Facing the phases of *Mycobacterium tuberculosis*

Hunting for better TB vaccines

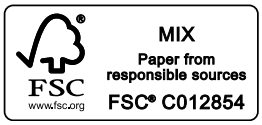
Susanna Commandeur

Facing the phases of *Mycobacterium tuberculosis*

Hunting for better TB vaccines

SUSANNA COMMANDEUR

ISBN: 978-94-6108-576-4
Cover: Amino acid sequences of *Mycobacterium tuberculosis* vaccine candidates analyzed in this thesis
Cover design: Susanna Commandeur - Nicole Nijhuis (Gildeprint)
Lay-out and printing: Gildeprint, Enschede, The Netherlands



Publication of this thesis was financially supported by KNCV Tuberculosis Foundation, U-CyTech Biosciences, Greiner Bio-One and BD Biosciences.

Facing the phases of *Mycobacterium tuberculosis*

Hunting for better TB vaccines

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 22 januari 2014
klokke 13.45 uur

door

Susanna Commandeur
geboren te Kreileroord in 1984

Promotiecommissie

Promotor:
Prof. dr. T.H.M. Ottenhoff

Co-promotor:
Dr. A. Geluk

Overige leden:
Prof. dr. W. Bitter *(Vrije Universiteit Amsterdam)*
Prof. dr. H.M. Dockrell *(London School of Hygiene and Tropical Medicine)*
Prof. dr. W. van Eden *(Universiteit Utrecht)*

Contents

CHAPTER 1

Introduction

Mycobacterium tuberculosis; too smart to handle?

Tuberculosis, a disease of all times

Tuberculosis (TB) is a disease that co-evolved with mankind for many thousands of years [1] and remains responsible for many deaths every year. Approximately 8.7 million new TB cases were identified in 2011 and 1.4 million individuals died of TB in the same year [2]. The first signs of human TB existence can be traced back over 9,000 years ago (7000 B.C.) as shown by DNA and mycolic acid markers detected in human skeletal remains [3]. TB has had many names over time, which refer to consequences of the disease. At the Hippocratic era (460-370 BC) TB was known as consumption or phthisis (Greek for consumption), referring to progressive emaciation or weight loss due to the disease. Later, during the middle ages TB was referred to as the white plague. At this time, TB was accountable for the death of one-fourth of the whole European population and it was even thought that the survival of the European race was at stake [4]. The mechanism and source of the disease remained unknown until the late 19th century. Continuing the work of many researchers who had tried to identify the source of TB, including Jakob Henle, Jean Antoine Villemin and Theodor Klebs, Robert Koch finally identified *Mycobacterium tuberculosis* (*Mtb*) to be the causative agent and was awarded the Nobel prize in 1905 for his work [5;6]. Unfortunately, despite intense efforts to reduce TB, including improved public health care and therapies [7-9], TB continues to remain one of the leading causes of death due to infectious disease worldwide [2].

Mycobacterium tuberculosis infection: From latent infection to active disease

Transmission occurs via inhalation of *Mtb* loaded aerosols that are spread by coughing pulmonary TB patients. The bacilli enter the alveolar space in the lungs where they are taken up by phagocytes including alveolar macrophages, dendritic cells but also pulmonary epithelial cells. *Mtb* is taken up in intracellular compartments called phagosomes. Both neutrophils and monocytes are then attracted to the site of infection, and monocytes subsequently mature into macrophages which are better at taking up and inhibiting *Mtb*. The precise functions of neutrophils remain unclear as they have protective properties at the time of infection but become detrimental at later stages [10-12]. Eventually, the attraction of innate cells to the site of initial infection results in the formation of the primary granuloma (Ghon complex) [13]. Pathogen loaded dendritic cells (DC) subsequently migrate to the mediastinal lymph nodes (MLN), which drain the lungs, where they start presenting antigens to the T and B cells [14;15]. Once the adaptive immune system is triggered, T-effector cells are recruited to the site of infection, where they can exert their function and further shape the granuloma [16].

Intriguingly, this triggering of adaptive immune responses in TB is significantly delayed compared to infections with other pathogens and, in addition, triggered innate and adaptive immune cells often cannot efficiently eliminate *Mtb*. Here, *Mtb* itself plays an important role as the bacilli possess several immune evasion mechanisms which include: inhibition of neutrophil

apoptosis [17], induction of anti-inflammatory responses, such as production of anti-inflammatory cytokines by innate cells and generation of regulatory T cells (Tregs) [18-20], delay in transport of infected, or antigen-loaded DC to the MLN [14;15;21], delay in priming and recruitment of innate and adaptive cells to the site of infection [18;22], preventing phago-lysosome fusion [23-25], detoxification of reactive oxygen species and reactive nitrogen intermediates [26], inhibition of MHC class II presentation [27], and reduced activation of antigen-specific T cells at the site of infection [28;29]. These mechanisms hamper a rapid response of adaptive immune cells, and enable *Mtb* to survive intracellularly [22;30;31]. Remarkably, *Mtb* can also infect bone marrow derived mesenchymal stem cells (BM-MSC) that reside in the bone marrow and use these cells as a hitherto unknown niche and hideout from immune patrol [32].

At first sight, the formation of granulomas seems to be beneficial for the host since it contains infection locally. However, recent data challenge this dogma and show that *Mtb* also benefits from granuloma formation, since it is able to use freshly recruited cells as novel niches, leading to dissemination [33]. Of special interest, the presence of *Mtb* ESAT-6 protein is required for well-formed granulomas as it triggers recruitment of macrophages via host matrix metalloproteinase 9 (MMP9) [34;35]. In addition, further spread is enhanced by *Mtb* (ESAT-6) induced necrosis of infected host cells e.g. [36-39] and inhibition of protective apoptosis e.g. [37;38;40;41], although other studies showed that (ESAT-6 induced) apoptosis is beneficial for *Mtb* e.g. [33;42-44], thus the exact effect of host cell death mechanisms on *Mtb* are yet undefined.

