go through a phase of latent infection before progression to active TB disease occurs; memory immune responses induced during this latent phase may remain detectable thereafter. In addition, during TB disease at least part of the bacilli are expected to encounter hypoxic conditions or to be exposed to nitric oxide which leads to upregulation of the dormancy regulon, as suggested by the finding of variable levels of HspX transcripts in different regions of the lungs of patients with chronic, active TB [22,23]. Furthermore, as recently has been observed for bacilli during aerated stationary phase in vitro, it is possible that in TB patients expression of the DosR is partial or altered, resulting in a more limited response to latency antigens [29].

We hypothesize that immune responses to latency antigens may contribute to the control of persistent *M. tuberculosis* infection and that changes in protein expression profile during latency result in an altered repertoire of *M. tuberculosis* antigens available for T-cell recognition. This could then be reflected in differences in antigen recognition patterns of responding T-cells. The observation that in TST+ individuals IFN-γ responses to the 4 most frequently recognized latency antigens were significantly higher than to CFP-10, while the opposite antigen recognition profile was found for TB patients, is consistent with this hypothesis. Furthermore, latently infected individuals recognized significantly more latency antigens and had a stronger IFN-γ production in response to the group of latency antigens when compared to TB patients. However, our study was not designed to specifically demonstrate a causal relationship between the observed immune responses and protection against TB disease, as this would require the follow-up of untreated latently infected persons and detailed analysis of the kinetics of responses in those who will progress to active TB

versus those who will remain healthy. The observed association between IFN-γ responses to latency antigens and natural protection needs to be further investigated, but we would like to suggest that these antigens may be interesting candidates for evaluation in post-exposure vaccination models.

An unexpected finding of the study was the recognition of latency antigens by some of the uninfected controls. None of the controls were BCG vaccinated, had any identified risk of exposure to TB or responded in vitro to the *M. tuberculosis*-specific antigens ESAT-6 or CFP-10. However, more than half of the controls recognized *M. tuberculosis* lysate, which is most likely explained by exposure to mycobacteria other than *M. tuberculosis.* Responses to latency antigens were nearly exclusively seen in this subgroup of controls, whereas virtually no responses were seen in the mycobacterium non-responsive group, suggesting that responses to latency antigens could result from cross-reactivity to non-tuberculous mycobacteria. It is interesting to note that other mycobacteria, like *M. bovis* and BCG, *M. microti* and *M. smegmatis* have a very similar dormancy regulon [30,31]. Moreover, there may be homologues in other bacteria than mycobacteria causing cross-reactivity. Of note, Rv1733c encodes a possible transmembrane protein, which bears homology with a hypothetical protein of *Streptomyces coelicolor;* and Rv2029c, which is a predicted phosphofructokinase involved in glycolysis, is expected to have homologues in other (myco) bacteria. It is not known whether the observed cross-reactive immune response to latency antigens among *M. tuberculosis* naïve persons contributes to the natural protection that develops in 90% of individuals who are infected with *M. tuberculosis* and remain free of disease; but this is may be an important topic for further studies [5,32].

In our study we tested proteins in equimolar concentrations, to allow for direct comparison of human T-cell responses to different antigens including the previously defined antigen HspX and newly identified latency antigens. The results indicated that several of the latter induced higher responses in a larger proportion of subjects than HspX did. We observed that HspX, at the concentration tested, was recognized by only 20% of TB patients whereas in TST+ persons IFN-γ responses were very infrequent and of a low level. Of note however, several T-cell lines strongly recognized the identical protein batch of HspX. The apparent discrepancy with a previous studie, in which recognition of HspX by T-cells of TST+ individuals was demonstrated [16,28], may be related to differences in readout methodology and to antigen concentrations tested, since relatively low concentrations of antigens were used. Finally, a difference in study populations could be an additional factor, as immune responses to HspX may have waned if the infection with *M. tuberculosis* was remote. The observed responses to HspX were all in recent converters, supporting the latter hypothesis.

In this study we focused on IFN-γ responses by T-cell lines and PBMC. The essential role of IFN-γ in the protective immunity to mycobacteria has been clearly demonstrated by studies in gene-knockout mice and by the increased susceptibility to mycobacteria in individuals with genetic defects in the IFN-γ/interleukin-12/23 pathway [33,34]. Nonetheless, future studies on latency antigens should also address production of other cytokines than IFN-γ [6]. In addition, besides CD4 responses, we are currently studying CD8 T-cells specific for mycobacterial latency antigens, as several studies indicate that CD8 T-cells are important for controlling latent *M. tuberculosis* infection [7,35].

In conclusion, the DosR regulon was found to encode a high number of *M. tuberculosis* antigens with strong T-cell and IFN-γ inducing capacity. A trend was observed towards stronger IFN-γ responses to a larger number of latency antigens in latently infected individuals as compared to individuals who developed TB disease, whereas the opposite profile was found for CFP-10. These findings are consistent with the hypothesis that immune responses to latency antigens may contribute to natural protection against TB disease. However, subsequent prospective studies of large cohorts of latently infected individuals are needed to determine how strongly T-cell responses to these newly identified latency antigens are associated with TB latency and with protection against progression towards active disease. If so, latency antigens may be promising tools for vaccination against TB reactivation.

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Chapter 3

Identification of CD4+ and CD8+ T cell responses to *Mycobacterium tuberculosis* DosR regulon encoded dormancy antigens and mapping of HLA class I and II restricted peptide-epitopes.

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Abstract

Effective global anti-tuberculosis (TB) strategies involves development of better vaccines, including preventive as well as post-exposure -or therapeutic- TB vaccines. The latter aim at preventing TB reactivation in already infected individuals, particularly since the widely used BCG-vaccine fails to protect against reactivation TB. Over 2 billion people are thought to be latently infected with *Mycobacterium tuberculosis* (*M. tuberculosis*). Target antigens suitable for post-exposure vaccination need to be expressed by *M. tuberculosis* during clinical latency, i.e. late stage infection. We previously reported human T cell activity against *M. tuberculosis* DosR regulon encoded antigens, which are strongly up-regulated during the *M. tuberculosis* stress response upon intracellular infection. Human T cell responses to *M. tuberculosis* DosR regulon encoded antigens were associated with control of latent TB infection, but the nature of the response remains unidentified. Protective cellular immunity to *M. tuberculosis* thrives on both $CD4^+$ and $CD8^+$ T cell mediated responses. Here, we identify the presence of both $CD4^+$ and $CD8^+$ T cell responses towards a series of immunodominant *M. tuberculosis* DosR regulon encoded antigens in *M. tuberculosis* responsive individuals. We further show that *M. tuberculosis* DosR regulon encoded antigens contain both $CD4^+$ and $CD8^+$ T cell epitopes, which are recognized in the context of HLA-class II or class I molecules, respectively. Proliferative responses were visualized using a CFSE based assay, allowing simultaneous detection of $CD4^+$ as well as $CD8^+$ proliferative T cell responses to the same antigens and peptides. These results significantly increase our understanding of the human immune response to *M. tuberculosis* phase/stage specific antigens, and may help in designing *M. tuberculosis* DosR regulon encoded antigen or peptide based vaccination approaches to TB.

Introduction

Annually, TB claims over 1.5 million lives and causes active (infectious) TB disease in over 9 million cases. It is estimated that over 2 billion people are latently infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of tuberculosis (TB). This vast reservoir of latently infected individuals forms a major source of new TB cases. One in every 10 *M. tuberculosis* infected individuals will eventually develop active TB during their lifetime whereas the remainder is able to contain the bacilli without developing any clinical symptoms. Current control of the TB pandemic is seriously impeded by the strong increase in TB morbidity and mortality during active HIV co-infection, and the continuous rise of multi-drug resistant (MDR) and extensively drug-resistant (XDR) *M. tuberculosis* strains (38).

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) is today's only available TB vaccine. BCG protects against severe forms of TB in young children and against leprosy, but does not efficiently and consistently protect against pulmonary TB in adults (8,13). Effective anti-TB strategies not only require better preventive vaccines, but also vaccines exerting post-exposure/therapeutic activity (25,40) since BCG is ineffective in latently infected individuals. Post-exposure TB vaccines should aim at preventing reactivation of TB infection in latently infected individuals by inducing robust immunity to antigens that are expressed by persisting *M. tuberculosis* bacilli during latent infection. Such immune responses are considered essential in controlling, or even better, eradicating persisting bacilli. This likely requires induction of adequate $CD4^+$ and $CD8^+$ T-cell responses against these dormancy associated *M. tuberculosis* antigens. Recently, we showed that genes of the *M. tuberculosis* DosR (Rv3133c) regulon encode antigens that can induce significant T cell responses in *M. tuberculosis* infected individuals (latently or actively infected)(18). The 48-gene DosR regulon is expressed by tubercle bacilli during in vitro exposure to hypoxia, low dose nitric oxide and carbon monoxide, conditions thought to be encountered by *M. tuberculosis* in vivo when persisting in immuno-competent hosts (36). Approximately half of the *M. tuberculosis* DosR regulon is also expressed over prolonged periods of time in the recently proposed enduring hypoxia response (EHR) model (30). Immunity to *M. tuberculosis* DosR regulon encoded antigens seems to be associated with control of latent TB infection, since several DosR regulon encoded antigens were (preferentially) recognized by individuals with latent TB infection (LTBI) (10,18,29). We therefore hypothesized that immune responses to these antigens might contribute to the control of persistent *M. tuberculosis* infection.

However, the exact nature of these human T cell responses to *M. tuberculosis* DosR regulon encoded antigens has not been studied in detail. Both $CD4^+$ and $CD8^+$ T cells participate in the optimal protective response to *M. tuberculosis*. Here, we describe a number of relevant and highly recognized *M. tuberculosis* DosR antigens that induce both $CD4^+$ and $CD8^+$ human T cell responses in mycobacteria primed individuals. Responses were mapped to a series of *M. tuberculosis* DosR regulon encoded peptides, which were recognized in the context of HLA class I and HLA class II molecules. Detailed epitope mapping offers the potential to elucidate possible subdominant epitopes; these might be exploited in designing improved vaccines by inducing

immunity to a broader epitope repertoire than would be seen following natural infection or protein vaccination (22,24).

Materials and Methods

Study subjects

Buffy coats from twenty-one in vitro PPD responsive (PPD^+) healthy anonymous, HLA typed blood bank donors were included in this study. No prior selection was made concerning HLA type. No clinical information is available from these anonymous healthy donors concerning TB infection/exposure, TB contact history or BCG vaccination, except that they were healthy and had no chronic viral infections. However, given the low TB incidence in the Netherlands, and the lack of a national BCG vaccination policy, none of these parameters would be expected to be of high significance in this cohort. Additionally, PBMC from 5 TB patients and 8 tuberculin skin test (TST) converters were studied. Blood of these 13 donors were obtained by venapuncture after written informed consent was obtained. The study protocol was approved by the Institutional Review Board of the Leiden University Medical Centre (LUMC). PBMC were isolated using Ficoll density gradient centrifugation and stored in liquid nitrogen until use.

M. tuberculosis antigens and peptides

Recombinant proteins were produced as described previously (14). Briefly, selected *M. tuberculosis* H37Rv genes were amplified by PCR from genomic H37Rv DNA and cloned by Gateway Technology (Invitrogen, San Diego, CA). Proteins were over-expressed in *Escherichia coli* strain BL21(DE3) and purified as described (14). All recombinant proteins were subjected to quality control assays including sequencing, size and purity check, residual endotoxin levels, non-specific T-cell stimulation and potential cellular toxicity (19). Purified protein derivative (PPD, batch RT49) was purchased from Statens Serum Institute, Denmark.

Synthetic peptides were synthesized as previously described (17). Peptides from *M. tuberculosis* DosR antigens Rv1733c, Rv2029c, Rv2031c, Rv2627c and reference antigen Ag85B were 20-mers peptides with 10 amino acids (aa) overlap except peptides 20-22 of Ag85B which were 15-mers with 10 aa overlap. The 20-mer peptides of Rv1733c, Rv2029c and Rv2627c were elongated with two lysine (K) residues at the C-terminal to improve solubility. Candidate HLA-A2 epitopes consisted of 9-mers and candidate HLA-DR3 epitopes were 15 or 16-mers. The HLA-A*0201-restricted, HIV-1 p17 Gag_{77-85} epitope (SLYNTVATL) was used as control peptide (34).

Generation of $CD4^+$ T cell lines

T cell lines were generated as previously described (2). In brief, PBMC were incubated at 1-2x10^6 cells/well in 24-well plates (Nunc, Roskilde, Denmark) in the presence of *M. tuberculosis* lysate (5 μg/ml) in IMDM supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen, Breda, The Netherlands) and 10% pooled human serum; further addressed as culture medium. After 6 days, IL-2 (25 U/ml) (Cetus,

Emeryville, CA, USA) was added and cultures were continued for another 2 to 3 weeks in presence of IL-2. T-cells were frozen and stored in liquid nitrogen until use.

Generation of CD8+ T cell lines

$CD8^+$ T cell lines were generated by using (autologous) dendritic cells (DC) as antigen presenting cell (APC) and three *M. tuberculosis* DosR regulon encoded candidate HLA-A2 epitopes. PBMC from a HLA-$A2^+$ TB patient (HLA-A2, A3, and DR11) were used: at day -7, $CD14^+$ cells were isolated by using MACS beads (Miltenyi, Germany) and plated in 6-wells plates at $3x10^6$ $CD14^+$ cells in RPMI, 10% FCS, containing 80 ng/ml GM-CSF (Biosource) and 500 U/ ml rIL-4 (Peprotech) further referred to as DC culture medium, at 37°C and 5% CO_2 to induce immature DCs (iDC) (35). $CD8^+$ T cells were isolated from the $CD14^-$ fraction. Both $CD8^+$ and $CD8^-$ fraction were frozen and stored in liquid nitrogen until use. iDCs were matured on day -1 in DC culture medium, supplemented with 50 ng/ml LPS. At day 0, mature DCs (mDCs) were harvested and resuspended in RPMI with 1% pooled human serum in the presence of 25 μg/ml per HLA-A2 candidate epitope and 3 μg/ml β2-microglobulin (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 4 hours at 37°C and 5% CO_2 with hourly resuspension of cells. After washing, peptide loaded mDCs were irradiated at 2500 rad. $CD8^+$ and $CD8^-$ T cell fractions were thawed and mixed in a 10:1 ratio and resuspended at $3x10^6$ cells in 1 ml in a 24-wells plate, forming the responding population. To the responders cells 1 ml stimulation culture (irradiated, peptide pulsed mDCs) was added (in a ratio 10:1 respectively) in culture medium supplemented with 10 ng/ml IL-7 and 50 pg/ml IL-12. At day 7, 1 ml medium was refreshed with culture medium and IL-7 and IL-2 in final concentration of 20 ng/ml and 50 U/ml respectively. At day 12, $4x10^6$ autologous PBMC were seeded at $4x10^6$ cells/ml in IMDM in a 24-wells plate. After two hours, non-adherent cells were washed away and 20 μg/ml per HLA-A2 candidate epitope and 3 μg/ml β2-microglobulin were added for an additional 4 hours in a volume of 0.5 ml/well. The responder CTL culture was resuspended at $1.5x10^6$ cells/ml in culture medium. Medium was removed from the peptide pulsed adherent cells and 1 ml CTL culture was added. At day 14, culture medium was added and IL-7 and IL-2 in final concentration of 20 ng/ml and 50 U/ml respectively. The CTL culture was restimulated every 7-8 days as described above, two days following restimulation, IL-7 and IL-2 were added as described above.

Lymphocyte stimulation assays

Proliferation based on CFSE dilution:

PBMC were thawed, washed and labeled at 10^7 cells/ml with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands) for 10 minutes at 37°C, dark in PBS/0.5% bovine serum albumin (BSA, Sigma-Aldrich, Zwijndrecht, The Netherlands). Labelling was stopped by adding 10% heat-inactivated fetal calf serum (FCS; Greiner, Alphen a/d Rijn, The Netherlands). Cells were washed in PBS/0.5% BSA and resuspended in culture medium. Cells were added to 96-well U-bottom microtiter plates (Nunc) at $1.5x10^5$ cells/well and stimulated in duplicate with antigens at 37°C and 5% CO_2. Antigens were tested in the following concentrations: DosR antigens, Ag85B and their respective single peptides, control peptide (HIV-1 p17 Gag_{77-85}): all at 10 μg/ml; PPD at 5 μg/ml; phytohaemagglutinin

(PHA, Remel, UK) at 2 μg/ml (positive control), and medium only as negative control. Cells of duplicates were pooled and washed with PBS with 0.1% BSA for evaluation by flow cytometry. Similar proliferation and readout assays were performed for the *M. tuberculosis* HLA-A2 candidate epitopes but with minor changes: cells were tested at $1x10^5$ cells/well (six-fold) in culture medium for either six or ten days. At day six, supernatants were harvested followed by staining cells for flow cytometry as described below. Ten day cultures included addition of recombinant IL-7 (5 ng/ml, AMDS, Benelux) on day 0 and recombinant IL-2 (10 U/ml, Cetus, Emeryville, CA, USA) on day seven; harvesting of cells occurred at day 10.

CD4 T cell line stimulation:

T cells from *M. tuberculosis* antigen specific-T cell lines (1.5×10^4/well) were cultured in triplicate in culture medium with autologous or HLA-DR matched/mismatched PBMC as APC (5×10^4/well, irradiated (2000 rad) in 96-wells flat-bottomed microtiter plates in the presence or absence of antigen and cultured at 37°C and 5% CO_2. At day three, supernatants were collected for the detection of IFNγ (2).

Flow Cytometry

Cells were stained with anti-CD3-PerCP, anti-CD4-APC and anti-CD8-PE (BD Biosciences, Erembodegem, Belgium) for 30 minutes at 4°C. Cells were washed in PBS/0.1% BSA and fixed in1% paraformaldehyde (Pharmacy LUMC, The Netherlands) and analyzed on a FACS Calibur (BD Biosciences). Analysis was done using CellQuest Pro software (BD Biosciences).

Analysis of proliferating lymphocytes (CFSE)

Cells gated on live lymphocytes combined with gating on $CD3^+$ T cells were analyzed for CFSE proliferation. The Δ geometric mean was used as a measure of proliferation and calculated as follows: Δ geometric mean = geometric mean (non-proliferated cells cells) - geometric mean (total cells). The Δ geometric mean was then used to calculate the relative proliferation which is in fact the percentage of the maximal proliferation (PHA), corrected for spontaneous proliferation (HIV-1 p17 Gag_{77-85}): ((Δ geometric mean sample- Δ geometric mean control peptide)/(Δ geometric mean PHA- Δ geometric mean control peptide))*100% = % of maximal proliferation. The cut off for a positive proliferation was set at 10% relative proliferation. This arbitrary high threshold value was chosen in order to limit the number of candidate epitopes to be evaluated in subsequent experiments.

IFNγ ELISA

The concentration IFNγ in the supernatants was measured by ELISA (U-CyTech, Utrecht, The Netherlands) according to the manufacturer's instructions. The detection limit of the assay is 20 pg/ml IFNγ. Samples were tested in duplicate; the mean value of unstimulated cultures was subtracted from the mean value of the stimulated cultures. An IFNγ response ≥ 100 pg/ml was considered positive for PBMC and for *M. tuberculosis* antigen specific T cell lines when IFNγ response was ≥ 50 pg/ml unless indicated otherwise.

Chromium release assay

As target cells, human EBV-BLCL JY (HLA-A*0201, -B7, -Cw7), EBV-BLCL JY-Rv1733c-GFP (EBV-BLCL JY transduced with an expression vector encoding the *M. tuberculosis* DosR gene Rv1733c and the GFP marker (31,33)) and EBV-BLCL JY-GFP (EBV-BLCL JY transduced with an empty control vector encoding only GFP) were incubated at 37°C for 1 hr with 0.1 mCi $Na_2{}^{51}CrO_4$ (Amersham, United Kingdom), washed, and plated in triplicate in 96-well round-bottom plates (2500 cells/well). Effector $CD8^+$ T cells were added in different effector-to-target (E:T) ratios, 12:1, 25:1 and 50:1 together with either medium, peptide (25 μg/ml), or 5% Triton X-100 and with of without pan HLA-class I or HLA-class II antibodies. After six hours the supernatants were harvested, and the percentage lysis was calculated as follows:
[(release - spontaneous release)/(maximum release- spontaneous release)] * 100%.

Results

Selection of recombinant *M. tuberculosis* proteins and corresponding peptides

For this study we selected four *M. tuberculosis* DosR regulon encoded antigens previously shown to be prominently recognized in humans, in order to identify potential $CD4^+$ and $CD8^+$ T cell responses as well as to identify peptide epitopes recognized in the context of specific HLA-class I and class II molecules. The immunodominant, early phase secreted antigen Ag85B was taken along as reference antigen. *M. tuberculosis* DosR antigens Rv1733c, Rv2029c and Rv2627c were chosen on the basis of T cell recognition profiles in *M. tuberculosis* infected individuals (TB patients and tuberculin skin test (TST) converters) from our previous work (18): all three antigens ranked among the top 10 most frequently recognized antigens in the *M. tuberculosis* infected groups. *M. tuberculosis* DosR antigen Rv2031c (HspX, hsp16, a-crystallin) was included since many studies have addressed the role of this heat-shock protein in anti-mycobacterial immunity (15,39,41). Recombinant proteins (Table 1) and overlapping sets of peptides of these five antigens were prepared (see supplementary file 1 for all peptide sequences).

Table 1. Selected *Mycobacterium tuberculosis* antigens tested in present study

	Rv number	Gene name [a]	Molecular mass (kDa)[a]	Product [a]	References
DosR genes	Rv1733c		22,4	conserved transmembrane protein	6, 10
	Rv2029c	*pfkB*	35,4	phosphofructokinase PfkB	6, 10, 14
	Rv2031c	*hspX*	16,3	heat shock protein HspX (alpha-crystallin)	6, 9, 10, 14, 21-23
	Rv2627c		46,3	conserved hypothetical protein	6, 10, 14
reference gene	Rv1886c	*fbpB*	34,6	secreted antigen 85-B fbpB (mycolyltransferase 85B, Ag85B)	25

[a] Annotations are from www.tbdb.org and http://genolist.pasteur.fr/TubercuList/

Identification of *M. tuberculosis* induced antigen specific CD4⁺ and CD8⁺ T cell responses based on CFSE proliferation

We used CFSE based proliferation of PBMC, since this method allows tracking of both CD4⁺ and CD8⁺ T cell subset responses in the same cell population (20). Figure 1 demonstrates typical CFSE based CD4⁺ and CD8⁺ T cell response profiles to *M. tuberculosis* antigens and control conditions when assaying PBMC from a PPD responsive donor. Following stimulation of cells with PPD, significant CD4⁺ and to a lesser extent also CD8⁺ T cell responses could be observed, underlining the significant advantage of this assay allowing responding subpopulations to be phenotyped and characterized. No response was seen to the negative control medium or to the irrelevant control peptide HIV-gag$_{77–85}$. Following stimulation of PBMC with *M. tuberculosis* DosR regulon encoded antigen Rv1733c or its corresponding peptides proliferative CD4⁺ T cell responses were observed (Figure 1a).

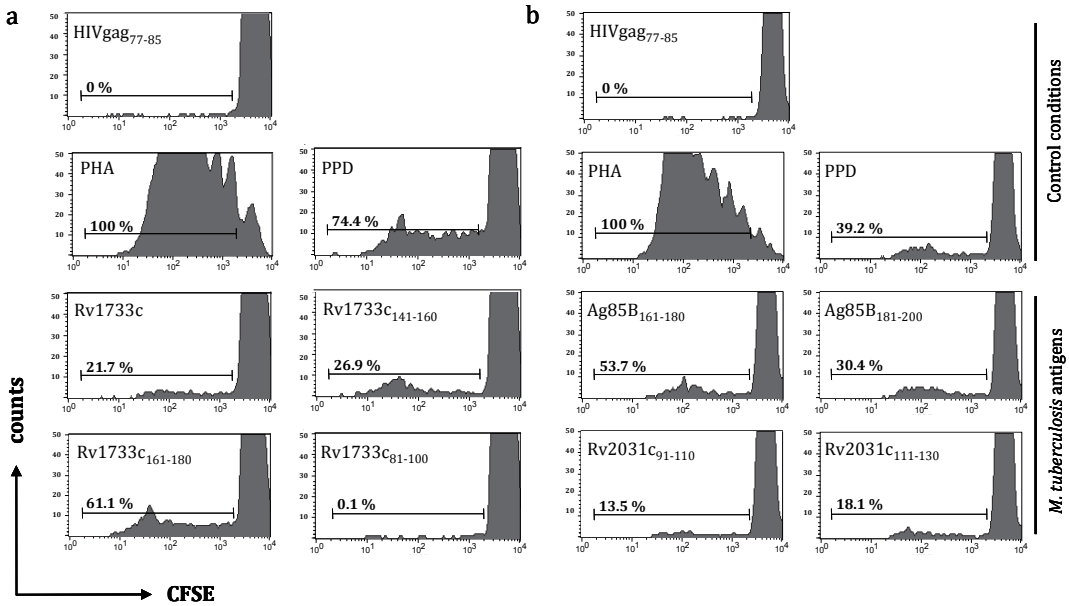


Figure 1. Proliferative T cell response profiles to M. tuberculosis antigens compared to control conditions. *PBMC from donor 9 (Table 2, HLA-A2, -A24, -B35, B57, -C4, C6, DR-7, DR-14) were stimulated with PHA or HIVgag$_{77-85b}$ as positive and negative controls, respectively, or with M. tuberculosis test antigens. After 6 days of culturing CFSE proliferation of CD4⁺ (a) or CD8⁺ T cells (b) was measured: CD4⁺ or CD8⁺ T cells were gated from a CD3⁺ T cell gate combined with a live lymphocyte gate. Individual histogram plots show the % relative proliferation ((Δ geometric mean sample - Δ geometric mean control peptide)/ (Δ geometric mean PHA- Δ geometric mean control peptide))*100% = % of maximal proliferation. PBMC were stimulated with Rv1733c and corresponding peptides, some of which induced CD4⁺ T cell proliferation while others did not (a) PBMC were also stimulated with four 20-mer peptides of Ag85B and Rv2031c encoding identified and published HLA-A2 restricted epitopes (see text) to which indeed CD8⁺ T cell proliferation was observed in this HLA-A2 positive donor (b). A relative proliferation ≥ 10% was considered positive.*

We wanted to establish whether our method was able to confirm already published epitopes of Ag85B and Rv2031c (HspX, α-crystallin), in order to further validate the CFSE based proliferation method. For the mature Ag85B protein, five HLA-A*0201 epitopes have been described ($Ag85B_{p45-53}$ GLPVEYLQV, $Ag85B_{p166-175}$ SMAGSSAMIL, $Ag85B_{p183-192}$ FIYAGSLSAL, $Ag85B_{p198-206}$ GMGPSLIGL and $Ag85B_{p239-247}$ KLVANNTRL (16)). We therefore included 20-mer peptides containing these five HLA-A*0201 restricted epitopes in our study. Four out of the five 20-mers were indeed recognized by $CD8^+$ T cells from this donor (Figure 1b) as well as by other HLA-$A2^+$ individuals (data not shown).
Also for Rv2031c three HLA-A*0201 restricted epitopes have previously been identified: $Rv2031c_{p21-29}$ LFAAFPSFA (6), $Rv2031c_{p91-105}$ SEFAYGSFVRTVSLP (15) and $Rv2031c_{p120-128}$ GILTVSVAV (4,6). Our data show that 20-mers encoding epitopes $Rv2031c_{p91-105}$ and $Rv2031c_{p120-128}$ (but not $Rv2031c_{p21-29}$ in this donor) were both recognized by several HLA-$A2^+$ individuals. Figure 1b demonstrates proliferative $CD8^+$ T cell responses following stimulation with several of the above mentioned 20-mer *M. tuberculosis* peptides.
Also peptides encoding previously published, HLA class II presented epitopes were recognized in this assay. These included: Rv2031c peptide 4 encoding $Rv2031c_{p31-50}$ (LRPTFDTRLMRLEDEMKEGR, HLA-DRB1*0301) (9,15,21) and Rv2031c peptides 3, 9, and 10 which encode several epitopes recognized in the context of HLA-DRB1*1501 (1,5) (data not shown). Taken together, the results from the CFSE assay agree well with results from other assays, including the ability to identify peptide epitopes recognized by $CD4^+$ and $CD8^+$ T cells.

Immunogenicity of *M. tuberculosis* DosR regulon encoded antigens based on CFSE proliferation and flow cytometry

CFSE labelled PBMC from randomly selected, healthy, PPD-responsive blood bank donors were stimulated with *M. tuberculosis* lysate; PPD; *M. tuberculosis* DosR regulon encoded proteins Rv1733c, Rv2029c, Rv2627c, Rv2031c; Ag85B protein; and all respective single peptides from each of the 5 recombinant antigens. Table 2 shows the HLA class-A, –B, -C and -DR genotypes and the corresponding *in vitro* PPD reactivity of the donors. IFNγ responses to PPD exceeding 100 pg/ml were considered positive, as used in our previous studies (2,18,19).
Immunogenic regions could be identified within the sequences of these *M. tuberculosis* DosR regulon encoded antigens (Table 3a-c). Table 3a and 3b show the comprehensive recognition profiles of $CD4^+$ and $CD8^+$ T cells of the tested antigens and their respective single peptides in all donors studied here. As anticipated, responses to the whole recombinant proteins were mostly confined to the $CD4^+$ T cells. In several cases $CD4^+$ T cell responses were accompanied by $CD8^+$ T cell responses (e.g. in donor 1), while isolated $CD8^+$ T cell responses to the recombinant proteins were not observed.
As expected, there were substantial inter-individual differences in antigen and peptide recognition, since some donors responded to many *M. tuberculosis* DosR regulon encoded proteins and corresponding peptides whereas others only responded to few proteins or peptides. Nevertheless, all 15 PPD^+ donors responded to at least one of the recombinant proteins and/or peptides investigated.

Table 2. HLA-class A, -B, -C and –DR genotypes and IFNγ responses to *M. tuberculosis* PPD of donors studied in current study

Donor	HLA type				PPD[a]
number	HLA-A	HLA-B	HLA-C	HLA-DR	pg/ml
1	1	7,8	7	2,3	+
2	2,31	8,44	5, 7	3,4	+
3	11,30	18,27	1, 5	3,4	+
4	2,29	45,50	3, 6	4,7	+
5	2	44,39	5, 7	7,15	+++
6	2,3	51,7	*	15,11	+
7	2	7,8	7	2,3	+
8	3,31	56,35	1, 4	1,4	++
9	2,24	35,57	4, 6	7,14	++
10	3,24	14,18	*	1,4	+++
11	1,31	51,52	*	11,15	+
12	2,68	44,51	1, 5	11,14	+
13	3	7,51	7, 4	1,15	++
14	3,24	7,62	7, 9	11,13	+
15	3,11	15,38	1, 12	3,13	+

*, HLA-C type unknown.

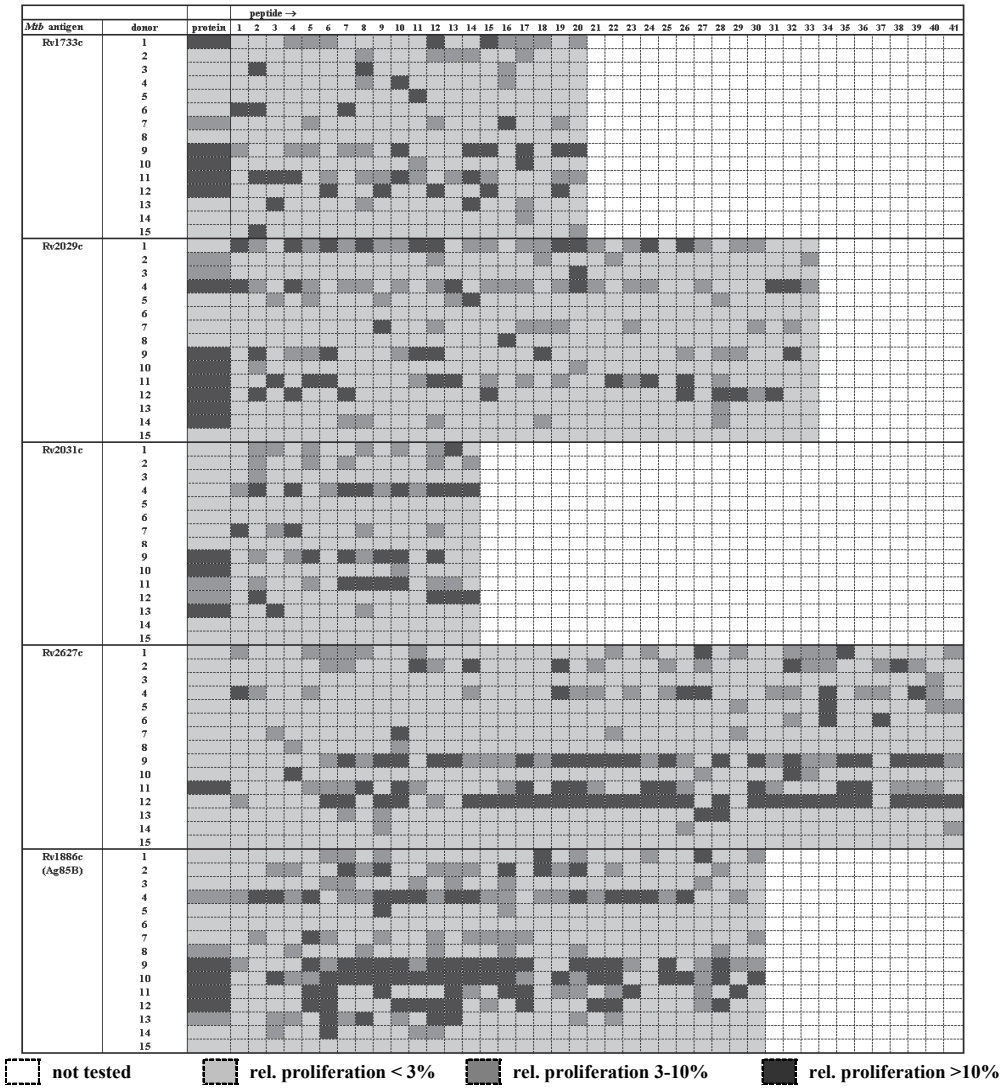
[a], Responses to PPD: +, 100-500 pg/ml; ++, 500-1000 pg/ml; +++, >1000 pg/ml.

In this series of donors, recombinant antigens Rv1733c, Rv2029c and Rv1886c (Ag85B) were recognized most efficiently: 7/15 PPD$^+$ donors recognized Rv2029c, 5/15 recognized Rv1733c and 4/15 recognized Ag85B. This contrasted to Rv2627c, which was recognized by only a single donor. In line with our previous observations, Rv2031c/hspX/*acr* was recognized by a minority of the donors (18).

Peptides in several instances appeared to be more able to induce CD4$^+$ or CD8$^+$ T cell responses when compared to recombinant proteins; this might be due to their preprocessed nature, facilitating high efficiency antigen presentation, and/or to their higher molarity, since proteins and peptides all were tested at 10 microgram/ml concentrations, regardless of molecular mass.

Importantly, a substantial number of immunogenic peptides recognized by CD4$^+$ T cells, CD8$^+$ T cells or both could be identified using this approach. Some peptides were recognized by CD4$^+$ T cells from at least one third of the donors, e.g. Ag85B peptides 9, 13 (6 donors), Ag85B peptides 5, 6 and Rv2627c peptide 19 (5 donors). Several other peptides were recognized by CD4$^+$ T cells from 4/15 donors, such as Rv1733c peptide 2, Rv2627c peptides 10, 19, 32, 34 and 35, and Ag85B peptides 10, 12, 16 and 22. A large number of peptides was recognized by at least three donors. Various CD8$^+$ T cell responses were detected as well, although the numbers of peptides recognized overall was lower than that for CD4$^+$ T cells. This might be related partly to the length of the peptides, since HLA class I alleles typically prefer 9-11-mer peptides for binding, whereas those assayed here were 20-mers. Nevertheless, particularly for Rv1733c and Ag85B, there were several peptides recognized by CD8$^+$ T cells from 4-5 donors like Rv1733c peptides 17 (5 donors), 2 and 19 (both 4 donors) and Ag85B peptides 5 and 13 (both 4 donors). Multiple peptides were recognized by at least 3 donors, still representing 20% of the panel tested.

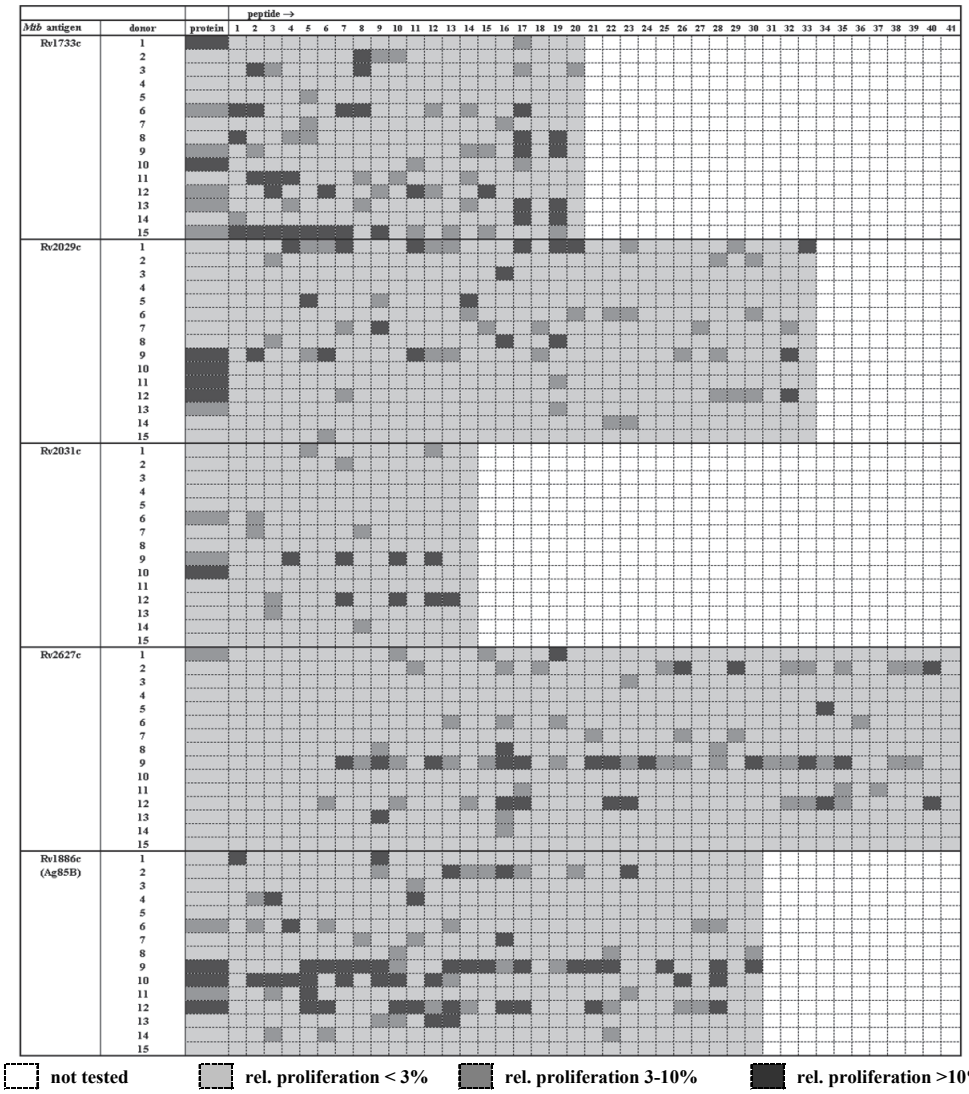
Table 3a. Proliferative CD4+ T cell response to *M. tuberculosis* peptides among all donors (n=15, numbers corresponding with donors in Table 2). A relative proliferation ≥ 10% was considered positive.



