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

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Chapter 1

Introduction and Outline of the thesis

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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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