Ultimately, the combination of host pressure and *Mtb* immune escape mechanisms can result in an established balance between host and pathogen, i.e. latent *Mtb* infection (LTBI). This balance between pathogen and host enables *Mtb* to reside for decades at the site of infection. LTBI individuals are thus *Mtb* infected but lack any clinical symptoms. However, latent infection can reactivate and lead to active disease: 5 to 10% of LTBI will develop TB during their lifetime and half of these cases will develop within two-five years after infection [45-47]. Nevertheless, reactivation after decades of latent infection has also been described [48]. Of note, these numbers are difficult to interpret partly because it is unknown what percentage of LTBI is able to clear the infection completely. It is believed that *Mtb* in LTBI mostly enters a dormant state, adapting to the harsh environment in the host while awaiting a possible chance to reactivate and spread. Once an imbalance occurs between host and pathogen, due to weakened host immunity, bacteria will be able to reactivate [31] and necrotic regions will develop within the granuloma, leading to caseous necrotic lesions. These lesions can liquefy which ultimately leads to leakage of caseum into the airways, resulting in a contagious TB case [13;16].

A very high risk of TB reactivation is observed in individuals that are immune deficient. These include: (i) HIV infected individuals particularly those with reduced (<200) $CD4^+$ T-cell counts [49-51], (ii) individuals undergoing anti-TNF treatment [52;53], and (iii) patients with genetic disorders in genes coding for receptors and cytokines involved in type-1 immunity and the Th1 cascade [54-56]. Thus, *Mtb* infection can result in a broad spectrum of stages, ranging from (likely) complete bacterial clearance to latent infection and reactivation or sometimes acute disease [57].

Efforts to Tackle TB

The timely detection and treatment of TB, and its prevention by vaccination are the two major tasks that the WHO has embraced to reduce the burden of TB. Here we will focus on immunological aspects of these tasks, and discuss immune-based detection techniques as well as vaccination against *Mtb*. Treatment of *Mtb* will not be further discussed in this chapter.

Detection of latent *Mtb* infection

Detection of *Mtb* infection is of major importance for adequate control of TB. *Mtb* infection can be determined in the sputum of active TB patients, using a smear microscopy test and bacterial culturing, or nucleic acid amplification tests to detect *Mtb* DNA (NAATs e.g: Xpert MTB/RIF® assay) complemented with chest X-rays [58;59]. Unfortunately, the presence of viable bacteria cannot be determined in LTBI. To verify *Mtb* exposure in this population an indirect test is required, analyzing the immune responses towards mycobacterial products.

Tuberculin skin test. Koch introduced tuberculin, an extract from tubercle bacilli dissolved in glycerine, as a new medicine to treat tuberculosis, however this unfortunately failed [60]. A few years later, Clemens Freiherr von Pirquet showed that Koch's tuberculin could be used as a tool to identify latent *Mtb* infection, by injecting the substance intracutaneously, which resulted in a delayed hypersensitivity (DTH) response in exposed individuals. Charles Mantoux further developed this diagnostic technique and in 1908, Florence Seibert improved tuberculin to purified protein derivative (PPD), which is still in use today [5;61]. This technique is currently known as the tuberculin skin test (TST) or Mantoux test. Unfortunately, cross-reactivity of PPD with nontuberculous mycobacteria (NTM) and the vaccine strain *Mycobacterium bovis* Bacille Calmette-Guérin *(*BCG) reduces the specificity of the TST [62-65]. Exposure to NTMs is very common as they reside in many sources in the host environment such as soil and natural waters [66;67]. One study found NTM to be abundantly present in showerhead biofilms [68]. A practical drawback of the TST is that it requires two visits, can induce boosting effects upon multiple TST testing and can be false negative in immunocompromised (e.g. HIV infected) individuals.

Interferon-Gamma Release Assays (IGRAs). To increase the specificity of *Mtb* diagnosis, a new technique was introduced. This technique is based on antigen specific interferon-gamma (IFN-γ) secretion upon stimulation with peptides from the highly specific and immunogenic *Mtb* antigens early-secreted antigen 6 (ESAT-6), culture filtrate protein 10 (CFP-10) and TB7.7. These tests are widely known as Interferon-Gamma Release Assays (IGRAs). In contrast to the TST, false negative responses due to *Mtb* specific anergy can be identified since both a positive and negative control are included [69]. Multiple studies have shown that IGRAs have enhanced specificity over TST [70-72], although most of these studies were performed in high-income countries such that the results cannot directly be extrapolated to low and middle income countries with higher TB incidences [73;74]. Indeed, IGRAs proved to be less powerful in high TB incidence countries

[75;76]. Due to the lack of sufficient evidence IGRAs are not recommended for use in low and middle income countries [74]. Furthermore, discordant results between TST and IGRA results have been documented which often remain poorly explained [77]. Despite the relative success of IGRAs in low endemic settings, it has not proved possible to use these tests to discriminate between recent and remote *Mtb* infection, and also not between active and latent *Mtb* infection. Recently an *Mtb* antigen, Rv2628, was described to be recognized by remote *Mtb* infected individuals, but not by recent *Mtb* infected individuals, suggesting a role as discriminatory marker [78]. Although the specificity is increased, the IGRA antigens are not fully restricted to *Mtb* as homologs of these proteins are also present in *Mycobacterium leprae* [79;80] *Mycobacterium kansasii*, *Mycobacterium szulgai*, *Mycobacterium riyadhense* and *Mycobacterium marinum*. Also, testing too soon after *Mtb* infection might result in false negative outcomes [81].

Importantly, as already indicated, these two tests determine *Mtb* exposure indirectly. Responses towards *Mtb* products can be detected, but this does not directly indicate that viable bacilli are still present. It may be that either the infection has been cleared but immune responses persist, or that *Mtb* bacilli are still present. Altogether, although latent *Mtb* infection detection methods are available, improvement is definitely required.

Vaccine development

Despite the availability of diagnostic tests and TB treatments, TB remains difficult to eradicate. One major concern is the rapid development of (extensively) multidrug resistant (XDR/MDR) *Mtb* strains. At this moment even strains are known which are resistant to all current drugs available (totally drug resistant *Mtb*, TDR) [82]. Therefore, robust *Mtb* vaccines are needed to prevent further development of TB outbreaks that cannot be controlled using current drugs.