Mtb, M. tuberculosis

Table 3c shows the cumulative recognition results for both CD4+ and CD8+ T cells to all tested antigens and peptides for all donors tested. The results show that many proliferative responses to the *M. tuberculosis* DosR regulon encoded antigens can be identified. This also underlines the immunogenicity of *M. tuberculosis* DosR regulon encoded antigens. Interestingly, several peptides were recognized by both CD4+ and CD8+ T cells (Rv1733c: peptide 2; Ag85B peptides 5 and 13). This issue is commonly not addressed in epitope mapping studies, since read-outs are mostly biased towards detecting CD4+ T cell responses. Furthermore, the results suggest some enrichment of

Table 3b. Proliferative CD8⁺ T cell response to *M. tuberculosis* peptides among all donors (n=15, numbers corresponding with donors in Table 2). A relative proliferation ≥ 10% was considered positive.



Mtb, *M. tuberculosis*

epitopes in particularly immunogenic regions, for example Rv1733c peptides 1-3, 17-19, and Ag85B peptides 5-13.

Taken together, we applied the cell tracking dye CFSE to visualize and simultaneously quantify both CD4⁺ and CD8⁺ T cell responses to specific *M. tuberculosis* antigens and individual peptides. We identify a large series of new possible peptide epitopes located on *M. tuberculosis* DosR regulon encoded antigens, which are recognized with high efficiency by CD4⁺ and/or CD8⁺ T cells.

Table 3c. Cumulative CD4$^+$ and CD8$^+$ T cell response profiles to *M. tuberculosis* proteins and their respective peptides among PPD responsive donors (n=15).

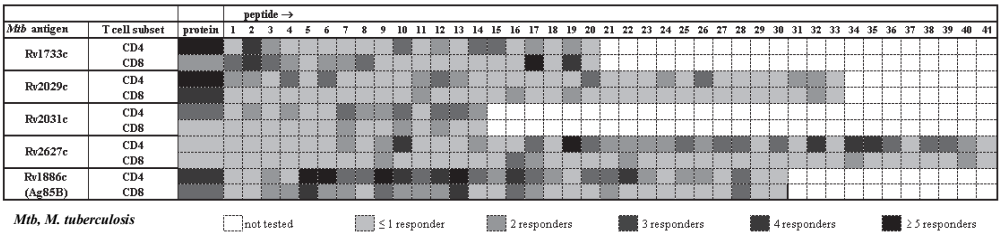
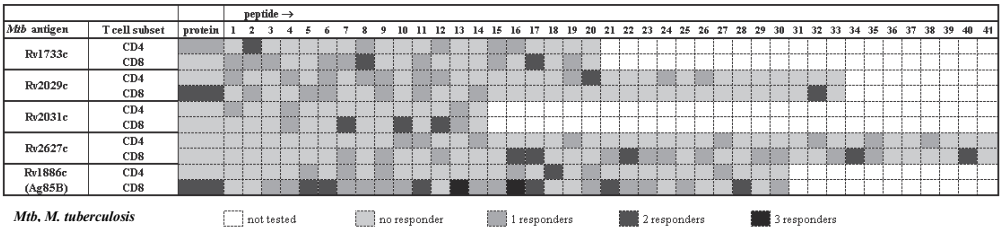


Table 3d. Cumulative CD4$^+$ and CD8$^+$ T cell response profiles to *M. tuberculosis* proteins and their respective peptides among heterozygous HLA-DR3$^+$ (n=5, CD4$^+$ T cell responses) and HLA-A2$^+$ (n=7, CD8$^+$ T cell responses) PPD responsive donors.



Identification of *M. tuberculosis* DosR regulon encoded peptide epitopes in the context of genetic HLA-A2 and HLA-DR3 polymorphism

The above described *M. tuberculosis* DosR regulon encoded peptide epitopes were recognized by different donors with varying HLA types. Many of the *in vitro* responses matched with *in silico* epitope motif searches for the relevant HLA types (26)(data not shown). This suggests that responses to *M. tuberculosis* DosR regulon encoded antigens can naturally occur in a wide range of HLA types. In order to better understand the molecular mechanisms of DosR regulon encoded epitope presentation, we examined peptide recognition in the context of HLA class I and class II polymorphism. Given the relatively limited number of individuals included in the panel, we decided to focus on 2 highly frequent alleles, HLA-A*0201 and HLA-DRB1*0301 (DR3/17) (frequencies are ~50% and ~20%, respectively among Caucasians)(23). Among the panel of 15 PPD$^+$ blood bank donors, 5 HLA-DR3 positive and 7 HLA-A2$^+$ donors were identified. T cell responses (CD4$^+$) to *M. tuberculosis* DosR regulon encoded peptides among the 5 HLA-DR3$^+$ donors were found in 2/5 donors, including to Rv1733c peptides 8 and 2, Rv2029c peptide 20 and Rv2627c peptide 19 (Table 2 and 3a). Many peptides were also recognized by (CD8$^+$ T cells from) HLA-A2$^+$ donors. Some peptides were recognized by up to 4/7 HLA-A2$^+$ donors such as Rv2627c peptides 19 and 34. A number of other peptides induced responses in 3/7 HLA-A2$^+$ donors: Rv2029c peptide 32, Rv2627c peptides 10, 26, 32, 38-40 and Rv2031c peptides 4, 7, 10 and 12 (Table 2 and Table 3b). Interestingly, Rv2627c protein was recognized by CD4$^+$ T cells of one donor whereas the single peptides of Rv2627c were recognized by many HLA-A2$^+$ and -DR3$^+$ donors implying that using peptides may be more sensitive in antigen discovery than using intact whole antigens.

Table 4a. List of *M. tuberculosis* DosR regulon encoded candidate HLA-DR3 epitopes

M. tuberculosis DosR antigen	epitope sequence[a]	start aa	end aa	score *in silico* prediction[a]
Rv1733c	TFRLRLPCRTILRVF	15	29	26
	ATMITFRLRLPCRTI	11	25	19
	VGIWVDSAGQLVDEPA	141	156	19
Rv2029c	TEKMRCGAPRYDPGG	35	49	11
	GINVARIVHVLGGCS	51	65	12
	YRFVLPGPSLTVAEQ	114	128	15
	KASVRELRECVGSEL	194	208	18
	VVSLGSQGALLATRH	231	245	14
	SIPMTAVSGVGAGDA	252	266	13
Rv2031c	TLPVQRHPRSLFPEF	4	18	26
	SVAVSEGKPTEKHIQ	125	139	12
Rv2627c	VAAGLEFYGNRRWLEK	93	108	17
	MGRAPLDLAVFRAWK	186	200	13
	RHTVKMAEPIGRMIS	316	330	13

[a], Epitope motif scores and candidate epitope sequences are obtained from www.syfpeithi.de

Table 4b. List of *M. tuberculosis* DosR regulon encoded candidate HLA-A2 epitopes

M. tuberculosis DosR antigen	epitope sequence[a]	start aa	end aa	score *in silico* prediction[a]	K_D <IC50> mM[b]	ELISA response[c]	CFSE proliferation[d]
Rv1733c	TRHPATATV	81	89	18	> 250	-	-
	HVYAHQAQT	73	81	11	5,5	+	-
	QAQTRHPAT	78	86	10	> 250	+	-
	GLWLSVAAV	170	178	29	0,11	+	-
	AALGLWLSV	167	175	24	3	-	-
	IADAALAAL	161	169	23	2.8 - 34	+	+
	ALLALTRAI	181	189	26	0,42	+	+
	LTRAILIRV	185	193	20	53	+	-
	AILIRVRNA	188	196	20	2,8	+	+
Rv2029c	ELAAEPTEV	314	322	21	1,8	+	-
	TEVGQDQYV	320	328	12	> 250	-	-
Rv2031c	AYGSFVRTV	94	102	19	13	-	-
	GSFVRTVSL	96	104	18	59	+	-
	RTVSLPVGA	100	108	12	2,5	+	-
Rv2627c	TANNREYAL	159	167	17	4,4	-	-
	GSQRWLSYT	151	159	9	209	-	-
	GLACAILGV	286	294	29	0,0041	-	+
	ILGVPVADL	291	299	29	0,72	-	-
	SLEEGLACA	282	290	25	0,24	-	-
	PIGRMISPL	324	332	19	200	-	-
	LSLTPLVPM	332	340	15	9	-	-
	SLTPLVPMP	333	341	19	49	-	+
	PMPGRFIYA	339	347	11	8,6	+	-
	AALEQSGLL	394	402	20	3	+	-
	GLLDAPRTQ	400	408	16	26	+	-

[a], Epitope motif scores and candidate epitope sequences are obtained from www.syfpeithi.de.
[b], control peptide (cold peptide) in binding assay had a K_D of 0.006
[c], positive IFNγ response to candidate epitope: +, positive responses exclusively found in HLA-A2$^+$ (heterozygous) donors (n=11);
-, positive responses found in donors with different HLA class A alleles (n=5).
[d], CD8$^+$ T cell proliferation following stimulation with candidate epitope (% proliferating CD8$^+$ cells): +, positive responses exclusively found in HLA-A2$^+$ (heterozygous)donors. When % proliferating CD8$^+$ cells was > [medium + 3*SD] it was considered positive.

Table 3d presents the cumulative $CD4^+$ and $CD8^+$ T cell response profiles to *M. tuberculosis* proteins and respective peptides among PPD responsive, heterozygous HLA-$DR3^+$ and HLA-$A2^+$ donors.
Next we further analyzed the *M. tuberculosis* DosR regulon encoded peptide epitopes in the context of HLA-DR3 and HLA-A2 presentation. First, we examined responses to control antigens, as above. Indeed, HLA-$A2^+$ donors recognized Ag85B peptides 13, 15, 16, 20, and Rv2031c peptides 10, 12, which all contained previously published HLA-A*0201 restricted epitopes. Similarly, Rv2031c peptide 4 that carries a HLA-DR3 epitope was indeed recognized by HLA-$DR3^+$ positive donors (Figure 1 and Table 3d), thus confirming once more that the method we use here is able to identify peptide specific responses.
A substantial number of peptides was recognized by the selected HLA-$DR3^+$ and HLA-$A2^+$ donors. These peptides were next subjected to further in silico minimal epitope motif searches (26) in the context of HLA-A2 or HLA-DR3 to select the precise epitopes embedded in the recognized 20-mers. Despite the limited number of individuals investigated, we were able to identify 14 *M. tuberculosis* candidate DosR regulon encoded HLA-DR3 presented epitopes (Table 4a) and 25 *M. tuberculosis* candidate DosR regulon encoded HLA-A2 presented epitopes (Table 4b).

In vitro verification of candidate *M. tuberculosis* DosR regulon encoded epitopes in the context of HLA-DR3

All fourteen candidate HLA-DR3 *M. tuberculosis* DosR regulon encoded epitopes were next synthesized as 15-mer peptides (Table 4a), and assessed for recognition by PBMC from two newly tested PPD responsive donors (#18: HLA-DR3, 5; #19: HLA-DR3, 2), both of which were not included in Table 2. Both donors were found to recognize a substantial number of these *M. tuberculosis* candidate HLA-DR3 presented epitopes (10 and 4, respectively) as shown in Figure 2a, whereas mycobacterium naïve donors 20 (HLA-DR1, 3) and 21 (HLA-DR3, 7) did not recognize any of the epitopes. Most importantly, these results show that responsiveness to *M. tuberculosis* candidate HLA-DR3 epitopes are clearly observed in individuals exposed/infected with mycobacteria but not in mycobacteria naïve, PPD negative donors.
Next, *M. tuberculosis* DosR regulon encoded antigens and selected epitopes were presented to a HLA-$DR3^+$ $CD4^+$ T cell line (TCL) which had been generated from PBMC of a treated TB patient (HLA-DR3, 15) by stimulation with *M. tuberculosis* lysate. Antigens were presented via (homozygous) HLA-DR3 matched or mismatched APC. For all antigens, except Rv1733c, both protein and peptide responses could be measured. For Rv1733c responses were seen against peptide-epitopes $Rv1733c_{p15\text{-}29}$ and $Rv1733c_{p141\text{-}156.}$ This result is compatible with the notion that peptide stimulation can visualize also a "subdominant" epitope repertoire which may not be visible following suboptimal stimulation with whole protein. Notably, no responses were found to peptide-epitope $Rv1733c_{p11\text{-}25}$ despite its large overlap with $Rv1733c_{p15\text{-}29;}$ this points to a C-terminal location of essential residues involved in either HLA-DR3 binding and/or TCR ligation.
In this TCL, responses to 11 out of the 14 *M. tuberculosis* candidate HLA-DR3 presented epitopes were observed. No or very low responses were observed when antigens or peptides were presented via HLA-DR3 mismatched APC (Figure 2b-e).

Only for peptide-epitope Rv2627cp$_{316-330}$ a remaining response, albeit strongly reduced, was observed when presented by HLA-DR3 mismatched APC. This could indicate that this peptide might be presented also by other HLA-class II molecules than HLA-DR3.

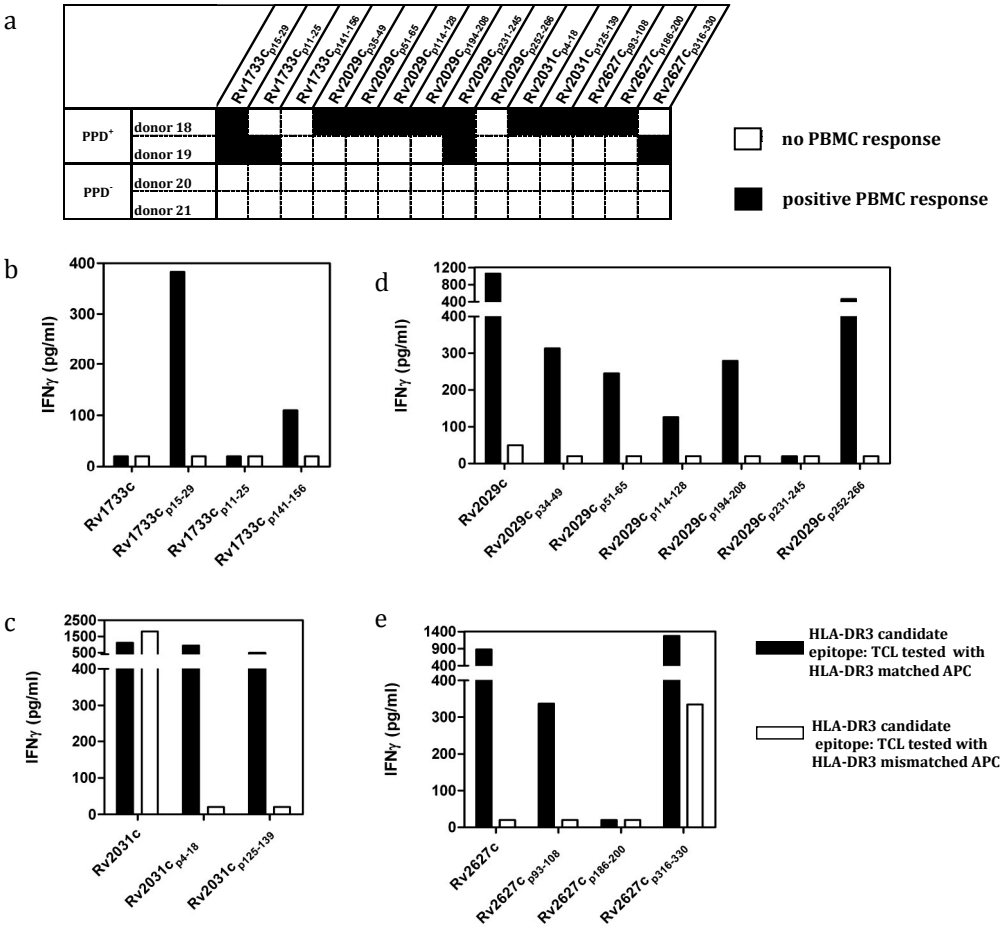


Figure 2. Recognition of candidate HLA-DR3 epitopes by assessment of IFNγ responses in PBMC and in CD4⁺ T cell lines*. PBMC from PPD⁺ HLA-DR3⁺ (heterozygous) donors (donor 18 and 19) both recognized a substantial number of candidate HLA-DR3 epitopes, in contrast to PPD⁻ HLA-DR3⁺ (heterozygous) donors (black box, response, white box, no response)* ***(a).*** *Immunodominant M. tuberculosis dosR antigens and their respective candidate HLA-DR3 epitopes were tested, using a CD4⁺ M. tuberculosis lysate induced T cell line (TCL) with HLA-DR3 matched (homozygous) APC (black bars)* ***(b-e)****. For Rv2029c* ***(d)****, Rv2031c* ***(c)*** *and Rv2627c* ***(e)*** *responses to both protein and candidate epitopes were observed. For Rv1733c (b) only responses to its respective candidate epitopes were observed (see text for discussion). When the TCL was tested with DR3 mismatched APC (white bars), no or strongly reduced responses were observed* ***(b-e)****. An IFNγ response ≥ 100 pg/ml was considered positive for PBMC and for M. tuberculosis antigen specific T cell lines when IFNγ response was ≥ 50 pg/ml.*

Confirming the above observations, responses to the longer 20-mer peptides of the *M. tuberculosis* DosR regulon encoded antigens containing the 15-meric HLA-DR3 candidate epitopes were also observed (data not shown). Thus, taken together these results strongly suggest that a series of *M. tuberculosis* DosR regulon encoded epitopes is presented to CD4+ T cells by HLA-DR3 molecules.

In vitro verification of candidate *M. tuberculosis* DosR regulon encoded epitopes in the context of HLA-A*0201

Taking a similar approach, 25 *M. tuberculosis* DosR regulon encoded HLA-A*0201 restricted candidate 9-meric epitopes were synthesized. First, their binding to HLA-A2 was measured (Table 4b). Several of the in silico predicted candidate HLA-A2 epitopes had intermediate or high binding affinity (IC50 <10 μM) for HLA-A*0201 molecules. Other candidate HLA-A2 epitopes, despite high prediction scores and in vitro CD8+ T cell recognition of the corresponding 20-mer peptide, had low (IC50>10 μM) or no measurable (IC50>100 μM) affinity for HLA-A*0201 molecules.

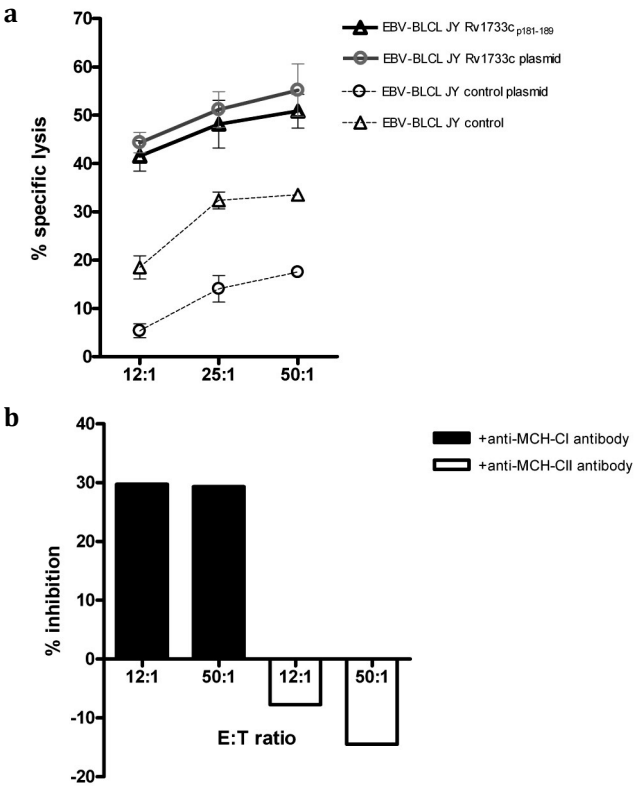


Figure 3. Cytotolytic activity of a CD8+ polyclonal T cell line against the HLA-A2 restricted M. tuberculosis epitope Rv1733c$_{p181-189}$. *The results show specific recognition of peptide epitope Rv1733c$_{p181-189}$ by CD8+ T cells when peptide was loaded onto EBV-BLCL JY target cells (compared to medium control). CD8+ T cells also recognized EBV-BLCL JY target cells that had been transduced with an expression plasmid encoding Rv1733c, thus facilitating endogenous expression and presentation of Rv1733c in the context of HLA-A*0201. In contrast EBV-BLCL JY target cells which were transduced with an empty plasmid* ***(a)*** *were killed to a much lesser extent. Specific lysis of EBV-BLCL JY target cells loaded with Rv1733c$_{p181-189}$ could be inhibited partly by addition of anti-HLA-class-I but not anti-HLA-class-II antibody* ***(b)****. The % specific lysis was calculated as follows: [(release-spontaneous release)/ (maximum release-spontaneous release)] x 100%.*

All 25 candidate HLA-A2 epitopes were tested in a lymphocyte stimulation test in 11 HLA-A2$^+$ and 5 HLA-A2$^-$ individuals (PPD$^+$ blood bank donors (either from Table 2 or newly tested), TST converters or TB patients) to examine their recognition in the context of HLA-A2 molecules. Five HLA-A2 candidate peptides were recognized solely by HLA-A2$^+$ but not HLA-A2$^-$ donors as assessed by CFSE proliferation (Table 4b). A polyclonal CD8$^+$ T cell line was generated to explore possible functional properties of CD8$^+$ T cells directed to these *M. tuberculosis* DosR regulon encoded epitopes. PBMC from a TB patient (HLA-A2, -A3, and -DR11) were stimulated with a combination of the candidate HLA-A2 restricted epitopes Rv1733c$_{p181-189}$, Rv2031c$_{p96-104}$ and Rv2627c$_{p151-159}$. All three peptides were recognized by this donor (data not shown). Effector CD8$^+$ T cells showed specific peptide dependent lysis of EBV-BLCL JY target cells (HLA-A*0201) when pulsed with peptide Rv1733c$_{p181-189}$ (but not with Rv2627c$_{p151-159}$ or Rv2031c$_{p96-104}$). Killing was observed at different effector-to-target ratios (E: T) (Figure 3a). As expected, the use of allogeneic EVB-BLCL target cells in combination with polyclonal effector cells resulted invariably in some background activity, even in such short term assays (5-35 % lysis of control targets). Nevertheless, peptide dependent lysis was clearly evident. CD8$^+$ T cells also recognized and lysed EBV-BLCL JY target cells that had been transduced with a plasmid encoding Rv1733c, while not recognizing EBV-BLCL JY target cells similarly transduced with control plasmid (Figure 3a).
Specific peptide dependent lysis of EBV-BLCL JY target cells loaded with HLA-candidate epitopes Rv1733c$_{p181-189}$ could be inhibited partly (~30 %) by the addition of a pan anti-HLA-class I antibody whereas no decrease in lysis was observed following addition of pan anti-HLA-class II antibody (Figure 3b).
Collectively these results show that CD8$^+$ T cells can recognize *M. tuberculosis* DosR antigen encoded peptide epitopes presented by HLA- A*0201 restricted. Furthermore, peptide specific CD8$^+$ T cells were capable of lysing peptide loaded- and endogenously processed antigen loaded- target cells in the context of HLA-A*0201 molecules. These results further document the presence of functional *M. tuberculosis* DosR regulon encoded epitopes for human CD8$^+$ T cells in the context of HLA class I molecules.

Discussion

Here we describe for the first time CD4$^+$ as well as CD8$^+$ T cell responses to a series of *M. tuberculosis* DosR regulon encoded antigens. These responses were detectable in mycobacteria primed (PPD$^+$) individuals and were HLA class II and class I restricted, respectively. A number of selected peptide epitopes was fine mapped in the context of the prevalent HLA alleles HLA-A*0201 and HLA-DR3. Responses to the here identified *M. tuberculosis* DosR regulon encoded antigenic peptides include CD4$^+$ and CD8$^+$ T cell proliferation, production of IFNγ (data not shown) and in the case of CD8$^+$ T cells also cytolytic activity.
We have hypothesized that *M. tuberculosis* DosR regulon encoded antigens (18) which are expressed during conditions mimicking intracellular stress as encountered during infection, represent rational targets for TB vaccination since immune responses to *M.*

tuberculosis DosR regulon encoded antigens will be activated during latent infection. Indeed, immune responses to *M. tuberculosis* DosR regulon encoded antigens have been shown to be associated with control of latent TB infection (18,29). It is against this background that we set out to identify *M. tuberculosis* DosR regulon encoded antigen specific $CD4^+$ and $CD8^+$ T-cell responses and to identify relevant epitopes.
We selected four *M. tuberculosis* DosR antigens, Rv1733c, Rv2029c, Rv2031c (HspX, α-crystallin) and Rv2627c, previously found to be prominently recognized by human T cells, for detailed $CD4^+$ and $CD8^+$ T-cell response profiling and epitope identification (Table 1). The use of overlapping peptides to examine T cell responses has been applied before (28,32). Although HLA-class I presented peptides are typically 8-11 amino acids long, and HLA-class II ligands can be between 10-25 amino acids (12,26,27), we nevertheless find efficient $CD8^+$ T cell responses using 20-mers, next to using 9-mer peptides. We tested 20-mer peptides with 10 aa overlap to limit the total numbers of peptides needed to allow us to visualize both $CD4^+$ and $CD8^+$ T cell responses in human samples (11). Our 6-10 day incubation period may have allowed internalization and processing of peptides for HLA-class I presentation, or allowed cross-presentation via alternative antigen presentation pathways (3,37). We were able to validate this strategy first by identification of $CD4^+$ as well as $CD8^+$ T cell responses to 20-mer peptides that contained already known epitopes (notably on Ag85B and Rv2031c (4,6,9,15,16,21)) (Figure 1b). We conclude that the use of 20-mer peptides in combination with CFSE based proliferation in human PBMC samples is a powerful and efficient approach to (i) visualize and quantitate both $CD4^+$ and $CD8^+$ T cell responses to the same antigen; and (ii) to identify and fine-map peptide epitopes for both $CD4^+$ and $CD8^+$ T cells simultaneously against the same peptides.
Peptides in several instances appeared to be more able to detect $CD4^+$ or $CD8^+$ T cell responses compared to recombinant proteins; this at first sight counter-intuitive observation, however, is readily explained by the preprocessed nature op peptides, facilitating high efficiency antigen presentation, as well as by their much higher molarity, since proteins and peptides all were tested at 10 microgram/ml concentrations, regardless of molecular mass. The lack of competition with otherwise dominant epitopes for processing when using whole recombinant proteins may also have permitted the identification of subdominant epitopes using peptides, which might have escaped detection otherwise.

A striking observation was the wealth of epitopes that could be identified in *M. tuberculosis* DosR regulon encoded antigens, following confirmation of known epitopes for $CD4^+$ and $CD8^+$ T cells in Ag85B and Rv2031c/hspX. This underscores the significant immunogenicity of *M. tuberculosis* DosR regulon encoded antigens in a wide variety of HLA class I and class II backgrounds. Moreover, within several *M. tuberculosis* DosR regulon encoded antigens highly immunogenic regions could be identified (Table 3a-c). Of further interest was that a substantial number of peptides elicited both $CD4^+$ and $CD8^+$ T cell responses.
We further investigated responses against a subset of epitopes, notably those that were most likely recognized in the context of HLA-A2 and HLA-DR3 molecules. Peptide presentation via HLA-DR3 matched vs. mismatched antigen presenting cells showed that indeed the majority of the predicted epitopes (Table 4a) was presented

to $CD4^+$ T cells by HLA-DR3 molecules. In parallel, HLA-A2 restriction of peptide recognition was verified by using a $CD8^+$ T cell line specific for the Rv1733c epitope $Rv1733c_{p181-189}$. The T cell line's cytotoxic activity towards target cells that expressed HLA-A*0201 could be inhibited partly by anti-HLA-class I antibody. Interestingly, these $CD8^+$ T cells also were able to recognize endogenously processed antigen, since they killed target cells that were transduced with Rv1733c.
Although *in silico* prediction is a valuable tool in epitope identification, our results showed a not very strict correlation between the *in silico* epitope prediction scores, *in vitro* affinity or the magnitude of the observed T cell responses (proliferation, IFNγ production, target cell killing). Using the functional methods described here, we were able to identify a set of *M. tuberculosis* DosR regulon encoded epitopes in the context of HLA-A2 and -DR3, some of which were investigated in detail. We intend to further characterize immune responses to these epitopes (e.g. using multiplex functional assays and polychromatic flow cytometry). Eventually, the protective efficacy of these epitopes need to be assessed in relevant in vivo models, including e.g. HLA transgenic mice (7).
In conclusion, we have identified $CD4^+$ and $CD8^+$ T cell responses to several immunodominant *M. tuberculosis* DosR regulon encoded antigens and corresponding HLA-class I and class II restricted peptide epitopes. These responses could be detected by applying the cell tracker dye CFSE, allowing simultaneous detection of $CD4^+$ and $CD8^+$ T cell responses to the same antigens and peptides. These results significantly increase our understanding of the human immune response to *M. tuberculosis* specific antigens, and may help in designing *M. tuberculosis* DosR regulon encoded antigen and/or peptide based vaccination approaches for TB.

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Supplementary file 1.

Table Ia-e. Overlapping peptide sets of antigens selected for this study. Table Ia-d, overlapping peptide sets from DosR antigens Rv1733c, Rv2029c, Rv2031c and Rv2627c, respectively. All DosR peptides are 20 aa long with 10 aa overlap. All DosR derived peptides, with exception of peptides derived from DosR antigen Rv2031c were elongated with two lysine (K) residues at the C-terminal to improve solubility. Table Ie shows the overlapping peptide set of reference (non-DosR) antigen Ag85B. Ag85B peptides are also 20 aa long with 10 aa overlap with exception of Ag85B peptide numbers 20-22 which were 15 aa long with 10 aa overlap (see text in Table Ie).

Table I-a. Overlapping peptide set of DosR antigen Rv1733c

peptide	DosR antigen Rv1733c	position
number	aa sequence	start-end
1	MIATTRDREGATMITFRLRLKK	1-20
2	ATMITFRLRLPCRTILRVFSKK	11-30
3	PCRTILRVFSRNPLVRGTDRKK	21-40
4	RNPLVRGTDRLEAVVMLLAVKK	31-50
5	LEAVVMLLAVTVSLLTIPFAKK	41-60
6	TVSLLTIPFAAAAGTAVQDSKK	51-70
7	AAAGTAVQDSRSHVYAHQAQKK	61-80
8	RSHVYAHQAQTRHPATATVIKK	71-90
9	TRHPATATVIDHEGVIDSNTKK	81-100
10	DHEGVIDSNTTATSAPPRTKKK	91-110
11	TATSAPPRTKITVPARWVVNKK	101-120
12	ITVPARWVVNGIERSGEVNAKK	111-130
13	GIERSGEVNAKPGTKSGDRVKK	121-140
14	KPGTKSGDRVGIWVDSAGQLKK	131-150
15	GIWVDSAGQLVDEPAPPARAKK	141-160
16	VDEPAPPARAIADAALAALGKK	151-170
17	IADAALAALGLWLSVAAVAGKK	161-180
18	LWLSVAAVAGALLALTRAILKK	171-190
19	ALLALTRAILIRVRNASWQHKK	181-200
20	IRVRNASWQHDIDSLFCTQRKK	191-210

Table I-b. Overlapping peptide set of DosR antigen Rv2029c

peptide	DosR antigen Rv2029c	position
number	aa sequence	start-end
1	MTEPAAWDEGKPRIITLTMNKK	1-20
2	KPRIITLTMNPALDITTSVDKK	11-30
3	PALDITTSVDVVRPTEKMRCKK	21-40
4	VVRPTEKMRCGAPRYDPGGGKK	31-50
5	GAPRYDPGGGGINVARIVHVKK	41-60
6	GINVARIVHVLGGCSTALFPKK	51-70
7	LGGCSTALFPAGGSTGSLLMKK	61-80
8	AGGSTGSLLMALLGDAGVPFKK	71-90
9	ALLGDAGVPFRVIPIAASTRKK	81-100
10	RVIPIAASTRESFTVNESRTKK	91-110
11	ESFTVNESRTAKQYRFVLPGKK	101-120
12	AKQYRFVLPGPSLTVAEQEQKK	111-130
13	PSLTVAEQEQCLDELRGAAAKK	121-140
14	CLDELRGAAASAAFVVASGSKK	131-150
15	SAAFVVASGSLPPGVAADYYKK	141-160
16	LPPGVAADYYQRVADICRRSKK	151-170
17	QRVADICRRSSTPLILDTSGKK	161-180
18	STPLILDTSGGGLQHISSGVKK	171-190
19	GGLQHISSGVFLLKASVRELKK	181-200
20	FLLKASVRELRECVGSELLTKK	191-210
21	RECVGSELLTEPEQLAAAHEKK	201-220
22	EPEQLAAAHELIDRGRAEVVKK	211-230
23	LIDRGRAEVVVVSLGSQGALKK	221-240
24	VVSLGSQGALLATRHASHRFKK	231-250
25	LATRHASHRFSSIPMTAVSGKK	241-260
26	SSIPMTAVSGVGAGDAMVAAKK	251-270
27	VGAGDAMVAAITVGLSRGWSKK	261-280
28	ITVGLSRGWSLIKSVRLGNAKK	271-290
29	LIKSVRLGNAAGAAMLLTPGKK	281-300
30	AGAAMLLTPGTAACNRDDVEKK	291-310
31	TAACNRDDVERFFELAAEPTKK	301-320
32	RFFELAAEPTEVGQDQYVWHKK	311-330
33	TEVGQDQYVWHPIVNPEASPKK	318-339

Table I-c. Overlapping peptide set of DosR antigen Rv2031c

peptide	DosR antigen Rv2031c	position
number	aa sequence	start-end
1	MATTLPVQRHPRSLFPEFSE	1-20
2	PRSLFPEFSELFAAFPSFAG	11-30
3	LFAAFPSFAGLRPTFDTRLM	21-40
4	LRPTFDTRLMRLEDEMKEGR	31-50
5	RLEDEMKEGRYEVRAELPGV	41-60
6	YEVRAELPGVDPDKDVDIMV	51-70
7	DPDKDVDIMVRDGQLTIKAE	61-80
8	RDGQLTIKAERTEQKDFDGR	71-90
9	RTEQKDFDGRSEFAYGSFVR	81-100
10	SEFAYGSFVRTVSLPVGADE	91-110
11	TVSLPVGADEDDIKATYDKG	101-120
12	DDIKATYDKGILTVSVAVSE	111-130
13	ILTVSVAVSEGKPTEKHIQI	121-140
14	SVAVSEGKPTEKHIQIRSTN	125-144

Table I-d. Overlapping peptide set of DosR antigen Rv2627c

peptide	DosR antigen Rv2627c	position
number	aa sequence	start-end
1	MASSASDGTHERSAFRLSPPKK	1-20
2	ERSAFRLSPPVLSGAMGPFMKK	11-30
3	VLSGAMGPFMHTGLYVAQSWKK	21-40
4	HTGLYVAQSWRDYLGQQPDKKK	31-50
5	RDYLGQQPDKLPIARPTIALKK	41-60
6	LPIARPTIALAAQAFRDEIVKK	51-70
7	AAQAFRDEIVLLGLKARRPVKK	61-80
8	LLGLKARRPVSNHRVFERISKK	71-90
9	SNHRVFERISQEVAAGLEFYKK	81-100
10	QEVAAGLEFYGNRRWLEKPSKK	91-110
11	GNRRWLEKPSGFFAQPPPLTKK	101-120
12	GFFAQPPPLTEVAVRKVKDRKK	111-130
13	EVAVRKVKDRRRSFYRIFFDKK	121-140
14	RRSFYRIFFDSGFTPHPGEPKK	131-150
15	SGFTPHPGEPGSQRWLSYTAKK	141-160
16	GSQRWLSYTANNREYALLLRKK	151-170
17	NNREYALLLRHPEPRPWLVCKK	161-180
18	HPEPRPWLVCVHGTEMGRAPKK	171-190
19	VHGTEMGRAPLDLAVFRAWKKK	181-200
20	LDLAVFRAWKLHDELGLNIVKK	191-210
21	LHDELGLNIVMPVLPMHGPRKK	201-220
22	MPVLPMHGPRGQGLPKGAVFKK	211-230
23	GQGLPKGAVFPGEDVLDDVHKK	221-240
24	PGEDVLDDVHGTAQAVWDIRKK	231-250
25	GTAQAVWDIRRLLSWIRSQEKK	241-260
26	RLLSWIRSQEEESLIGLNGLKK	251-270
27	EESLIGLNGLSLGGYIASLVKK	261-280
28	SLGGYIASLVASLEEGLACAKK	271-290
29	ASLEEGLACAILGVPVADLIKK	281-300
30	ILGVPVADLIELLGRHCGLRKK	291-310
31	ELLGRHCGLRHKDPRRHTVKKK	301-320
32	HKDPRRHTVKMAEPIGRMISKK	311-330
33	MAEPIGRMISPLSLTPLVPMKK	321-340
34	PLSLTPLVPMPGRFIYAGIAKK	331-350
35	PGRFIYAGIADRLVHPREQVKK	341-360
36	DRLVHPREQVTRLWEHWGKPKK	351-370
37	TRLWEHWGKPEIVWYPGGHTKK	361-380
38	EIVWYPGGHTGFFQSRPVRRKK	371-390
39	GFFQSRPVRRFVQAALEQSGKK	381-400
40	FVQAALEQSGLLDAPRTQRDKK	391-410
41	AALEQSGLLDAPRTQRDRSAKK	394-413

Table I-e. Overlapping peptide set of early secreted antigen Ag85B (Rv1886c)

peptide	antigen Ag85B	position
number	aa sequence	start-end*
1	FSRPGLPVEYLQVPSPSMGR	41-60
2	LQVPSPSMGRDIKVQFQSGG	51-70
3	DIKVQFQSGGNNSPAVYLLD	61-80
4	NNSPAVYLLDGLRAQDDYNG	71-90
5	GLRAQDDYNGWDINTPAFEW	81-100
6	WDINTPAFEWYYQSGLSIVM	91-110
7	YYQSGLSIVMPVGGQSSFYS	101-120
8	PVGGQSSFYSDWYSPACGKA	111-130
9	DWYSPACGKAGCQTYKWETF	121-140
10	GCQTYKWETFLTSELPQWLS	131-150
11	LTSELPQWLSANRAVKPTGS	141-160
12	ANRAVKPTGSAAIGLSMAGS	151-170
13	AAIGLSMAGSSAMILAAYHP	161-180
14	SAMILAAYHPQQFIYAGSLS	171-190
15	QQFIYAGSLSALLDPSQGMG	181-200
16	ALLDPSQGMGPSLIGLAMGD	191-210
17	PSLIGLAMGDAGGYKAADMW	201-220
18	AGGYKAADMWGPSSDPAWER	211-230
19	GPSSDPAWERNDPTQQIPKL	221-240
20	NDPTQQIPKLVANNT #	231-245
21	QIPKLVANNTRLWVY #	236-250
22	VANNTRLWVYCGNGT #	241-255
23	VANNTRLWVYCGNGTPNELG	241-260
24	CGNGTPNELGGANIPAEFLE	251-270
25	GANIPAEFLENFVRSSNLKF	261-280
26	NFVRSSNLKFQDAYNAAGGH	271-290
27	QDAYNAAGGHNAVFNFPPNG	281-300
28	NAVFNFPPNGTHSWEYWGAQ	291-310
29	THSWEYWGAQLNAMKGDLQS	301-320
30	YWGAQLNAMKGDLQSSLGAG	306-325

15-mers, 10 aa overlap

* sequence Ag85B consists of 325 aa. First 40 aa is leader sequence; not tested in study. Here, start of aa at position 41

Chapter 4

Lack of immune responses to *Mycobacterium tuberculosis* DosR regulon proteins following *Mycobacterium bovis* BCG vaccination.

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Abstract

BCG is widely used as a vaccine against tuberculosis (TB) despite its variable protective efficacy. Relatively little is known about the immune response profiles following BCG vaccination in relation to protection against TB. Here we tested whether BCG vaccination results in immune responses to DosR (Rv3133c) regulon encoded proteins. These so-called TB latency antigens are targeted by the immune system during persistent *Mycobacterium tuberculosis* infection and have been associated with immunity against latent *M. tuberculosis* infection.

In silico analysis of the DosR regulon in BCG and *M. tuberculosis* showed at least 97% homology in amino acid sequence, with 41 out of 48 genes being identical. Transcriptional profiling of 14 different BCG strains, under hypoxia and nitric oxide exposure *in vitro,* revealed a functional DosR regulon similar to that observed in *M. tuberculosis*.

Next, we assessed human immune responses to a series of immuno-dominant TB latency antigens and found that BCG vaccination fails to induce significant responses to latency antigens. Similar results were found in BCG vaccinated BALB/c mice. In contrast, responses to latency antigens were observed in individuals with suspected exposure to TB (as indicated by positive IFNγ responses to TB specific antigens ESAT-6 and CFP-10), and in mice vaccinated with plasmid DNA encoding selected latency antigens.

Since immune responses to TB latency antigens have been associated with control of latent *M. tuberculosis* infection, our findings support the development of vaccination strategies incorporating DosR regulon antigens to complement and improve the current BCG vaccine.

Introduction

Tuberculosis (TB) remains a major global health threat. Each year about eight million new TB cases occur and two million people die from TB. It is estimated that one third of the world population is latently infected with *Mycobacterium tuberculosis*. From this vast latent reservoir about 10% of infected people are expected to develop overt TB disease during their lifetime. However, with the expanding HIV-1/AIDS pandemic this number is expected to soar in the next few decades (11,42).

The current TB vaccine is the live attenuated bacterium *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG). BCG is known to protect against severe forms of TB in young children and against leprosy. However, it does not efficiently and consistently protect against pulmonary TB in adults, the most prevalent and contagious form of TB; neither does BCG offer protection from reactivation of latent TB infection. This partly explains why BCG has little impact on the global TB epidemic despite its widespread use as a prophylactic TB vaccine (43). Over the years many hypotheses have been put forward to explain the apparent variability in protective efficacy of BCG, which varies from 0 to 80% (16). Explanations for this inconsistency include differences in trial methodology, population host genetics, use of different BCG vaccine strains (2) and heterogeneous immunity to a variety of environmental mycobacteria that may interfere with, or mask the protection provided by BCG (7,26).

Immune response profiles following BCG vaccination comprise myriad effector mechanisms, multiple T-cell subsets and many targeted antigens. BCG is capable of inducing Th1 responses (38), which are critical in mycobacterial infections (17). In addition, BCG is also capable of inducing both $CD4^+$ and $CD8^+$ T cell responses to antigens shared with *M. tuberculosis*, such as secreted antigens of the mycolyl transferase family (Ag85) (19,20,33) and non-deleted members of the ESAT-6 family (*e.g.* TB10.4) (32), but also heat shock proteins like Hsp65 and Hsp70 (15). However, it is still not completely known how these and other antigen-specific immune responses contribute to protection against TB.

Recently we studied human T-cell responses to DosR (Rv3133c) regulon encoded antigens (referred to as TB latency antigens) of *M. tuberculosis* (24). We observed preferential recognition of latency antigens by Mantoux skin test positive individuals with latent TB compared to patients with TB disease, suggesting that these immune responses are associated with latent TB disease (14,24). The DosR regulon is expressed by tubercle bacilli under *in vitro* conditions of hypoxia and low dose nitric oxide exposure (40), and in IFNγ-activated macrophages (30). These conditions are thought to mimic the environment encountered by tubercle bacilli *in vivo* when persisting in immuno-competent hosts (31).

One of the most abundantly produced proteins during hypoxia is the 16-kDa α-crystallin homolog HspX (Rv2031c) (12). HspX is part of the DosR regulated genes and is targeted by both $CD4^+$ T cells (9,41) and $CD8^+$ T cells (10). Interestingly, infants vaccinated with BCG do not mount immune responses to HspX, whereas they are capable of generating immune responses to other TB antigens (39). The absence of immune responses to this particular latency antigen led us to the formulation of our hypothesis. In this study we tested the hypothesis that BCG vaccination in humans and

in a mouse model fails to induce immune responses to not only HspX but generally to TB latency antigens that are targeted by the immune system during latent *M. tuberculosis* infection.

Materials and Methods

In vitro expression of the BCG DosR regulon

Expression of the DosR regulon by *M. tuberculosis* strain H37Rv and 14 different BCG strains (Connaught (JPG), Pasteur 140, Sweden, Connaught, Japan, Canada, Vietnam, Danish SSI 1331, Russian, Brazil, Tice, Moscow, Pasteur 133A and Pasteur 1173, (kindly provided by Dr. Angelo Izzo, Colorado State University, USA) was determined using standard methods of microarray RNA expression analysis as previously described (40). Briefly, mycobacteria were grown in 7H9 medium (supplemented with bovine serum albumin, NaCl, glucose, and glycerol) in 250 ml vented tissue culture flasks and shaking 90 rpm. At a culture OD 0.15, 30 ml cultures were placed in an anaerobic GasPak chamber (Becton, Dickinson and Company) and continued to shake. Reference cultures were treated in the same manner but not placed in the anaerobic chamber. After four hours RNA was isolated and microarray analysis was conducted as previously reported (40) with TB oligonucleotide microarrays provided by Colorado State University through the Tuberculosis Vaccine Testing and Research Materials contract.

Study subjects

Cross sectional study (The Netherlands) comprising 43 healthy adult persons (10 male, 33 female) who were vaccinated with BCG between 1 and 37 years before this study (median time after vaccination 25 years). The age at the time of blood sampling ranged from 20-60 years. Thirty-eight individuals were of Dutch origin; the remaining 5 individuals were of German, British, Netherlands Antillean, Philippine and South Korean descent. Blood samples were obtained from all study subjects after written informed consent was obtained, using standard venous puncture and blood collection in heparinized tubes. Subsequently, peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll density gradient and stored in liquid nitrogen as described previously (24). The study protocol (P207/99) was approved by the ethical review board of the Leiden University Medical Centre.

Longitudinal BCG vaccination study (United Kingdom) comprising adolescents who were recruited through the UK schools' BCG vaccination Programme (4). Year 8 pupils (age 12-13 years) were informed verbally and by an information sheet of the study and recruitment proceeded following written informed consent from parent or guardian and verbal consent from the child. Exclusion criteria were evidence of previous BCG vaccination (BCG scar or vaccination records) or serious illness. Ethnical backgrounds of participants were as follows: White British (30%), White Irish (3%), Black Caribbean (22%), Black African (13%), Black other (7%), Asian Pakistan (12%), Asian other (5%) and mixed/other (8%).

Ethical approval for this study was given by the Local Research Ethics Committee of Redbridge and Waltham Forest Health Authority (R&WFHA) and by the Ethics Committee of the London School of Hygiene & Tropical Medicine.
Baseline blood samples were collected from participants at the time of tuberculin skin testing. Skin testing was carried out on the volar surface of the forearm using the Heaf technique with tuberculin PPD. A baseline of 10 ml of intravenous blood was taken and transferred into tubes containing 100 U of preservative-free sodium heparin (Monoparin, CP Pharmaceuticals Ltd. Wrexham, UK). Blood samples were set up in the whole blood assay as soon as possible on the same day as venapuncture. The Heaf test induration was inspected after 7 days and graded appropriately by experienced nurses. Those due to receive BCG following a negative Heaf test received BCG immediately (n=22). Two to four weeks following vaccination, depending upon school availability, recruits were revisited for a follow-up, 10 ml blood sample.

M. tuberculosis antigens

Recombinant proteins were produced as previously described (18). Briefly, nucleotide sequences of selected *M. tuberculosis* H37Rv genes were obtained from http://genolist.pasteur.fr/TubercuList. Genes were amplified by PCR from genomic DNA of *M. tuberculosis* H37Rv and cloned by Gateway Technology (Invitrogen, San Diego, CA) in pDEST™17, a bacterial expression vector containing an N-terminal hexa-histidine tag for rapid purification with nickel-chelating resin. The proteins were over-expressed in *Escherichia coli* BL21(DE3) and purified as previously described (18). Sequencing was performed to confirm the identity of the cloned DNA fragments. Size and purity were checked by gel electrophoresis and Western blotting with anti-His antibodies (Invitrogen). Residual endotoxin levels were determined with a Limulus Amebocyte Lysate assay (Cambrex) and were found to be below 50 IU/mg recombinant protein. Protein batches were subsequently tested for non-specific T cell stimulation and for potential cellular toxicity in lymphocyte stimulation assays using PBMC of *M. tuberculosis* unexposed, BCG unvaccinated, Mantoux skin test negative healthy donors.
Mycobacterium tuberculosis lysate, grown under low oxygen conditions (MTB), was obtained from growing *M. tuberculosis* H37Rv for 24 hours in tubes with tightly screwed caps as previously described (29). The low oxygen derived *M. tuberculosis* lysate was kindly provided by Dr Karen Weldingh and Dr Peter Andersen (Statens Serum Institute (SSI), Copenhagen, Denmark).

In vitro proliferation assays

Lymphocyte stimulation assays (cross-sectional BCG vaccination study) were performed using isolated PBMC as previously described (24). Briefly, PBMC (1.5×10^5/well) were cultured in Iscove's modified DMEM (Gibco, Paisley, UK) supplemented with 10% pooled human serum and 40 U/ml penicillin and 40 μg/ml streptomycin, in 96-well round-bottom microtiter plates (Nunc, Roskilde, Denmark) at 37°C, 5% CO_2, in the absence or presence of stimulant. Antigens were tested in the following concentrations: latency antigens, ESAT-6, CFP-10 and Ag85B all at 0.33 μM, *M. tuberculosis* hypoxic lysate and purified protein derivative (PPD) of *M. tuberculosis* (batch RT49 SSI, Denmark) at 5 μg/ml and positive control phytohaemagglutinin

(PHA) (Remel, UK) at 2 μg/ml. Total volume was 200 μl/well. All stimulations were performed in triplicate. At day 6, supernatants were harvested (75 μl/well, pooled per triplicate) and stored at maximally –20°C until use in IFNγ detection assay.
Diluted whole blood assay (longitudinal BCG vaccination study), was used as previously described (3). In summary, whole blood was diluted 1 in 5 with serum-free medium (RPMI 1640 supplemented with 2 mM L-Glutamine, Invitrogen) and 100 μl was plated in 96-well, round-bottomed tissue culture plates (Nunc, Roskilde, Denmark). Antigen was also added in 100 μl to give a final whole blood dilution of 1:10 and culture volume of 200 μl. Medium and PHA (5 μg/ml; Remel, UK) containing wells were included as negative and positive controls respectively, in parallel with *M. tuberculosis* antigens at 5 μg/ml: purified protein derivative (PPD) of *M. tuberculosis* (batch RT49, lot 210; SSI, Denmark), Rv1733c, Rv2029c, Rv2623, Rv2627c, Rv2628, Ag85A and ESAT6/CFP10 fusion protein. Cell cultures were incubated on the day of blood collection at 37˚C with 5% CO_2. Supernatants were harvested on day 6 and stored at maximally -20˚C prior to ELISA.

Detection of IFNγ by ELISA
Cross-sectional study: IFNγ concentrations in the supernatants were measured by ELISA (U-CyTech, Utrecht, The Netherlands). The detection limit of the assay was 20 pg/ml. ELISA samples were tested in duplicate. The mean value of unstimulated cultures was subtracted from the mean value of the stimulated cultures. A positive IFNγ response was predefined as ≥100pg/m (24) in the cross-sectional study (The Netherlands). Positive *in vitro* responses of the study subjects to the TB specific antigens ESAT-6 and/or CFP-10 were used as a marker for previous TB exposure (21,37).
Longitudinal study: quantitative IFNγ ELISAs were done in single wells (100 μl) using commercially available antibody pairs (BD Pharmingen) as previously described (3). Recombinant IFNγ BD Pharmingen) was used for the standard curve with lowest detection limit of 31 pg/ml. Negative control (medium) values were subtracted from all results. A positive IFNγ response was predefined as >62 pg/ml (3) in this longitudinal study (United Kingdom). A positive control supernatant was included in duplicate on each ELISA plate to control for interplate and intraplate variation.

In vivo studies
BALB/c (H-2^d) mice were bred at the Animal Facilities of the WIV-Pasteur Institute of Brussels, from breeding couples originally obtained from Bantin & Kingman (United Kingdom). All animals were 8-10 weeks old at the start of the experiments. Experiments were performed in agreement with the Ethical Committee of CODA-PIB-WIV regulations (permit no. 060202-02).
Male BALB/c mice were vaccinated with 0.2 mg (10^6 CFU) of freshly prepared solution of *M. bovis* BCG vaccine (strain GL2) grown as a surface pellicle on synthetic Sauton medium for 14 days and homogenized by ball mill (22). Mice were immunized subcutaneously with 0.2 ml. Female BALB/c mice were anesthetized with ketamine/xylazine and injected intramuscularly in both quadriceps muscles with 2 times 50 μg of V1J.ns-tPA vector encoding one of the latency antigens Rv1733c, Rv1738, Rv2029c, HspX (Rv2031c), Rv2032, Rv2626c, Rv2627c, Rv2628 or ESAT-

6/CFP10 fusion protein (21) or Ag85A (13,28). Mice were vaccinated three times at three-week intervals and immune responses were analyzed four weeks after the third immunization.
BCG vaccinated mice were sacrificed at 1 or 3 months after vaccination and spleens were removed aseptically and homogenized using a loosely fitting Dounce homogenizer. Plasmid DNA vaccinated mice were sacrificed four weeks after the third DNA immunization. Splenocytes (4×10^6 cells/ml) from three mice per group were tested as a pool (month 1 after BCG) or individually (3-4 mice, month 3 after BCG or after last DNA vaccination) for cytokine response to purified recombinant his-tagged latency antigens, Ag85A or ESAT-6-CFP-10 (all 5 μg/ml). Pooled supernatants from at least three wells were harvested after 72h when peak IFNγ values can be measured and stored at -20°C until assayed. IFNγ activity was quantified by sandwich ELISA using coating antibody R4-6A2 and biotinylated detection antibody XMG1.2 (both Pharmingen). Assay sensitivity was 5 pg/ml. Samples were tested undiluted, and at 1:10 or 1:100 dilution. Cytokine content was calculated for the dilution with OD value in the linear part of the standard curve.

Statistical analysis
For comparison of proportion of responders between test groups in the cross-sectional study the Chi Square Test (χ^2 Test) for independent samples was used. However, Fisher's exact test for independent samples was applied if expected values were lower or equal to 5 ($E \leq 5$). Comparison of proportion of responders in the longitudinal study was performed by McNemar's test for paired samples. For all assays $P < 0.05$ was considered statistically significant. For statistical analysis SPSS 11.0 for Windows was used.

Results

In silico sequence analysis and in vitro expression of the DosR regulon of BCG.
The DosR regulon of *M. tuberculosis* is expressed *in vitro* under conditions of hypoxia and low-dose nitric oxide stimulation (40). To evaluate whether BCG is similarly capable of expressing the DosR regulon, we first assessed available genome sequences of *M. tuberculosis*, *M. bovis* and BCG for the level of conservation and then performed *in vitro* transcription profiling of DosR regulon encoded genes.
BLAST searches (http://ncbi.nlm.nih.gov/BLAST/) of DosR encoded sequences showed that all 48 DosR regulon encoded genes were highly conserved between *M. tuberculosis* H37Rv and CDC1551 strains, *M. bovis* AF2122/97 and BCG Pasteur 1173P2. Homology of amino acid sequences was at least 97%, with 85% (41 of the 48) genes being identical (*data not shown*).
Subsequently, we assessed RNA expression profiles of 14 different BCG vaccine strains and *M. tuberculosis* H37Rv under *in vitro* conditions of hypoxia and nitric oxide stimulation. Results showed that all tested BCG strains have a functional DosR regulon with similar expression profiles as compared to *M. tuberculosis* (40). Figure 1 shows the RNA profiles of nine DosR regulated genes of *M. tuberculosis* H37Rv and BCG

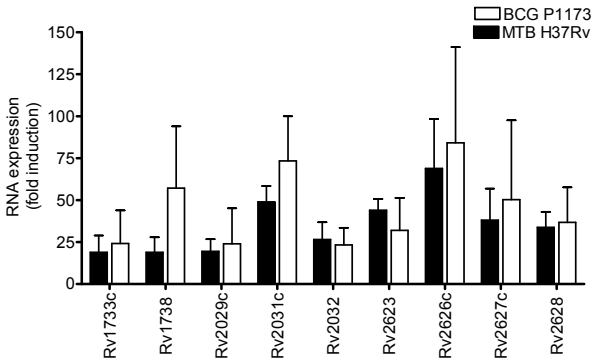

Figure 1. Expression of DosR genes (RNA). *RNA transcript expression profiles (fold induction) of nine DosR encoded genes induced during low oxygen conditions in Mycobacterium tuberculosis H37Rv (n=4) and BCG Pasteur 1173 (n=4). The RNA expression profiles of both strains are highly similar, except for Rv1738. Data shown as mean + SD*

Pasteur strain 1173 when exposed to low oxygen levels. Selection of these nine genes was based on results from our previous study (24) and contains the four most recognized latency antigens in humans. These genes were further analyzed in this study.

Immune responses to DosR regulon encoded antigens in human BCG vaccinees.
Next we addressed the question whether BCG vaccination in human beings leads to the induction of immune responses to DosR regulon encoded latency antigens that are targeted during natural *M. tuberculosis* infection.
IFNγ responses to immuno-dominant latency antigens were assessed in a longitudinal BCG vaccination study in young adolescents in the United Kingdom. Twenty-two tuberculin skin test (TST) negative adolescents were included in the vaccination group; blood was taken before pre- and post (2-4 weeks) BCG vaccination. Results showed that responses to the latency antigens of the participants before BCG vaccination were minimal: median responses to tested latency antigens Rv1733c, Rv2029c, Rv2623, Rv2627c and Rv2628 were below the predefined cutoff level of 62 pg/ml IFNγ as used in this study (Figure 2A). BCG vaccination did not alter the response profiles of the adolescents to the latency antigens: proportion of responders in the pre-vaccination group ranged from 4.5% to 33.3% compared to 0% to 31.8 % post-BCG vaccination. Statistical analysis of the proportion of responders to TB latency antigens showed no significant difference between pre- and post BCG vaccination (McNemar's test, $P = 0.500$ to $P = 1.000$).
Following BCG vaccination there was a significant increase in the IFNγ response to PPD from 46% to 96% responders ($P = 0.001$). There was no significant difference for responses to Ag85A, only a trend towards an increase in response from 64% to 77% responders ($P = 0.375$). Although all eligible subjects were tested negative in the tuberculin skin test (Heaf test) prior to BCG vaccination, *in vitro* stimulation with PPD or Ag85A showed that a considerable proportion of the tested adolescents gave positive whole blood assays (46% and 64% respectively), which is most likely

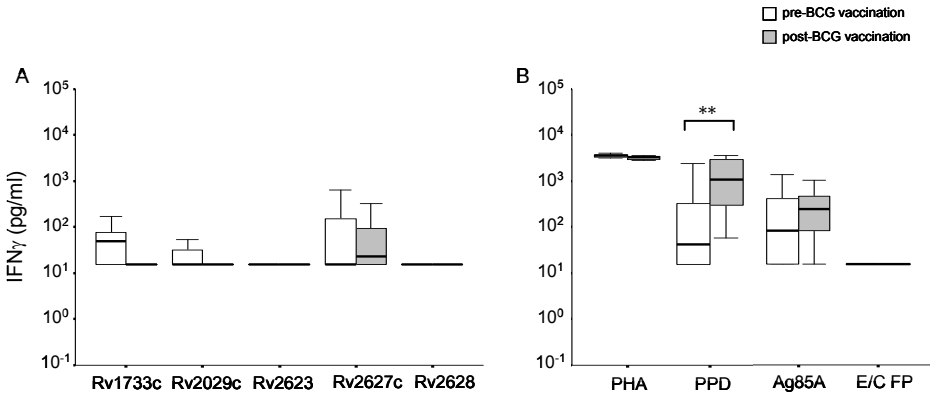

Figure 2. IFNγ responses to M. tuberculosis latency antigens in a longitudinal BCG study. *Box and Whisker plot of IFNγ responses to the latency antigens in schoolchildren pre- and post-BCG vaccination. Horizontal black line in the box represents the median; lower boundary of the box represents the 25th percentile and upper boundary the 75th percentile. Whiskers extend from the box to the highest and lowest value with exclusion of the extreme values and the outlier values. Supernatants were taken after 6 days for measurement of IFNγ production. (A) IFNγ responses of the pre-BCG vaccinated group to tested latency antigens. (B) Responses to PHA and control antigens PPD, Ag85A and ESAT-6/CFP-10 fusion protein. E/C FP, ESAT-6/CFP-10 fusion protein. Vaccinated adolescents, n=22, except for antigen Rv1733c in the pre-vaccinated group where 12 individuals were tested. An IFNγ response ≥ 62.5 pg/ml was considered positive. The McNemar's test for paired samples was used to compare the proportions of responders per group, ** P=0.001.*

as a result of exposure to cross-reactive environmental mycobacteria. As expected, none of the participants responded to the *M. tuberculosis* specific ESAT-6/CFP-10 fusion protein (Figure 2B). Thus, these results show that in adolescents following BCG vaccination virtually no increases in responses to *M. tuberculosis* latency antigens are observed.

Next, we carried out a cross-sectional study (The Netherlands) including BCG vaccinated adults without exposure to TB (n=27) compared to subjects with likely exposure to TB (n=16). For the purpose of this study, individuals with an IFNγ response >100 pg/ml to *M. tuberculosis* specific antigens ESAT-6 and CFP-10 (21,37) were considered previously exposed to TB.

Figure 3 shows the immune recognition profiles of both BCG vaccinated groups to the latency antigens. Overall, BCG vaccinated individuals without evidence of exposure to TB showed significantly lower IFNγ production to the latency antigens compared to the group that had positive responses to ESAT-6 and/or CFP-10. The median IFNγ responses to all tested latency antigens in the unexposed group were all below the cut-off level of 100 pg/ml, whereas this was the opposite in the TB exposed group. In the latter group median IFNγ values to all tested latency antigens, except for protein Rv2628, were above 100 pg/ml; indicating that over half of the individuals strongly recognized the antigens (Figure 3A).

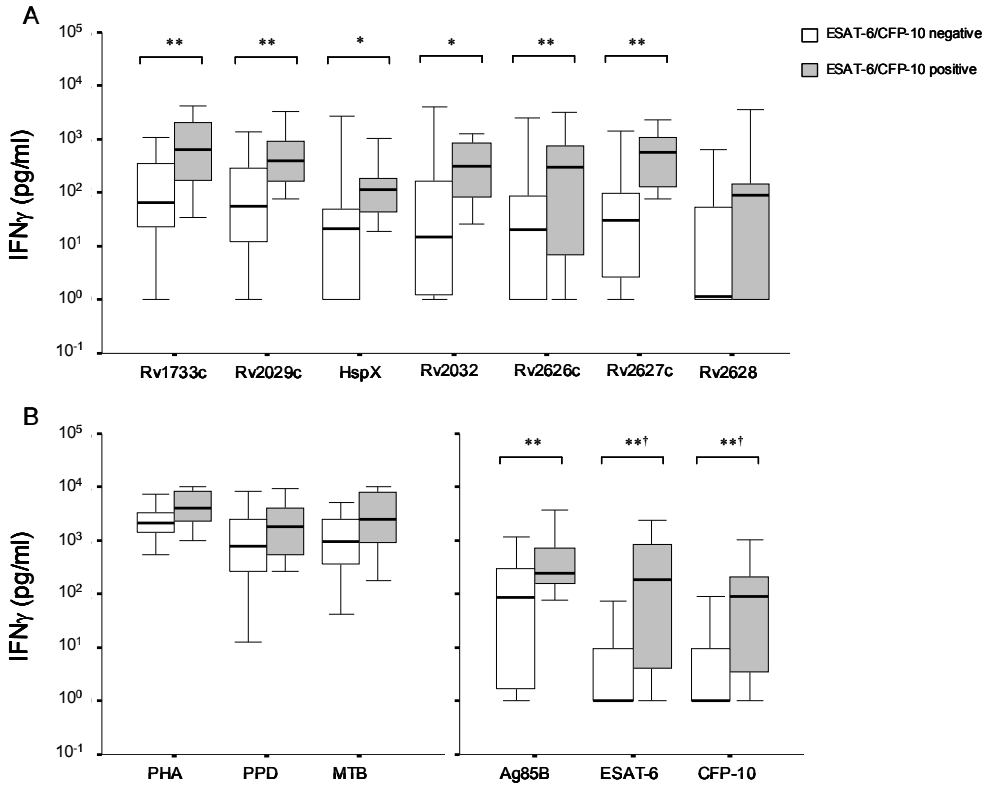

Figure 3. IFNγ responses to M. tuberculosis latency antigens in a cross-sectional BCG study. *Box and Whisker plot of IFNγ responses to the latency antigens by BCG vaccinated individuals. Horizontal black line in the box represents the median; lower boundary of the box represents the 25th percentile and upper boundary the 75th percentile. Whiskers extend from the box to the highest and lowest value with exclusion of the extreme values and the outlier values. Supernatants were taken after 6 days for measurement of IFNγ production (A) IFNγ responses to the latency antigens of the group that is BCG vaccinated without TB exposure (no IFNγ production to ESAT-6 or CFP-10) and with exposure to TB (positive IFNγ production to ESAT-6 or CFP-10). (B) IFNγ responses to control stimuli PHA, PPD, MTB, Ag85B, ESAT-6 and CFP-10.*
MTB, lysate of M. tuberculosis cultured under low oxygen conditions. BCG vaccinated group without TB exposure, n=23, BCG vaccinated group with TB exposure, n=16. An IFNγ response ≥100 pg/ml was considered positive. χ^2-test, P<0.05, ** P<0.01, † Fishers exact test.*

The proportion of responders (i.e. >100 pg/ml IFNγ) per antigen was as follows (BCG vaccinated without TB exposure vs. BCG vaccinated with TB exposure): Rv1733c 44.4 % vs. 93.8 % (P = 0.001), Rv2029c 37.0 % vs. 81.3 % (P = 0.005), HspX 22.2 % vs. 56.3 % (P = 0.024), Rv2032 29.6 % vs. 68.8 % (P = 0.013), Rv2626c 20.0 % vs. 68.8 % (P = 0.003), Rv2627c 25.9 % vs.75 % (P = 0.002) and Rv2628 25.9 % vs. 43.8 % (P = 0.228). Furthermore, the levels of IFNγ production in the BCG vaccinated group with suspected TB exposure were similar to those we previously found in Mantoux positive individuals (24). In addition to the responses to latency antigens, exposure to TB was also associated with higher IFNγ production to Ag85B (P = 0.005) which is shared between BCG and *M. tuberculosis.* The data also suggest that the responses to mycobacterial complex antigens like PPD (P = 0.139) and *M. tuberculosis* lysate (from

bacteria cultured under hypoxic conditions) (P = 0.279) are higher but no significant difference was detected (Figure 3B).
Time after BCG vaccination and moment of sampling varied considerably in our study population. We therefore attempted to seek a correlation between the level of IFNγ production and time since vaccination. However, no time dependent association was observed (data not shown).
Taken together, BCG vaccination in the skin in general appears to induce poor responses to not only HspX but also to other latency antigens of *M. tuberculosis*. In contrast, following exposure to TB, significant responses to latency antigens are seen.

Immune responses to DosR regulon antigens in mice.
In order to determine whether BCG vaccination also fails to induce adequate T-cell responses to *M. tuberculosis* latency antigens in mice, BALB/c mice were vaccinated subcutaneously with BCG (n=3). The mice were sacrificed 4 weeks and 3 months after BCG vaccination in order to test splenocytes for IFNγ production in response to latency antigens, the homologous or shared mycobacterial antigen Ag85A and the fusion protein of ESAT-6 and CFP-10 of *M. tuberculosis* (21).

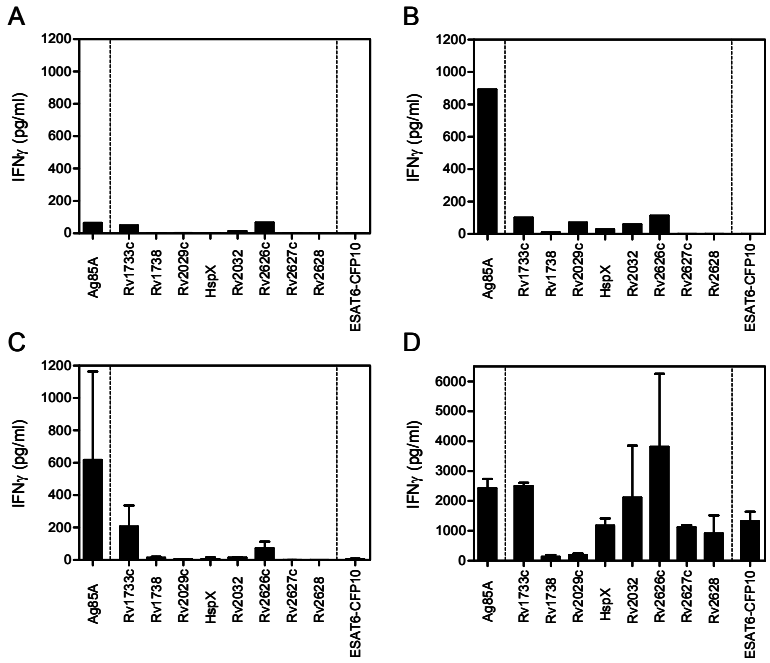

Figure 4. IFNγ responses to M. tuberculosis latency antigens in BALB/c mice following BCG vaccination (s.c.) or vaccination (i.m.) with plasmid DNA*. Supernatants were taken after 3 days (72 hours) for measurement of IFNγ production. (A) Naïve, non-BCG vaccinated, tested as a pool, n=3. (B) Animals 4-weeks post BCG vaccination, mice tested as a pool, n=3. (C) Animals 3-months post BCG vaccination, n=3. (D) Mice were immunized 3x with plasmid DNA encoding for one the eight latency antigens, Ag85A or ESAT-6/CFP-10. Splenocytes were in vitro restimulated with their respective antigens, n=4.*

Four weeks post-BCG vaccination, splenocytes showed very low to no production of IFNγ when stimulated with latency antigens (Figure 4B). The levels of IFNγ were similarly low as those of naïve control mice (Figure 4A). At this same time point cells produced significant amounts of IFNγ to Ag85A (Figure 4B) and PPD (data not shown). As expected, responses to the fusion protein ESAT6-CFP-10 were absent. When responses were assessed at 3 months post-immunization, similar results were obtained: IFNγ responses of splenocytes of vaccinated mice to latency antigens remained at levels comparable to those in naïve mice (Figure 4A), and only to Ag85A significant IFNγ production was found (Figure 4C). To rule out that absence of IFNγ responses to latency antigens was due to an inherent inability of BALB/c mice to respond to these latency antigens, mice were vaccinated with DNA. BALB/c mice (n=4) were vaccinated three times with three weeks intervals with naked DNA plasmid encoding one of eight different latency antigens, Ag85A or ESAT-6/CFP-10. Four weeks after the last immunization spleen cell IFNγ production was analyzed. Vaccination with six out of eight DNA plasmids induced significant antigen specific IFNγ production (Figure 4D). Robust levels of IFNγ were produced after *in vitro* re-stimulation with Rv1733c, HspX, Rv2032c, Rv2626c, Rv2627c and Rv2628. Antigens Rv1738 and Rv2029c appeared to be not very immunogenic for T cells in BALB/c mice, albeit significant antibody responses to the latter protein could be induced (29a). Vaccination with DNA plasmids encoding Ag85A or ESAT6-CFP-10 fusion protein was also very effective in inducing strong IFNγ responses. The results thus demonstrate that mice are able to generate strong Th1 type immune responses to latency antigens after immunization with DNA plasmid but that subcutaneous BCG administration fails to induce immune responses to *M. tuberculosis* latency antigens in BALB/c mice.

Discussion

Relatively little is known about the antigen specificity of immune response profiles following BCG vaccination in relation to protection against TB. In this study we addressed the question whether BCG vaccination induces immune responses to DosR regulon encoded antigens, in particular to those TB latency antigens that are targeted during persistent *M. tuberculosis* infection (24).

Comparison of available genome sequences of *M. tuberculosis, M. bovis* and BCG showed that the entire set of DosR regulon coding sequences is conserved in BCG; except for a few minor point mutations, which are not expected to have a major impact on expression or immunologic recognition of these antigens. Subsequently, transcriptional analysis of 14 different BCG vaccine strains under *in vitro* conditions of low oxygen and nitric oxide exposure were studied. Our results show that RNA expression profiles of the DosR regulon latency antigens reported in this study, were highly similar to those previously reported for *M. tuberculosis* (40). In addition, BCG is reported to be capable of adapting to anaerobiosis *in vitro* by shifting down to a non-replicating persistent state similar to *M. tuberculosis* (25). When tested in the so-called Wayne model or in standing (*i.e.* hypoxic) cultures it was previously shown that BCG is

capable of producing at least four DosR regulon encoded proteins: Rv2623, Rv2626c, HspX (Rv2031c, *acr*) and DosR (Rv3133c) (5,6). We conclude from these *in vitro* observations that BCG has a functional DosR regulon, although no complete proteomics data is available to demonstrate that every single protein encoded by the regulon is indeed expressed.

Next we monitored immune responses to a series of immuno-dominant TB latency antigens following BCG vaccination, both in a longitudinal and a cross-sectional study in two different human cohorts, and corroborated our findings in a BALB/c mouse model.

As mentioned before, neonates vaccinated with BCG at birth do not develop immune responses to the latency antigen HspX, whereas they are capable of mounting responses to other non-dormancy associated TB antigens (including PPD) (39). BCG vaccinated adolescents (longitudinal study) and BCG vaccinated adults who have not been exposed to TB (*i.e. in vitro* negative for production of IFNγ to ESAT-6 and/or CFP-10) (cross-sectional study), mounted immune responses to secreted antigens of the Ag85 complex and PPD, but did not develop responses to HspX and other tested latency antigens (Figure 2, 3).

In contrast to the above, BCG vaccinated adults with previous exposure to TB (*i.e. in vitro* positive production of IFNγ to ESAT-6 and/or CFP-10) in the cross-sectional study showed significantly higher IFNγ responses to selected TB latency antigens next to increased and significant (only Ag85B) responses to common TB antigens. These results agree with our recent findings that T cells from individuals with latent TB infection preferentially recognize a set of TB latency antigens (*i.e.* Rv1733c, Rv2029c and Rv2627c) while patients with (past or active) TB disease preferentially recognized secreted antigens such as CFP-10 and ESAT-6 (14,24).

The absence of *in vitro* detectable responses to *M. tuberculosis* latency antigens in BCG vaccinated adults may in part be explained by the common belief that BCG-induced protection to TB wanes over a period of 10-15 years (34). Since time after BCG vaccination in our study group varied between 1 and 37 years we attempted to correlate the level of responses to latency antigens to time elapsed since vaccination, but found no significant associations.

Somewhat unexpectedly, 46% of the adolescents who tested negative in the Heaf skin test prior to BCG vaccination, showed *in vitro* responses to PPD. Our own observations (unpublished observations) and those of others (4) show that *in vitro* detectable immune responses to PPD can be observed in a significant group of Mantoux negative, non-BCG vaccinated and TB unexposed, healthy individuals. This phenomenon is likely caused by previous exposure to cross-reactive environmental mycobacteria (4, 7, 16, 26). In addition, previous exposure to environmental mycobacteria might also have induced low level of responses (4.5%-33% responders) in the pre-vaccinated BCG group in the longitudinal study (Figure 2) since DosR encoded antigens have not been reported to be solely expressed in *M. tuberculosis.*

Our findings in humans were corroborated in BCG vaccinated BALB/c mice. Upon subcutaneous vaccination with BCG, the mice generated immune responses to the common *M. tuberculosis* antigens Ag85A and PPD. In contrast, poor responses were detectable against *M. tuberculosis* latency antigens both one and three months post-vaccination. Yet, vaccination with recombinant DNA plasmids induced a strong T cell

response to several latency antigens including Rv1733c, HspX, Rv2032, Rv2626c, Rv2627c and Rv2628, as well as Ag85A and ESAT6-CFP-10 fusion protein. DNA plasmids encoding Rv1738 and Rv2029c were not immunogenic in mice. This demonstrated that the mice were able to generate strong Th1 type immune responses to *M. tuberculosis* latency antigens when immunized with DNA plasmids, but that subcutaneous BCG administration failed to induce immune responses to such antigens in BALB/c mice. Using HLA-transgenic mice (19, 20) a similar observation concerning HspX was recently made in our laboratory: mice vaccinated with BCG did not develop immune responses to HspX whereas immunization with HspX did induce responses to HspX and its predefined T cell epitopes (10, 20a).

Of interest is our observation that the recognition pattern of latency antigens seems to be different between humans and BALB/c mice. It appears that humans responded highest to antigens Rv1733c, Rv2029c and Rv2627c whereas mice responded most to Rv1733c, Rv2032 and Rv2626c (although both not statistically significant). However this may be related to different mode of antigen exposure being natural infection in humans versus DNA vaccination in mice. This observation might have implications for proof of concept studies which involve TB latency antigens in mouse models and their extrapolation to latent TB in humans.

Finally, two other animal models confirmed the lack of T cell responses to *M. tuberculosis* latency antigens following BCG vaccination. First, adult male rhesus monkeys were vaccinated with BCG (23) and *in vitro* immune responses to *M. tuberculosis* latency antigens were monitored at nine weeks post-vaccination. Similar to observations in humans and mice, significant immune responses to PPD were seen following BCG vaccination, but no responses to *M. tuberculosis* latency antigens (Dr F Verreck, personal communication). Second, cattle were vaccinated with BCG or infected with *M. bovis* (8) and immune responses were measured against a series of latency antigens, (bovine) PPD as well as ESAT6-CFP-10 fusion protein. Significant responses to PPD were observed and animals infected with virulent *M. bovis* also responded significantly to ESAT6 and CFP-10, but no significant responses to any of the tested latency antigens were observed (Dr M Denis and Dr B Buddle, personal communication). Although it is difficult to establish positive controls for responses to TB latency antigens in these two animal models, the results are likely accounted for by the same mechanisms underlying the observed effects in humans and mice following BCG vaccination.

The studies summarized above have been performed with different strains of BCG and this may potentially confound interpretation and comparison of results. However micro-array expression profiling of 14 different BCG vaccine strains showed highly similar regulation of the DosR regulon. This rather suggests that lack of immune responses to TB latency antigens following BCG vaccination is not a strain-specific phenomenon, but an intrinsic property of dermal vaccination with BCG.