Current TB vaccine. In 1921, Albert Calmette and Camille Guérin developed the first vaccine against TB. They cultured *Mycobacterium bovis* (*M. bovis*) until it became avirulent (due to the loss of genetic regions, including RD1[83]) and designated the vaccine as *M. bovis* Bacille Calmette-Guérin (BCG) [5;84]. BCG is still the only vaccine available at this moment. The effect of BCG has widely been studied in many countries and showed its protective value towards disseminated and meningeal childhood TB [85] as well as to a certain level against leprosy, which is caused by *Mycobacterium leprae* [86;87]. Unfortunately, BCG vaccination results in highly variable protection against pulmonary TB in adults [88], which is the most common form of TB. Importantly, pulmonary TB is, together with the rare laryngeal TB, the only form of TB accountable for further transmission of disease. The effect of BCG can be diminished due to co-infections with NTM or helminthes [89-91]. In addition to the highly variable protection, BCG vaccination, which is a live attenuated vaccine, can lead to severe infection in immune deficient individuals [92] [54]. The lack of BCG's efficacy against pulmonary TB and its severe adverse effects in immune deficiencies clearly show that there is a significant need for improved TB vaccines which are both safer and more effective than BCG.

Novel TB vaccines. Mtb vaccines need to trigger cellular immunity to induce protection against *Mtb* as the bacterium resides intracellular in phagosomes such that antibodies cannot easily reach the pathogen. Importantly, Th1 immunity was previously shown to play an important role in the control of *Mtb* infection [16]. New preventive TB vaccines can either be used as booster vaccines on top of previous BCG vaccination, or as individual, priming vaccines. The three different types of vaccines currently under study are (i) live attenuated and/or genetically modified mycobacterial vaccines, (ii) killed (fragmented) whole bacteria and (iii) subunit vaccines. Notably, the development of therapeutic vaccines is of significant interest. The goal of this vaccine is to prevent reactivation of *Mtb* in LTBI by enhancing the control of infection. At this moment, over 12 vaccines already entered clinical trials, whereas over 40 vaccines are currently in the pre-clinical phase [16].

The live attenuated and/or genetically modified mycobacteria are developed to be used as priming vaccines, meant to replace BCG. These improved vaccines should be more immunogenic, maintain this immune response for a longer period of time and be safer in immune-deficient individuals. Recombinant BCG (rBCG) modifications include i) introduction or over-expression of specific *Mtb* antigens which are lacking from BCG, and ii) improvement of antigen presentation by genetic modification of important pathways [16]. One of these rBCG vaccines is rBCGΔUrec:Hly$^+$ (VPM1002). rBCGΔUrec:Hly$^+$ expresses listeriolysin (Hly) which is responsible for rBCGs escape from phagosomes into the cytosol. Urease C (UreC), a protein involved in preventing phagosomal acidification, was removed to improve the effect of Hly, which functions most optimal under acidic conditions. This rBCG had a better vaccine potential compared to its parental strain [93]. Apoptosis of infected cells was promoted and increased CD4$^+$ as well as CD8$^+$ T-cell responses was observed, possibly due to enhanced cross priming. The vaccine has entered phase IIa trials [94]. Other rBCG vaccines include strains that overexpress antigens such as Ag85B (rBCG30) [95] and RD1 antigens [96]. Aeras-422, a vaccine that resembles rBCGΔUrec:Hly$^+$, expresses perfringolysin, but also overexpresses Ag85A, Ag85B and Rv3407 [97]. The combination of induced escape into the cytosol and overexpression of immunogenic antigens in one vaccine appears to have an improved efficacy. However this vaccine induced side effects such as the occurrence of shingles urging termination of the phase I trial [16;98;99].

Using *Mtb* strains for vaccine purposes requires special attention, it is important that attenuated strains are not virulent and therefore at least two independent loci, e.g. involved in essential metabolic pathways (autotrophy) or virulence, should be deleted, preventing reversion of virulence [100;101]. Currently several attenuated *Mtb* strains are under study as potential vaccines. The attenuated *Mtb* ΔsecA2ΔlysA [102] and *Mtb* ΔfbpAΔsapM [103] are examples of such mutant strains and are in a very early stage of analysis. Two other attenuated *Mtb* strains, *Mtb* ΔRD1ΔpanCD [104] and *Mtb* ΔPhoP (SO2), have already been studied for some time, showing promising immunogenicity and safety results. In order to comply to a consensus reached at a WHO Geneva meeting in 2004 [100;101;105], the SO2 strain has been further improved by deletion of the FadD26 gene, generating *Mtb* ΔPhoPΔfad or MTBVAC. The MTBVAC vaccine

has now entered phase I clinical trial to analyze its safety in humans. Interestingly, a novel potential vaccine strain was developed consisting of an attenuated *Mtb* strain expressing HIV antigens to prevent both *Mtb* and HIV infections [106].

In contrast to live attenuated and/or genetically modified mycobacteria vaccines, non-live subunit vaccines were initially developed to be used as booster on top of BCG vaccination or to enable novel live attenuated and/or genetically modified mycobacterial vaccines to enhance the long-lived immune memory response against *Mtb*. Subunit vaccines consist of protein(s) and/or (poly)epitopes delivered using a viral vector system or as recombinant product together with an (Th1) adjuvant [16;107]. One of the most advanced subunit vaccine is MVA85A, a recombinant (replication deficient) strain of Modified Vaccinia Ankara (MVA) virus that expresses the *Mtb* Ag85A antigen. MVA85A was shown to be immunogenic and safe in use in children and adults located at multiple sites [108-111]. Unfortunately, although MVA85A was safe, the phase IIb trial, published in February 2013, resulted in insignificant efficacy against TB and *Mtb* infection in children [112]. Another vaccine, AdAg85A which is currently studied in phase I trial, resembles MVA85A in that it also expresses Ag85A but expressed in an (replication deficient) Adenovirus (type 5) [113;114]. Ad35 (AERAS-402) is a virus-based vaccine expressing both *Mtb* Ag85A, Ag85B and TB10.4 as a fusion protein using a replication deficient Adenovirus (type 35) vector. Again, this vaccine also showed strong immunogenic potential in BCG primed individuals and also at different test sites [115;116]. This Ad35 based vaccine is currently in a phase IIb trial [117]. Importantly, some concerns have been raised over the use of adenovirus as vaccine vectors including the existence of neutralizing host antibodies that can influence the functioning of the vaccine, which can be avoided by using other viral vectors [118-120].