Upon administration in the skin, BCG is ordinarily not expected to persist in immuno-competent humans, although rare cases have been reported of persistence and reactivation of BCG in individuals who developed immune deficiencies many years after vaccination (1, 27, 35, 36). It therefore seems unlikely that BCG in the skin will encounter the necessary environmental triggers needed to express the latency antigens and subsequently to trigger immune responses Moreover, absence of T cell

responses are not due to restrictions in T cell repertoire since mice respond to these antigens when immunized with DNA plasmids and BCG vaccinated individuals exposed to TB also respond to latency antigens by IFNγ production. Further studies will be aimed at identification of exact mechanisms that are responsible for regulation and expression of the DosR regulon and consecutively immunity to latency antigens following BCG vaccination.
Since the start of its first use in the 1920s, 3 billion doses of BCG have been delivered throughout the world, unfortunately with relatively little impact on the global TB epidemic, underlining the need for a better vaccine regimen. Our findings suggest future studies evaluating the protective efficacy of latency antigens against (latent) TB and potential use of latency antigens as vaccine candidates, either alone, with BCG or alternatively, as post-exposure TB vaccines.

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Chapter 5

T-cell recognition of the HspX protein of *Mycobacterium tuberculosis* correlates with latent *M. tuberculosis* infection but not with *M. bovis* BCG vaccination

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Abstract

During stationary growth or in vitro conditions mimicking relevant aspects of latency such as hypoxia, the HspX protein (Rv2031c) is specifically upregulated by *M. tuberculosis*. In this study we compared T cell responses against HspX and the secreted *M. tuberculosis* protein Ag85B (Rv1886c), in tuberculosis (TB) patients, tuberculin skin test positive individuals (TST+), BCG-vaccinated individuals and healthy negative controls. IFN-γ responses against HspX were significantly higher in *M. tuberculosis* exposed individuals than in *M. tuberculosis* unexposed BCG vaccinees. In contrast, no such differences were found with respect to T cell responses against Ag85B. Therefore, BCG-based vaccines containing relevant fragments of HspX, may induce improved responses against this TB latency antigen. To identify relevant MHC class I- and class II-restricted HspX-specific T cell epitopes, we immunized HLA-A2/K^b and HLA-DR3.Ab^0 transgenic (tg) mice with HspX. Two new T cell epitopes were identified, p91-105 and p31-50, restricted via HLA-A*0201 and HLA-DRB1*0301, respectively. These epitopes were recognized by human T cells as well, underlining the relevance of HspX T cell recognition both in vivo and in vitro. In line with the data in humans, BCG immunization of both tg strains did not lead to T cell responses against HspX-derived epitopes, whereas nonlatency antigens were efficiently recognized. These data support the notion that BCG vaccination per se does not induce T cell responses against the latency antigen, HspX. Thus, we suggest that subunit vaccines incorporating HspX and/or other latency antigens, as well as recombinant BCG strains expressing latency antigens need to be considered as new vaccines against TB.

Introduction

Tuberculosis (TB) is one of the leading causes of mortality (two million annually) from an infectious disease, particularly in the developing world (36). It is estimated that 2 billion people are latently infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), the etiologic agent of TB which causes active disease in 5-10% of infected individuals. Most individuals initially control the infection by mounting cell-mediated immunity. However, residual mycobacteria remain viable for many years in healthy immunocompetent hosts in the absence of disease (4) and the majority of contagious TB disease cases will arise from this enormous source of latent TB infection.

The currently available vaccine against TB, *M. bovis* BCG, is largely ineffective at protecting against pulmonary disease in adults (16, 23). The precise nature of the T cell response needed for protection against adult pulmonary TB is incompletely defined, as is the cause for the lack of protection by BCG vaccination (3).

The 16kDa heat shock protein HspX (Rv2031c) is required for mycobacterial persistence within the macrophage and is a dominant protein produced during static growth or under oxygen deprivation (37). Under these conditions, it can account for up to 25% of the total bacillary protein expression. It is proposed that HspX plays an active role in slowing the growth of *M. tuberculosis* in vivo immediately after infection, as *M. tuberculosis* mutants lacking the hspX gene exhibited increased growth both in mice and in macrophages (24). In addition to the presence of specific humoral responses against HspX in sera of cavitary TB patients (29), T cell responses to HspX were also found to be associated with latent *M. tuberculosis* infection (13, 14) pointing to the importance of HspX as an antigenic target of immune responses during latent TB infection.

Since new vaccines containing relevant fragments of HspX, may induce improved responses against this TB latency antigen, we have generated and characterized HspX-specific, human $CD8^+$ and $CD4^+$ T cells, restricted by common HLA class I and class II alleles. In addition, PBMC from *M. tuberculosis* infected individuals (active as well as latent infection) and BCG vaccinated individuals with and without exposure to *M. tuberculosis* were examined for their in vitro response to HspX. Finally, the effect of BCG- or HspX immunization on induced immunity against HspX was analyzed in HLA-$A2/K^b$ and HLA-$DR3.Ab^0$ transgenic (tg) mice.

Materials and Methods

Antigens

BCG (*M. bovis* bacillus Calmette Guérin, Danish 1331) was purchased from the Statens Serum Institute (Copenhagen, Denmark), killed *M. tuberculosis* H37Rv sonicate was obtained from Dr. D. van Soolingen (RIVM, The Netherlands). The antigen 85B gene (Rv1886c) and the hspX gene (Rv2031c) of *M. tuberculosis* were amplified by PCR and cloned by Gateway Technology (Invitrogen, San Diego, CA) in a bacterial expression vector containing an N-terminal histidine tag. The proteins were overexpressed in *Escherichia coli* BL21(DE3) and purified, as described (17).

Study subjects

The study included 63 individuals, amongst whom 17 TB patients, 18 tuberculin skin test (TST) positive individuals, 17 BCG vaccinated individuals and 11 non-BCG vaccinated, TST negative, healthy Dutch controls. All individuals gave informed consent before blood sampling. The study protocol was approved by the institutional review board of the Leiden University Medical Centre.

ELISPOT assay for single-cell IFN-γ release

Venous blood was obtained from study participants in heparinized tubes and PBMC isolated by Ficoll density centrifugation. PBMC ($1x10^6$) were pulsed (16h) in 48-well plates with antigen (10 μg/ml) in IMDM (Life Technologies) with 10% pooled human serum. PVDF-backed 96-well plates (MAIPS45, Millipore, Bedford, MA) were pre-coated with 5 μg/ml anti-IFN-γ mAb 1-D1K (Mabtech, Stockholm, Sweden), washed 6x with IMDM and blocked (2 h) with IMDM containing 10% FCS. Antigen-pulsed PBMC ($2.5x10^5$ cells/well; 150 μl) were incubated in the PVDF-backed 96-well plates at 37°C, 5% CO_2. After 16 h plates were washed (PBS 0.05% Tween-20), incubated with 100 μl biotinylated anti-IFN-γ mAb (0.3 μg/ml) for 3 h at RT, washed, incubated with streptavidin-alkaline phosphatase conjugate (1:1000; Mabtech) for 2 h, washed and incubated with 100 μl NBT/BCIP substrate (Sigma, St. Louis, MO). The reaction was stopped by addition of water. Plates were dried and analyzed using a Zeiss Axioplan 2 microscope and KS ELISPOT software (CarlZeiss Vision, Hallbergmoos, Germany). A positive response to antigen was taken as twice the background.

Synthetic peptides

15-mer and 20-mer peptides were synthesized by simultaneous multiple peptide synthesis as described (30). Homogeneity was confirmed by analytical HPLC and by mass spectrometry. Fluorescence labeled peptides were synthesized as described previously (19).

HLA-DR/ peptide binding assay

As a source of HLA-DR molecules EBV-transformed B lymphoblastoid cell lines (EBV-BLCL) homozygous for HLA-DR were used. HLA-DR molecules were purified by affinity chromatography and peptide binding to purified HLA-DR3 molecules (60-600 nM) was determined as described (21). As standard fluorescent peptides influenza hemagglutinin (HA) p308-319 (PKYVKQNTLKLAT) was used for HLA-DR1 and HLA-DR2, and hsp65 p3-

13 (KTIAYDEEARR) for HLA-DR3. Peptide binding affinity was defined as high (IC_{50} < 1μM), intermediate (1μM - 10 μM), weak (10 μM - 100 μM) or absent (> 100 μM) (21).

Generation and epitope mapping of human, HspX-reactive $CD8^+$ T cell lines

PBMC ($1x10^5$cells/ well) derived from an HLA-A*0201$^+$ donor were stimulated with peptide pools containing four 15-mer HspX peptides overlapping 10 AA (10 μg/ ml of each peptide) for 7 days in IMDM (10% HS) in the presence of r-IL7 (5 ng/ ml, Biocarta) and r-IL15 (5 ng/ ml, Biocarta) in 96 wells round bottom plates. After 2 days r-IL2 (25 U/ ml, Cetus) was added to the cultures.

Intracellular IFN-γ staining

Seven days later, cells were collected and co-cultured for 6 h with the HLA-A*0201-positive EBV-BLCL JY, that had been pulsed overnight with single HspX peptides in IMDM (10% FCS) and washed twice with IMDM (10% FCS). During the last 2 h of co-culture, Brefeldin A was added (10 μg/ ml, Sigma). Cell-surface staining was performed using CD19-FITC, CD4-PerCP and CD8-APC (all Becton Dickinson, CA) after which the cells were permeabilized with Perm buffer (Pharmingen, CA) and stained with anti-IFN-γ-PE (Becton Dickinson, CA). Stimulation with phorbol myristate acetate-ionomycine was used as a positive control and unstained cells as a negative control.

Generation and epitope mapping of human, HspX-reactive $CD4^+$ T cell lines

PBMC (1.5×10^6 cells/ well) of healthy human donors were stimulated with HspX (10 μg/ ml) in IMDM (10% human serum) in 24-well plates. After 6 days r-IL-2 (25 U/ ml (Chiron, CA) was added and cells were additionally cultured for 10 to 15 days, frozen and stored in liquid nitrogen until further use. Human $CD4^+$ T cell lines (1.5×10^5 cells/ well) and HLA-DR matched irradiated PBMC (5×10^5 cels/ well) were incubated with either HspX (10, 1.0, 0.1 or 0.01 μg/ ml) or HspX-derived peptides (10, 1.0, 0.1 or 0.01 μg/ ml) in flat-bottom 96-well plates. After 72 h, 0.5 μCi of [^{3}H]thymidine was added to each well. After 18 h, cells were collected on glass fiber filter strips and the radioactivity incorporated into the DNA was determined by liquid scintillation counting. Results are the mean of triplicate cultures. SEM were < 20%.

HLA transgenic (tg) mice

HLA-DRB1*0301/DRA tg, murine class II deficient (HLA-DR3.Ab^0) mice were generated as described (18). During breeding, PBMCs were typed for expression and segregation of the transgene by flow cytometry and PCR (27). HLA-A2/K^b mice expressed, in addition to the murine class I alleles H2-K^b and H2-D^b, a chimeric HLA-A*0201/K^b gene encoding H2-K^b α3 domain and the HLA-A*0201 α1 and α2 domains (20). HLA-A2/K^b mice were bred under specific pathogen-free conditions at TNO-PG (Leiden, The Netherlands). Surface expression of HLA-A*0201/K^b molecule was confirmed by flow cytometry.

Immunizations

Emulsions comprising equal volumes of HspX recombinant protein in PBS and incomplete Freund's adjuvant (IFA; DIFCO, Detroit, MI) were prepared and administered as subcutaneous (s.c.) injections into the flanks (in total 10 μg protein per mouse, n = 4).

Live *M. bovis* BCG (Montreal strain) was diluted with PBS. Each mouse was s.c. injected with 50 μl around each hind leg (in total $1x10^6$ CFU/ mouse). Seven days post-injection, spleens were removed and cell suspensions prepared for in vitro culture. For DNA immunizations mice were injected intramuscularly 3 times (at 3-wk intervals) in both quadriceps (2x 50 μl) with HspX plasmid (1 mg/ml) or control DNA (empty vector) in PBS. Splenocytes were harvested three weeks after the last DNA injection.

In vitro culture

Splenocytes from each mouse (1.5×10^5 cells/ well) were stimulated in triplicate cultures with antigen in round bottom 96-well plates in 200 μl RPMI 1640 (Life Technologies) supplemented with 2mM L-glutamine (Life Technologies), 100 U/100 μg/ml penicillin/ streptomycin solution (Life Technologies) and 10 % heat-inactivated FCS (Life Technologies). HspX and *M. tuberculosis* sonicate were tested at 5 μg/ ml, peptides at 25 μg/ ml. After 6 days, 0.5 μCi of [^{3}H]thymidine was added to each well. After 18 h cells were collected on glass fiber filter strips and the radioactivity incorporated into the DNA was determined by liquid scintillation counting. Results are the mean of triplicate cultures. SEM were <20 %.

ELISA murine IFN-γ

Splenocytes were resuspended in RPMI-1640 medium (Life Technologies, Rockville, MD, USA) supplemented with 10% heat-inactivated FCS (INTEGRA Biosciences AG, Switzerland) were added to 96-well U-bottom plates (Corning B.V. Life Sciences, The Netherlands) and stimulated in triplicates with antigens. After 72 hours, culture supernatants were taken and evaluated for their IFN-γ content using murine IFN-γ CytoSet™ ELISA kit (Biosource, Camatillo, CA, USA). The assay was performed according to the manufacturer guidelines.

Cytotoxicity assays

The human EBV-BLCL JY (HLA-A*0201, -B7, -Cw7) was incubated at 37° C for 1 hour with 0.1 mCi $Na_2{}^{51}CrO_4$ (Amersham, UK), washed and plated with pooled splenocytes from immunized mice (n = 4), in triplicates in 96-well round-bottomed plates (2500 cells/ well) together with medium, peptide (2 μg) or 5 % Triton-X100 (20). After 6 h supernatants were harvested and % specific lysis was calculated as: [(release – spontaneous release) / (maximum release – spontaneous release)] x 100%.

Statistical analysis

Differences in IFN-γ responses to HspX between different test groups were analyzed with the two-tailed Mann-Whitney U test for non-parametric distribution using Graph Pad Prism (version 4). *P*-values were corrected for multiple comparisons. The statistical significance level used was $P<0.05$.

Results

T cell responses against HspX do not correspond to BCG vaccination per se

Individuals with different stages of *M. tuberculosis* infection were previously reported to respond differentially to HspX (14). Since the effect on T-cell responses in BCG vaccination in areas where TB is and not endemic may vary (5), we analyzed HspX responses in IFN-γ ELISPOT assays using PBMC from BCG-vaccinated individuals and from non-BCG vaccinated, healthy controls in addition to TB patients and TST+ individuals (Figure 1).

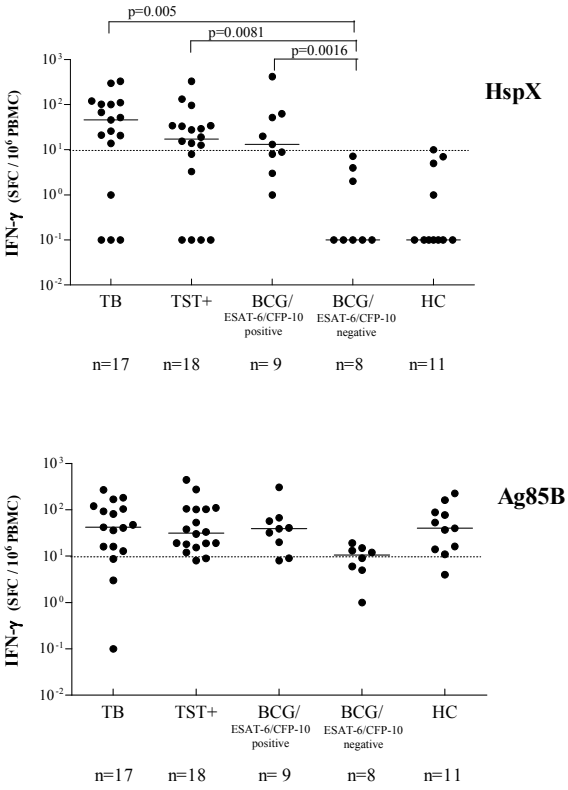

Figure 1. *IFN-γ production against HspX and Ag85B of M. tuberculosis by PBMC of 17 TB patients (TB), 18 tuberculin skin test positive (TST+) individuals, 17 BCG vaccinated individuals (nine in vitro positive for ESAT-6 and/or CFP-10 and eight in vitro negative for ESAT-6 and/or CFP-10), and 11 non-BCG vaccinated, healthy controls (HC). Spot forming cells (SFC) per 10⁶ PBMC corrected for medium values are given on the y axis. Median values are indicated by horizontal lines. P values are calculated by the non-parametric Mann Whitney U test. T cell responses were considered positive if the medium-corrected number of SFC was > 10/10⁶ cells.*

Based on their in vitro IFN-γ response to the *M. tuberculosis*-specific proteins ESAT-6 and/ or CFP-10 (32), we divided BCG vaccines into *M. tuberculosis*-unexposed individuals (<10 spot-forming cells [SFC]/10^6 PBMC; n=8) and individuals with likely exposure to *M. tuberculosis* (> 10 SFC/ 10^6 PBMC; n = 9; ranging from 16 to 116 SFC/10^6 PBMC).

Table 1. HspX/Ag85B ratios

Test group	Median HspX/ Ag85B value
TB patients	0.55
TST^+	0.33
BCG, ESAT-6/CFP-10 positive[a]	0.35
BCG, ESAT-6/CFP-10 negative[b]	0.060
Controls, *M. tuberculosis* unexposed	0.0062

[a] *In vitro responsive to ESAT-6 and/or CFP-10*
[b] *In vitro unresponsive to ESAT-6 and/or CFP-10*

HspX induced high numbers of IFN-γ producing T cells in three of the five groups (TB, TST^+ and BCG vaccinees responding to ESAT-6 and/or CFP-10). These T cell responses differed significantly ($P \leq 0.0081$) from BCG-vaccinated individuals without a response to ESAT-6 and/or CFP-10 and from healthy controls. As a control, the number of IFN-γ producing T cells induced by the secreted Ag85B was analysed in the same individuals. Interestingly, no significant differences between the five groups were detected in the number of SFC induced by Ag85B. This indicates that the lack of IFN-γ production in response to HspX in BCG vaccinees and controls is not caused by the absence of anti-mycobacterial responses. The median values of the HspX/Ag85B response ratio were 0.060 and 0.0062 in BCG-vaccinated, *M. tuberculosis*-unexposed individuals and the healthy control group, respectively, whereas *M. tuberculosis* infected or exposed groups had 10-fold higher HspX/Ag85B response ratios ranging between 0.55 and 0.33 (Table 1).

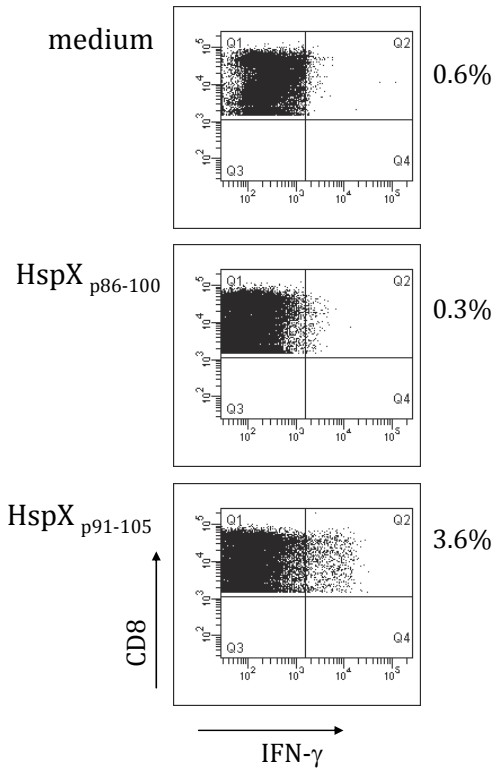

Figure 2. *Intracellular IFN-γ staining of HLA-A*0201+, CD8+ human T cell lines generated by stimulation of PBMC with seven peptide pools, each containing four HspX 15-mers. T cell lines were co-cultured for 6 h with the HLA-A*0201-positive EBV-BLCL JY, that had been pulsed overnight with one single HspX peptide (indicated on the left). Cells were incubated with PE-labeled anti-IFN-γ and APC-labeled anti-CD8 MAb. For the analysis, only lymphocytes were used with the exclusion of CD19-positive cells to avoid contamination of the JY cells.*

HLA-A*0201-restricted T cell epitopes

Since mounting evidence from human studies and murine models of TB points toward a role of $CD8^+$ T cells in controlling (latent) *M. tuberculosis* infection (22, 28, 31), we set out to identify HLA-class I-restricted T cell responses to the HspX *M. tuberculosis* latency antigen. PBMC derived from an HspX responding HLA-A*0201+ donor were stimulated with seven peptide pools, each containing four HspX-derived 15-mer peptides with an overlap of 10 amino acids. After 7 days, cells were

collected and co-cultured for 6 h with HLA-A*0201-positive EBV-BLCL, that had been pulsed overnight with single HspX peptides, and induction of intracellular IFN-γ was assessed by FACS analysis (Figure 2). Intracellular IFN-γ producing $CD8^+$ T cells were detected in the T cell line generated with pool 5. In this pool only one peptide, HspX p91-105, induced significant levels of intracellular IFN-γ production (Figure 2).

Murine HLA-A*0201-restricted epitopes

HLA-A2/K^b mice represent a suitable model for the induction and detection of high affinity HLA-A*0201-restricted $CD8^+$ CTL responses in vivo(11, 20). Thus, these mice were immunized three times with plasmid DNA encoding *hspX* or empty vector DNA. Three weeks after the last DNA injection, splenocytes were incubated with peptide pulsed, ^{51}Cr-labeled HLA-A*0201-positive JY cells (which express HLA-A*0201 but not H2-K^b or H2-D^b) in order to analyze their cytolytic potential. JY cells pulsed with HspX p91-105 were lysed by splenocytes of immunized mice (Figure 3). Splenocytes derived from mice immunized similarly with empty vector DNA did not show any lysis (data not shown). These data show that the same HLA-A*0201-restricted cytotoxic T lymphocyte (CTL) epitope is identified in HLA-A*0201 humans and in HLA-A2/K^b.

The relatively low level of lysis by the CTL raised in HLA-A2/K^b mice (maximal 25 %) could be due to the presence of the chimeric HLA-A*0201/K^b gene in the tg mice. This expresses the H2-K^b α3- and the HLA-A*0201 α1 and α2 molecules allowing efficient CD8 interaction of murine $CD8^+$ T cells and thus interacts less efficiently with the human HLA-A*0201 molecule expressed on the JY target cells.

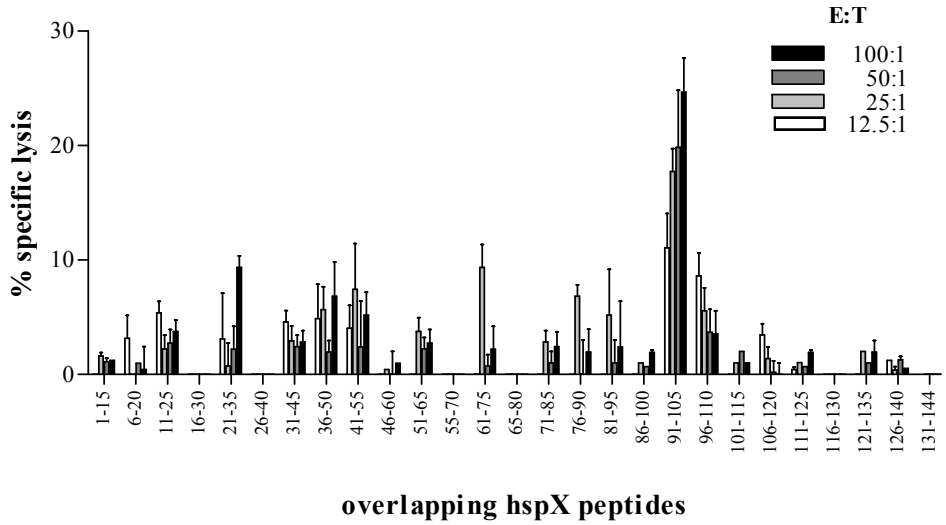

Figure 3. *Cytotoxic activity of splenocytes derived from HLA-A2/K^b mice, immunized with HspX plasmid DNA. M. tuberculosis-derived peptides used to pulse HLA-A*0201$^+$ target cells (JY) are indicated on the x axis. The percentage specific lysis is indicated on the yaxis. E:T, effector to target ratio.*

HLA-DR binding of HspX-derived peptides and HLA-DR-restricted T cell epitopes

Previously, several studies have shown that different regions of the *M. tuberculosis* HspX latency antigen can provoke human CD4+ T cell responses. Especially the immunodominant region p91-105 is permissively recognized, since peptide epitopes were defined in the context of HLA-DRB1*0101, -DRB1*1101, -DRB1*1301, -DRB1*1501, and DRB1*0401 (1, 2). Other peptide sequences recognized by human T cells are: p21-40 (HLA-DRB1*0401) (1), p21-29 and p120-128 (both HLA-A*0201) (10).

To study CD4+ T cell responses to HspX, we first assessed HLA-binding affinity of HspX-derived peptides. Ten amino acids overlapping 20-mer peptides, covering the entire sequence of the HspX protein of *M. tuberculosis* were used to analyze peptide binding to purified HLA molecules (Table 2). HLA-DR1 and HLA-DR2 showed similar binding affinities for the HspX peptides, particularly p11-30 and p91-105 bound with high affinity to these alleles. HLA-DR3, on the other hand, bound especially well to p31-50, which was confirmed by the presence of the HLA-DR3 peptide binding motif (21) with L (Leucine) at position n, E (Glutamate) at position n+2 and K (Lysine) at position n+4 (Table 2).

HspX 20-mers were used for epitope mapping in three human, HspX-specific T cell lines from individuals homozygous for HLA-DRB1*0101, -*01501 or -*0301 (Figure 4). The HLA-DRB1*0101+ T cell line recognized p91-105 (Figure 4A), which also binds with high affinity to both HLA-DR1 and HLA-DR2. Another peptide that bound with high affinity to these alleles, p21-40 (Table 2), induced high proliferative responses, whereas p71-90, which bound with lower affinity, induced moderate responses in the HLA-DRB1*01501+ T cell line (Figure 4B). The HLA-DR3-restricted epitope identified here, p31-50 (Figure 4C), corresponded well with the high binding affinity for HLA-DR3 as described for other HLA-DR3-restricted, mycobacterial peptide epitopes (18). T cell responses were HLA-DR-restricted as MAbs directed against HLA-DR (B8.11.2) decreased proliferation of all three T cell lines (data not shown).

Table 2. HLA-DR binding of overlapping HspX peptides[a]

Amino acids	Sequence	IC50 (μM)		
		HLA-:DR*0101	DR*1501	DR*0301
1-20	MATTLPVQRHPRSLFPEFSE	15	22	>100
11-30	PRSLFPEFSELFAAFPSFAG	< 0.7	< 0.7	42
21-40	LFAAFPSFAGLRPTFDTRLM	5	**0.9**	68
31-50	LRPTFDTRLMR**L**ED**EM**K**E**GR	24	35	**0.5**
41-60	RLEDEMKEGRYEVRAELPGV	8	13	41
51-70	YEVRAELPGVDPDKDVDIMV	12	17	35
61-80	DPDKDVDIMVRDGQLTIKAE	5	3	4
71-90	RDGQLTIKAERTEQKDFDGR	14	9	>100
81-100	RTEQKDFDGRSEFAYGSFVR	6	1	>100
91-110	SEFAYGSFVRTVSLPVGADE	**< 0.7**	**< 0.7**	45
101-120	TVSLPVGADEDDIKATYDKG	>100	>100	>100
111-130	DDIKATYDKGILTVSVAVSE	>100	70	>100
121-140	ILTVSVAVSEGKPTEKHIQI	>100	>100	>100
125-144	SVAVSEGKPTEKHIQIRSTN	>100	>100	>100

[a] *T cell epitopes and associated values are depicted in boldface.*

Murine HLA-DR3 restricted epitopes

To address the in vivo immunogenicity and epitope specificity of the HspX protein, we have used HLA-DR3.Ab^0 tg mice. After immunization of the mice with HspX protein in IFA, splenocytes were restimulated in vitro with the overlapping HspX 20-mer peptides. Since the HLA-DR3.Ab^0 mice are devoid of any murine class II molecules expressed at the cell surface, all CD4$^+$ T cells are restricted by HLA-DR3. Figure 4D shows that murine HLA-DR3-restricted T cells recognize the same immunodominant HspX peptide (p31-50) as human HLA-DR3-restricted T cells, indicating that p31-50 is an in vivo processed and presented HLA-DR3-restricted T cell epitope.

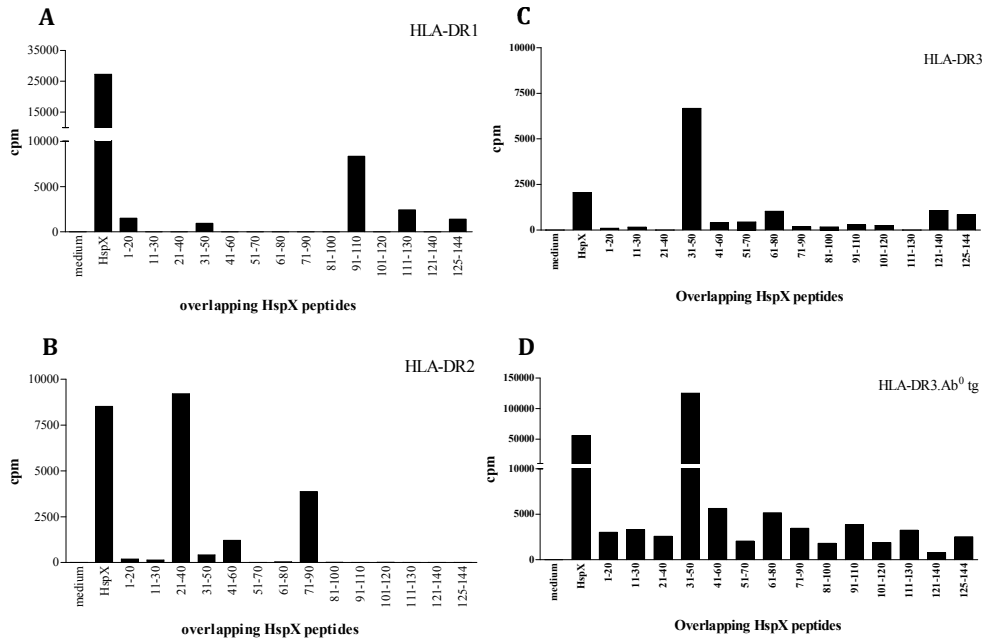

Figure 4. *T cell proliferation of HLA-DR1$^+$ (**A**), -DR2$^+$ (**B**) or -DR3$^+$ (**C**) human T cell lines responsive to the HspX protein, or of splenocytes from HLA-DR3.Ab^0 tg mice immunized with recombinant protein of HspX (**D**). T cell proliferation (in counts per minute [cpm]) corrected for medium values is shown on the y axis. The SEM were < 12%. The amino acid numbering of the overlapping HspX peptides from Table 2 is given on the x axis.*

BCG immunization of HLA-A2/K^b and HLA-DR3.Ab^0 does not induce T cell responses against HspX

Since BCG vaccinated, *M. tuberculosis* unexposed individuals in a cross-sectional comparison did not show T cell responses directed against HspX (Figure 1 and (33), we decided to evaluate this in HLA-tg mice. Splenocytes were harvested ten days post BCG-vaccination, and analyzed for their ability to lyse the human target cell JY, which expresses HLA-A*0201. Target cells were pulsed with one of seven known HLA-A*0201-epitopes (10 μg/ml per peptide): hsp65 p369-377 (11), Ag85B p143-152 and

Ag85B p199-207(20), HspX p91-105 (the present study), HspX p21-29 and HspX p120-128 (10) or the HLA-A2-binding influenza A matrix peptide: Flu p58-66 (34).
The hsp65 and Ag85 epitopes were strongly recognized by CTL in a dose-dependent fashion (Figure 5A). However, none of the target cells pulsed with HspX peptides were lysed. No lysis was observed for control cells: splenocytes pulsed with HLA-A*0201-binding influenza virus p58-66 (Figure 5A) or splenocytes derived from unimmunized A2/K^b mice (data not shown). Similarly, we investigated the T cell responses in HLA-DR3.Ab^0 tg mice after immunization with BCG. Splenocytes of BCG-immunized mice were restimulated in vitro with the HspX protein, its HLA-DR3-restricted epitope p31-50, *M. tuberculosis* hsp65, its HLA-DR3-restricted epitope p3-13, Ag85B or its HLA-DR3-restricted epitope p51-70 (Figure 5B). Again, no IFN-γ responses were detected in response to HspX or its HLA-DR3-restricted epitope. In contrast, both other proteins and their HLA-DR3-restricted epitopes induced significant levels of IFN-γ. No T cell responses could be detected against any of the antigen in mock-immunized HLA-DR3.Ab^0 mice.

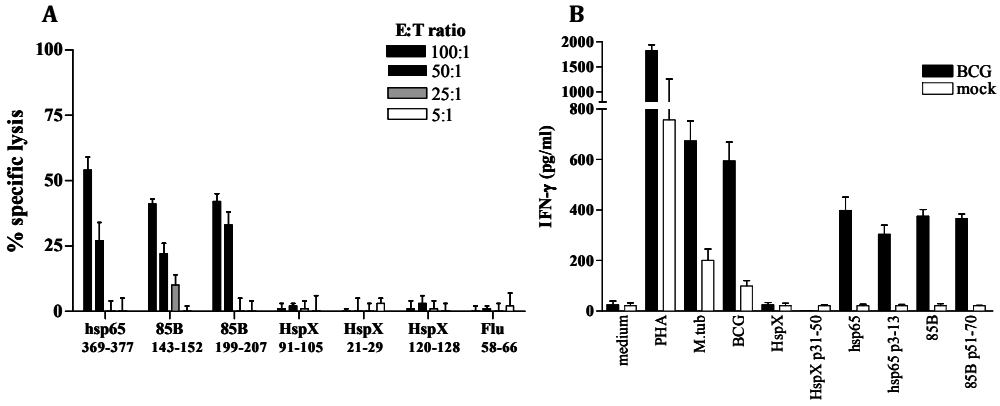

Figure 5. (A) *Cytotoxic activity of splenocytes derived from HLA-A2/K^b mice, immunized with BCG. Ten days after immunization, splenocytes were harvested and analyzed for their ability to lyse human HLA-A*0201-positive target cells (JY). HLA-A*0201-restricted peptides used to pulse target cells are indicated on the x axis. The HLA-A*0201-binding peptide of influenza A matrix (F58-66) was used as a control. E:T, effector to target ratio. The sequences of peptides used in the CTL assay were as follows: hsp65p369-377: KLAGGVAVI, Ag85B p143-152: FIYAGSLSAL, Ag85B p199-207: KLVANNTRL, HspX p91-105: SEFAYGSFVRTVSLP, HspX p21-29: LFAAFPSFA, HspX p120-128: GILTVSVAV and influenza A matrix p58-66: GILGFVFTL. (**B).** IFN-γ production of splenocytes of HLA-DR3.Ab^0 mice immunized with BCG in PBS (BCG) or with PBS only (mock). The antigens used for in vitro challenge are given on the x axis.*

Discussion

Since BCG vaccination does not protect against reactivation of TB, mycobacterial antigens that induce differential T cell responses in individuals latently infected with *M. tuberculosis* compared to unexposed BCG-vaccinated individuals may help to identify protective antigens and immune responses against TB. Such antigens may be applied in development of post-exposure vaccines as well as for new specific diagnostic tools.
By means of whole genome DNA microarray expression profiling and proteomic analysis 48 so-called latency antigens have been identified (35). In a previous study, our group analyzed the immunogenicity of 25 of these latency antigens and observed strong IFN-γ responses particularly in latently infected individuals (25).
In the present study we have focussed on the latency antigen HspX, as it is required for bacterial growth within the macrophage and is predominantly present during stationary growth of *M. tuberculosis* (37). Moreover, strong T cell responses to HspX in African populations were observed which were mostly restricted to latently infected individuals (14). In addition, T cells from TB patients recognizing the HspX protein showed a switch from Th0 towards Th1 after chemotherapy, indicating their potential to induce protective T cell responses (9, 15). These characteristics of HspX make it an interesting target for post-exposure TB vaccination as well as for the possible diagnosis of preclinical infection.
We here describe HspX-specific T cell responses in *M. tuberculosis* (likely)-exposed individuals (TB patients, TST^+ asymptomatic individuals, BCG vaccinees with positive ESAT-6 and/ or CFP-10 T cell responses) in comparison to *M. tuberculosis* unexposed individuals (BCG vaccinees and healthy controls lacking a T cell response to ESAT-6 and/ or CFP-10). Our results show that most (24 of 34, [71%]), *M. tuberculosis* infected or –exposed individuals responded well in the ELISPOT assay to HspX whereas sporadic and significantly lower responses were observed in *M. tuberculosis* unexposed individuals including BCG vaccinated individuals without any known exposure to *M. tuberculosis.* This suggests that BCG vaccination alone does not induce T cell responses against the HspX antigen. Similar findings come from a study in The Gambia where neonatal BCG immunization did not lead to IFN-γ responses to HspX or CFP-10, whereas these proteins were well-recognized in *M. tuberculosis*-exposed household contacts and health care workers (33).
Several studies have indicated that BCG can express the HspX homologue during oxygen depletion (6, 12, 26); however, our data suggest that vaccination with BCG in humans does not induce immune responses against HspX. We hypothesize that the expression of HspX by BCG in vivo after vaccination is limited and insufficient to induce an immune response.
To confirm this assumption in vivo, we applied BCG immunization to an HLA-class I and HLA-class II tg mouse models -HLA-A2/K^b and HLA-DR3.Ab^0, respectively- which provide powerful models to help characterizing in vivo T cell responses against mycobacterial antigens in the context of HLA polymorphism (18, 20). Furthermore, the HLA-A*0201 and HLA-DRB1*0301 alleles are major HLA alleles as frequencies are 42% and 24%, respectively in Caucasian, African/ Afro-Caribbean, and Oriental populations (8).

Splenocytes derived from BCG-immunized HLA-A*0201.K^b mice failed to lyse target cells pulsed with HLA-A*0201-restricted HspX peptides, whereas target cells pulsed with HLA-A*0201-restricted epitopes derived from hsp65 or Ag85B of *M. tuberculosis* were lysed up to 54% (Figure 5A). Similarly splenocytes from BCG-immunized DR3.Ab^0 mice were challenged in vitro with several mycobacterial antigen and their HLA-DR3-restricted peptides. Whereas immunization of the HLA-DR3.Ab^0 mice with the HspX protein induced responses against HspX p31-50 and the HspX protein (Figure 4D), such responses were not detected after BCG immunization of HLA-DR3.Ab^0 mice (Figure 5B). In contrast, both the cytosolic hsp65 and the secreted Ag85B of *M. tuberculosis* and their respective HLA-DR3-restricted epitopes, p3-13 and p51-70, induced significant levels of IFN-γ in these mice, a finding consistent with our previous findings (18). These data indicate that BCG immunization does not provoke T cell responses against the HspX latency antigen, while it is possible to induce $CD4^+$- and $CD8^+$ T cell responses to HspX using protein- or DNA vaccination. Thus, these data suggest that expression of HspX by BCG after in vivo vaccination is probably low compared to the expression of its homologue in *M. tuberculosis* under latent conditions and thus will not lead to a significant immune response directed against HspX.

This study shows that BCG vaccination alone does not induce T cell responses against the HspX antigen but that HspX is an immunogenic antigen that harbors several T cell epitopes. Thus, we anticipate that improved (BCG) vaccines, expressing relevant fragments of *M. tuberculosis* latency antigens (7, 25), may have potential as vaccines against latent TB.