Also different subunit vaccines consisting of protein and adjuvants are currently studied. M72/AS01, consisting of Rv1196 (PPE18) and Rv0125 (serine protease, pepA) just finished phase IIa trial testing and showed to be safe and immunogenic [121;122]. Hybrid 1 (H1) (Ag85B and ESAT-6 fusion protein) administered using IC31 as adjuvant induced strong immunity in healthy donors and mycobacteria exposed individuals [123;124]. HyVac4 (or H4, consisting of Ag85B and TB10.4 fusion protein) shows immunogenic and protective effects in various animal models, and has now entered clinical trials as H4 administered together with IC31 [125]. Of note, H4 was developed to avoid H1 vaccine induction of ESAT-6 responses which interferes with IGRA outcome. Other potential subunit vaccines, including H56 (H1 backbone including Rv2660) [126] are still in pre-clinical phase e.g.: [127;128]. Instead of proteins, immunogenic epitopes can be combined to form a polyepitope and this also shows vaccine potential [107]. These vaccines can be used in heterologous prime-boost settings, thus combining live or attenuated vaccines with subunit vaccines to induce optimal protection.

Early secreted antigens such as ESAT-6 and Ag85 are often selected for TB subunit vaccines, based on expression in active replicating *Mtb*, secretion and strong (*in vitro*) immunogenicity [129]. Previous studies showed a protective effect of *Mtb* short culture filtrate which contained

both ESAT-6 and Ag85B [130]. Nonetheless, the antigens chosen for vaccination should also be significantly expressed by *Mtb* during *in vivo* infection, preferably over extended periods of time, preferably in different phases of *Mtb* infection and in both resistant and susceptible host backgrounds, such that the vaccine would induce T cells that directly recognize infected cells. This will be discussed in the next section.

Knowing *Mtb's* lifestyle

Phases of *Mycobacterium tuberculosis*

As already indicated, after infection *Mtb* is able to reside for decades inside the host without causing any clinical symptoms. To accomplish this, *Mtb* has to evade host induced pressures such as nutrient and oxygen shortage, acidic environment, toxic products produced by host cells and immune pressure. Influenced by these factors, *Mtb* encounters a dormant state which is characterized by: lipid body loaded, (phenotypic) drug-resistant, non-replicating bacteria with low metabolic activity (dormant bacteria) [131;132]. The granuloma will function to contain the infection [31]. Most probably both active replicating and dormant bacteria will be present during infection [133], but only dormant bacteria are tolerant to drugs and therefore difficult to eliminate by antibiotic treatment [16]. For improved diagnosis of latent *Mtb* infection and for optimal (therapeutic) vaccine development, a better understanding of *Mtb*'s lifecycle is of critical importance, particularly better insights in how *Mtb* adapts to *in vivo* environment upon infection. The change of *Mtb*'s genetic and proteomic makeup could have major impact on what antigens can be used as vaccine candidates.

In the beginning of the 21st century, *in vitro Mtb* culture studies captured the first differences in *Mtb's* transcriptomics response by comparing bacteria cultured under different stress conditions [134-136]. These studies focused on stress factors that *Mtb* is thought to encounter upon infection, including hypoxia, low pH, nutrient deprivation and free radicals. Oxygen tension is an important factor in the activation state of *Mtb* as the bacteria prefer to inhabit aerated parts of the host. [137]. Though, the transcriptional analysis showed that *Mtb* is able to adapt to oxygen depletion, to nitric oxide and to carbon monoxide [138-142], environmental conditions which are thought to occur within host cells and granulomas. Importantly, hypoxic regions were identified at the infection site *in vivo* [143]. Particularly interesting was the upregulation of a cluster of 48 genes, known as the dormancy regulon (DosR), which is controlled by regulator *Rv3133c* (DosR or DevR) [144;145]. Interestingly, a second set of genes is expressed at a later stage of hypoxia, known as the enduring hypoxic response (EHR). This set includes ~230 genes (including several DosR genes), which are upregulated considerably longer than most of the genes expressed by the DosR regulon [146].

Starvation conditions also trigger differential *Mtb* gene expression [140;147]. Here, *Mtb* has to compete for nutrients or adapt to the lack of nutrients and has to switch to the use of different

carbon sources for energy [148]. Interestingly, a substantial number of EHR genes overlap with genes associated with the starvation response [146]. Indeed, *In vivo*, *Mtb* will be exposed to multiple stress conditions at once. Dormancy was further simulated by Deb *et al.* [149] who cultured *Mtb* at low oxygen, high CO_2, acidic pH and low nutrient conditions. This resulted in dormant *Mtb* that had lost acid-fastness, contained lipid inclusion bodies and had become drug tolerant. Interestingly, genes encoding enzymes involved in the glyoxylate shunt were significantly expressed by *Mtb* cultured under multiple stress conditions. This cycle plays an essential role in the use of fatty acids as carbon source, and is essential for *Mtb's* survival *in vivo* [150]. Thus, this study verified the role of the glyoxylate shunt in stress conditions that *Mtb* may encounter *in vivo* [148;151;152]. All studies described here, however, mimicked the host environment in *in vitro* systems, but it would be more informative and relevant to directly analyze *Mtb's* responses *in vivo* in infected lung tissue. Several studies were performed to analyze *Mtb* transcripts *in vivo* (reviewed by Waddell *et al.* [153]), however, no animal models exist that fully resemble human TB disease, although some models show several specific TB phenotypes such as hypoxia [143] and lesion/granuloma formation [154;155]. Intriguingly, multiple studies show a role for lipid metabolism in *in vivo* survival of *Mtb* [149;156;157].

Thus *Mtb* is able to adapt to host environmental conditions and appears to express particular sets of genes in each condition, with some overlap in genes expressed between the different conditions identified and investigated. The proteins encoded by these sets of genes can be considered to be expressed in an *Mtb* infection phase related way, and thus may be of considerable interest as novel subunit vaccine candidates [16;158]. They may have an additional or even superior effect compared to antigens that are early secreted only. Indeed, the expression of *Mtb* proteins *in vivo* is of substantial significance as one study showed that upon *Mtb* infection the expression of Ag85B decreased, resulting in reduced activation of Ag85B specific T cells [29]. Thus Ag85B based vaccines might induce limited protection due to the loss of *Mtb* Ag85B expression in chronic phase infection. This might also be an explanation for the low efficacy induced by MVA85A [112]. The phase related antigens can therefore also be of use in therapeutic vaccines. A few novel *Mtb* phase related antigens are currently incorporated into TB vaccines. H56 contains next to ESAT-6 and Ag85B, also Rv2660, encoded by *Rv2660*, a gene highly expressed during starvation [126]. Furthermore, a polyepitope (consisting of immunogenic epitopes from Hsp65, Ag85B, 19Kda lipoprotein, HspX and Rv1733c) contains latency antigens HspX and Rv1733c [107]. Both studies revealed protective value of the vaccines. Lastly, the protective value of resuscitation promoting factor (Rpf) antigens as subunit vaccines was studied [159]. Rpfs are a set of genes encoding proteins suggested to be involved in reactivation or resuscitation of dormant bacteria. Five Rpfs are known in *Mtb*: *RpfA-E* (*Rv0867c, Rv1009, Rv1884c, Rv2389c* and *Rv2450c*). The proteins encoded by these genes resemble the Rpf protein encoded by *Micrococcus luteus*, which is expressed and secreted by actively replicating *M. luteus* bacteria. This protein showed to be able to resuscitate dormant *M. luteus*, but also stimulated the growth of mycobacteria [160]. Importantly,

the *Mtb* specific Rpfs also had a stimulating effect on the growth of *M. luteus* and mycobacteria [161].

Thus understanding *Mtb's* lifestyle opens doors to novel vaccine candidates that can be of value for both prophylactic and therapeutic vaccination strategies.

Understanding TB immunity

Besides the aforementioned stress conditions, immune pressure is also a hurdle *Mtb* has to encounter. Understanding this is essential for future development of novel TB vaccines. Vaccine induced humoral immunity is a well-known correlate of protection for many viral vaccines such as hepatitis and influenza [162]. However, cell mediated immunity (CMI) is essential in protection against *Mtb* infection. Although the role of B cells in TB immunity should not be neglected [163]. *Mtb* primarily resides in phagosomes which results in processing and presenting of *Mtb* antigens via the MHC class II route.

As previously mentioned, genetic defects in IFN-γ and IL-12 pathways, cytokines specific for Th1 differentiation, as well as depletion of $CD4^+$ T cells showed an increased risk of tuberculosis [49-51;56], confirming that Th1 cells play an important role in TB protection. Analyzing Th1 immunity is currently the most common approach to study TB vaccine potential [164]. Furthermore, IGRAs are also based on Th1 T cell analysis as antigen-specific IFN-γ production is measured. An important role for IFN-γ in *Mtb* control is activation of macrophages. Besides IFN-γ, other Th1 cytokines are of importance in protection against TB. As mentioned above, patients receiving anti-TNF therapy as treatment against different inflammatory diseases such as psoriasis and rheumatoid arthritis, have a higher risk of developing TB [52;53;165;166], indicating the need of TNF-α in protection against TB. Both IFN-γ and TNF-α activate APC antimicrobial effector mechanisms. Although we now know that Th1 immunity is of particular importance, exact insights in TB immune regulation remain limited. In contrast to Th1 cells, Th2 cells are generally thought not to have protective value in TB infection, and helminth-induced Th2 responses can have a detrimental effect on protection against TB [90] and BCG vaccination efficacy. Interestingly, a recent mouse model showed that $CD4^+$ T cells can exert protective functions even in the absence of IFN-γ and/or TNF-α [167], indicating that other factors may play an important role in protection as well. Indeed, functional, IFN-γ deficient, $CD4^+$ T cells still contribute to initial *Mtb* control [168], however, IFN-γ producing $CD4^+$ T cells are required for long-term control [168].

The study of novel T-cell subsets could shed further light upon the continued (though suboptimal) protection in the absence of IFN-γ and/or TNF-α. Multiparameter flowcytometric analysis has enabled detection of multiple markers on immune cells. Such analysis allows detection of multiple T-cell subsets that might play a role in infection and vaccination induced immunity. Recent new subsets include Th17 and regulatory T cells [169]. Human IL-1β/IL-6/IL-23 induced $CD4^+$ Th17 cells produce different cytokines and chemokines including IL-17, IL-22, CCL-20 but

also IFN-γ production has been described [169-172]. Vaccine induced Th17 cells were involved in TB protection in mice [173]. Specifically, IL-17 plays a role in maturation of granuloma formation [174]. Conversely, Th17 cells can be damaging as repeated antigen exposure results in IL-17-dependent lung pathology and enhanced neutrophil influx [175]. Thus, the net effect of Th17 cells on TB protection remains unclear. Regulatory T cells (Tregs) are also induced upon *Mtb* infection and are responsible for delayed priming of $CD4^+$ and $CD8^+$ T cells in the MLN [18] and direct suppression of effector T cell activity [176], thus having an anti-inflammatory effect. Therefore, they can be involved in bacterial persistence, next to limitation of host tissue damage [177]. Interestingly, differentiation of Th17 to Th1 and Treg to Th17 T cell subsets have been detected and indicate plasticity of unstable T-cell subsets [172;178;179].

Importantly, *Mtb* not only resides in the phagosome, but can also translocate to the cytosol, a mechanism which is RD1 region dependent [180;181]. Translocation of mycobacterial products to the cytosol enables loading of immunogenic epitopes to MHC class I and presentation to $CD8^+$ T cells, although many other mechanisms might also be involved in MHC-I loading such as cross-presentation and efferocytosis [40;182;183]. $CD8^+$ T cells produce multiple cytokines upon activation but can exert direct cytotoxic functions as well [31]. In addition to $CD4^+$ T cells, $CD8^+$ T cells also contribute to TB protection [184-186].

The current belief is that T cells producing multiple cytokines, chemokines and/or effector molecules are more effective compared to single cytokine producing cells. In more detail, T cells producing IFN-γ, TNF-α and IL-2 are believed to be of higher quality compared to double or single cytokine producing Th1 T cells and provide optimal protection and effector function [187]. In this study, polyfunctional T cells expressing IFN-γ, TNF-α and IL-2 correlated with protection against the intracellular pathogen *Leishmania major*. Subsequently, this was confirmed in mouse TB vaccine studies [188-190]. Also, higher frequencies of polyfunctional T cells were observed in LTBI compared to TB patients [191], based on mycobacterial load [192]. However, polyfunctional T cells correlated also to active TB disease [193;194]. In addition, several other studies showed no correlation of these cells with vaccination induced protection [195;196]. Interestingly, the antigen dose may be of importance for the development of protective polyfunctional T cells [197]. Furthermore, the duration of antigen stimulation and cytokine accumulation (due to Golgi transport inhibition) also influences the detection of polyfunctional T cells [198]. Thus, overall, the role of polyfunctional T cells in TB remains unclear.