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Chapter 6

Cross-reactive immunity to *Mycobacterium tuberculosis* DosR regulon encoded antigens in individuals infected with environmental, non-tuberculous mycobacteria

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Abstract

Mycobacterium tuberculosis (*M. tuberculosis*) DosR-regulon encoded antigens are highly immunogenic in *M. tuberculosis*-infected humans and associated with latent TB infection. We have investigated the hypothesis that infection with or exposure to non-tuberculous mycobacteria (NTM) can induce cross-reactive immunity to *M. tuberculosis* DosR-regulon encoded antigens since responsiveness has been observed in non-*M. tuberculosis* exposed but PPD responsive individuals.

M. tuberculosis DosR-regulon encoded antigen specific T-cell responses were studied in PBMC of NTM infected/exposed individuals. BLASTP was used to determine the presence of *M. tuberculosis* DosR-regulon encoded protein orthologs among environmental mycobacteria and non-mycobacteria.

Significant IFNγ production was observed in PBMC from NTM infected/exposed individuals in response to *M. tuberculosis* DosR-regulon encoded antigens. DosR-regulon encoded protein orthologs were prominently present in tuberculous- and environmental mycobacteria, and surprisingly also in non-mycobacteria. The ubiquitous presence of the highly conserved DosR master regulator-protein Rv3133c suggests that this is a general adaptive bacterial response-regulator.

We report a first series of *M. tuberculosis* antigens to which cross-reactive immunity is induced by NTM infection/exposure. The high conservation of *M. tuberculosis* DosR-regulon encoded antigens most likely enables them to induce cross reactive T-cell responses.

Introduction

Yearly, tuberculosis (TB) claims 1.7 million lives. Its global incidence exceeds 9 million new cases. TB morbidity and mortality are likely to increase further as a result of TB reactivation in HIV-1/*Mycobacterium tuberculosis* (*M. tuberculosis*) co-infected individuals, as well as the rising frequencies of multi-drug resistant (MDR) and extensively drug resistant (XDR) *M. tuberculosis* strains (41). It is estimated that 2 billion people carry a latent TB infection (LTBI). This vast reservoir forms a major source of new TB cases: one out of every ten *M. tuberculosis* infected individuals will develop active TB disease at one point in their lifetime, while the remainder is able to contain the bacilli without any clinical symptoms.

In a series of recent *M. tuberculosis* antigen discovery studies, aiming at identifying new *M. tuberculosis* biomarker and vaccine antigens, we found that genes from the *M. tuberculosis* DosR (Rv3133c) regulon encode antigens that can induce specific T-cell immunity in *M. tuberculosis* infected individuals (20, 32). Tubercle bacilli express the DosR regulon under *in vitro* conditions of hypoxia, low dose nitric oxide (40) and carbon monoxide, (19, 37) conditions thought to be encountered by persisting intracellular bacilli in the immuno-competent host. Studies have also reported up-regulated expression of DosR regulon genes in infected murine macrophages and infected murine lung tissues (19, 35-37). Importantly, immunity to *M. tuberculosis* DosR-regulon encoded antigens might contribute to the control of persistent *M. tuberculosis* infection since several DosR-regulon encoded antigens were preferentially recognized by subjects with LTBI (20, 32). Finally, we also found that BCG vaccination does not induce immune responses to *M. tuberculosis* DosR-regulon encoded antigens, which might partly underlie its inefficacy against late *M. tuberculosis* infection (16, 21).

During these studies, we unexpectedly observed that some *M. tuberculosis* DosR-regulon encoded antigens were also recognized by healthy controls (HC) who had a positive in vitro IFNγ response to purified protein derivative (PPD) or *M. tuberculosis* lysate. However, none of these tuberculin skin test (TST) negative HC had had any known exposure to or infection with *M. tuberculosis*, or had received BCG (20). We therefore hypothesized that these responses could be due to exposure to environmental, non-tuberculous mycobacteria (NTM).

NTM comprise all mycobacterial species that are not included within the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *Mycobacterium africanum*, *Mycobacterium microti*, and *Mycobacterium canetti*) or *Mycobacterium leprae* (8, 28). NTM are facultative intracellular bacteria with specific niches in the environment, are ubiquitously present and opportunistic. Diagnosis and treatment of lung infections caused by NTM involves careful evaluation of the infection since no reference standard or parameter exists by which the different NTM can be characterized (5). It has long been suspected that exposure to NTM can shape host immunity. For example, neonatal BCG vaccination results in significant protection against pediatric TB, but generally fails to protect adequately against pulmonary TB in adults; this latter failure has been attributed in part to the immunomodulatory effects

of NTM exposure/infection (2, 7, 10, 26), although the kinetics of the latter as well as the underlying mechanisms involved remain undefined.
Here, we have investigated whether infection with or exposure to NTM can induce cross-reactive immunity to *M. tuberculosis* DosR-regulon encoded antigens. We also studied the presence of *M. tuberculosis* DosR regulon protein orthologs and homologs among mycobacteria and several non-mycobacterial species.

Materials and Method

Study subjects

One hundred and thirty-seven Dutch subjects were included in this study: 80 blood bank donors; 33 healthy, *M. tuberculosis* unexposed, BCG non-vaccinated individuals; 12 NTM infected subjects; and 12 NTM exposed subjects. Full characteristics of the latter two groups have been described in detail elsewhere (4). In brief, we included patients for whom *M. marinum* (n=7) had been cultured from a typical lesion on the hand or forearm. To discriminate between infection and colonization, *M. kansasii* (n=5) infection was defined by characteristic radiographic abnormalities in combination with a positive culture from a bronchoscopic washing or lung biopsy specimen or at least 2 positive sputum cultures, in the absence of an alternative diagnosis. Currently, no reference standard or parameter exists by which exposure to environmental mycobacteria can be characterized or quantified. Individuals exposed to NTM were selected by reasoning that exposure would be most pronounced during repeated and intense contact with the natural habitat of environmental bacteria, due to professional or recreational activities. Owners of tropical fish tanks (n=4), veterinarians (n=3) and professional flower growers (n=5) were selected as representative groups. Study subjects were recruited at the LUMC and at the Regional Health Service in Geleen (NL). The study protocols (P207/99 and P136/97) were approved by the Institutional Review Board of the LUMC.
All study subjects except blood bank donors, answered a questionnaire about BCG vaccination, travel history and historic TB/NTM contact. None of the study subjects used or had used immunosuppressive treatment or belonged to a risk group for HIV infection.
Blood was obtained by venipuncture after written informed consent was obtained. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll density gradient centrifugation and stored in liquid nitrogen until use.

M. tuberculosis antigens

Recombinant proteins (Table 1) were produced as described previously (15). Briefly, selected *M. tuberculosis* H37Rv genes were amplified by PCR from genomic H37Rv DNA and cloned by Gateway Technology (Invitrogen, San Diego). Proteins were over-expressed in *Escherichia coli* strain BL21(DE3) and purified as described (15). Recombinant proteins were subjected to quality assurance and quality control (QA/QC) assays as described in (21) including testing for residual endotoxin levels, absence of non-specific T cell stimulation in PPD nonresponsive donors and absence of cellular toxicity as assessed by lack of inhibition of mitogen induced proliferation.

As reference antigens PPD (RT49; SSI, Denmark) and *M. tuberculosis* 'hypoxic' lysate (31,43,44) were used (provided by Dr. Peter Andersen, SSI, Denmark).
Synthetic peptides from ESAT-6 and CFP-10 were produced as previously described (17). For both antigens, nine peptides (20 aa with 10 aa overlap) spanning the complete protein sequence were synthesized.

Lymphocyte stimulation assay
Lymphocyte stimulation assays were performed as previously described (20). Briefly, PBMC (1.5×10^5/well) were cultured in IMDM (Gibco, Paisley, UK) supplemented with 10% pooled human serum, 50 U/ml penicillin and 50 μg/ml streptomycin, in 96-well round-bottom microtiter plates (Nunc, Roskilde, Denmark) at 37°C, 5% CO_2, in the absence or presence of stimulant (in triplicate). The following antigen concentrations were used: all *M. tuberculosis* recombinant proteins at 0.33 μM (average concentration 8 μg/ml, range 4.3-16.2), *M. tuberculosis* hypoxic lysate and PPD both at 5 μg/ml and peptide pools of ESAT-6 and CFP-10 at 1 μg/ml for each peptide. Phytohaemagglutinin (PHA; Remel, UK, 2 μg/ml) and medium were used as positive and negative control respectively. At day 6, supernatants were harvested and stored at –20°C until use.

Detection of IFNγ by ELISA
IFNγ concentrations were measured by ELISA (U-CyTech, Utrecht, NL) as previously described (20). The detection limit of the assay was 20 pg/ml. Values of unstimulated cultures (median concentration 20 pg/ml, range 20-184) were subtracted from stimulated cultures to obtain the antigenic responses. Responses were regarded positive if the response was ≥100pg/ml.

Assessment of DosR protein orthologs in environmental (myco) bacteria
Gene homologs are genes in different species that descended from a common ancestral DNA sequence. Gene homologs are further specified as orthologs when they have retained the same function in the different species. Here, protein orthologs for *M. tuberculosis* DosR-regulon encoded antigens were identified as reciprocal best matches. Protein sequences that were sourced from National Center for Biotechnology Information (NCBI) and annotated at Tuberculosis Database (30) were used in this analysis. Using basic local alignment search tool (BLAST) program (1), protein sequences from *M. tuberculosis* DosR-regulon encoded antigens were compared with other (myco)bacterial species and vice-versa. Reciprocal best matching pairs between *M. tuberculosis* DosR-regulon encoded antigens and those of others were identified as ortholog pairs. When there was no reciprocal best match, a hit that produced the highest percent similarity score was taken as best homolog. A cut-off value of 40% similarity score was used.

Statistical analysis
The Wilcoxon signed rank test was used to compare the responses per antigen in the study population to the unstimulated (medium) condition. The Bonferroni correction (corrected *P*-value $=1-(1-P)^n$) was applied as post-hoc adjustment. The Kruskal-Wallis test was used for comparison between groups and followed by Dunns post-hoc test when appropriate. All statistical analyses were performed on the raw data (i.e. not

corrected for the medium values). Statistical analyses were performed with SPSS 14.0 and Graph Pad Prism 4.02; $P < 0.05$ was considered to be statistically significant.

Results

Selection of *M. tuberculosis* DosR-regulon encoded antigens

Eight of the most interesting *M. tuberculosis* DosR-regulon encoded antigens were selected for further investigation (Table 1). Selection was based on T-cell recognition profiles in TB patients, TST converters and healthy un-infected controls (HC) observed in our earlier study (20): Rv1733c, Rv2029c, Rv2032, Rv2627c, Rv3129 ranked among the top 10 most frequently recognized antigens in all three groups. Antigens Rv2626c and Rv2628 were selected as they ranked among the top 10 antigens in at least two study groups, including the TST converters. Rv2031c (16kD heat-shock protein, HspX, a-crystallin)(42) was selected since it has been relatively well studied in anti-mycobacterial immunity. All antigens were tested in equimolar concentrations to allow for direct comparison of immunogenicity between the different proteins, since they varied in size (12.3-46.3 kDa).

Table 1. Selected *M. tuberculosis* DosR antigens tested in present study

DosR gene	Gene name [a]	Molecular mass (kDa)[a]	Product [a]	Reference
Rv1733c		22.4	Conserved transmembrane protein	20, 21, 32
Rv2029c	*pfkB*	35.4	Phosphofructokinase PfkB	20, 21, 32
Rv2031c	*hspX*	16.3	Heat shock protein HspX (alpha-crystallin)	11, 16, 20, 21, 32, 42-44
Rv2032	*acg*	36.6	Hypothetical protein Acg	14, 20, 29, 32
Rv2626c		15.5	Conserved hypothetical protein	6, 20, 21, 31, 32, 36
Rv2627c		46.3	Conserved hypothetical protein	20, 21, 32
Rv2628		13.1	Hypothetical protein	20, 21, 32
Rv3129		12.3	Conserved hypothetical protein	20

[a] *Annotations are from www.tbdb.org and http://genolist.pasteur.fr/TubercuList/*

In vitro PPD responses in healthy individuals: a survey amongst healthy *M. tuberculosis*-unexposed donors

Given the ubiquitous nature of NTM, it is likely that humans are exposed to, and immunologically primed by NTM (39a). We therefore assessed the in vitro responsiveness to PPD in two different groups of healthy individuals and for comparison, in NTM exposed/infected individuals. Of note, PPD is not *M. tuberculosis*-specific since it contains many antigens that are cross-reactive with other mycobacteria, including BCG and NTM.

The first group of HC comprised *M. tuberculosis* uninfected/unexposed, BCG unvaccinated individuals (n=27). Twenty individuals had a negative TST (the remaining seven were not TST-tested). Secondly, a group of healthy Dutch blood bank donors (n=66) was included (unknown TST-status). This group was less well defined since no specific information was available concerning BCG vaccination and TB exposure/contact history. However, given the low TB incidence in the Netherlands,

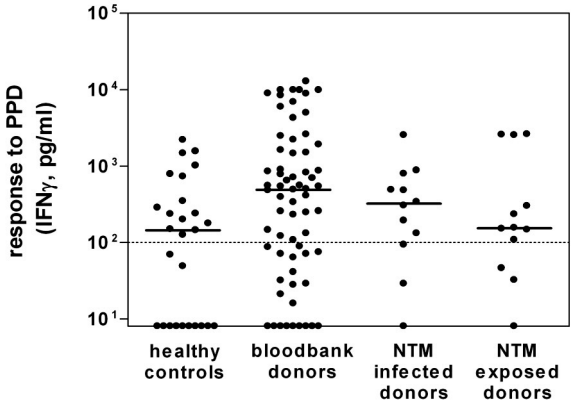

Figure 1. Results of an in vitro PPD survey in healthy individuals and NTM infected/exposed individuals. *Healthy controls were either a group of well defined healthy, M. tuberculosis-uninfected, non-BCG vaccinated individuals (n=27) or a group of random healthy blood bank donors (n=66). IFNγ responses to PPD were measured as an indication of exposure to mycobacteria. In both the healthy control and the healthy blood bank donor groups, over half or the individuals, 56% and 68% respectively, responded (IFNγ > 100 pg/ml) to PPD. The proportions of PPD responders in the NTM infected (n=12) or exposed (n=12) individuals were both 75 %, and were slightly higher compared to the healthy control groups. Horizontal bars show median values.*

and the lack of a national BCG vaccination policy, none of these parameters would be expected to be of high significance here.

Figure 1 shows in vitro IFNγ responses to PPD in both groups of healthy controls. In both groups, over half of the individuals, 56% and 68% respectively, responded in vitro to PPD (IFNγ>100 pg/ml). Although these responses are most likely the result of exposure to NTM, in the case of older blood bank donors we cannot formally exclude a contribution of remote TB exposure or BCG vaccination. The proportions of in vitro PPD responders in the NTM infected/exposed group (18/24 of these individuals had been TST-tested; overall with negative TST result) were slightly higher, both 75%. In general, the high levels of in vitro responsiveness to PPD in these selected study groups may be due to the higher sensitivity of in vitro six-day assays compared to the TST.

Table 2. Characteristics of NTM infected or exposed study subjects included in this study

Subject group	No of subjects	Sex, no. of men/ no.of women	Mean age, years (range)	vaccinated with BCG	Exposed to TB[a]	Clinical symptoms
M. marinum skin patients	7	6/1	58.3 (43.1-74.8)	1/7	0	typical aquarium granuloma[b]
M. kansasii lung patients	5	4/1	54.6 (40.5-76.4)	0/5	0	cavitary pneumonia[b]
						Median duration of exposure, years (range)
Aquarium owners	4	3/1	54.4 (40.6-62.3)	0/4	0	30.5 (10-40)
Veterinarians	3	3/0	38.9 (34.6-41.6)	0/3	0	11 (8-30)
Flower growers	5	5/0	46.7 (28.3-56.4)	0/5	0	38 (15-44)

[a], Received a diagnosis of active TB, was exposed to a patient with pulmonary TB, or was included in a contact investigation.
[b], All *M. marinum* and *M. kansasii* infections were culture proven.

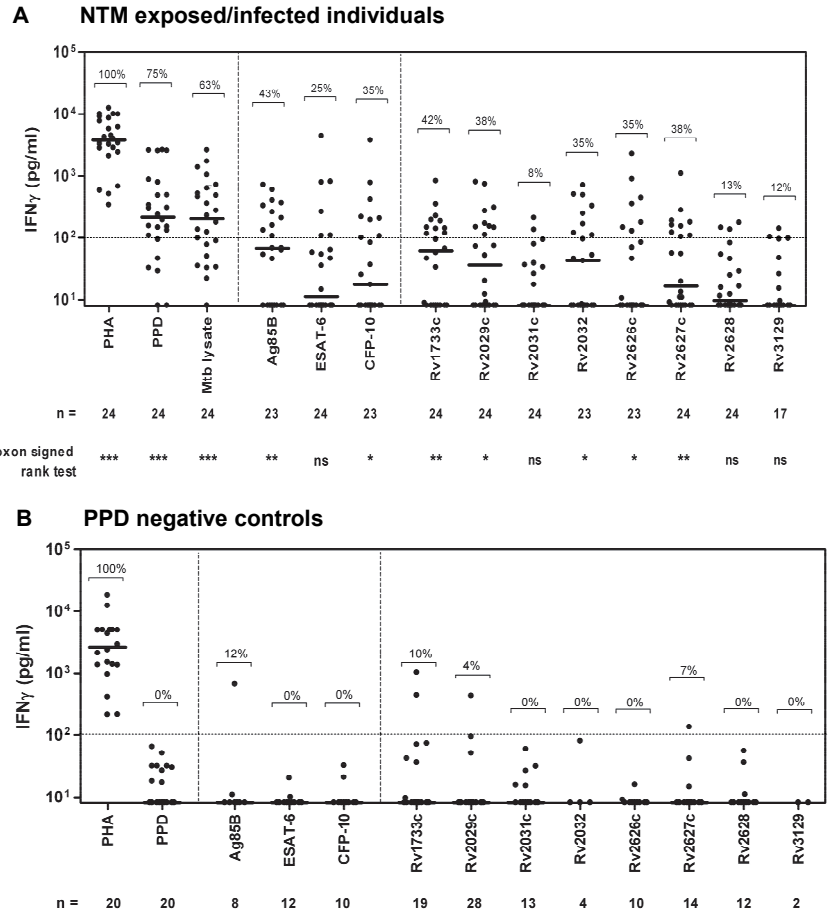

Figure 2. IFNγ responses to M. tuberculosis antigens in NTM primed individuals and in healthy, PPD negative (PPD⁻) individuals. *Responses to M. tuberculosis antigens: Mtb lysate, Ag85B, ESAT-6, CFP-10, and a series of eight DosR-regulon encoded antigens were measured in individuals infected with, or exposed intensely to environmental mycobacteria (n=24-17). Significant IFNγ production was measured in the NTM primed individuals* ***(A)****. Also responses to different batches of recombinant M. tuberculosis antigens Ag85B, ESAT-6, CFP-10 and eight DosR-regulon encoded antigens were tested in different PPD⁻ individuals (maximally 20 different PPD⁻ individuals). No or low (statistically non-significant) responses to the various recombinant M. tuberculosis antigens were observed in PPD⁻ individuals* ***(B)****. The percentage of responders is indicated above each column for each antigen. The Wilcoxon signed rank test was used for comparison of the height of the antigenic response to the negative control (medium) condition in (A). IFNγ responses ≥100 pg/ml were considered positive. Horizontal bars represent median values. PHA, phytohaemagglutenin; Mtb lysate, lysate of M.tuberculosis cultured under low oxygen condition. ***, P<0.0001; **, P<0.01; *, P <0.05; ns, not significant. P <0.05 was considered statistically significant.*

In vitro IFNγ responses to selected *M. tuberculosis* DosR-regulon encoded antigens in individuals infected with or exposed to environmental mycobacteria

We next studied 24 subjects with either proven NTM infection (n=12) or significant exposure to NTM (n=12) (Table 2). Antigens were tested in all 24 study subjects except for Ag85B, Rv2626c, Rv2032 and CFP-10 (all four in n=23), and Rv3129

(n=17), due to insufficient available PBMC from some donors. For five DosR-regulon encoded antigens a significant response was found (Rv1733c, *P*=0.0026; Rv2029c, *P*=0.011; Rv2032, *P*= 0.0341; Rv2626c, *P*= 0.0226 and Rv2627c, *P*= 0.0025; Wilcoxon signed rank test), Figure 2A. The Bonferroni correction was performed following the Wilcoxon signed rank test. The Bonferroni correction is regarded as conservative; combined with the small study population it may result in an underestimation of the significance of the results. Nevertheless, following this correction the responses to two *M. tuberculosis* DosR-regulon encoded antigens remained significant: Rv1733c (*P*= 0.033) and Rv2627c (*P*= 0.032).

The highest frequency of responders was detected following stimulation with Rv1733c (42% response), while only 8% responded to Rv2031c (confirming our previous observations (20)).

A significant proportion of the donors responded to control antigens Ag85B (43%), ESAT-6 (25%) and CFP-10 (35%). All responses to ESAT-6 and CFP-10 were confined to the individuals who also responded to PPD or *M. tuberculosis* lysate. As expected, many subjects responded to PPD and *M. tuberculosis* lysate, 75% and 63%, respectively.

Importantly, the responses and the proportion of responders to *M. tuberculosis* DosR-regulon encoded antigens were low (non-significant) or absent in healthy TST-negative *and* in vitro PPD negative individuals (Figure 2B) and *M. tuberculosis* lysate non-responsive individuals (20) showing that responses to *M. tuberculosis* DosR-regulon encoded antigens are associated with mycobacterium responsiveness and exposure.

Taken together, our results show that individuals infected with, or exposed to environmental mycobacteria, have high and significant IFNγ responses to individual antigens of the *M. tuberculosis* DosR-regulon encoded antigens. The majority of these individuals responded to the complex mycobacterial antigens PPD and *M. tuberculosis* hypoxic lysate in the absence of any detectable *M. tuberculosis* exposure, compatible with NTM infection/exposure.

IFNγ responses to *M. tuberculosis* DosR-regulon encoded antigens in individual groups with specific NTM infection or exposure

In the previous section, all NTM infected/exposed individuals were analyzed as a single group. Here, although numbers are relatively small, results are analyzed per subgroup: Figure 3 shows IFNγ responses to PPD, Ag85B, ESAT-6, CFP-10 and to *M. tuberculosis* DosR-regulon encoded antigens Rv1733c, Rv2029c, Rv2626c and Rv2627c in *M. marinum* patients (n=7), *M. kansasii* patients (n=5) and NTM exposed individuals (n=12).

A number of individuals responded to ESAT-6 and CFP-10 although these responses were low in *M. kansasii* infected individuals. Responses to CFP-10 were significantly higher in the *M. marinum* infected group (*P*<0.05), in line with our previous work (4).

Overall, responses to *M. tuberculosis* DosR-regulon encoded antigens are found in all different subgroups. Particularly to Rv2029c and Rv2626c higher responses were found in NTM exposed rather than NTM infected individuals.

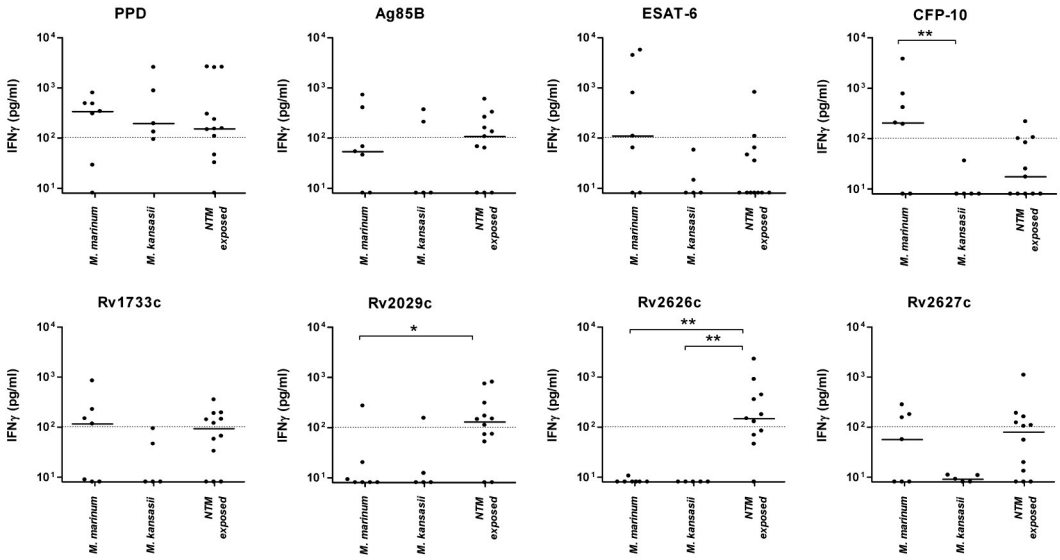

Figure 3. *IFNγ responses to M. tuberculosis antigens PPD, Ag85B, ESAT-6, CFP-10 and M. tuberculosis DosR-regulon encoded antigens Rv1733c, Rv2029c, Rv2626c and Rv2627c in individuals infected with M. marinum (n=7); M. kansasii (n=5); or intensely exposed to environmental mycobacteria (aquarium owners, flower growers and veterinarians, n= 12 (n=11 in the case of CFP-10, Ag85B, Rv2626c)). Horizontal bars represent median values. As indicated earlier, IFNγ responses ≥100 pg/ml were considered positive. Statistical analysis was performed with the Kruskal-Wallis test. If P <0.05, analysis was continued by performing Dunn's post-hoc test, *, P <0.05, **, P <0.01. P <0.05 was considered statistically significant.*

The presence of *M. tuberculosis* DosR regulon protein orthologs in environmental (myco) bacteria

Twenty-seven bacterial genomes were analyzed for the presence of orthologs of the 48 *M. tuberculosis* H37Rv DosR-regulon encoded antigens. The selection encompassed four *M. tuberculosis* strains (CDC1551, 'C', 'F11' and 'Haarlem'), three tuberculous, non-*M. tuberculosis* strains (*M. bovis*, *M. bovis* BCG and *M. leprae*), eight NTM strains (e.g. *M. marinum*) and twelve other (environmental) bacteria (e.g. *Streptomyces spp*) phylogenetically related to mycobacteria. Results are summarized in Table 3. When an ortholog was lacking the best homolog was selected. As expected, high degrees of similarities for all *M. tuberculosis* DosR-regulon sequences were found in *M. tuberculosis* strains: identities were close to or exactly 100% and few DosR-regulon orthologs scored below 99% similarity. In contrast, *M. leprae* had orthologs/homologs for only six *M. tuberculosis* DosR-regulon encoded antigens, compatible with its well-known genome downsizing (9). Nevertheless, *M. leprae* shared a high degree of similarity for other essential, *M. tuberculosis* non-DosR-regulon encoded proteins, including Rv0440 (Hsp65; 96% identity) and Rv3804 (Ag85A; 90% identity). *M. bovis* and *M. bovis* BCG both contain virtually identical orthologs of all 48 *M. tuberculosis* DosR-regulon encoded antigens (similarities from 98%-100%). Six non-DosR-regulon encoded antigens, TB10.4 (Rv0288), Hsp65 (Rv0440), Ag85B (Rv1886c), TB10.3 (Rv3019c), Ag85A (Rv3804c) and ESAT-6 (Rv3875), were

Table 3 (part 1). Analysis of orthologs or homologs of *Mycobacterium tuberculosis* H37Rv DosR encoded antigens in tuberculous mycobacteria and *M. leprae*

	% Similarity to H37Rv antigen[a]						
	Tuberculous mycobacteria						
H37Rv DosR antigens	*Mycobacterium tuberculosis* CDC1551	*Mycobacterium tuberculosis* strain "C"	*Mycobacterium tuberculosis* F11	*Mycobacterium tuberculosis* (Haarlem)	*Mycobacterium bovis* AF2122/97	*Mycobacterium bovis* BCG str. Pasteur 1173P2	*Mycobacterium leprae* TN
Rv0079	100	100	100	100	100	100	
Rv0080	100	100	100	100	99	98	
Rv0081	100	100	100	100	100	100	
Rv0569	100	100	100	71*	100	100	
Rv0570	99	100	100	100	100	100	46*
Rv0571c	100	100	100	100	100	99	
Rv0572c	99	99	99	99	99	99	
Rv0573c	100	100	100	100	100	100	
Rv0574c	100	100	100	100	99	99	
Rv1733c	98	99	99	99	99	99	
Rv1734c		100	100	100	98	100	
Rv1735c	100	100	100	100	100	100	
Rv1736c	100	100	100	100	99	99	
Rv1737c	100	100	100	97	100	100	43*
Rv1738	100	100	100	100	100	100	
Rv1812c	99	99	99	99	99	99	46*
Rv1813c	100	100	100	100	100	100	
Rv1996	99	100	100	100	100	100	
Rv1997	100	98	100	100	100	100	48*
Rv1998c	100	100	100	100	99	99	
Rv2003c	100	100	100	100	100	100	
Rv2004c	100	100	100	100	100	100	
Rv2005c	100	100	100	100	100	100	
Rv2006	100	57*	100	100	99	99	66*
Rv2007c	100	100	100	100	100	100	
Rv2028c	100	100	100	100	100	100	
Rv2029c	100	100	100	100	100	100	
Rv2030c	100	100	100	100	100	100	
Rv2031c	100	100	100	100	100	100	
Rv2032	100	100	100	100	99	99	
Rv2623	100	100	100	100	100	100	
Rv2624c	100	100	100	100	100	100	
Rv2625c	100	100	100	100	100	100	
Rv2626c	100	100	100	100	100	100	
Rv2627c	99	99	99	99	99	99	
Rv2628	100	100	100	100	98	99	
Rv2629	100	100	100	100	100	100	
Rv2630	100	100	100	100	100	100	
Rv2631	99		100	99	100	100	
Rv3126c	100	100	100	100	100	100	
Rv3127	100	100	100	100	99	99	
Rv3128c	62*	100	100	100	99	100	
Rv3129	100	100	100	100	100	100	
Rv3130c	99	100	99	100	100	100	44
Rv3131	100	100	100	100	100	100	
Rv3132c	100	100	100	100	99	99	
Rv3133c	100	100	100	100	100	100	
Rv3134c	100	100	100	100	100	100	
H37Rv non-DosR antigens							
Rv0288 (*esxH* /TB10.4)	100	100	100	100	100	100	85
Rv0440 (*groEL2* /Hsp65.2)	100	99	100	100	100	100	96
Rv1886c (*fbpB*/ Ag85B)	100	100	100	100	100	99	88
Rv3019c (*esxR* /TB10.3)	100	100	100	90*	100	100	82
Rv3804c (*fbpA* /Ag85A)	100	100	100	100	100	100	89
Rv3875 (*esxA* /esat-6)	100	100	100	100	100		65

[a] Orthologs and homologs (indicated with *) are expressed by percent similarity. A cutoff value score of 40% was used. Gene identities of corresponding orthologs or homologs in the assessed bacterial species are described in the Supplemental material

Table 3 (part 2). Analysis of orthologs or homologs of *Mycobacterium tuberculosis* H37Rv DosR encoded antigens in NTM.

	% Similarity to H37Rv antigen[a]							
	Non tuberculous mycobacteria (NTM)							
H37Rv DosR antigens	Mycobacterium sp. KMS	Mycobacterium avium 104	Mycobacterium marinum M	Mycobacterium ulcerans Agy99	Mycobacterium avium subsp. paratuberculosis str. K10	Mycobacterium sp. MCS	Mycobacterium smegmatis str. MC2 155	Mycobacterium vanbaalenii PYR-1
Rv0079	54		73	71		54	51	53
Rv0080	57		55*	55*		57	56	52*
Rv0081	69	78	87			69	66	68
Rv0569	88					87	71*	
Rv0570	87	42*	41*	41*	42*	87	42*	88
Rv0571c	62*	60*	78	77	60*	62*	65*	82
Rv0572c								84
Rv0573c	55*	53*	56*		53*	55*	56*	54*
Rv0574c								
Rv1733c	53	56	52			53	58	49
Rv1734c								
Rv1735c								
Rv1736c	87				75*	87	87	89
Rv1737c	88	41*	41*		40*	88	88	90
Rv1738			75*	73*				76
Rv1812c	46	42	83	83	42	46	44	46
Rv1813c			66	66*				
Rv1996		72*	77	76	66*	67*	65*	63*
Rv1997	82	49*	86	86	49*	82	62	62
Rv1998c	49		49	49		49	49	50
Rv2003c								
Rv2004c	63		75		48	63	68	67
Rv2005c	70*	79	87	87	80	70*	65*	67*
Rv2006	72	65*	71	69*	66*	72	73	74
Rv2007c	88	83	85	79	83	88	80	83
Rv2028c	60	42	57*	57*	59*	60	61	61
Rv2029c	74		79			74	77	77
Rv2030c	87	73*	89	58*	73*	87	75*	85
Rv2031c	76	59*	86		59*	64*	78	81
Rv2032	73	66	68*	67*	66	73	75	72
Rv2623	71	57	86	86	71*	71	68*	70
Rv2624c	68		84	55*	58*	68	60	61
Rv2625c			86	86				69
Rv2626c			60	60				84
Rv2627c	80	47	84	44*	47	80	46*	79
Rv2628		70			70			69
Rv2629	66	66	81	81	66	66		65
Rv2630								
Rv2631								
Rv3126c								
Rv3127	66	52*	81	81	65*	66	66	66*
Rv3128c								50*
Rv3129	73	80	80	80	80	73	76	66
Rv3130c	71	65*	78	79	65*	71	71	69
Rv3131	76	75	83	82	76	76	77	77
Rv3132c	78	67*	89	89	63	77	80*	77*
Rv3133c	92	94	95	95	94	92	94	94
Rv3134c	55*	66	79	78	66	59*	54*	55*
H37Rv non-DosR antigens								
Rv0288 (*esxH* /TB10.4)	86	89*	93*	92*	89*	86	85	85
Rv0440 (*groEL2* /Hsp65.2)	95	96	96	96	96	95	95	95
Rv1886c (*fbpB*/ Ag85B)	80	92	94	94	92	80	82	83
Rv3019c (*esxR* /TB10.3)	86*	91	94	93	91	86*	85*	81*
Rv3804c (*fbpA* /Ag85A)	80	89	90	90	89	80	80	83*
Rv3875 (*esxA* /esat-6)	74		96			74	80	58

[a] Orthologs and homologs (indicated with *) are expressed by percent similarity. A cutoff value score of 40% was used. Gene identities of corresponding orthologs or homologs in the assessed bacterial species are described in the Supplemental material

Table 3 (part 3). Analysis of orthologs or homologs of *Mycobacterium tuberculosis* H37Rv DosR encoded antigens in environmental bacteria.

	% Similarity to H37Rv antigen[a]											
	Environmental bacteria (non-mycobacterial)											
H37Rv DosR antigens	Streptomyces avermitilis MA-4680	Streptomyces coelicolor A3(2)	Corynebacterium diphtheriae NCTC 13129	Corynebacterium efficiens YS-314	Corynebacterium glutamicum ATCC 13032	Corynebacterium jeikeium K411	Rhodococcus sp. RHA1	Rhodobacter sphaeroides 2.4.1	Nocardia farcinica IFM 10152 DNA	Propionibacterium acnes KPA171202	Acidothermus cellulolyticus 11B	Bifidobacterium longum NCC2705
Rv0079									56			
Rv0080	52	57							68			
Rv0081		79		67	71		57					
Rv0569												
Rv0570	53	53	41*	40*	41*	41*	41*	57	41*	54	55	40*
Rv0571c	60*						66		63		72	
Rv0572c												
Rv0573c	56*	63	55*	54*	53*	54*	52*		52*	53*	55*	55*
Rv0574c	45	42					67				42	42
Rv1733c	43	46					51		43			
Rv1734c												
Rv1735c							64					
Rv1736c		72	72	72	74				71	66	80	
Rv1737c	43*	62	49	49	40*		43*		40*	40*	51	
Rv1738	53*						76					
Rv1812c	45	45	45*	46*	45*	42*	79	45*	43	47*	46*	
Rv1813c												
Rv1996	48*	49*	52	57*	55*	57*	60*		61*	44*		41*
Rv1997	48*	49*	52*	58	57	53*	53	60	51*	49*	48*	58
Rv1998c	60	56			42		51		48			
Rv2003c											51	
Rv2004c		56					61	46				
Rv2005c	50	50*	59*	59	58	54*	62		63	46		48
Rv2006		63		62		44*	72		61	46*		
Rv2007c	76*	75*	78*	80*	80*	78*	70	67*	72*	78*	85	73*
Rv2028c	42*	40*	40*	41*	41*	42*	47*		43*	41*		
Rv2029c	48	50	48	50	50		71	54	50	50		
Rv2030c	60*						61*		60*		58*	
Rv2031c											62	
Rv2032		46*					59		59			
Rv2623	47*	48*	55*	56*	56*	58	60*		59*	44*		49*
Rv2624c	42*	51*	43*	43*	43*	43*	48*		45*		50	43*
Rv2625c	44	45									45	
Rv2626c	74	56							70			
Rv2627c												
Rv2628												
Rv2629		46							50			
Rv2630												
Rv2631	44	45	44	45	47		44		45			
Rv3126c												
Rv3127		44*					56		56*			
Rv3128c												
Rv3129			58	56	58					67		
Rv3130c	46*	45*					61		51*		43*	
Rv3131		47					51		63			
Rv3132c	57*	64*	48*			47	74		70*	53*	72	
Rv3133c	80	80	60	62	58	58	87	49	85	61	77	63
Rv3134c	40*	44*	41*	42*	42*	42*	45*		47*	43*		
H37Rv non-DosR antigens												
Rv0288 (*esxH* /TB10.4)												
Rv0440 (*groEL2* /Hsp65.2)	90	89	88	89	89	89	93	76	94	87	90	84
Rv1886c (*fbpB*/ Ag85B)		42*	50*	53	51*	47*	55		53*			
Rv3019c (*esxR* /TB10.3)												
Rv3804c (*fbpA* /Ag85A)		42*	51	53*	53	56	56*		50*			
Rv3875 (*esxA* /esat-6)			60*	55*	55*	56						

[a] Orthologs and homologs (indicated with *) are expressed by percent similarity. A cutoff value score of 40% was used. Gene identities of corresponding orthologs or homologs in the assessed bacterial species are described in the Supplemental material

included as reference genes. These genes had orthologs in all seven tuberculous mycobacterial strains, with very high similarity to complete identity. TB10.3 and ESAT-6 formed the exception: for TB10.3 no ortholog was found in *M. leprae*, and as expected, no ESAT-6 ortholog was present in *M. bovis* BCG.
Forty-one out of 48 *M. tuberculosis* DosR-regulon encoded antigens appeared to have orthologs (or homologs) in the NTM species analyzed. The number of DosR regulon orthologs/homologs in the eight NTM species varied from the presence of 27 orthologs/homologs in *M. avium* to 39 in *Mycobacterium vanbaalenii*. Interestingly, almost half of the DosR-regulon encoded antigens were present in all eight NTM species. Orthologs of all five reference genes were found in the NTM.
Currently, the genome sequence of *M. kansasii* is incomplete and therefore was not included in Table 3. However, nucleotide BLAST searches with the available sequences from NCBI resulted in the identification of 13 *M. tuberculosis* DosR antigen homologs/orthologs (similarities 73%-84%) in *M. kansasii*, including Rv2627c (80% similarity) which was tested in this study.
Variable numbers of *M. tuberculosis* DosR regulon orthologs and homologs were also found in non-mycobacterial species such as *Streptomyces coelicolor* (present in soil) and *Bifidobacterium longum* (a gastrointestinal commensal). When assessing the bacterial species with regard to the average number of orthologs per single DosR-regulon encoded antigen, NTM had significantly higher numbers of DosR regulon encoded protein orthologs (average of 3.8 orthologs per single DosR regulon encoded antigen among eight examined NTM) when compared to the non-mycobacterial species (average of 2.5 orthologs per single DosR regulon encoded antigen among 12 examined non-mycobacteria).
In contrast, most reference antigens had few or no orthologs in environmental non-mycobacterial species, with the anticipated exception of the highly conserved gene *hsp65* (Rv0440).
An interesting observation concerned the *M. tuberculosis* DosR sequence (Rv3133c) itself: orthologs of this protein were found in almost all species assessed, *M. leprae* forming the exception. Equally interesting is the fact that these Rv3133c orthologs had a strikingly high degree of similarity among all mycobacterial species (from 93%–100%); and to a lesser extent also a considerable degree of similarity in more distantly related, non-mycobacterial species (ranging from 50%- 87%).
Taken together, our results demonstrate that *M. tuberculosis* DosR-regulon encoded proteins have orthologs in both environmental mycobacterial and non-mycobacterial species, with a remarkably high degree of similarity for the dormancy response regulator gene Rv3133c.

Discussion

This study was designed to investigate the hypothesis that infection with or exposure to NTM can induce cross-reactive immune responses to a recently identified set of *M. tuberculosis* antigens, namely *M. tuberculosis* DosR-regulon encoded antigens. Previously, we reported that *M. tuberculosis* infected individuals (active and latently)

can respond to *M. tuberculosis* DosR-regulon encoded antigens (11, 20, 32), and that responsiveness to these late stage antigens was associated with control of latent *M. tuberculosis* infection (20, 32). Unexpectedly, we observed that a significant proportion of healthy, *M. tuberculosis* uninfected, BCG unvaccinated individuals also responded to *M. tuberculosis* DosR-regulon encoded antigens, next to *M. tuberculosis*-lysate (20) and PPD in vitro (21). We therefore hypothesized that these responses might have resulted from exposure to NTM, with concomitant induction of cross-reactive immune responses to *M. tuberculosis* DosR-regulon encoded antigens.

Of note, very few in vitro PPD non-responsive individuals responded to the tested recombinant *M. tuberculosis* antigens (Figure 2B). Possible contamination of the recombinant proteins is difficult to exclude in the absence of a gold standard control, but our rigorous QA/QC procedures and the strong correlation between responses to *M. tuberculosis* lysate/PPD and test *M. tuberculosis* recombinant antigens strengthen the conclusion that responses observed are indeed truly specific. Moreover, the presence of *M. tuberculosis* DosR-regulon encoded homologs in non-mycobacterial species (Table 3) suggests that also non-mycobacteria might evoke responses to *M. tuberculosis* DosR-regulon encoded antigens.

From early to recent publications it is evident that environmental mycobacteria such as *M. kansasii, M. smegmatis* and *M. avium* are capable of adapting to conditions of starvation (3, 23, 38). *M. smegmatis* not only behaved similarly to slow growing *M. tuberculosis* under in vitro oxygen depletion and reactivation conditions (12) but also expressed orthologs of *M. tuberculosis* DosR antigens Rv2031c (α-crystallin, HspX), Rv3132c, Rv3133c (*DosR, devR*) and Rv3134c (22, 24). In addition, *M. bovis* BCG adapted to oxygen starvation in the same fashion as *M. tuberculosis* by up-regulating DosR-regulon encoded antigens Rv2031c, Rv3133c, Rv2623 and Rv2626c (6), although it was recently shown that *M. bovis* BCG is defective in the induction of the *M. tuberculosis* dormancy genes Rv1736c and Rv1737c (18).

The observed IFNγ responses of NTM infected/exposed individuals to *M. tuberculosis* antigens, including *M. tuberculosis* DosR-regulon encoded antigens, support our hypothesis. They also provide indirect evidence that NTM express DosR protein orthologs/homologs in vivo (Figure 2A and 3).

Recently, a number of mycobacterial genomes has been sequenced, including NTM genomes. We systematically analyzed these genomes for the presence of *M. tuberculosis* H37Rv DosR-regulon encoded orthologs (or when lacking, the best possible homolog) (Table 3). Results showed that the majority of mycobacterial genomes indeed encompass orthologs of *M. tuberculosis* DosR-regulon encoded genes. As expected, the highest similarities (analyzed at the amino acid sequence level in order to assess potential immunological cross-reactivity) were detected among members of the *M. tuberculosis* complex. Most relevant for our hypothesis was the fact that orthologs of 41 out of 48 different *M. tuberculosis* DosR-regulon encoded genes were detected in the genomes of NTM. Rather unexpectedly, several environmental *non*-mycobacterial species were also found to have appreciable numbers of *M. tuberculosis* DosR-regulon encoded orthologs. Of interest, the DosR response regulator itself, Rv3133c, was found in almost all species examined (with the exception of *M. leprae*, which is notorious for its downsized genome (9)). Rv3133c encodes a transcription factor that mediates the hypoxic response of *M. tuberculosis* (14, 27, 33,

34, 39). The widespread presence of Rv3133c orthologs and other members of the dormancy regulon suggest that adaptation to hypoxia is a characteristic shared by both pathogenic and non-pathogenic mycobacteria, and even by species outside the *Mycobacterium* genus.

In the current study, patients with documented *M. kansasii* and *M. marinum* infections were included. Both mycobacteria can cause disease in otherwise healthy, immunocompetent individuals. Although *M. avium* is more ubiquitously present in the environment, patients with *M. avium* infection were not included since *M. avium* infections are often associated with pre-existing disease or immune deficiency (5). Due to its ubiquitous nature, exposure to *M. avium* in our study groups can however not be excluded.

Analysis of the responses in the *M. marinum* or *M. kansasii* infected patients showed that the former patients seemed to respond equally well to Rv1733c and Rv2627c despite the fact that *M. marinum* orthologs of these two *M. tuberculosis* genes vary in the degree of similarity (52.3% and 84.5% respectively). In contrast, responses to Rv2029c, Rv2626c (Figure 3) and Rv2032 (data not shown) (29) were practically absent despite the presence of orthologs of these genes in *M. marinum*. High responses to *M. tuberculosis* DosR-regulon encoded antigens Rv1733c, Rv2029c, Rv2626c, and Rv2627c were observed in those who were intensely exposed to environmental mycobacteria. Our study is not sufficiently powered to explain these particular observations. One limitation is that levels of exposure to NTM in the latter group are unquantified as no valid set of criteria is available. Although the small numbers may have caused some of the observed differences, it also remains possible that different immune recognition profiles may have developed specifically following NTM infection or exposure, due to possible differences in expression of antigens. Additionally, immuno-modulatory effects of repeated mycobacterial infection/exposure may have shaped the observed recognition profile. In any case, further studies will be needed to confirm and extend these findings.

In conclusion, we document T-cell immunity (IFNγ responses) to *M. tuberculosis* DosR-regulon encoded antigens in individuals infected with or exposed to NTM, in the absence of *M. tuberculosis* infection, *M. tuberculosis* exposure or BCG vaccination. The results lend support to the hypothesis that *M. tuberculosis* DosR-regulon encoded antigen directed responses can be the result of exposure to or infection with cross-reacting NTM. The results are corroborated by the presence of orthologs of many different *M. tuberculosis* DosR antigens in environmental mycobacteria as well as non-mycobacteria. Prior studies have suggested significant effects of prior sensitization by NTM on BCG vaccination and host immunity to *M. tuberculosis*, but the antigens involved have not been identified (13, 25, 26). Our results warrant more specific studies to analyse the contribution and influence of cross-reactive immunity following NTM infection on host responses to *M. tuberculosis*, BCG and potential novel TB vaccines.

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Supplementary file

List of Organisms/Strains assessed for orthologs or homologs of *M. tuberculosis* H37Rv DosR antigens

MT_H37RV_V2 *Mycobacterium tuberculosis* H37Rv complete genome (V2) AL123456
MT_CDC1551 *Mycobacterium tuberculosis CDC1551* , complete genome
MT_C_V1 *Mycobacterium tuberculosis* strain "C" assembly 1
MT_F11_V4 *Mycobacterium tuberculosis* F11 assembly 4 (finished)
MT_H_V1 *Mycobacterium tuberculosis* (Haarlem) assembly 1
MB_AF2122_2 *Mycobacterium bovis* AF2122/97, complete genome
MBCG1 *Mycobacterium bovis BCG* str. Pasteur 1173P2, complete genome
ML_TN2 *Mycobacterium leprae* TN, complete genome
KMS *Mycobacterium* sp. KMS assembly, from Genbank
MA104 *Mycobacterium avium* 104, complete genome
MN1 *Mycobacterium marinum* M, complete genome
MU1 *Mycobacterium ulcerans* Agy99, complete genome
MA_K10 *Mycobacterium avium* subsp. paratuberculosis str. k10, completegenome
MT_MCS *Mycobacterium* sp. MCS, complete genome
MT_Smeg *Mycobacterium smegmatis* str. MC2 155, complete genome
MV1 *Mycobacterium vanbaalenii* PYR-1, complete genome
SM_AVER2 *Streptomyces avermitilis* MA-4680 assembly, from Genbank
SM_COEL_V2 *Streptomyces coelicolor* A3(2) assembly, from Genbank
CD1 *Corynebacterium diphtheriae* NCTC 13129, complete genome
CE1 *Corynebacterium efficiens* YS-314, complete genome
CGB1 *Corynebacterium glutamicum* ATCC 13032, complete genome
CJ1 *Corynebacterium jeikeium* K411 assembly, from Genbank
RHA1 *Rhodococcus sp* . RHA1 assembly, from Genbank
RSP1 *Rhodobacter sphaeroides* 2.4.1 assembly, from Genbank
NCF1 *Nocardia farcinica* IFM 10152 DNA, complete genome
PBA1 *Propionibacterium acnes* KPA171202, complete genome
ATC1 *Acidothermus cellulolyticus* 11B, complete genome
BFL1 B*ifidobacterium longum* NCC2705, complete genome

M. tuberculosis **H37Rv DosR orthologs in different mycobacterial and non-mycobacterial species. Homologs are indicated in a coloured box. Orthologs and homologs are expressed as % similarity (part 1).**

H37Rv DosR antigens	MT_CDC1551	MT_C_V1	MT_F11_V4	MT_H_V1	MB_AF2122_2	MBCG1	ML_TN2	KMS	MA104	MN1
Rv0079	MT0086 (100.00)	TBCG_00080 (100.00)	TBFG_10080 (100.00)	TBHG_00080 (100.00)	Mb0082 (100.00)	BCG_0112 (100.00)		Mkms_1112 (54.15)		MMAR_3705 (73.85)
Rv0080	MT0087 (100.00)	TBCG_00081 (100.00)	TBFG_10081 (100.00)	TBHG_00081 (100.00)	Mb0083 (99.34)	BCG_0113 (98.68)		Mkms_3695 (57.14)		MMAR_1521 (55.28)
Rv0081	MT0088 (100.00)	TBCG_00082 (100.00)	TBFG_10082 (100.00)	TBHG_00082 (100.00)	Mb0084 (100.00)	BCG_0114 (100.00)		Mkms_4402 (69.23)	MAV_5108 (78.85)	MMAR_1653 (87.96)
Rv0569	MT0595 (100.00)	TBCG_00564 (100.00)	TBFG_10579 (100.00)	TBHG_02240 (71.05)	Mb0584 (100.00)	BCG_0614 (100.00)		Mkms_1101 (88.10)		
Rv0570	MT0596 (99.86)	TBCG_00565 (100.00)	TBFG_10580 (100.00)	TBHG_00566 (100.00)	Mb0585 (100.00)	BCG_0615 (100.00)	ML1734 (46.16)	Mkms_1102 (87.21)	MAV_3919 (42.10)	MMAR_1642 (41.92)
Rv0571c	MT0597 (100.00)	TBCG_00566 (100.00)	TBFG_10581 (100.00)	TBHG_00567 (100.00)	Mb0586c (100.00)	BCG_0616c (99.77)		Mkms_3308 (62.56)	MAV_2347 (60.58)	MMAR_2069 (78.67)
Rv0572c	MT0599 (99.12)	TBCG_00567 (99.12)	TBFG_10582 (99.12)	TBHG_00568 (99.12)	Mb0587c (99.12)	BCG_0617c (99.12)				
Rv0573c	MT0601 (100.00)	TBCG_00568 (100.00)	TBFG_10583 (100.00)	TBHG_00569 (100.00)	Mb0588c (100.00)	BCG_0618c (100.00)		Mkms_3928 (55.30)	MAV_1550 (53.88)	MMAR_4068 (56.28)
Rv0574c	MT0602 (100.00)	TBCG_00569 (100.00)	TBFG_10584 (100.00)	TBHG_00570 (100.00)	Mb0589c (99.74)	BCG_0619c (99.74)				
Rv1733c	MT1774 (98.99)	TBCG_01688 (99.05)	TBFG_11750 (99.52)	TBHG_01690 (99.52)	Mb1762c (99.52)	BCG_1772c (99.52)		Mkms_3466 (53.96)	MAV_1986 (56.92)	MMAR_3501 (52.33)
Rv1734c		TBCG_01689 (100.00)	TBFG_11751 (100.00)	TBHG_01691 (100.00)	Mb1763c (98.75)	BCG_1773c (100.00)				
Rv1735c	MT1776 (100.00)	TBCG_01690 (100.00)	TBFG_11752 (100.00)	TBHG_01692 (100.00)	Mb1764c (100.00)	BCG_1774c (100.00)				
Rv1736c	MT1778 (100.00)	TBCG_01692 (100.00)	TBFG_11754 (100.00)	TBHG_01693 (100.00)	Mb1765c (99.69)	BCG_1775c (99.69)		Mkms_1277 (87.61)		
Rv1737c	MT1779 (100.00)	TBCG_01693 (100.00)	TBFG_11755 (100.00)	TBHG_01694 (97.26)	Mb1766c (100.00)	BCG_1776c (100.00)	ML0844 (43.14)	Mkms_1281 (88.74)	MAV_4892 (41.50)	MMAR_0526 (41.85)
Rv1738	MT1780 (100.00)	TBCG_01694 (100.00)	TBFG_11756 (100.00)	TBHG_01695 (100.00)	Mb1767 (100.00)	BCG_1777 (100.00)				MMAR_2068 (75.00)
Rv1812c	MT1860 (99.75)	TBCG_01765 (99.75)	TBFG_11842 (99.75)	TBHG_01769 (99.75)	Mb1842c (99.75)	BCG_1846c (99.75)	ML2061 (46.39)	Mkms_4356 (46.11)	MAV_1130 (42.69)	MMAR_2688 (83.75)
Rv1813c	MT1861 (100.00)	TBCG_01766 (100.00)	TBFG_11843 (100.00)	TBHG_01770 (100.00)	Mb1843c (100.00)	BCG_1847c (100.00)				MMAR_1426 (66.43)
Rv1996	MT2052 (99.68)	TBCG_01948 (100.00)	TBFG_12028 (100.00)	TBHG_01954 (100.00)	Mb2019 (100.00)	BCG_2013 (100.00)			MAV_2494 (72.33)	MMAR_5410 (77.10)
Rv1997	MT2053 (100.00)	TBCG_01949 (98.08)	TBFG_12029 (100.00)	TBHG_01955 (100.00)	Mb2020 (100.00)	BCG_2014 (100.00)	ML2671 (48.57)	Mkms_1118 (82.31)	MAV_4235 (49.13)	MMAR_0860 (86.44)
Rv1998c	MT2054 (100.00)	TBCG_01950 (100.00)	TBFG_12030 (100.00)	TBHG_01956 (100.00)	Mb2021c (99.61)	BCG_2015c (99.61)		Mkms_0266 (49.03)		MMAR_0514 (49.03)
Rv2003c	MT2059 (100.00)	TBCG_01955 (100.00)	TBFG_12035 (100.00)	TBHG_01961 (100.00)	Mb2026c (100.00)	BCG_2020c (100.00)				
Rv2004c	MT2060 (100.00)	TBCG_01956 (100.00)	TBFG_12036 (100.00)	TBHG_01962 (100.00)	Mb2027c (100.00)	BCG_2021c (100.00)		Mkms_1090 (63.77)		MMAR_3476 (75.63)
Rv2005c	MT2061 (100.00)	TBCG_01957 (100.00)	TBFG_12037 (100.00)	TBHG_01963 (100.00)	Mb2028c (100.00)	BCG_2022c (100.00)		Mkms_1121 (70.82)	MAV_2507 (79.32)	MMAR_2993 (87.80)
Rv2006	MT2062 (100.00)	TBCG_03316 (57.14)	TBFG_12038 (100.00)	TBHG_01964 (100.00)	Mb2029 (99.85)	BCG_2023 (99.92)	ML0414 (66.16)	Mkms_3470 (72.44)	MAV_4338 (65.05)	MMAR_2257 (71.51)
Rv2007c	MT2063 (100.00)	TBCG_01959 (100.00)	TBFG_12039 (100.00)	TBHG_01965 (100.00)	Mb2030c (100.00)	BCG_2024c (100.00)		Mkms_2763 (88.07)	MAV_3500 (83.64)	MMAR_2994 (85.45)
Rv2028c	MT2087 (100.00)	TBCG_01981 (100.00)	TBFG_12065 (100.00)	TBHG_01987 (100.00)	Mb2053c (100.00)	BCG_2047c (100.00)		Mkms_4202 (60.59)	MAV_4010 (42.85)	MMAR_1515 (57.70)
Rv2029c	MT2088 (100.00)	TBCG_01982 (100.00)	TBFG_12066 (100.00)	TBHG_01988 (100.00)	Mb2054c (100.00)	BCG_2048c (100.00)		Mkms_3475 (74.60)		MMAR_3482 (79.19)
Rv2030c	MT2089 (100.00)	TBCG_01983 (100.00)	TBFG_12067 (100.00)	TBHG_01989 (100.00)	Mb2056c (100.00)	BCG_2049c (100.00)		Mkms_1097 (87.31)	MAV_2347 (73.95)	MMAR_3483 (89.84)
Rv2031c	MT2090 (100.00)	TBCG_01984 (100.00)	TBFG_12068 (100.00)	TBHG_01990 (100.00)	Mb2057c (100.00)	BCG_2050c (100.00)		Mkms_1098 (76.06)	MAV_4906 (59.78)	MMAR_3484 (86.52)
Rv2032	MT2091 (100.00)	TBCG_01985 (100.00)	TBFG_12069 (100.00)	TBHG_01991 (100.00)	Mb2058 (99.70)	BCG_2051 (99.70)		Mkms_1091 (73.01)	MAV_2505 (66.36)	MMAR_3487 (68.00)
Rv2623	MT2698 (100.00)	TBCG_02565 (100.00)	TBFG_12642 (100.00)	TBHG_02561 (100.00)	Mb2656 (100.00)	BCG_2650 (100.00)		Mkms_0733 (71.53)	MAV_2491 (57.31)	MMAR_2077 (86.78)
Rv2624c	MT2699 (100.00)	TBCG_02566 (100.00)	TBFG_12643 (100.00)	TBHG_02562 (100.00)	Mb2657c (100.00)	BCG_2651c (100.00)		Mkms_1104 (68.58)		MMAR_2076 (84.41)
Rv2625c	MT2700 (100.00)	TBCG_02567 (100.00)	TBFG_12644 (100.00)	TBHG_02563 (100.00)	Mb2658c (100.00)	BCG_2652c (100.00)				MMAR_2075 (86.96)
Rv2626c	MT2701 (100.00)	TBCG_02568 (100.00)	TBFG_12645 (100.00)	TBHG_02564 (100.00)	Mb2659c (100.00)	BCG_2653c (100.00)				MMAR_2620 (60.00)
Rv2627c	MT2702 (99.76)	TBCG_02569 (99.76)	TBFG_12646 (99.76)	TBHG_02565 (99.76)	Mb2660c (99.76)	BCG_2654c (99.76)		Mkms_4343 (80.79)	MAV_1605 (47.42)	MMAR_2072 (84.50)
Rv2628	MT2703 (100.00)	TBCG_02570 (100.00)	TBFG_12647 (100.00)	TBHG_02566 (100.00)	Mb2661 (98.33)	BCG_2655 (99.17)			MAV_3503 (70.65)	
Rv2629	MT2704 (100.00)	TBCG_02571 (100.00)	TBFG_12648 (100.00)	TBHG_02567 (100.00)	Mb2662 (100.00)	BCG_2656 (100.00)		Mkms_2789 (66.76)	MAV_0244 (66.12)	MMAR_2070 (81.82)
Rv2630	MT2705 (100.00)	TBCG_02572 (100.00)	TBFG_12649 (100.00)	TBHG_02568 (100.00)	Mb2663 (100.00)	BCG_2657 (100.00)				
Rv2631	MT2707 (99.77)		TBFG_12650 (100.00)	TBHG_02569 (99.77)	Mb2664 (100.00)	BCG_2658 (100.00)				
Rv3126c	MT3211 (100.00)	TBCG_03063 (100.00)	TBFG_13146 (100.00)	TBHG_03057 (100.00)	Mb3149c (100.00)	BCG_3148c (100.00)				
Rv3127	MT3212 (100.00)	TBCG_03064 (100.00)	TBFG_13147 (100.00)	TBHG_03058 (100.00)	Mb3150 (99.71)	BCG_3149 (99.71)		Mkms_3473 (66.56)	MAV_2502 (52.03)	MMAR_1522 (81.96)
Rv3128c	MT2874 (62.5)	TBCG_03065 (100.00)	TBFG_13149 (100.00)	TBHG_03059 (100.00)	Mb3152c (99.48)	BCG_3151c (100.00)				
Rv3129	MT3215 (100.00)	TBCG_03066 (100.00)	TBFG_13150 (100.00)	TBHG_03060 (100.00)	Mb3153 (100.00)	BCG_3152 (100.00)		Mkms_1100 (73.91)	MAV_4009 (80.20)	MMAR_1521 (80.20)
Rv3130c	MT3216 (99.78)	TBCG_03067 (100.00)	TBFG_13151 (99.78)	TBHG_03061 (100.00)	Mb3154c (100.00)	BCG_3153c (100.00)	ML1244 (44.26)	Mkms_3472 (71.81)	MAV_4336 (65.00)	MMAR_1519 (78.83)
Rv3131	MT3217 (100.00)	TBCG_03068 (100.00)	TBFG_13152 (100.00)	TBHG_03062 (100.00)	Mb3155 (100.00)	BCG_3154 (100.00)		Mkms_1094 (76.95)	MAV_4111 (75.85)	MMAR_1518 (83.08)
Rv3132c	MT3218 (100.00)	TBCG_03069 (100.00)	TBFG_13153 (100.00)	TBHG_03063 (100.00)	Mb3156c (99.83)	BCG_3155c (99.83)		Mkms_1093 (78.00)	MAV_2508 (67.35)	MMAR_1517 (89.42)
Rv3133c	MT3219 (100.00)	TBCG_03070 (100.00)	TBFG_13154 (100.00)	TBHG_03064 (100.00)	Mb3157c (100.00)	BCG_3156c (100.00)		Mkms_4201 (92.82)	MAV_4109 (94.84)	MMAR_1516 (95.79)
Rv3134c	MT3220 (100.00)	TBCG_03071 (100.00)	TBFG_13155 (100.00)	TBHG_03065 (100.00)	Mb3158c (100.00)	BCG_3157c (100.00)		Mkms_1122 (55.00)	MAV_4010 (66.42)	MMAR_1515 (79.01)
H37 Rv non-DosR antigens										
Rv0288 (*esxH* / TB10.4)	MT0301 (100.00)	TBCG_00285 (100.00)	TBFG_10295 (100.00)	TBHG_00287 (100.00)	Mb0296 (100.00)	BCG_0328 (100.00)	ML2531 (85.42)	Mkms_0386 (86.32)	MAV_4865 (89.60)	MMAR_0547 (93.75)
Rv0440 (*groEL2* / hsp65.2)	MT0456 (100.00)	TBCG_00432 (99.80)	TBFG_10445 (100.00)	TBHG_00436 (100.00)	Mb0448 (100.00)	BCG_0479 (100.00)	ML0317 (96.48)	Mkms_0620 (95.56)	MAV_4707 (96.48)	MMAR_0759 (96.30)
Rv1886c (*fbpB* / Ag85B)	MT1934 (100.00)	TBCG_01838 (100.00)	TBFG_11914 (100.00)	TBHG_01841 (100.00)	Mb1918c (100.00)	BCG_1923c (99.69)	ML2028 (88.89)	Mkms_1620 (80.12)	MAV_2816 (92.28)	MMAR_2777 (94.75)
Rv3019c (*esxR* / TB10.3)	MT3104 (100.00)	TBCG_02955 (100.00)	TBFG_13035 (100.00)	TBHG_00287 (90.62)	Mb3045c (100.00)	BCG_3042c (100.00)	ML2531 (82.29)	Mkms_0386 (86.4)	MAV_4865 (91.67)	MMAR_0547 (94.79)
Rv3804c (*fbpA* / Ag85A)	MT3911 (100.00)	TBCG_03730 (100.00)	TBFG_13838 (100.00)	TBHG_03742 (100.00)	Mb3834c (100.00)	BCG_3866c (100.00)	ML0097 (89.97)	Mkms_5103 (80.18)	MAV_0214 (89.35)	MMAR_5368 (90.83)
Rv3875 (*esxA* / esat-6)	MT3989 (100.00)	TBCG_03802 (100.00)	TBFG_13910 (100.00)	TBHG_03813 (100.00)	Mb3905 (100.00)		ML0049 (65.93)	Mkms_0080 (74.74)		MMAR_5450 (96.84)

M. tuberculosis **H37Rv DosR orthologs in different mycobacterial and non-mycobacterial species. Homologs are indicated in a coloured box. Orthologs and homologs are expressed as % similarity (part 2).**

H37Rv DosR antigens	MU1	MA_K10	MT_MCS	MT_Smeg	MV1	SM_AVER2	SM_COEL_V2	CD1	CE1	CGB1
Rv0079	MUL_3647 (71.84)		Mmcs_1095 (54.15)	MSMEG_3935 (51.45)	Mvan_1377 (53.25)					
Rv0080	MUL_2418 (55.28)		Mmcs_3622 (57.14)	MSMEG_5136 (56.82)	Mvan_5621 (52.63)	SAV1271 (52.74)	SCJ12.26 (57.48)			
Rv0081			Mmcs_4316 (69.23)	MSMEG_6451 (66.35)	Mvan_5692 (68.52)		SCH35.02 (79.09)		CE0945 (67.62)	cg0993 (71.58)
Rv0569			Mmcs_1084 (87.50)	MSMEG_4361 (71.42)						
Rv0570	MUL_1878 (41.92)	MAP3100c (42.10)	Mmcs_1085 (87.21)	MSMEG_2299 (42.10)	Mvan_1398 (88.21)	SAV2461 (53.86)	SCO5805 (53.86)	DIP1867 (41.74)	CE2423 (40.81)	cg2786 (41.15)
Rv0571c	MUL_3276 (77.75)	MAP1887 (60.57)	Mmcs_3246 (62.56)	MSMEG_4215 (65.34)	Mvan_1399 (82.21)	SAV5710 (60.29)				
Rv0572c					Mvan_2913 (84.93)					
Rv0573c		MAP2430 (53.88)	Mmcs_3854 (55.29)	MSMEG_4911 (56.23)	Mvan_4297 (54.87)	SAV5158 (56.89)	SCJ1.20 (63.68)	DIP1858 (55.12)	CE2412 (54.63)	cg2774 (53.65)
Rv0574c						SAV7293 (45.71)	SCM10.29c (42.51)			
Rv1733c			Mmcs_3403 (53.96)	MSMEG_1127 (58.55)	Mvan_1379 (49.16)	SAV1512 (43.86)	SCJ1.26 (46.78)			
Rv1734c										
Rv1735c										
Rv1736c		MAP2617c (75.63)	Mmcs_1260 (87.61)	MSMEG_5137 (87.55)	Mvan_4543 (89.32)		SCO6532 (72.03)	DIP0497 (72.49)	CE1293 (72.29)	cg1341 (74.43)
Rv1737c	MUL_3265 (32.61)	MAP2102c (39.69)	Mmcs_1264 (88.74)	MSMEG_5141 (88.57)	Mvan_4547 (90.18)	SAV5119 (43.73)	SCJ12.25c (62.79)	DIP0502 (49.09)	CE1416 (49.71)	cg1345 (39.61)
Rv1738	MUL_3277 (73.75)				Mvan_1414 (76.92)	SAV7348 (53.77)				
Rv1812c	MUL_3068 (83.75)	MAP0947c (42.69)	Mmcs_4270 (46.11)	MSMEG_6818 (44.38)	Mvan_4805 (46.73)	SAV425 (45.98)	SCJ1.07 (45.82)	DIP1748 (45.00)	CE0339 (46.04)	cg1656 (45.60)
Rv1813c	MUL_4016 (66.27)									
Rv1996	MUL_5033 (76.45)	MAP1742c (66.90)	Mmcs_3621 (67.60)	MSMEG_3940 (65.72)	Mvan_1963 (63.82)	SAV1275 (48.76)	SCJ12.12c (49.14)	DIP2276 (52.84)	CE2791 (57.63)	cg3255 (55.32)
Rv1997	MUL_0612 (86.22)	MAP0843 (49.13)	Mmcs_1101 (82.31)	MSMEG_3926 (62.50)	Mvan_1648 (62.22)	SAV5331 (48.61)	SCG20A.26 (49.35)	DIP2286 (52.63)	CE1670 (58.70)	cg1744 (57.54)
Rv1998c	MUL_1177 (49.49)		Mmcs_0256 (49.03)	MSMEG_0422 (49.03)	Mvan_0289 (50.97)	SAV2103 (60.41)	SC1A9.07 (56.33)			cg1206 (42.64)
Rv2003c										
Rv2004c		MAP2082 (48.21)	Mmcs_1074 (63.77)	MSMEG_3942 (68.49)	Mvan_1384 (67.58)		SCJ1.12 (56.84)			
Rv2005c	MUL_2234 (87.12)	MAP1741c (80.00)	Mmcs_1104 (70.83)	MSMEG_3940 (65.54)	Mvan_1378 (67.58)	SAV1275 (50.00)	SCJ1.29c (50.72)	DIP2276 (59.02)	CE2791 (59.15)	cg3255 (58.80)
Rv2006	MUL_0921 (69.16)	MAP3478 (66.27)	Mmcs_3407 (72.44)	MSMEG_3954 (73.22)	Mvan_4107 (74.58)		SCF55.40c (63.02)		CE1089 (62.07)	
Rv2007c	MUL_3264 (79.09)	MAP2726c (83.64)	Mmcs_2719 (88.07)	MSMEG_1124 (80.00)	Mvan_0299 (83.64)	SAV3129 (76.47)	SC9E12.20 (75.49)	DIP0973 (78.43)	CE1160 (80.39)	cg1252 (80.39)
Rv2028c	MUL_2424 (57.70)	MAP3179c (59.30)	Mmcs_4127 (60.59)	MSMEG_5245 (61.57)	Mvan_2549 (61.36)	SAV1300 (42.30)	SCJ1.30c (40.13)	DIP2276 (40.76)	CE2791 (41.11)	cg3255 (41.46)
Rv2029c			Mmcs_3412 (74.60)	MSMEG_3947 (77.14)	Mvan_4105 (77.10)	SAV3689 (48.90)	SCE22.14c (50.17)	DIP1430 (48.96)	CE1828 (50.34)	cg2119 (50.00)
Rv2030c	MUL_3276 (58.36)	MAP1887 (73.63)	Mmcs_1081 (87.31)	MSMEG_4215 (75.11)	Mvan_1393 (85.42)	SAV5710 (60.59)				
Rv2031c		MAP3701c (59.78)	Mmcs_0257 (64.55)	MSMEG_3932 (78.87)	Mvan_1374 (81.16)					
Rv2032	MUL_2417 (67.66)	MAP1743c (66.36)	Mmcs_1075 (73.01)	MSMEG_3943 (75.47)	Mvan_2550 (72.39)		SCJ1.11 (46.39)			
Rv2623	MUL_3267 (86.44)	MAP1741c (71.20)	Mmcs_0719 (71.53)	MSMEG_3940 (68.97)	Mvan_1963 (70.31)	SAV1300 (47.58)	SCJ1.16c (48.97)	DIP2276 (55.90)	CE2791 (56.94)	cg3255 (56.25)
Rv2624c	MUL_2424 (55.38)	MAP3179c (58.96)	Mmcs_1087 (68.58)	MSMEG_3939 (60.38)	Mvan_1387 (61.74)	SAV1275 (42.90)	SCJ12.19c (51.35)	DIP2276 (43.75)	CE2791 (43.81)	cg3255 (43.83)
Rv2625c	MUL_3269 (86.96)				Mvan_1412 (69.92)	SAV6673 (44.17)	SCJ41.35c (45.26)			
Rv2626c	MUL_3142 (60.00)				Mvan_1401 (84.51)	SAV6263 (74.07)	SCM1.29c (56.03)			
Rv2627c	MUL_1215 (44.89)	MAP2375c (47.42)	Mmcs_4257 (80.79)	MSMEG_0625 (46.98)	Mvan_1418 (79.23)					
Rv2628		MAP2728 (70.65)			Mvan_1397 (69.70)					
Rv2629	MUL_3275 (81.82)	MAP0246c (66.39)	Mmcs_2745 (66.76)		Mvan_1404 (65.84)		SC1E6.22c (46.44)			
Rv2630										
Rv2631						SAV4753 (44.78)	SCE15.16c (45.48)	DIP2025 (44.99)	CE2835 (45.05)	cg3329 (47.64)
Rv3126c										
Rv3127	MUL_2417 (81.35)	MAP1743c (65.94)	Mmcs_3410 (66.56)	MSMEG_3952 (66.25)	Mvan_1383 (66.87)		SCJ12.27c (44.26)			
Rv3128c					Mvan_2415 (50.48)					
Rv3129	MUL_2418 (80.20)	MAP3177 (80.20)	Mmcs_1083 (73.91)	MSMEG_5243 (76.60)	Mvan_5621 (66.34)			DIP1874 (58.89)	CE2430 (56.25)	cg2794 (58.33)
Rv3130c	MUL_2420 (79.05)	MAP3477 (65.21)	Mmcs_3409 (71.81)	MSMEG_5242 (71.24)	Mvan_4104 (69.65)	SAV7256 (46.92)	SCM11.13c (45.64)			
Rv3131	MUL_2421 (82.48)	MAP3273c (76.47)	Mmcs_1078 (76.95)	MSMEG_3955 (77.09)	Mvan_2547 (77.26)		SCJ12.27c (47.19)			
Rv3132c	MUL_2422 (89.42)	MAP3270c (63.22)	Mmcs_1077 (77.79)	MSMEG_5241 (80.07)	Mvan_1395 (77.41)	SAV4257 (57.43)	SCH22A.01 (64.28)	DIP2326 (48.01)		
Rv3133c	MUL_2423 (95.79)	MAP3271c (94.84)	Mmcs_4126 (92.82)	MSMEG_5244 (94.26)	Mvan_2548 (94.88)	SAV4375 (80.48)	SCJ12.16c (80.75)	DIP0600 (60.20)	CE1356 (62.62)	cg2200 (58.74)
Rv3134c	MUL_2424 (78.63)	MAP3179c (66.42)	Mmcs_4127 (59.82)	MSMEG_3939 (54.02)	Mvan_2549 (55.75)	SAV1300 (40.06)	SCJ1.29c (44.97)	DIP2276 (41.69)	CE2791 (42.47)	cg3255 (42.08)
H37 Rv non-DosR antigens										
Rv0288 (*esxH* / TB10.4)	MUL_1210 (92.70)	MAP3784 (89.58)	Mmcs_0377 (86.32)	MSMEG_0621 (85.26)	Mvan_0418 (85.26)					
Rv0440 (*groEL2* / hsp65.2)	MUL_1393 (96.11)	MAP3936 (96.48)	Mmcs_0607 (95.56)	MSMEG_0880 (95.74)	Mvan_0764 (95.56)	SAV3931 (90.00)	SCD95A.29 (89.07)	DIP2020 (88.70)	CE2561 (89.41)	cg3011 (89.22)
Rv1886c (*fbpB* / Ag85B)	MUL_2970 (94.44)	MAP1609c (92.59)	Mmcs_1596 (80.12)	MSMEG_2078 (82.97)	Mvan_1902 (83.97)		SC1f2.06 (42.62)	DIP2193 (50.36)	CE2709 (53.29)	cg3186 (51.05)
Rv3019c (*esxR* / TB10.3)	MUL_1210 (93.75)	MAP3784 (91.67)	Mmcs_0377 (86.31)	MSMEG_0621 (85.26)	Mvan_0418 (81.15)					
Rv3804c (*fbpA* / Ag85A)	MUL_4987 (90.83)	MAP0216 (89.05)	Mmcs_5015 (80.18)	MSMEG_6398 (80.55)	Mvan_1902 (83.90)		SC1F2.06 (42.32)	DIP2193 (51.94)	CE2709 (53.92)	cg3182 (53.62)
Rv3875 (*esxA* / esat-6)			Mmcs_0071 (74.74)	MSMEG_0066 (80.00)	Mvan_0079 (58.43)			DIP0559 (60.71)	CE0583 (55.00)	cg0672 (55.00)

M. tuberculosis **H37Rv DosR orthologs in different mycobacterial and non-mycobacterial species. Homologs are indicated in a coloured box. Orthologs and homologs are expressed as % similarity (part 3).**

H37Rv DosR antigens	CJ1	RHA1	RSP1	NCF1	PBA1	ATC1	BFL1
Rv0079				nfa19090 (56.20)			
Rv0080				nfa28920 (68.15)			
Rv0081		RHA1_ro11232 (57.14)					
Rv0569							
Rv0570	jk0478 (41.18)	RHA1_ro06441 (41.55)	RSP_3547 (57.04)	nfa43070 (41.69)	PPA1026 (54.29)	Acel_1476 (55.76)	BL0670 (39.96)
Rv0571c		RHA1_ro06036 (66.83)		nfa11240 (63.27)		Acel_0137 (72.60)	
Rv0572c							
Rv0573c	jk0492 (54.63)	RHA1_ro01442 (52.92)		nfa10850 (52.16)	PPA1681 (53.05)	Acel_1692 (55.68)	BL0287 (55.67)
Rv0574c		RHA1_ro00281 (67.30)				Acel_2058 (42.33)	BL1567 (42.06)
Rv1733c		RHA1_ro00046 (51.11)		nfa35490 (43.29)			
Rv1734c							
Rv1735c		RHA1_ro02252 (64.94)					
Rv1736c				nfa45520 (71.86)	PPA0507 (66.95)	Acel_0510 (80.00)	
Rv1737c		RHA1_ro08515 (43.93)		nfa44520 (40.72)	PPA1978 (40.64)	Acel_1747 (51.06)	
Rv1738		RHA1_ro00038 (76.25)					
Rv1812c	jk0914 (42.61)	RHA1_ro07103 (79.65)	RSP_3707 (45.12)	nfa52780 (43.86)	PPA0139 (47.56)	Acel_0803 (46.79)	
Rv1813c							
Rv1996	jk0100 (57.04)	RHA1_ro00026 (60.66)		nfa29970 (61.70)	PPA1028 (44.77)		BL0009 (41.69)
Rv1997	jk0638 (53.20)	RHA1_ro03758 (53.53)	RSP_4078 (60.25)	nfa27140 (51.20)	PPA2240 (49.90)	Acel_1545 (48.81)	BL0683 (58.78)
Rv1998c		RHA1_ro02162 (51.58)		nfa49070 (48.54)			
Rv2003c						Acel_1780 (51.23)	
Rv2004c		RHA1_ro00055 (61.23)	RSP_0470 (46.30)				
Rv2005c	jk0100 (54.51)	RHA1_ro00050 (62.37)		nfa30400 (63.03)	PPA1028 (46.45)		BL1664 (48.94)
Rv2006	jk1930 (44.29)	RHA1_ro00045 (72.72)		nfa27660 (61.09)	PPA1108 (46.58)		
Rv2007c	jk1395 (78.43)	RHA1_ro08284 (70.18)	RSP_2424 (67.27)	nfa47510 (72.54)	PPA0627 (78.43)	Acel_0499 (85.45)	BL1563 (73.00)
Rv2028c	jk0100 (42.50)	RHA1_ro00026 (47.27)		nfa28970 (43.85)	PPA1028 (41.04)		
Rv2029c		RHA1_ro00056 (71.79)	RSP_2334 (54.93)	nfa28550 (50.35)	PPA0143 (50.35)		
Rv2030c		RHA1_ro06036 (61.16)		nfa46810 (60.00)		Acel_0137 (58.13)	
Rv2031c						Acel_0609 (62.14)	
Rv2032		RHA1_ro00031 (59.94)		nfa27670 (59.42)			
Rv2623	jk0100 (58.10)	RHA1_ro00026 (60.66)		nfa30400 (59.23)	PPA1028 (44.40)		BL1664 (49.28)
Rv2624c	jk0100 (43.79)	RHA1_ro02261 (48.27)		nfa30400 (45.32)		Acel_1774 (50.37)	BL1664 (43.85)
Rv2625c						Acel_1181 (45.53)	
Rv2626c				nfa790 (70.37)			
Rv2627c							
Rv2628							
Rv2629				nfa27130 (50.56)			
Rv2630							
Rv2631		RHA1_ro06876 (44.78)		nfa13690 (45.71)			
Rv3126c							
Rv3127		RHA1_ro00027 (56.44)		nfa27670 (56.53)			
Rv3128c							
Rv3129					PPA1465 (67.02)		
Rv3130c		RHA1_ro00024 (61.74)		nfa46000 (51.18)		Acel_0484 (43.90)	
Rv3131		RHA1_ro00031 (51.72)		nfa28960 (63.55)			
Rv3132c	jk0888 (47.76)	RHA1_ro00049 (74.07)		nfa28940 (70.26)	PPA0925 (53.76)	Acel_0516 (72.38)	
Rv3133c	jk1161 (58.05)	RHA1_ro00048 (87.38)	RSP_0087 (49.51)	nfa19100 (85.58)	PPA2028 (61.14)	Acel_0518 (77.31)	BL0930 (63.41)
Rv3134c	jk0100 (42.91)	RHA1_ro00050 (45.52)		nfa29970 (47.03)	PPA1028 (43.89)		
H37 Rv non-DosR antigens							
Rv0288 (*esxH* / TB10.4)							
Rv0440 (*groEL2* / hsp65.2)	jk0267 (89.78)	RHA1_ro02146 (93.89)	RSP_2311 (76.21)	nfa52750 (94.07)	PPA0453 (87.73)	Acel_0101 (90.47)	BL0002 (84.70)
Rv1886c (*fbpB* / Ag85B)	jk0134 (47.79)	RHA1_ro04059 (55.49)		nfa1830 (53.39)			
Rv3019c (*esxR* / TB10.3)							
Rv3804c (*fbpA* / Ag85A)	jk0133 (56.51)	RHA1_ro04960 (56.45)		nfa1830 (50.91)			
Rv3875 (*esxA* / esat-6)	jk1747 (56.04)						

Chapter 7

Pulmonary delivery of DNA encoding *Mycobacterium tuberculosis* latency antigen Rv1733c associated to PLGA-PEI nanoparticles enhances T cell responses in a DNA prime/protein boost vaccination regimen in mice

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Abstract

During persistent infection and hypoxic-stress, *Mycobacterium tuberculosis* (*M. tuberculosis*) expresses a series of *M. tuberculosis* latency antigens. The aim of this study was to evaluate the immunogenicity of a DNA vaccine encoding the *M. tuberculosis* latency antigen Rv1733c and to explore the effect of pulmonary delivery and co-formulation with poly (D,L-lactide-*co*-glycolide) (PLGA) - polyethyleneimine (PEI) nanoparticles (np) on host immunity. Characterization studies indicated that PLGA-PEI np kept their nanometer size after concentration and were positively charged. The np were able to mature human dendritic cells and stimulated them to secrete IL-12 and TNF-α comparable to levels observed after lipopolysaccharide (LPS) stimulation. *M. tuberculosis* latency antigen Rv1733c DNA prime combined with Rv1733c protein boost enhanced T cell proliferation and IFN-γ secretion in mice in response to Rv1733c and *M. tuberculosis* hypoxic lysate. Rv1733c-DNA adsorbed to PLGA-PEI np and applied to the lungs increased T cell proliferation and IFN-γ production more potently compared to the same vaccinations given intramuscularly. The strongest immunogenicity was obtained by pulmonary priming with np-adsorbed Rv1733c DNA followed by boosting with Rv1733c protein. These results confirm that PLGA-PEI np is an efficient DNA vaccine delivery system to enhance T cell responses through pulmonary delivery in a DNA prime/protein boost vaccine regimen.