Outline of this thesis

Efforts to decrease the number of TB cases by using latest *Mtb* detection methods, treatments and BCG vaccination campaigns have failed to result in eradication of the disease. TB remains one of the leading causes of death on our planet. The main focus of this thesis is to identify *Mtb* infection phase related antigens, and to evaluate these as potential antigens for TB vaccines.

Mtb Rpfs proteins are likely involved in reactivation of dormant mycobacteria, and thus may play an important role in TB disease. We therefore hypothesized that immunity directed against Rpf proteins could play a role in the control of reactivating bacteria. In **chapter 2** T-cell responses towards two *Mtb* resuscitation promoting factors (Rpfs), Rv0867c (RpfA) and Rv2389c (RpfD), are described in mycobacterium exposed individuals.

A second group of *Mtb* antigens, DosR antigens, previously was shown to be preferentially recognized by LTBI. From this perspective, we hypothesized that enhancing DosR specific immunity may be beneficial in controlling LTBI. However, only very few data on the *Mtb* DosR regulon encoded antigen-specific T-cell responses were available. We therefore analyzed the precise *Mtb* DosR antigen responding T cells and determined immunogenic peptide epitopes (**chapter 3)**.

The thus far described phase related antigens are all selected from initial *in vitro* studies which tried to mimic environmental stress conditions that *Mtb* encounters upon infection. We hypothesized that *Mtb* vaccine candidates should be expressed by *Mtb in vivo,* in infected tissue, and therefore analyzed *Mtb* gene expression profiles in the lungs of four genetically related but distinct mouse strains that represents a spectrum of TB susceptibility controlled by the *super-susceptibility to TB 1* locus. The immunogenicity of a subset of *in vivo* expressed *Mtb* (IVE-TB) antigens, associated to *Mtb* infection phenotypes, was subsequently determined (**chapter 4**).

Besides the criterion that vaccine candidates should be expressed *in vivo*, they should also be immunogenic *in vivo* and provide protection against *Mtb* infection. To determine the immunogenic and protective value of one of the IVE-TB antigens, Rv2034, HLA transgenic mice were vaccinated with Rv2034 as an experimental TB vaccine as described in **chapter 5**.

Studying the Rv2034 specific T-cell responses in more detail, we used a specific CD154 T-cell cloning method that allows more detailed analysis of these responses and in addition the development of novel tools to dissect the precise nature of the IVE-TB specific T-cell response (**chapter 6**).

The results of all studies included in this thesis are summarized and discussed in **chapter 7**.

References

1. **Gagneux,S.,** Host-pathogen coevolution in human tuberculosis. *Philos.Trans.R.Soc. Lond B Biol.Sci.* 2012. **367**: 850-859.
2. **WHO**. The global plan to stop TB 2011-2015. 2011.
3. **Hershkovitz,I., Donoghue,H.D., Minnikin,D.E., Besra,G.S., Lee,O.Y., Gernaey,A.M., Galili,E., Eshed,V., Greenblatt,C.L., Lemma,E., Bar-Gal,G.K., and Spigelman,M.,** Detection and molecular characterization of 9,000-year-old Mycobacterium tuberculosis from a Neolithic settlement in the Eastern Mediterranean. *PLoS.One.* 2008. **3**: e3426.
4. **Dubos,R. and Dubos, J.,** *The white plague: tuberculosis, man and society*. Boston: Little, Brown, 1952.
5. **Daniel,T.M.,** The history of tuberculosis. *Respir.Med.* 2006. **100**: 1862-1870.
6. **Herzog,H.,** History of tuberculosis. *Respiration* 1998. **65**: 5-15.
7. **Daniel,T.M.,** Hermann Michael Biggs and tuberculosis as a public health concern. *Int.J.Tuberc.Lung Dis.* 2011. **15**: 1572-1573.
8. **Daniel,T.M.,** Hermann Brehmer and the origins of tuberculosis sanatoria. *Int.J.Tuberc.Lung Dis.* 2011. **15**: 161-2, i.
9. **Mitchison,D. and Davies,G.,** The chemotherapy of tuberculosis: past, present and future. *Int.J.Tuberc.Lung Dis.* 2012. **16**: 724-732.
10. **Yang,C.T., Cambier,C.J., Davis,J.M., Hall,C.J., Crosier,P.S., and Ramakrishnan,L.,** Neutrophils exert protection in the early tuberculous granuloma by oxidative killing of mycobacteria phagocytosed from infected macrophages. *Cell Host.Microbe* 2012. **12**: 301-312.
11. **Lowe,D.M., Redford,P.S., Wilkinson,R.J., O'Garra,A., and Martineau,A.R.,** Neutrophils in tuberculosis: friend or foe? *Trends Immunol.* 2012. **33**: 14-25.
12. **Blomgran,R. and Ernst,J.D.,** Lung neutrophils facilitate activation of naive antigen-specific CD4+ T cells during Mycobacterium tuberculosis infection. *J.Immunol.* 2011. **186**: 7110-7119.
13. **Reece,S.T. and Kaufmann,S.H.,** Floating between the poles of pathology and protection: can we pin down the granuloma in tuberculosis? *Curr.Opin.Microbiol.* 2012. **15**: 63-70.
14. **Wolf,A.J., Desvignes,L., Linas,B., Banaiee,N., Tamura,T., Takatsu,K., and Ernst,J.D.,** Initiation of the adaptive immune response to Mycobacterium tuberculosis depends on antigen production in the local lymph node, not the lungs. *J.Exp.Med.* 2008. **205**: 105-115.
15. **Reiley,W.W., Calayag,M.D., Wittmer,S.T., Huntington,J.L., Pearl,J.E., Fountain,J.J., Martino,C.A., Roberts,A.D., Cooper,A.M., Winslow,G.M., and Woodland,D.L.,** ESAT-6-specific CD4 T cell responses to aerosol Mycobacterium tuberculosis infection are initiated in the mediastinal lymph nodes. *Proc.Natl. Acad.Sci.U.S.A* 2008. **105**: 10961-10966.
16. **Ottenhoff,T.H. and Kaufmann,S.H.,** Vaccines against tuberculosis: where are we and where do we need to go? *PLoS.Pathog.* 2012. **8**: e1002607.
17. **Blomgran,R., Desvignes,L., Briken,V., and Ernst,J.D.,** Mycobacterium tuberculosis inhibits neutrophil apoptosis, leading to delayed activation of naive CD4 T cells. *Cell Host.Microbe* 2012. **11**: 81-90.
18. **Shafiani,S., Tucker-Heard,G., Kariyone,A., Takatsu,K., and Urdahl,K.B.,** Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. *J.Exp.Med.* 2010. **207**: 1409-1420.
19. **Geijtenbeek,T.B., Van Vliet,S.J., Koppel,E.A., Sanchez-Hernandez,M., Vandenbroucke-Grauls,C.M., Appelmelk,B., and Van,K.Y.,** Mycobacteria target DC-SIGN to suppress dendritic cell function. *J.Exp.Med.* 2003. **197**: 7-17.