Introduction

Tuberculosis (TB) is the second leading cause of death from an infectious disease, following HIV-1 infection. Two million people die from TB each year while one-third of the world's population is latently infected with the tubercle bacillus. Although the majority (90-95%) of these people does not become ill, they present an immense reservoir of latently infected individuals who may progress to active TB disease when their immune system becomes affected (e.g. upon HIV-1 infection) (1, 2). Therefore, it is highly important to explore the development of post-exposure or therapeutic TB vaccines that can protect already infected individuals against endogenous reactivation of TB disease (3-5).

Persisting tubercle bacilli are ordinarily contained within granulomatous lesions in infected tissues and shift to a dormancy state with reduced metabolic activity that enables them to survive in conditions of nutrient and oxygen deprivation and nitric oxide stimulation (6-8). In vitro expression profiling studies of tubercle bacilli have shown that in this state, a regulon is modulated of about 48 genes that are under the control of its putative regulatory factor DosR (Rv3133c) (8, 9). Recently, we observed preferential recognition of these so-called TB latency antigens in individuals with latent *Mycobacterium tuberculosis* (*M. tuberculosis*) infection compared to TB-patients. In particular the DosR regulon encoded antigens Rv1733c, Rv2029c, Rv2627c and Rv2628 induced strong IFN-γ responses in latently infected individuals. These results suggested that these antigens are targets of the immune system during persistent *M. tuberculosis* infection and may thus be of interest as potential vaccine candidates to help protecting individuals already infected with *M. tuberculosis* (10, 11). DosR antigen Rv1733c was shown to be the most immunodominant amongst the *M. tuberculosis* infected individuals tested and responses to this single antigen were significantly higher in individuals with latent *M. tuberculosis* infection compared to those in TB patients (10). Recent vaccination studies of plasmid DNA encoding latency antigens confirmed their immunogenicity also in mice models (12). Based on the human data and the preliminary mice studies, Rv1733c was selected as a DNA vaccine antigen for detailed studies here.

DNA vaccines against tuberculosis are widely explored by virtue of their ability to induce strong cellular immunity, which is needed for TB control (13, 14). Although there are some conflicting reports regarding the efficiency of DNA vaccines for the immunotherapy of tuberculosis in animal infection models, it is believed that the combination of DNA vaccines with drug treatment can help prevent re-infection and reactivation of the disease in already infected populations (15).

Despite the fact that DNA vaccines have shown promising results in animal models (13, 16, 17), low immunogenicity has been observed in human clinical trials so far (18, 19). Thus, strategies are needed to enhance the immunogenicity of DNA vaccines. These include: improving transfection of host cells and antigen expression; augmenting antigen presentation; enhancing co-stimulation and increasing T lymphocyte expansion (reviewed in (20)). Several novel delivery systems and application methods are being developed to potentiate the performance of DNA vaccines, aiming at enhanced transfection of host cells (21). One of the best-studied

materials for vaccine delivery is the biocompatible and biodegradable polymer poly-D,L-lactide-*co*-glycolide (PLGA). PLGA microparticles prepared with the cationic surfactant cetyltrimethylammonium bromide (CTAB) enhanced the potency of anti-HIV DNA vaccines in a non-human primate model (22) and increased the potency of a tuberculosis DNA vaccine in mice (23). The immuno-potentiating effect of these particles was caused by increasing DNA persistence and recruiting phagocytes to the injection site, activating antigen-presenting cells (APCs) and directly priming dendritic cells (DCs) (24, 25). In a similar approach, we previously synthesized PLGA nanoparticles (np) with polyethyleneimine (PEI) moieties on their surface that could efficiently adsorb DNA. These np were internalized by human bronchial epithelial cells in culture and resulted in protein expression, indicating their ability to serve as a DNA delivery system for pulmonary administration (26).
In this study PLGA-PEI np were further characterized as to their stability and their effect on human-derived DCs. The immunogenicity of plasmid DNA encoding the *M. tuberculosis* latency antigen Rv1733c was explored in mice in a prime-boost vaccination regimen, by combining pulmonary delivery and the use of PLGA-PEI np formulation.

Materials and Methods

Materials

D, L-Lactide/glycolide copolymer (PLGA, PURASORB®, DL663FL, molar ratio: 53/47, inherent viscosity 0.69dl/g) was a generous gift from PURAC (Gorinchem, The Netherlands). Twenty-five kDa branched water-free polyethyleneimine (PEI) and Tween-80® were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Poloxamer 188 (Lutrol F68) was purchased from BASF (Ludwigshafen, Germany). Acetone of analytical grade was from Fisher chemicals (Leicestershire, UK) and dichloromethane of analytical grade from Biosolve BV (Valkenswaard, The Netherlands). Tris/Boric acid/EDTA buffer (TBE), agarose gel and loading buffer were obtained from Bio-Rad Laboratories (Veenendaal, The Netherlands).

Preparation of DNA loaded PLGA-PEI np

PLGA-PEI np were prepared as previously described (26). A solution of 10% (w/v) PLGA in dichloromethane was stirred for 30 minutes. PEI solution in acetone was prepared to a final concentration of 1% (w/v). PEI was added to the PLGA solution to achieve a PLGA-PEI ratio of 10:1, Tween-80® added to a final concentration of 1% (w/v) and acetone added up to 10ml. This organic phase was mixed and poured into an aqueous phase of 20ml of 0.5% (w/v) Poloxamer-188 in milli-Q®. The suspension was stirred overnight at room temperature to evaporate the organic solvents and filtered through 4-7μm retention size paper filter ($595^{1/2}$ folded filters, Schleicher & Schuell, s'Hertogenbosch, The Netherlands) to remove large polymer particles. The filtered np were loaded with V1Jns encoding *M. tuberculosis* Antigen 85B (Ag85B) by adding np suspension to 25μg/ml DNA plasmid solution (the amount of np was determined according to the requested PEI-DNA ratio). The mixture was vortexed for 30 seconds and left at room temperature for at least 1hr before use. We used Ag85B

plasmid as a *M. tuberculosis* DNA vaccine model for our *in vitro* characterization studies because of previous work performed in our laboratory and reagents available for analysis (27). The size, zeta potential, morphology, loading efficiency and ability to transfect human epithelial cells were extensively studied and previously reported (26).

Particle size and zeta potential measurements of concentrated formulations

Formulations were concentrated by centrifugation at 13,000 rpm for 20 minutes. The np were resuspended in saline to achieve a final concentration of 1µg/µl. To verify the physical stability of the np suspension after concentration, particle size and zeta potential measurements were performed using Zetasizer® 3000 HSA (Malvern Instruments, Bergen op Zoom, The Netherlands). Particle size was measured by photon correlation spectroscopy (PCS) at 25°C and a fixed 90° scattering angle. Zeta potential determinations were based on electrophoretic mobility of the np in the aqueous medium.

Generation of human monocyte derived dendritic cells (mo-DCs)

Immature mo-DCs were prepared by incubating human peripheral blood mononuclear cells (PBMC) for 15 minutes at 4°C with CD14 beads (MACS, Miltenyi Biotec, Germany). CD14 positive cells were separated using positive selection LS+ columns (Miltenyi Biotec, Germany) and cultured for 6 days in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF, 1000 U/ml, Biosource, Etten-Leur, The Netherlands) and IL-4 (500 U/ml, Sanvertech, The Netherlands). Cells were harvested on day 7 and were applied with the DNA formulations.

Analysis of mo-DCs surface marker expression following exposure to PLGA-PEI formulation using fluorescence-activated cell sorter (FACS)

Immature mo-DCs were plated at a density of $5x10^5$ cells/well in 24-well plates and incubated with Ag85B plasmid DNA solution (1µg/well), PLGA-PEI np without DNA and np with Ag85B plasmid DNA (1µg/well) for 48 hours. Cells were incubated with culture medium in the absence of any formulation as negative control whereas cells incubated with lipopolysaccharide (LPS, 100ng/ml, Sigma, St. Louis, MO, USA) were used as a positive control. Surface marker expression was measured using saturation concentrations of PE conjugated antibodies for CD83 and CD80 and of FITC conjugated antibody for CD40 and CD86 (Becton Dickinson, Erembodegem, Belgium). Acquisition and analysis were performed using a FACS Calibur and with CellQuest™ software (Becton Dickinson, Erembodegem, Belgium).

Detection of IL-12p40 and TNF-α secreted from mo-DCs following exposure to PLGA-PEI formulations

Immature mo-DCs were plated at a density of $5x10^5$ cells/well in 96-well plates and incubated for 24 hours with the formulations described above. In addition, the effect of DNA dose on cytokine secretion was evaluated and 1, 2 or 5 µg DNA was added to the wells associated to PLGA-PEI np or as a solution. LPS (100 ng/mL) and medium

treated wells were used as a positive and negative control, respectively. Each experimental condition was set-up in triplicate and supernatants were collected and stored at -20°C until analysis. The IL-12p40 and TNF-α production were analyzed by Enzyme-Linked Immunosorbent Assay (ELISA; CytoSet,BioSource, Nivelles, Belgium) according to the manufacturer's instructions.

Loading PLGA-PEI np with plasmid DNA for in vivo studies

PLGA-PEI np were loaded with a Gateway® (Invitrogen) adapted pV1J.ns-tPA DNA plasmid encoding *M. tuberculosis* gene Rv1733c (13). Mock DNA plasmid control vector consisted of the same backbone, but with no insert sequence. 200µl PLGA-PEI np suspension was added to 250µl of 200 µg/ml DNA plasmid solution and vortexed for 30 seconds. This mixture gave a final PLGA-PEI ratio of 10:1 and a PEI-DNA ratio of 1:1 (w/w). Formulations were left overnight at room temperature and then centrifuged for 15 minutes at 12,000 rpm. Final DNA concentration adsorbed to np for *in vivo* application was 1 mg/ml.

Immunizations

BALB/c female mice, 6-8 weeks old at the first vaccination, were vaccinated three times at three weeks intervals. Mice were immunized intramuscularly (n=3) or by endotracheal aerosol application (n=5) with 50µg of DNA in solution or adsorbed to 550µg PLGA-PEI np. Non-invasive aerosol application was performed using the technique described by Bivas-Benita et al. (28) where mice were endotracheally intubated with the Penn-Century Microsprayer® (Penn-Century Inc., Philadelphia, Pennsylvania, USA) and the formulation sprayed directly in the airways. Boosting was performed three weeks after the last vaccination by intramuscular injection of 20µg of the Rv1733c protein in incomplete Freund's adjuvant (IFA; Difco Laboratories, Detroit, Michigan, USA) (29). Control mice were immunized subcutaneously with 20µg of the Rv1733c protein in IFA using the same vaccination regimen.

Preparation of antigens

Recombinant Rv1733c was produced as previously described (30). Briefly, the nucleotide sequence of Rv1733c was obtained from http://genolist.pasteur.fr/TubercuList. The gene was amplified by PCR from genomic DNA of *M. tuberculosis* H37Rv and cloned by Gateway Technology (Invitrogen, San Diego, CA, USA) in pDEST™17, a bacterial expression vector containing an N-terminal hexa-histidine tag for rapid purification with nickel-chelating resin. The proteins were over-expressed in *Escherichia coli* BL21(DE3) and purified as previously described (30). Sequencing was performed to confirm the identity of the cloned DNA fragment. Size and purity were checked by gel electrophoresis and Western blotting with anti-His antibodies (Invitrogen, Breda, the Netherlands). The residual endotoxin level was determined with a Limulus Amebocyte Lysate assay (Cambrex, Verviers, Belgium) and was found to be below 50 IU/mg recombinant protein. The protein batch was subsequently tested for non-specific T cell stimulation and for potential cellular toxicity in lymphocyte stimulation assays using PBMC of *M. tuberculosis* unexposed,

BCG unvaccinated, Mantoux skin test negative healthy donors. Recombinant Rv1733c was dissolved in PBS and used in a final concentration of 20 μg/ml.
In vitro, *M. tuberculosis* adapts to low oxygen stress, a condition the bacilli are exposed to during latent infection, by modulating the *dosR* regulon. For this study we used a lysate, which was prepared from *M. tuberculosis* H37Rv cultured under low oxygen conditions as a proxy for the antigenic repertoire that may be presented to the immune system during latent infection. Bacteria were grown for 24 hours in tubes with tightly screwed caps (low-oxygen cultures). Culturing was continued until an OD_{650} of ca. 0.5 was reached. Bacilli were harvested by centrifugation and lysed by using 0.1 mm glass beads as previously described (31-33). This lysate was precipitated with acetone and dialysed against phosphate-buffered saline. The total protein concentration of the resulting preparation was determined by the BCA test (Pierce, Rockford, Illinois, USA). The lysate was used in an end concentration of 1 μg/ml. The *M. tuberculosis* hypoxic lysate was kindly donated by Dr. Karen Weldingh and Dr. Peter Andersen (SSI, Denmark).

Proliferation assay
Spleens were harvested 3 weeks after the last vaccination or 10 days after the protein boost and ground with a 70μm nylon cell strainer (BD biosciences, Erembodegem, Belgium) to obtain a uniform single cell-suspension. Splenocytes were labeled at 10^7 cells/ml with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands) for 10 minutes at 37°C in PBS with 0.5% BSA (Sigma-Aldrich, Zwijndrecht, The Netherlands). Following labeling, 10% heat-inactivated fetal bovine serum (FBS; Greiner, Alphen a/d Rijn, The Netherlands) was added to the cell suspension and washed in PBS with 0.5% BSA. Splenocytes were resuspended in RPMI-1640 medium (Gibco, Breda, The Netherlands) supplemented with 10% FBS. Cells were added to 96-well U-bottom plates (Corning, Schiphol-Rijk, The Netherlands) at $3x10^5$ cells/well and stimulated in triplicate with antigens. Seventy-two hours later, supernatants were taken; triplicates were pooled and stored at -20°C until IFN-γ was assayed. Cells of triplicates were pooled and washed with PBS with 0.1% BSA. Cells were evaluated by flow cytometry where cells gated from a live lymphocyte population were analyzed for CFSE proliferation. The Δ geometric mean was used as a measure of proliferation. The Δ geometric mean was obtained by subtracting the geometric mean CFSE fluorescence of the total population from the geometric mean CFSE fluorescence of the undivided cell population: Relative proliferation (or Δ geometric mean) = geometric mean (unproliferating cells) - geometric mean (total cells).
Relative proliferation was expressed as a percentage of the maximal proliferation measured using phytohaemagglutinin (PHA) stimulation (end concentration 2 μg/ml) and was corrected for spontaneous proliferation:

$$\left(\frac{[\Delta \text{ geometric mean sample} - \Delta \text{ geometric mean medium}]}{[\Delta \text{ geometric mean PHA} - \Delta \text{ geometric mean medium}]} \right) * 100 = \% \text{ of maximal proliferation}$$

IFN-γ analysis

Supernatants were evaluated for their IFN-γ content using IFN-γ CytoSet™ ELISA kit (Biosource, Etten-Leur, The Netherlands). The assay was performed according to the manufacturer's instructions. ELISA samples were tested in duplicate and the mean value of the unstimulated cells was subtracted from the mean value of the samples.

Statistical analysis

Statistical analysis of proliferation and IFN-γ secretion was performed using one-way ANOVA test. Multiple comparisons between the 9 immunized mice groups were performed by a subsequent Tukey's correction test. Statistical significance is represented by * (P<0.05), ** (P<0.01) and *** (P<0.001).

Results

Stability of concentrated formulations loaded with plasmid DNA

Concentration of the formulations is a first, but necessary step to prepare for the in vivo studies in which 50μg of DNA needs to be applied in 50μl volume to the airways. After centrifugation, resuspension of the np was performed using water or saline. Loading and concentration of the np resulted in a small mean size increase from 235nm to 275nm when resuspended in water and 271nm in saline (Table 1). After concentration, np remained positively charged, however, mean zeta potential was reduced from +64.3mV to +38.8mV after resuspension in water and to +40.6mV after resuspension in saline.

Table 1. Size and zeta (ζ) potential of np 10:1 PLGA-PEI ratio and 1:1 PEI-DNA ratio after concentrating formulations for in vivo application

	Size (nm)	ζ Potential (mV)
Not loaded, not concentrated	235	64.3
Loaded, concentrated, resuspended with H2O	27 ± 39	38.8 ± 10.9
Loaded, concentrated, resuspended with Saline	271 ± 20	40.6 ± 4.0

Values are mean averages ± S.D. of 3 separately loaded samples. All size measurements had a polydispersity index <0.2.

Effect of DNA loaded PLGA-PEI np on mo-DC maturation

PLGA-PEI np were evaluated for their ability to stimulate and induce maturation of human DCs in culture. The latter was evaluated by measuring the up-regulation of surface expression of the molecules CD40, CD80, CD83 and CD86 compared to unstimulated cultures. Exposure of mo-DCs to Ag85B DNA in solution or loaded onto PLGA-PEI np resulted in increased surface expression of all four markers to a level comparable to the increase noted in response to the positive control LPS (Figure 1). Incubation with non-loaded PLGA-PEI np did not cause DC maturation as levels of surface CD40, CD80, CD83 and CD86 were similar to the base line expression (Figure 1, 4th row).

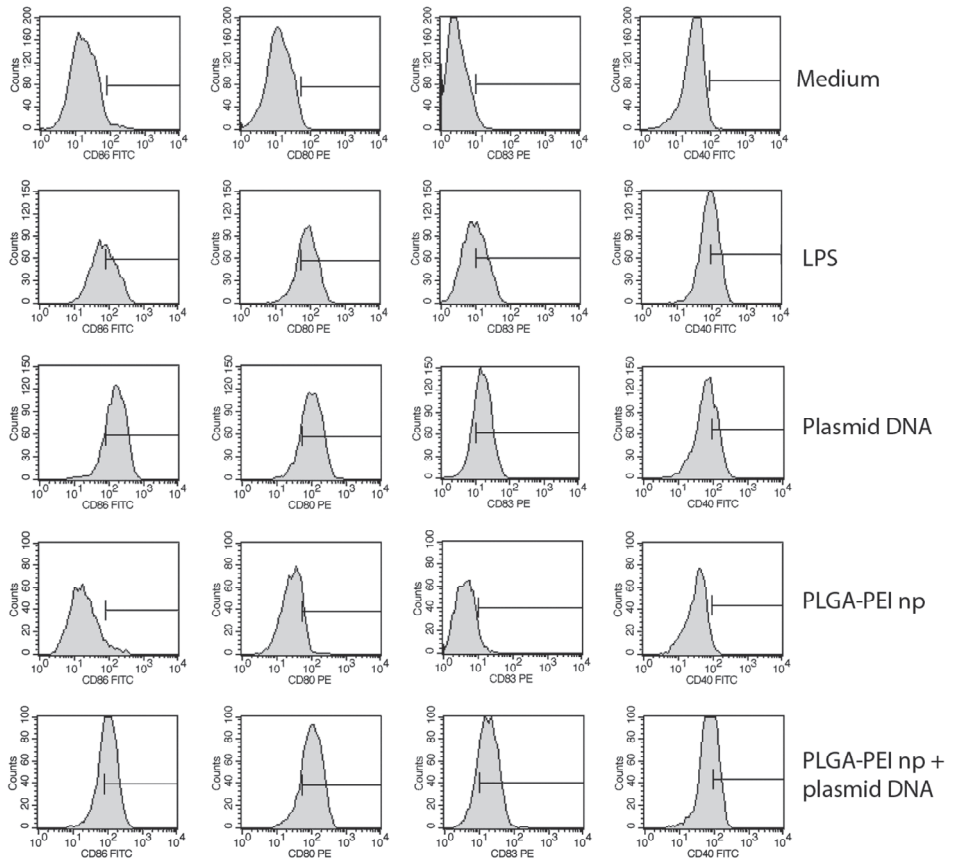

Figure 1. Maturation of mo-DCs following incubation with PLGA-PEI np formulations. *Surface markers on DCs were detected by FACS following incubation with FITC-labeled CD86 and CD40 MAb and PE-labeled CD80 and CD83 MAb.*

IL-12 and TNF-α secretion from mo-DCs following exposure to PLGA-PEI formulations

Activation of DCs can also be evaluated functionally by the secretion of cytokines. The levels of the stimulatory cytokines IL-12 (p40 subunit) and TNF-α were evaluated in 24 hours DCs culture supernatants by ELISA with detection limits of 0.4 and 0.28 ng/ml, respectively. IL-12 secretion was the highest after application of 1μg plasmid DNA adsorbed to PLGA-PEI np with values similar to those obtained after LPS stimulation (Figure 2). Increasing the dose of DNA adsorbed to the np resulted in reduced IL-12 production and after application of the 5μg DNA dose there was no detectable IL-12. Applying DNA in solution resulted in lower IL-12 levels than those obtained by 1μg DNA adsorbed to np but DCs exposed to PLGA-PEI np without DNA did not secrete any IL-12. Similar results were obtained for TNF-α where application of 1μg DNA adsorbed to PLGA-PEI np induced the highest TNF-α levels which were comparable to those obtained by the positive control, LPS (Figure 3).

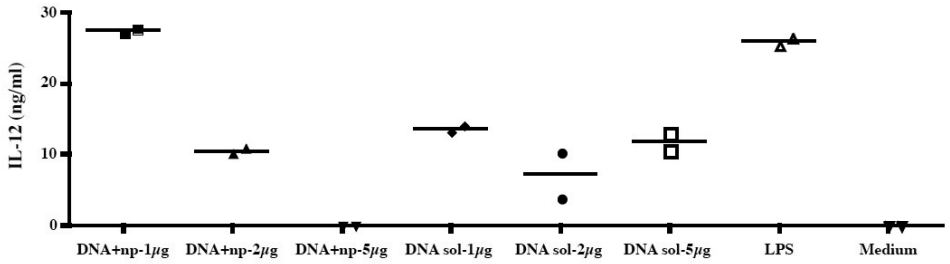

Figure 2. IL-12 secretion from mo-DCs following incubation (24hrs) with different formulations. *IL-12 levels were measured using ELISA and LPS was used as a positive control. The results are the mean of duplicate samples from one representative experiment out of two performed.*

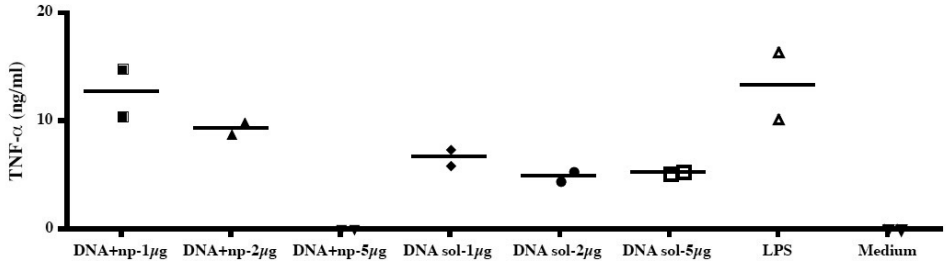

Figure 3. TNF-α secretion from mo-DCs following incubation (24 hrs) with different formulations. *TNF-α levels were measured using ELISA and LPS was used as a positive control. The results are the mean of duplicate samples from one representative experiment out of two performed.*

Immunogenicity of *M. tuberculosis* Rv1733c administered in different delivery regimens and formulations: proliferative responses

Proliferative responses of the splenocytes from the different groups of immunized animals were evaluated using CFSE based cell proliferation measured by flow cytometry. In vitro restimulation with recombinant Rv1733c antigen was used to evaluate antigen specific responses. In addition, *M. tuberculosis* hypoxic lysate was used as a proxy for the antigenic repertoire that may be presented to the immune system during latent infection. The responsiveness of the splenocytes to this stimulation indicates in vivo expression of the Rv1733c in bacilli grown under low oxygen conditions. The total proliferation of splenocyte cultures restimulated with Rv1733c was increased in all the groups that received the recombinant Rv1733c protein boost after the homologous DNA immunization, compared to DNA vaccination without protein boost (Figure 4a). Of interest was that the group that received Rv1733c plasmid DNA adsorbed to PLGA-PEI np endotracheally followed by a subsequent protein boost had significantly higher proliferative responses than when the same vaccination regimen was administered intramuscularly ($P<0.05$). In addition, this pulmonary vaccination regimen induced the strongest Rv1733c total

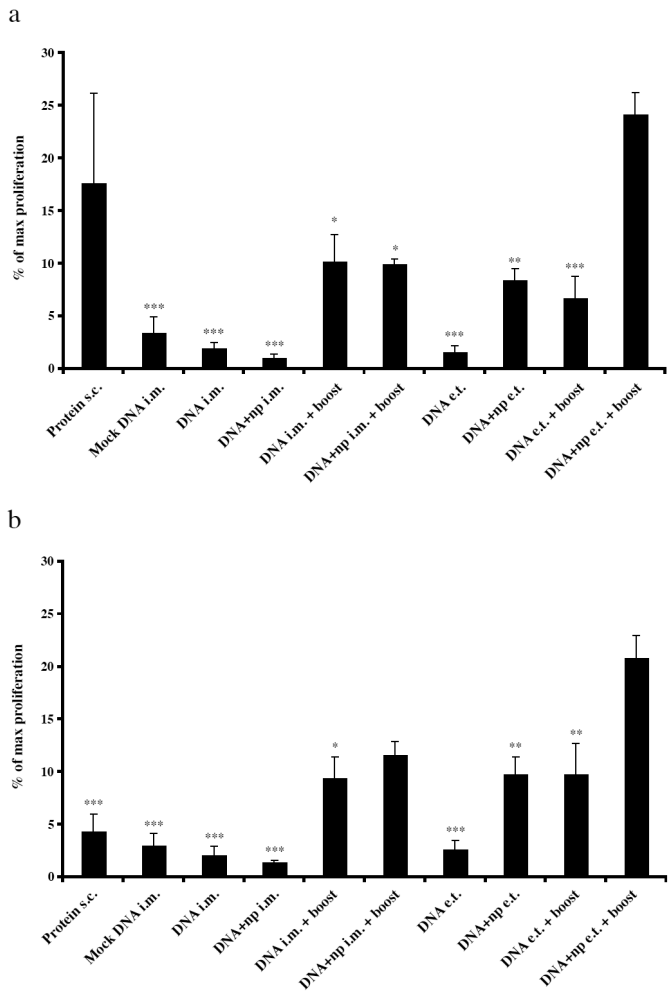

Figure 4. T cell proliferation of immunized mice in response to Rv1733c protein (a) and M. tuberculosis hypoxic lysate (b) restimulation. *Relative proliferation is represented as the mean percentage of the measured relative proliferation per group ± SEM (n=3-5). Statistical significant differences as compared to the DNA+np e.t. + boost vaccinated group are represented by * (P < 0.05), ** (P < 0.01) and *** (P < 0.001).*

proliferative T cell responses among all groups studied here. In order to exclude that inherent adjuvant activity through TLR-9 triggering by CpG motifs carried by the plasmid vector might have accounted for the observed Rv1733c responses, we also used empty non-coding vector DNA as a control. This did not induce significant proliferative (Figure 4a) responses, which indicates that responses are induced by the specific antigen encoding sequence in the context of the delivery platform.
Similar results for total proliferation were obtained after restimulation of splenocytes from the same mice with *M. tuberculosis* hypoxic lysate (Figure 4b). T cell proliferation

was again significantly increased after endotracheal immunization with Rv1733c DNA adsorbed to PLGA-PEI np followed by protein boost, in comparison to all the other groups studied.

Immunogenicity of Rv1733c administered in different vaccine regimens and formulations: IFN-γ responses

To further evaluate the cellular immune responses initiated in the vaccinated groups, splenocytes from immunized mice were restimulated with Rv1733c or *M. tuberculosis* hypoxic lysate for 72 hours, followed by IFN-γ analysis using ELISA.

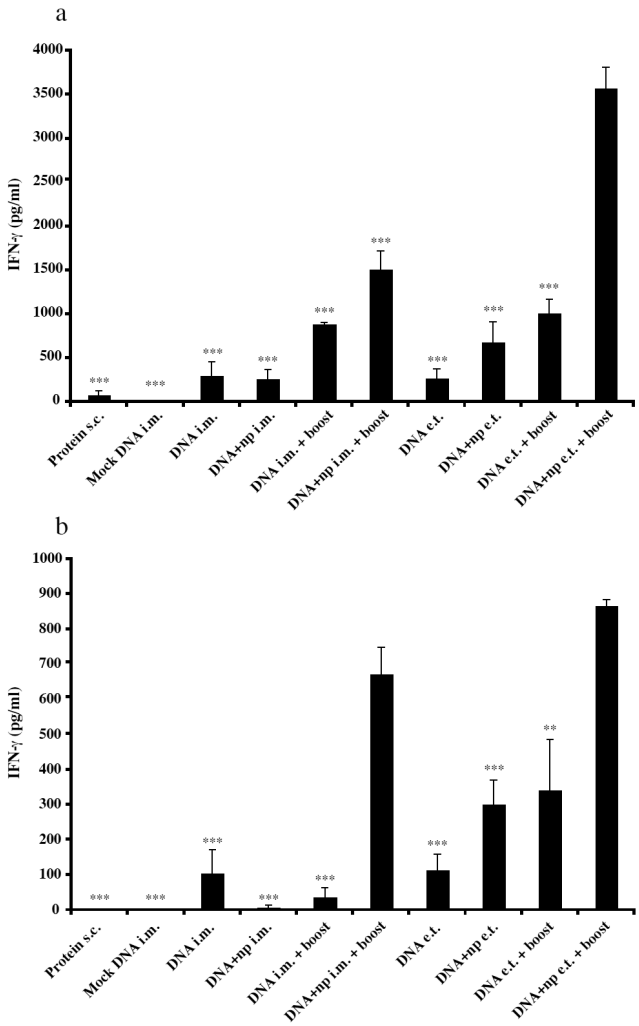

Figure 5. IFN-γ production from spleen cell cultures of immunized mice in response to Rv1733c protein (a) and M. tuberculosis hypoxic lysate (b)*. IFN-γ levels from splenocytes culture supernatants were measured by ELISA and represented as mean concentration values ± SEM (n=3-5). Statistical significant differences as compared to the DNA+np e.t. + boost vaccinated group are represented by * (P < 0.05), ** (P < 0.01) and *** (P < 0.001).*

IFN-γ production in response to restimulation with recombinant Rv1733c protein was elevated in the groups that received DNA followed by protein boost in both intramuscular and pulmonary applications, when compared to the corresponding groups without the protein boost (Figure 5a).
Moreover, PLGA-PEI np increased IFN-γ production in the protein-boosted groups, in comparison to the same vaccination regimen without the particles. The vaccination regimen of pulmonary priming with Rv1733c DNA vaccine adsorbed to PLGA-PEI np followed by a protein boost gave the highest levels of IFN-γ and was significantly different from all other immunized groups ($P<0.001$). Splenocytes from mice immunized with only recombinant Rv1733c protein produced low levels of IFN-γ, although they proliferated strongly (Figure 4a), suggesting the involvement of a non IFN-γ associated proliferative T cell component in the response to the protein only immunization.
The secretion of IFN-γ in response to *M. tuberculosis* hypoxic lysate restimulation was lower in magnitude but indicated a comparable trend (Figure 5b). In the vaccine regimens that included the protein boost, PLGA-PEI np showed higher production of IFN-γ in comparison to the groups receiving the DNA vaccine as a solution. Also here, pulmonary delivery of Rv1733c plasmid DNA induced higher levels of IFN-γ than intramuscular delivery after priming with DNA associated to np and boosting with protein. Like the results obtained after Rv1733c restimulation, splenocytes of protein-immunized animals responded poorly to stimulation with *M. tuberculosis* hypoxic lysate. Additionally, intramuscular administration of a mock DNA plasmid resulted in no IFN-γ secretion from splenocytes in response to either Rv1733c or *M. tuberculosis* hypoxic lysate.

Discussion

The 2 billion latently *M. tuberculosis* infected individuals worldwide represent an enormous reservoir of potential TB cases, since TB reactivation occurs in about 5-10% of latently infected people. Targeting mycobacterial antigens expressed by persisting *M. tuberculosis* bacilli represents a new strategy to help control latent infection and prevent TB disease reactivation. The *M. tuberculosis* DosR regulon encoded genes are such late stage specific genes. We have previously shown that responses to *M. tuberculosis* DosR antigen Rv1733c were significantly higher in individuals with latent *M. tuberculosis* infection compared to TB patients (10). These results suggest that responses to this antigen are associated with control of latent *M. tuberculosis* infection. The present study therefore evaluates the immunogenicity of a DNA vaccine encoding *M. tuberculosis* latency antigen Rv1733c in mice in a prime-boost setting, employing mucosal application of a novel DNA formulation (26, 28).
We have previously developed PLGA-PEI np as a novel delivery platform for pulmonary DNA vaccination (26). We have now further improved this system by concentrating np to reach the desired DNA dose for mice. Particles kept their nanometer size and positive surface charge, which will enable adherence to the pulmonary mucosal membranes and subsequent cellular uptake. The effect of PLGA-

PEI np formulations on human DC maturation was evaluated next, by measuring the expression of the surface molecules CD40, CD80, CD83 and CD86. We showed that PLGA-PEI np by themselves were inert and did not cause an increase in surface markers while DNA-loaded np increased the expression of these markers to levels comparable to those seen after LPS activation. Similar stimulation was induced by empty control plasmid DNA, suggesting that plasmid DNA itself caused the activation, probably through Toll-like receptor 9 (TLR9) recognition and TANK-binding kinase-1 (TBK1)-dependent signalling (34). We observed equivalent DC maturation using different plasmid DNAs (encoding *M. tuberculosis* Ag85B, a mock plasmid vector and a comparable polyepitope plasmid DNA vector (35)), demonstrating that this stimulation is intrinsic to the bacterial DNA vector, independent of the insert characteristics. Franco et al. reported similar observations with *Mycobacterium leprae* HSP65 (36). These results thus bridge our human DC studies, using plasmid encoding *M. tuberculosis* Ag85B, with our mouse immunization studies using DNA plasmids encoding *M. tuberculosis* latency antigen Rv1733c.

The stimulatory function of DCs also depends on cytokine secretion. IL-12 and TNF-α are two essential cytokines for efficient microbial resistance (37-41). Our results show that PLGA-PEI np adsorbed with 1μg of plasmid DNA are able to induce similar levels of IL-12 and TNF-α secretion as LPS. Application of higher DNA doses adsorbed to PLGA-PEI np abolished the cytokine secretion, suggesting PEI induced toxicity. Since the PEI-DNA ratio was constant, an increased particles/DNA dose resulted in a higher PEI dose, which could lead to cell toxicity and decreased cell viability (26). This was strengthened by the observation that when DNA alone was given in the same amounts, it induced moderate cytokine secretion in each of the three DNA doses. These results imply that the enhanced cytokine secretion seen for PLGA-PEI np adsorbed with 1μg DNA is likely due to better cellular uptake (42).