20. **Chieppa,M., Bianchi,G., Doni,A., Del,P.A., Sironi,M., Laskarin,G., Monti,P., Piemonti,L., Biondi,A., Mantovani,A., Introna,M., and Allavena,P.,** Cross-linking of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program. *J.Immunol.* 2003. **171**: 4552-4560.

21. **Gallegos,A.M., Pamer,E.G., and Glickman,M.S.,** Delayed protection by ESAT-6-specific effector CD4+ T cells after airborne M. tuberculosis infection. *J.Exp.Med.* 2008. **205**: 2359-2368.

22. **Shaler,C.R., Horvath,C., Lai,R., and Xing,Z.,** Understanding delayed T-cell priming, lung recruitment, and airway luminal T-cell responses in host defense against pulmonary tuberculosis. *Clin.Dev.Immunol.* 2012. **2012**: 628293.

23. **Vergne,I., Chua,J., Singh,S.B., and Deretic,V.,** Cell biology of mycobacterium tuberculosis phagosome. *Annu.Rev.Cell Dev.Biol.* 2004. **20**: 367-394.

24. **Mishra,A.K., Driessen,N.N., Appelmelk,B.J., and Besra,G.S.,** Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in Mycobacterium tuberculosis physiology and host-pathogen interaction. *FEMS Microbiol.Rev.* 2011. **35**: 1126-1157.

25. **Welin,A., Winberg,M.E., Abdalla,H., Sarndahl,E., Rasmusson,B., Stendahl,O., and Lerm,M.,** Incorporation of Mycobacterium tuberculosis lipoarabinomannan into macrophage membrane rafts is a prerequisite for the phagosomal maturation block. *Infect.Immun.* 2008. **76**: 2882-2887.

26. **Ehrt,S. and Schnappinger,D.,** Mycobacterial survival strategies in the phagosome: defence against host stresses. *Cell Microbiol.* 2009. **11**: 1170-1178.

27. **Pennini,M.E., Pai,R.K., Schultz,D.C., Boom,W.H., and Harding,C.V.,** Mycobacterium tuberculosis 19-kDa lipoprotein inhibits IFN-gamma-induced chromatin remodeling of MHC2TA by TLR2 and MAPK signaling. *J.Immunol.* 2006. **176**: 4323-4330.

28. **Egen,J.G., Rothfuchs,A.G., Feng,C.G., Horwitz,M.A., Sher,A., and Germain,R.N.,** Intravital imaging reveals limited antigen presentation and T cell effector function in mycobacterial granulomas. *Immunity.* 2011. **34**: 807-819.

29. **Bold,T.D., Banaei,N., Wolf,A.J., and Ernst,J.D.,** Suboptimal activation of antigen-specific CD4+ effector cells enables persistence of M. tuberculosis in vivo. *PLoS.Pathog.* 2011. **7**: e1002063.

30. **Ernst,J.D.,** The immunological life cycle of tuberculosis. *Nat.Rev.Immunol.* 2012. **12**: 581-591.

31. **Gupta,A., Kaul,A., Tsolaki,A.G., Kishore,U., and Bhakta,S.,** Mycobacterium tuberculosis: immune evasion, latency and reactivation. *Immunobiology* 2012. **217**: 363-374.

32. **Das,B., Kashino,S.S., Pulu,I., Kalita,D., Swami,V., Yeger,H., Felsher,D.W., and Campos-Neto,A.,** CD271+ Bone Marrow Mesenchymal Stem Cells May Provide a Niche for Dormant Mycobacterium tuberculosis. *Sci. Transl.Med.* 2013. **5**: 170ra13.

33. **Davis,J.M. and Ramakrishnan,L.,** The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell* 2009. **136**: 37-49.

34. **Volkman,H.E., Pozos,T.C., Zheng,J., Davis,J.M., Rawls,J.F., and Ramakrishnan,L.,** Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. *Science* 2010. **327**: 466-469.

35. **Volkman,H.E., Clay,H., Beery,D., Chang,J.C., Sherman,D.R., and Ramakrishnan,L.,** Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. *PLoS.Biol.* 2004. **2**: e367.

36. **Chen,M., Gan,H., and Remold,H.G.,** A mechanism of virulence: virulent Mycobacterium tuberculosis strain H37Rv, but not attenuated H37Ra, causes significant mitochondrial inner membrane disruption in macrophages leading to necrosis. *J.Immunol.* 2006. **176**: 3707-3716.

37. **Gan,H., Lee,J., Ren,F., Chen,M., Kornfeld,H., and Remold,H.G.,** Mycobacterium tuberculosis blocks crosslinking of annexin-1 and apoptotic envelope formation on infected macrophages

to maintain virulence. *Nat.Immunol.* 2008. **9**: 1189-1197.