Our strategies to improve DNA vaccine immunogenicity included DNA prime-protein boost vaccine regimens, previously shown to enhance immunogenicity for mycobacterial antigens such as Ag85A (29), HSP65 (43, 44), ESAT-6 (45) and MPB 70 (46). Using our new delivery platform, we observed enhanced cellular proliferation and IFN-γ secretion in mice that received the DNA prime followed by Rv1733c protein boost, in response to either Rv1733c protein or *M. tuberculosis* hypoxic lysate. These results emphasize the importance of protein boosting in DNA based vaccination regimens in order to optimize stimulation of cellular immunity. In future studies we plan to validate this strategy by evaluating its protective efficacy in (latent) *M. tuberculosis* infection models in mice.

In case of mucosal infectious diseases such as TB, immunization via the mucosal linings of the lung seems a highly relevant approach, since this can induce both systemic and local immune responses at the site of mycobacterial entry (47). Recent studies suggested that airway antigen delivery could also restore protective mucosal immunity following intramuscular DNA vaccine delivery (48). In the case of latent *M. tuberculosis* infection, boosting DCs and resident T cells in the lungs with relevant *M. tuberculosis* latency antigens could increase local immunity, and help control local dormant bacteria, thus preventing reactivation of the infection. We show here for the first time that Rv1733c DNA adsorbed to PLGA-PEI np applied to the lungs increased

proliferation and IFN-γ secretion in comparison to the same vaccination regimen given intramuscularly. By contrast, the immunogenic capacity of DNA alone was not affected by the application method. This strongly suggests that np are taken up more efficiently by the lung mucosa compared to muscle tissue, thus enhancing immunity to *M. tuberculosis* Rv1733c. Since PLGA-PEI np are positively charged they adhere better to negatively charged cellular membranes, thus promoting intracellular uptake and subsequent protein expression in human airway epithelial cells as we have observed previously (26). Our in vivo results support the in vitro observations, suggesting that DNA adsorbed to the delivery system could promote its survival in the mucosal environment against degradative enzymes, and thus result in protein expression within the pulmonary tissue.

The immunogenic potential of the DNA/PLGA-PEI np applied to the lungs followed by a protein boost could be a result of different T cell subsets stimulation, like $CD4^+$ T cells, which are stimulated more vigorously following a protein boost (29) and could synergize to achieve higher antigen specific responses. Another population that could contribute to the increased cell proliferation and IFN-γ secretion is the antigen specific memory T cell population (49). The effect of pulmonary application of PLGA-PEI np carrying DNA vaccine on DCs in vivo and on specific T cell populations will be an important aspect of future studies.

Taken together, these data demonstrate that the immunogenicity of DNA vaccines can be strongly enhanced in case of pulmonary delivery by formulating the DNA with PLGA-PEI np, followed by protein boosting. Further studies will need to elucidate the exact mechanisms responsible for the increased immunogenicity following pulmonary vaccination via PLGA-PEI np. This work may help to design better vaccines against mucosal infectious diseases, such as TB.

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Chapter 8

Summary and General Discussion

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Summary

Mycobacterium tuberculosis is one of the worlds' most successful pathogens. At present, around 2 billion people harbor latent *M. tuberculosis* infection without experiencing any clinical symptoms. Many, if not most, new cases of active TB arise from this large number of latently infected individuals. The exact factors that tilt the balance from latent to active TB disease are largely unknown. One evident factor for driving latent disease into progression to active disease is co-infection with HIV. The ever growing numbers of TB casualties imparted by the current HIV pandemic and rise of drug resistant *M. tuberculosis* strains, urge measures to control TB reactivation which include more effective drugs and new TB vaccines.

Effective anti-TB vaccination will not only require better preventive, but also post-exposure or therapeutic vaccines since *M. bovis* BCG (BCG) is ineffective in already latently infected individuals. We argue that detailed analysis of *M. tuberculosis* genes whose expression is induced or upregulated predominantly during the latent stage of infection may lead to the identification of new *M. tuberculosis* genes for drug and vaccine development. Post-exposure vaccines based on late stage antigens would aim at inducing robust immunity to these antigens in order to prevent reactivation of TB infection in latently infected individuals. Such immune responses encompassing both $CD4^+$ and $CD8^+$ T-cell responses are considered essential in controlling, or preferably, eradicating persisting bacilli.

Interestingly, late stage antigen-based vaccines may be combined with early stage antigens, intended to prevent establishment of TB infection, to generate multi-stage vaccines allowing usage in pre-exposure as well as post-exposure setting.

The recently identified 48-gene *M. tuberculosis* DosR (dormancy) regulon is expressed by tubercle bacilli during in vitro exposure to hypoxia, low dose nitric oxide or carbon monoxide, conditions thought to be encountered by persisting, intracellular bacilli in granulomas in immuno-competent hosts. Here, we hypothesize that genes expressed by dormant and persisting *M. tuberculosis* bacilli may represent attractive targets for post-exposure or therapeutic vaccination against TB.

This thesis focuses on the immunologic evaluation of *M. tuberculosis* DosR regulon genes in humans and in mice regarding immunogenicity, (latent) *M. tuberculosis* infection, induction of immune responses following BCG and exposure to environmental, non-tuberculous mycobacteria (NTM).

General Discussion

Exploring immunogenicity of *M. tuberculosis* DosR regulon encoded genes

During the course of infection, *M. tuberculosis* changes its gene expression pattern as a result of intricate host-pathogen interaction. This naturally implies that the immune system faces different sets of mycobacterial antigens during the different phases of infection, including latent *M. tuberculosis* infection. The identification of *M. tuberculosis* stage specific genes has been accelerated by the application of genome wide pathogen gene-expression arrays. The DosR regulon is up-regulated during conditions which are encountered by intracellular mycobacteria, and may thus represent a first example of *M. tuberculosis* stage specific genes (38, 45).
Our first question was to determine the potential immunogenicity of the *M. tuberculosis* DosR antigens. Our results indeed indicate their in vivo relevance and immunogenic nature, since *M. tuberculosis* DosR proteins were recognized both by *M. tuberculosis* specific $CD4^+$ T-cell lines and PBMC from *M. tuberculosis* infected individuals (Chapter 2)(27).
Important further support for our hypothesis was provided by the differential recognition of the *M. tuberculosis* DosR antigens: TST^+ individuals not only recognized more dormancy antigens but also had a stronger cumulative IFNγ response to *M. tuberculosis* DosR antigens compared to individuals who had developed TB disease. The preferred recognition by TST converters appeared to be restricted to the group of late stage DosR antigens since IFNγ responses to CFP-10, an early secreted *M. tuberculosis*-specific antigen, were significantly stronger in TB patients. Similar results have been obtained by Demissie and colleagues (14). They also observed preferential recognition of dormancy antigen HspX by latently infected individuals whereas ESAT-6, another early secreted *M. tuberculosis* specific protein, was shown to be most responsive in TB patients (14). Moreover, Schuck and colleagues identified a set of T-cell antigens specific for latent *M. tuberculosis* infection which included three of the *M. tuberculosis* DosR encoded antigens (36). T cell recognition profiles likely mirror the antigenic spectrum the immune system has experienced during *M. tuberculosis* infection. Thus, it is likely that these in vitro results suggest in vivo expression, processing and presentation of the DosR regulon encoded proteins to the human immune system during latent *M. tuberculosis* infection.
Immunogenicity of *M. tuberculosis* DosR antigens was corroborated and extended further in mice by Roupie and colleagues (34). Immunization of Balb/c and C57/B6 mice with DNA encoding DosR encoded genes resulted in recall responses displaying high levels of dormancy antigen specific IFNγ production. Importantly, and in line with the above observations in humans, strong cellular immunity to dormancy antigens was associated with chronically persistent but not acute *M. tuberculosis* infection in Balb/c mice. Additionally, several *M. tuberculosis* DosR $CD4^+$ and $CD8^+$ T cell epitopes were identified. These congruent results in mice and humans suggest that further exploration of the DosR antigens can also be done productively in mice for proof of principle or proof of concept studies.

Measuring specific CD4+ and CD8+ T cell responses to *M. tuberculosis* DosR antigens

Strong anti-mycobacterial immunity is essential for control and eradication of *M. tuberculosis* infection. For several immunodominant mycobacterial antigens such as the Ag85 complex, Hsp65 and CFP-10, T cell epitopes have been identified in the context of both MCH class I and class II molecules (12, 18, 19, 26, 30, 37, 40). In line with the aforementioned antigens, several class I and II epitopes have been documented for dormancy antigen HspX (16kDa, α-crystallin, Rv2031c) (1, 11, 16). We showed that other members of the *M. tuberculosis* DosR encoded regulon are also recognized by a broad spectrum of MHC alleles in humans (Chapter 3) and in mice (34). More specifically, in the human setting, several DosR epitopes could be identified in the context of HLA-A02*01 and HLA-DR3. Peptide specific CD4+ and CD8+ T cell responses included T cell proliferation and IFNγ production and in the case of CD8+ T cells also cytolytic activity. Many more HLA-A2 and HLA-DR3 candidate epitopes have been identified and require further validation of HLA restriction. These studies encourage further follow-up studies including assessing epitopes from other DosR antigens in various HLA backgrounds.
Detailed epitope mapping increases our understanding of the human immunity and offer insights into how this might be exploited for therapeutic T cell immunity to *M. tuberculosis* specific antigens. The use of peptides offers the potential to elucidate also subdominant epitopes next to dominant epitopes; these might be exploited in designing more effective subunit vaccines by inducing immunity to a broader epitope repertoire than would be seen following natural infection or protein vaccination.

Induction of cross-reactive immunity to *M. tuberculosis* DosR encoded genes by mycobacteria other than *M. tuberculosis*

Members of the DosR regulon encoded genes are not encoded by any of the chromosomal regions of difference (RD) (4, 10, 21, 29) indicating that they are not unique to *M. tuberculosis*. Most dormancy antigens have unknown functions, and encode hypothetical (or conserved hypothetical) proteins. A number of DosR genes has been characterized in more detail such as Rv2029c encoding a phosphofructokinase and Rv2007c encoding a possible ferredoxin fdxA, both molecules involved in a range of general metabolic reactions (glycolysis and electron transport, respectively)(13, 33) and are likely ubiquitously expressed amongst most (myco) bacteria.
Over the years, several mycobacterial genomes have been sequenced, including genomes of BCG and NTM. Systematic analysis of these genomes for the presence of *M. tuberculosis* (strain H37Rv) DosR gene orthologs led us to the understanding that the majority of mycobacterial genomes indeed encompass several to many orthologs of the *M. tuberculosis* DosR genes (Chapter 6). Not unexpectedly, the highest similarities were detected among members of the *M. tuberculosis* complex. Interestingly, in silico

analysis compared to the *M. tuberculosis* DosR regulon orthologs in BCG revealed that all 48 genes were either identical or highly (> 97% homology) identical.
Thus far, actual production of a few DosR proteins has been shown to occur in BCG during in vitro oxygen starvation (7, 8). In vitro, almost all of the dormancy genes proved functional in different BCG strains as judged by expression profiles under conditions of low oxygen levels (Chapter 4) (23). This is compatible with DosR (Rv3133c, *devR*) being essential for long term survival of BCG under hypoxic conditions (7). Only *narK2* (Rv1737c, involved in nitrate transport) and *narX* (Rv1736c, nitrate reductase) were hardly expressed in BCG (23, 41). As of yet, lack of expression of the DosR regulon in BCG under hypoxic conditions is limited to *narK2* and *narX*.
The limited differences in RNA expression patterns of DosR genes in BCG and *M. tuberculosis* are in contrast with the large differences observed in the in vitro recognition of DosR proteins following BCG or *M. tuberculosis* immunization/infection. Differential recognition of DosR antigen HspX following BCG vaccination and *M. tuberculosis* exposure had been reported before: no immune responses (IFNγ) to HspX could be detected following BCG vaccination (Chapter 5) (17, 44). Later, this observation was extended to several more members of the DosR regulon such as Rv1733c and Rv2029c in humans and in mice. Importantly, immunity to non-DosR encoded antigens (such as Ag85B, CFP-10/ESAT-6) could be measured indicating that generation of specific anti-microbial immunity was not impaired (Chapters 4&5) (17, 28, 44). By contrast, significant IFNγ production to *M. tuberculosis* DosR antigens was only detected in BCG vaccinees who had a positive in vitro ESAT-6/CFP-10 response, indicative of exposure to *M. tuberculosis* (Chapters 4&5) (17, 28).
Collectively, these studies suggest that BCG immunization fails to induce adequate immune responses to *M. tuberculosis* dormancy antigens despite having, and in vitro expressing, an almost identical DosR regulon. BCG is not expected to persist in immuno-competent individuals; following administration in the normal skin, it is cleared by the host immune system. BCG may thus not be able to induce (sufficient) expression of its DosR regulon to facilitate persistence as well as induction of immunity against DosR regulon encoded antigens. Underlying mechanisms and conditions that result in the lack of or too little/short expression of BCG DosR antigens following BCG vaccination need to be investigated. Furthermore, it remains to be seen whether this might relate to BCG's incomplete induction of protection against TB reactivation in the adult.
NTM have been proposed to contribute to BCG's inconsistent protective efficacy (3, 9, 32). NTM are present in high abundance in the environment and are able to induce cross-reactive, non-specific *M. tuberculosis* tuberculin responsiveness (15). Reactivity may be caused by many shared mycobacterial antigens between NTM and BCG, but these have not been identified. More specifically, cross-reactivity of the *M. tuberculosis* DosR encoded antigens at the immunologic level between *M. tuberculosis* and NTM would in fact be expected, and underlie -at least in apart- the influence of NTM on anti-mycobacterial immunity.
Several studies including ours (Chapter 6) (27) have reported in vitro PPD reactivity in healthy, BCG unvaccinated, *M. tuberculosis* naive individuals (6, 28). Responsiveness in such healthy controls indeed stretched beyond PPD or *M. tuberculosis* (lysate), since

reactivity to *M. tuberculosis* DosR antigens was also found (Chapter 2)(27). Also in individuals with proven NTM infection (*M. marinum/ M. kansasii*) or in those with high exposure to NTM, significant responses to dormancy antigens are observed. These results are concordant with the large number of *M. tuberculosis* DosR orthologs present in NTM as mentioned above (Chapter 6). *M. tuberculosis* DosR antigen directed responses may indeed be the result of exposure to, or infection with, cross-reacting NTM as implicated here. However, it remains unknown how DosR is exactly regulated in NTM and to which extent NTM induced cross-reactivity to DosR encoded antigens may influence in vitro surveys in *M. tuberculosis* infected individuals and importantly, whether this might contribute to natural protection to *M. tuberculosis*.

M. tuberculosis DosR antigens: novel targets for the development of post-exposure/ therapeutic TB vaccines?

Most new generation TB vaccines in the pipeline are designed as prophylactic vaccines to be used as BCG-booster vaccines or are recombinant BCG vaccines, designed to replace the current BCG vaccine (2,39). These vaccines are almost exclusively based on antigens that are expressed during early (growth) phase of *M. tuberculosis* infection (e.g. ESAT-6, Ag85) and are intended for prophylactic purposes. However, prophylactic vaccines may not work in individuals harboring latent TB infection (43) since in the already infected host during later stages of infection, immunity to other antigens including DosR regulon encoded may be more relevant. It would be advantageous for those at risk for TB reactivation, to develop post-exposure- or therapeutic vaccines that adequately activate T cell responses specific for antigens associated with late stage infection (when bacteria are in dormant state or resuscitation state). Such vaccines should be capable of directly targeting dormant or early reactivating *M. tuberculosis* organisms, aiming to help preventing reactivation or even to eliminate the pathogen.

During latent TB infection, *M. tuberculosis* is contained within typical granulomatous structures. It is assumed that in these lesions dormant bacilli are present that persist in a metabolically reduced or inactive state (25). However, newly available information provided evidence that in the course of chronic infection in mice, *M. tuberculosis* actually replicates albeit under restraint by the host immune system (20). Regardless, in both cases latent infection arises, representing a balance between host defense and bacterial dormancy/ replicating persistence, which can be maintained for many decades. During bacterial dormancy, in vivo expression of the DosR regulon is modulated and prepares bacilli for long periods of in vitro dormancy (45). This role was recently discussed by Rustad and colleagues (35). In a defined in vitro hypoxia model, they found strong expression of genes of the so-called endured hypoxic response (EHR) and suggested these to be involved in the control of bacteriostasis. The EHR was defined as a set of *M. tuberculosis* genes that was not induced initially upon hypoxia but was significant up-regulated at four and seven days of hypoxia. The thus defined EHR regulon encompasses 230 genes. However, it should be noted that comparison of DosR gene expression profiles in the EHR model and in the Wayne

model (originally used in the identification of the DosR regulon) revealed significant overlap of expressed DosR genes, as expected. Indeed, over half of the DosR genes are still abundantly expressed in the EHR model at late time-points of hypoxia (at day four and seven). The reason why they were not classified as EHR genes was that the EHR criteria did not include genes that were also expressed at early time points (< four days); these early time points may actually be highly relevant for the initiation of the bacterial adaptive response cascade to hypoxia. Interestingly, in our studies, the four DosR genes found to be immunodominant in humans and in mice (Rv1733c, Rv2626c, Rv2627c, Rv2628) were present among the EHR genes.
Only future research will be able to confirm whether EHR genes also regulate long term bacterial dormancy. In any case, fact is that recognition of the DosR genes occurs in *M. tuberculosis* infected individuals indicative of in vivo expression and presentation to the immune system. Importantly, the association of DosR responses to latent TB infection underscores a significant and relevant role for these genes of this regulon in latent TB infection, providing a basis as targets for novel tuberculosis intervention strategies.

Many TB vaccination strategies can be followed as excellently reviewed and summarized (2, 22, 39). One vaccination approach is DNA subunit vaccination. DNA vaccines against tuberculosis have been explored given their ability to induce strong cellular immunity, a requirement for TB control (24, 42). Optimizing the administration route and formulation of DNA will be critical in enhancing immunogenicity, since DNA vaccines in humans have thus far been fairly disappointing. For TB, immunization via the mucosal linings of the lung may be a much more relevant approach than classical intramuscular vaccination, since mucosal vaccination can induce both systemic and local immune responses at the site of mycobacterial entry (5).
Recent studies with plasmid DNA encoding latency antigens confirmed immunogenicity of the *M. tuberculosis* DosR antigens in mice (Chapter 5) (34). In man, *M. tuberculosis* DosR gene Rv1733c was shown to be the most immunodominant amongst *M.* tuberculosis infected individuals (27). We explored in more depth the immunogenicity of Rv1733c following DNA immunization in mice by including strategies to improve DNA vaccine immunogenicity (Chapter 7). Immunogenicity could be improved by a heterologous prime boost combination: we observed enhanced cellular proliferation and IFNγ secretion in mice that received Rv1733c DNA prime followed by Rv1733c protein boost. Immunogenicity was even more pronounced when co-formulated with nanoparticles and finally, immune responses were highest and most significant upon pulmonary delivery compared to similar vaccinations given intramuscularly.
In the case of latent *M. tuberculosis* infection, boosting DCs and resident T cells in the lungs with relevant *M. tuberculosis* latency antigens could increase local immunity, and help control local dormant bacteria in order to help preventing reactivation of the infection.
Overall, the DosR antigen based studies discussed above encourage further in depth characterization of underlying host cellular immune mechanisms including infection of host cells and antigen expression, augmenting antigen presentation, co-stimulation

and driving T lymphocyte expansion and effectiveness in controlling *M. tuberculosis* during late stage infection. Development of new tools and new anti-TB strategies to identify correlates of protection and disease will also be an essential step in this goal (31). Future studies must focus not only on evaluating DosR based subunit vaccines but also include evaluation of other vaccine approaches (peptide or whole protein based vaccines) to the actual protective efficacy of DosR directed immunity in (latent) *M. tuberculosis* infection in mouse models and humans.

Concluding remarks and Future Perspectives

First data pointing to in vivo relevance of *M. tuberculosis* DosR encoded antigens came from their immunogenic nature in *M. tuberculosis* infected individuals. Significant differential T cell recognition in individuals with latent TB (and corroborated in mice) hinted towards an association with protection from reactivation TB. The latter is something BCG fails to accomplish. Remarkably, BCG vaccination does not induce immunity to dormancy antigens, neither in humans nor in mice whilst BCG does possess a functional homologous DosR regulon which is induced under low oxygen conditions. NTM infection or exposure on the contrary, appeared to induce cross-reactive responses to *M. tuberculosis* DosR antigens. Future research will need to provide better insights into the distribution and expression of DosR encoded genes among NTM and their influences on immunity to both *M. tuberculosis* and BCG.
Any post-exposure- or therapeutic TB vaccines that are to be administrated to ethnically different groups at different geographical sites would need to work against prevalent and local *M. tuberculosis* strains. Different *M. tuberculosis* lineages however, may have shaped and selected different genetic polymorphisms associated with host resistance in specific human populations which may translate into different disease epidemiology and immune response profiles. Possible strain heterogeneity together with host-specific pathogen adaptation, repeated *M. tuberculosis* re-infections and naturally co-infections such as with HIV will need to be considered in the development of any future TB vaccine.
Large scale longitudinal and cross-sectional studies focusing on identification of biomarkers of protective immunity and evaluation of novel TB vaccine candidates, based on *M. tuberculosis* DosR and other antigens are currently being carried by the Grand Challenges in Global Health partnership in different endemic countries. These studies may eventually provide valuable insight and answers into these issues.
Obviously, the exact nature of human and murine T cell responses to *M. tuberculosis* DosR antigens has not been studied exhaustively yet. Immunological signaling pathways should be explored in relation to host resistance during bacterial dormancy. Importantly, next to the role of IFNγ additional correlates of protective immunity (e.g. multifunctional cells producing IFNγ, TNFα and IL-2) should be investigated.
Another interesting and attractive option to pursue is the design and construction of multi-stage vaccines. Sub-unit vaccines based on the genes of the DosR regulon might be combined with TB early stage antigens, reactivation antigens or resuscitation antigens. The advent of high throughput sequencing allows and facilitates identification of other *M. tuberculosis* stage or phase specific antigens. Such vaccines

would exploit the advantage of immune protection against both early and late stages of infection.

Future studies need to build on the current results and should continue the exploration and evaluation of the potential of *M. tuberculosis* DosR encoded genes as TB vaccine candidates in order to halt the growing global burden of tuberculosis.

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Nederlandse Samenvatting

Algemene inleiding

Tuberculose (TB) is een infectieziekte die wordt veroorzaakt door de bacterie *Mycobacterium tuberculosis (M. tuberculosis)*. In de volksmond wordt TB ook wel de 'tering' of de 'witte pest' genoemd. *M. tuberculosis* komt verspreid over de gehele wereld voor. In totaal is bijna één derde van de wereldbevolking (ruwweg 2 miljard mensen) besmet met de tuberkel bacil. Jaarlijks ontwikkelen ongeveer negen miljoen mensen daadwerkelijk de ziekte TB.
TB eist ongeveer 2 miljoen doden per jaar, vooral in Azië, Afrika en Latijns-Amerika, en in toenemende mate in Oost-Europa. TB is met AIDS en malaria de infectieziekte die wereldwijd de meeste dodelijke slachtoffers eist.
TB is een ziekte die in principe alle organen kan aantasten maar longtuberculose is de meest voorkomende vorm. Andere getroffen lichaamsdelen zijn bijvoorbeeld de gewrichten, botten, hersenen en lymfeklieren.
Na de infectie, die zich via de luchtwegen verspreid, zorgt de bacterie ervoor dat er kleine ontstekingen ontstaan in het lichaam: er worden kleine knobbeltjes (granulomas) gevormd die kenmerkend zijn voor TB. In deze knobbeltjes bevinden zich 'slapende' ofwel latente bacteriën. De meeste mensen (90%) die besmet zijn vertonen geen ziektesymptomen en zullen deze ook niet ontwikkelen; zij dragen de ziekte latent met zich mee en vormen derhalve geen besmettingsbron. De overige 5-10% van de geïnfecteerde mensen worden wel ziek. Het ziekteproces treedt in een klein deel (1%) al vrij snel op na de infectie terwijl de rest op een later moment in hun leven ziek wordt na een aanvankelijk latente periode van infectie; deze 'reactivatie' van infectie kan zelfs na 50 jaar nog optreden. Tijdens de 'actieve' ziekte (open TB) is de patiënt een zeer besmettelijke bron van bacteriën.
De precieze factoren die kunnen voorspellen of verklaren wanneer iemand actieve ziekte ontwikkelt of welke markers (zogenaamde 'biomarkers') juist typerend zijn voor de latente fase van infectie, zijn helaas nog grotendeels onbekend. Wel is bekend dat een verzwakte afweer, bijvoorbeeld als gevolg van besmetting met het Humaan Immuundeficiëntie Virus (HIV), een groot risico vormen voor het ontwikkelen van TB. De samenloop van TB met de huidige HIV-pandemie en het verschijnen van steeds meer (extreem) antibiotica ongevoelige TB stammen zorgt helaas voor nog meer slachtoffers. Deze situatie vraagt om nieuwe, effectievere medicijnen en vaccins waarmee we TB reactivatie kunnen voorkomen, (latente) TB beter kunnen behandelen en de ziekte daarmee kunnen indammen.

Nieuwe anti-TB vaccins omvatten niet alleen betere preventieve vaccins maar ook nieuwe vaccins die werkzaam zijn in de reeds geïnfecteerde mens. Veel nieuwe actieve TB gevallen zijn afkomstig uit de groep van latent geïnfecteerde personen. Zogenaamde 'post-exposure' of therapeutische vaccins zouden kunnen verhinderen dat de latente infectie reactiveert. Het vaccin zou immers het lichaam kunnen helpen de infectie onder controle te houden, of beter nog, de latente infectiehaard op te ruimen. Tot op heden bestaan er geen post-exposure vaccins tegen TB, noch betrouwbare biomarkers die specifiek zijn voor latente TB. Bovendien weten we nog

niet precies welke afweerreacties er echt toe doen tijdens de natuurlijke bescherming tegen reactivatie TB. Het enige beschikbare vaccin tegen TB is een verzwakte vorm van de runder TB bacil, en heet *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). BCG is beschermend en effectief in kinderen tegen ernstige vormen van TB. BCG biedt helaas onvoldoende bescherming tegen longtuberculose en is niet werkzaam in mensen die al geïnfecteerd zijn met *M. tuberculosis*.

Een vaccin heeft als doel het immuunsysteem te leren effectief te reageren tegen bepaalde stukjes eiwit (antigenen) van een bacterie of virus. Wanneer het afweersysteem dat eenmaal geleerd heeft kan het de echte ziekteverwekker snel en effectief uitschakelen.
Er zijn diverse benaderingen om geschikte vaccinkandidaten te vinden. Deze moeten echter ook geschikt zijn voor de ontwikkeling van post-exposure / therapeutische vaccins zoals wij die voor ogen hebben. Eén benadering is het bestuderen van karakteristieke genen die door *M. tuberculosis* veel gebruikt worden of hoog afgeschreven (geëxpresseerd) worden tijdens de latente, 'slapende' fase van de infectie. Deze genen, die vertaald worden naar eiwitten (antigenen), zouden kunnen worden gebruikt als doelwit voor het afweersysteem, en aldus betrokken kunnen zijn bij het voorkomen van TB reactivatie. Een nieuw vaccin zou hiervan gebruik kunnen maken en het afweersysteem helpen de tuberkel bacterie te herkennen nog gedurende de latente fase van infectie, en op die manier de bacterie kunnen stoppen. Een goed vaccin moet er in elk geval voor zorgen dat diverse witte bloedcellen (de T cellen) deze latentie antigenen goed herkennen.

Dit proefschrift

De genen van het DosR ('dormancy') regulon zijn een specifieke set van genen die door *M. tuberculosis* hoog geëxpresseerd worden in modellen die de latente fase van infectie nabootsen. Dit regulon bestaat uit een set van 48 genen, die wordt gereguleerd door de dormancy respons master regulator, DosR. Bij het bestuderen van de genexpressie profielen van de bacterie tijdens deze simulaties werd de genexpressie van het gehele dormancy regulon sterk opgereguleerd. Deze set genen vormen het onderwerp van ons onderzoek.

Centraal in dit proefschrift staat het bestuderen van de DosR gereguleerde genen met betrekking tot het vermogen om afweerreacties te induceren en het zoeken naar biomarkers voor latente TB infectie. In de eerste instantie werd gekeken of de genen van het DosR regulon als eiwitten een immuunrespons kunnen induceren in de mens (**Hoofdstuk 2**). In totaal werden 25 van de 48 dormancy genen geselecteerd en vertaald naar het bijbehorende eiwit. Deze 25 behoorden tot de genen die het sterkst tot expressie kwamen in *M. tuberculosis*. Diverse cellen werden getest op herkenning van de 25 dormancy eiwitten. De cellen waren afkomstig van mensen met: latente TB infectie, actieve TB infectie of geen TB infectie. Uit de resultaten bleek dat alle geteste antigenen herkend werden door minstens één, maar vaak meerdere donoren. Bovendien herkende de groep van personen met een latente TB infectie meer dormancy antigenen dan de groep met actieve TB: een sterke en brede respons op de

dormancy antigenen leek zodoende geassocieerd te zijn met een goede natuurlijke afweer tegen *M. tuberculosis*.

Hierna onderzochten we in detail welke witte bloedcellen reageerden op de dormancy antigenen. **Hoofdstuk 3** beschrijft de reacties van twee soorten witte bloedcellen, $CD4^+$ en $CD8^+$ T cellen, op *M. tuberculosis* dormancy antigenen. Ook bestudeerden we welke brokstukjes van deze antigenen (peptiden) deze immuunreactie induceerden. Relatief veel peptiden bleken in staat om T cel responsen uit te lokken; sommige peptiden bleken door $CD4^+$ T cellen herkend te worden, andere door $CD8^+$ T cellen en een kleiner aantal door beide subsets van T cellen.

De precieze afweerreacties die ten grondslag liggen en karakteristiek zijn voor de beschermende immuniteit tegen TB, en het gebrek daaraan bij BCG vaccinatie zijn nog grotendeels onbekend. In **Hoofdstuk 4 en 5** hebben we gekeken of de DosR antigenen een rol hebben in de immuunreactie die volgt na BCG vaccinatie. Verrassend was dat BCG vaccinatie in zowel humane cellen als muizencellen niet in staat leek een afweerreactie op te wekken tegen de *M. tuberculosis* dormancy antigenen! Dit bleek niet te kunnen worden verklaard door genetische deletie van deze genen in BCG, noch door een gebrekkige capaciteit van BCG om genen van het DosR regulon *in vitro* tot expressie te brengen. Kennelijk leidt BCG vaccinatie in de mens dus niet tot een adequate reactie tegen *M. tuberculosis* dormancy antigenen.

Een andere onverwachte bevinding was het voorkomen van afweerreacties op de dormancy antigenen in sommige gezonde, niet *M. tuberculosis* geïnfecteerde mensen. De *M. tuberculosis* dormancy genen bleken niet uniek voor *M. tuberculosis*: ze komen ook voor in andere (minder gevaarlijke) mycobacteriën en soms ook in bacteriën die men vrij veel aantreft in het milieu. Dit laten we zien in **Hoofdstuk 6**. Hier beschrijven we dat mensen die niet besmet zijn met *M. tuberculosis* maar met een verwante minder gevaarlijke mycobacterie ook kunnen reageren op de dormancy antigenen van *M. tuberculosis*. Dit laat zien dat dormancy antigenen van *M. tuberculosis* en omgevings mycobacteriën kruisreactief zijn. Eerder onderzoek heeft laten zien dat kruisreactiviteit van omgevingsmycobacteriën met het BCG vaccin niet altijd een positieve invloed had op de werking van BCG, hoewel de precieze mechanismen daarvan nog onbekend zijn. Toekomstig onderzoek zal moeten uitwijzen wat de invloed en relatie is van kruisreactiviteit van omgevingsmycobacteriën met de *M. tuberculosis* dormancy antigenen.

DNA vaccins zijn bijzonder goed in het induceren van sterke, beschermende afweerreacties. In **Hoofdstuk 7** bestudeerden we het eiwit Rv1733c, één van de meest potente dormancy antigenen, als DNA vaccin. In muizen bleek dit inderdaad een goede afweerreactie te induceren. Rv1733c als DNA vaccin kon nog krachtiger worden gemaakt door een specifieke formulering toe te passen (met nanopartikel-moleculen) en het direct toe te dienen via de luchtwegen (het doelwitorgaan van *M. tuberculosis*) i.p.v. de huid.

Conclusies

Onze hypothese was dat *M. tuberculosis* dormancy eiwitten potentiële antigenen zouden kunnen zijn voor het menselijk afweersysteem en daarmee nieuwe kandidaten zijn voor de ontwikkeling van nieuwe post-exposure / therapeutische vaccins tegen TB.
Onze experimenten tonen aan dat *M. tuberculosis* DosR regulon gecodeerde dormancy eiwitten daadwerkelijk herkend worden door het afweersysteem van mensen die geïnfecteerd zijn (geweest) met *M. tuberculosis*. Ten tweede, een sterkere en bredere afweerreactie tegen de dormancy antigenen lijkt geassocieerd te zijn met een natuurlijke bescherming tegen *M. tuberculosis*. Deze afweerreactie kan wellicht een rol spelen bij het voorkomen van reactivatie van latente TB naar actieve TB. Omgevingsmycobacteriën bleken ook in staat om reacties te induceren tegen *M. tuberculosis* dormancy antigenen, waarschijnlijk als gevolg van kruisreactiviteit met de *M. tuberculosis* dormancy antigenen. Daar tegenover staat het opmerkelijke gebrek van BCG om maar enige reactie te induceren tegen de dormancy antigenen, terwijl de gensequenties van de DosR regulon genen tussen BCG en *M. tuberculosis* zo goed als 100% overeenkomen, en BCG *in vitro* ook het DosR regulon tot expressie kan brengen.

Deze bevindingen vormen de eerste stap op weg naar de ontwikkeling van nieuwe TB vaccins en van biomarkers die kenmerkend zijn voor de diverse fasen van TB infectie. Toekomstig onderzoek zal uiteraard rekening moeten houden met de verschillende immuunreactieprofielen die aanwezig zijn in verschillende etnische groepen. Grootschalige studies die de immuunreacties in de tijd volgen kunnen belangrijke en doorslaggevende antwoorden geven op de vraag of *M. tuberculosis* dormancy antigenen geschikte vaccin kandidaten zijn tegen TB. Grote populatiestudies zoals uitgevoerd in Afrika door het 'Grand Challenges in Global Health Initiative' zullen hier een belangrijke rol in spelen. Gecombineerd met het definiëren van beschermende immuniteit, is het in principe een kwestie van tijd voordat het duidelijk is welk antigen -of juist meerdere antigenen- geselecteerd zal worden voor opname in toekomstige TB-vaccins. Dit werk zal ook rekening moeten houden met het voorkomen en de verspreiding van verschillende *M. tuberculosis* stammen, die wellicht kunnen verschillen in de expressieniveaus van DosR regulon antigenen. Het zal ook mogelijk zijn DosR antigenen te combineren met andere antigenen die belangrijk zijn gedurende de *verschillende* fases die *M. tuberculosis* infectie doorloopt in tijd (vroege infectie, latente infectie, actieve ziekte). Vaccins bestaande uit de belangrijkste antigenen van iedere fase van de infectie worden ook wel multi-stage vaccins genoemd, en zouden als groot voordeel hebben dat ze niet alleen post-exposure / therapeutisch maar ook preventief gebruikt kunnen worden.
Vervolgonderzoek zal moeten uitwijzen wat de exacte rol is van de *M. tuberculosis* dormancy antigenen in relatie tot de bescherming tegen reactivatie van latente TB, en hoe deze kennis kan worden uitgebuit in de vorm van betere vaccins en biomarkers van TB.

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 20 oktober 1978 te Groningen. In 1997 werd het VWO diploma aan het Praedinius Gymnasium in Groningen gehaald.
In hetzelfde jaar begon zij aan de studie Biologie aan de Rijksuniversiteit Groningen. Na het tweede studiejaar koos zij voor de hoofdrichting Medische Biologie. Voor deze hoofdrichting werden twee stages voltooid: de eerste wetenschappelijke stage werd gelopen op de afdeling Medische Microbiologie (sectie Moleculaire Virologie) van de Rijksuniversiteit Groningen onder begeleiding van Dr. Anke Huckriede en de tweede werd uitgevoerd de afdeling Neurowetenschappen (sectie Medische Fysiologie) aan de Rijksuniversiteit Groningen onder begeleiding van Dr. Sjef Copray.
Na het behalen van het doctoraal examen in 2002 begon zij in 2003 als promovenda op de afdeling Immunohematologie en Bloedtransfusie en de afdeling Infectieziekten van het Leids Universitair Medisch Centrum. Hier werkte zij onder begeleiding van eerst Dr. M.R. Klein en later Prof. Dr. T.H.M. Ottenhoff aan het in dit proefschrift beschreven onderzoek.
Sinds mei 2009 is zij werkzaam als post-doctoraal onderzoeker onder begeleiding van Prof. Dr. J.P.M. van Putten op de afdeling Infectieziekten en Immunologie (sectie Infectiebiologie) aan de Universiteit Utrecht.

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1.) **Lin MY**, van Meijgaarden KE, Friggen AH, Franken KLMC, Geluk A, Drijfhout JW, Arend SM, Klein MR, and Ottenhoff THM. Identification of $CD4^+$ and $CD8^+$ T cell responses to *Mycobacterium tuberculosis* DosR regulon encoded dormancy antigens and mapping of HLA class I and II restricted peptide-epitopes. *Manuscript in preparation*

2.) **Lin MY,** Reddy TBK , Arend SM, Friggen AH, Franken KLMC, van Meijgaarden KE, Verduyn MJC, Schoolnik GK, Klein MR, and Ottenhoff THM. Cross-reactive immunity to *Mycobacterium tuberculosis* DosR regulon-encoded antigens in individuals infected with environmental, non-tuberculous mycobacteria. *Infection and Immunity* 77(11):5071-5079, **2009**

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4.) **Lin MY** and Ottenhoff THM. Not to wake a sleeping giant: new insights into host-pathogen interactions identify new targets for vaccination against latent *Mycobacterium tuberculosis* infection. *Biological Chemistry* 389(5):497-511, **2008**

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***** These authors contributed equally

List of Abbreviations

Ag	antigen
APC	antigen presenting cell *and* allophycocyanin
BCG	*Mycobacterium bovis* bacille Calmette-Guérin
BLAST (p/n)	basic local alignment search tool (protein/nucleotide)
CFP-10	culture filtrate protein-10
CFSE	carboxyfluorescein succinimidyl ester
CHP	conserved hypothetical protein
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
Dormancy antigens	antigens encoded by the dormancy regulon
DosR	dormancy survival regulator (*encoded by Rv3133c)*
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
ESAT-6	early secreted antigenic target-6
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HP	hypothetical protein
HspX	heat shock protein X
IFNγ	interferon gamma
IL	interleukin
IMDM	Iscove's Modified Dulbecco's Media
Latency antigens	see Dormancy antigens
LPS	lipopolysaccharide
LST	lymphocyte stimulation test
LTBI	latent tuberculosis infection
M. tuberculosis	*Mycobacterium tuberculosis*
NO	nitric oxide
NRP	non replicating persistence
NTM	non tuberculous mycobacteria
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PLGA-PEI	poly (D, L-lactide-*co*-glycolide)-polyethyleneimine
PPD	purified protein derivative
PRR	pattern recognition receptors
RD	region of deletion
TB	tuberculosis
TCR	T cell receptor
Th	T helper cell

TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
TST	tuberculin skin test