38. **Divangahi,M., Desjardins,D., Nunes-Alves,C., Remold,H.G., and Behar,S.M.,** Eicosanoid pathways regulate adaptive immunity to Mycobacterium tuberculosis. *Nat.Immunol.* 2010. **11**: 751-758.
39. **Welin,A., Eklund,D., Stendahl,O., and Lerm,M.,** Human macrophages infected with a high burden of ESAT-6-expressing M. tuberculosis undergo caspase-1- and cathepsin B-independent necrosis. *PLoS.One.* 2011. **6**: e20302.
40. **Martin,C.J., Booty,M.G., Rosebrock,T.R., Nunes-Alves,C., Desjardins,D.M., Keren,I., Fortune,S.M., Remold,H.G., and Behar,S.M.,** Efferocytosis is an innate antibacterial mechanism. *Cell Host.Microbe* 2012. **12**: 289-300.
41. **Behar,S.M., Martin,C.J., Booty,M.G., Nishimura,T., Zhao,X., Gan,H.X., Divangahi,M., and Remold,H.G.,** Apoptosis is an innate defense function of macrophages against Mycobacterium tuberculosis. *Mucosal.Immunol.* 2011. **4**: 279-287.
42. **Derrick,S.C. and Morris,S.L.,** The ESAT6 protein of Mycobacterium tuberculosis induces apoptosis of macrophages by activating caspase expression. *Cell Microbiol.* 2007. **9**: 1547-1555.
43. **Ramakrishnan,L.,** Revisiting the role of the granuloma in tuberculosis. *Nat.Rev.Immunol.* 2012. **12**: 352-366.
44. **Choi,H.H., Shin,D.M., Kang,G., Kim,K.H., Park,J.B., Hur,G.M., Lee,H.M., Lim,Y.J., Park,J.K., Jo,E.K., and Song,C.H.,** Endoplasmic reticulum stress response is involved in Mycobacterium tuberculosis protein ESAT-6-mediated apoptosis. *FEBS Lett.* 2010. **584**: 2445-2454.
45. **Sutherland,I.,** Recent studies in the epidemiology of tuberculosis, based on the risk of being infected with tubercle bacilli. *Adv.Tuberc.Res.* 1976. **19**: 1-63.
46. **Harries,A.D. and Dye,C.,** Tuberculosis. *Ann.Trop.Med.Parasitol.* 2006. **100**: 415-431.
47. **Taylor,Z., Nolan,C.M., and Blumberg,H.M.,** Controlling tuberculosis in the United States. Recommendations from the American Thoracic Society, CDC, and the Infectious Diseases Society of America. *MMWR Recomm.Rep.* 2005. **54**: 1-81.
48. **Arend,S.M. and van Dissel,J.T.,** Evidence of endogenous reactivation of tuberculosis after a long period of latency. *J.Infect.Dis.* 2002. **186**: 876-877.
49. **Pawlowski,A., Jansson,M., Skold,M., Rottenberg,M.E., and Kallenius,G.,** Tuberculosis and HIV co-infection. *PLoS.Pathog.* 2012. **8**: e1002464.
50. **Saunders,B.M., Frank,A.A., Orme,I.M., and Cooper,A.M.,** CD4 is required for the development of a protective granulomatous response to pulmonary tuberculosis. *Cell Immunol.* 2002. **216**: 65-72.
51. **Kwan,C.K. and Ernst,J.D.,** HIV and tuberculosis: a deadly human syndemic. *Clin.Microbiol.Rev.* 2011. **24**: 351-376.
52. **Keane,J., Gershon,S., Wise,R.P., Mirabile-Levens,E., Kasznica,J., Schwieterman,W.D., Siegel,J.N., and Braun,M.M.,** Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N.Engl.J.Med.* 2001. **345**: 1098-1104.
53. **Harris,J. and Keane,J.,** How tumour necrosis factor blockers interfere with tuberculosis immunity. *Clin.Exp.Immunol.* 2010. **161**: 1-9.
54. **Fortin,A., Abel,L., Casanova,J.L., and Gros,P.,** Host genetics of mycobacterial diseases in mice and men: forward genetic studies of BCG-osis and tuberculosis. *Annu.Rev.Genomics Hum.Genet.* 2007. **8**: 163-192.
55. **Ottenhoff,T.H., Verreck,F.A., Hoeve,M.A., and van,d., V,** Control of human host immunity to mycobacteria. *Tuberculosis.(Edinb.)* 2005. **85**: 53-64.
56. **Ottenhoff,T.H., Verreck,F.A., Lichtenauer-Kaligis,E.G., Hoeve,M.A., Sanal,O., and van Dissel,J.T.,** Genetics, cytokines and human infectious disease: lessons from weakly pathogenic

mycobacteria and salmonellae. *Nat.Genet.* 2002. **32**: 97-105.

57. **Barry,C.E., III, Boshoff,H.I., Dartois,V., Dick,T., Ehrt,S., Flynn,J., Schnappinger,D., Wilkinson,R.J., and Young,D.,** The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat.Rev.Microbiol.* 2009. **7**: 845-855.

58. **Dheda,K., Ruhwald,M., Theron,G., Peter,J., and Yam,W.C.,** Point-of-care diagnosis of tuberculosis: Past, present and future. *Respirology.* 2013. **18**: 217-232.

59. **Lawn,S.D. and Zumla,A.I.,** Tuberculosis. *Lancet* 2011. **378**: 57-72.

60. **Gradmann,C.,** Robert Koch and the white death: from tuberculosis to tuberculin. *Microbes.Infect.* 2006. **8**: 294-301.

61. **Daniel,T.M.,** Florence Barbara Seibert and purified protein derivative. *Int.J.Tuberc.Lung Dis.* 2009. **13**: 281-282.

62. **Farhat,M., Greenaway,C., Pai,M., and Menzies,D.,** False-positive tuberculin skin tests: what is the absolute effect of BCG and non-tuberculous mycobacteria? *Int.J.Tuberc.Lung Dis.* 2006. **10**: 1192-1204.

63. **Pinto,L.M., Grenier,J., Schumacher,S.G., Denkinger,C.M., Steingart,K.R., and Pai,M.,** Immunodiagnosis of tuberculosis: state of the art. *Med.Princ.Pract.* 2012. **21**: 4-13.

64. **Latorre,I., De Souza-Galvao,M., Ruiz-Manzano,J., Lacoma,A., Prat,C., Altet,N., Ausina,V., and Dominguez,J.,** Evaluating the non-tuberculous mycobacteria effect in the tuberculosis infection diagnosis. *Eur.Respir.J.* 2010. **35**: 338-342.

65. **Wolf,T., Goetsch,U., Oremek,G., Bickel,M., Khaykin,P., Haberl,A., Bellinger,O., Gottschalk,R., Brodt,H.R., and Stephan,C.,** Tuberculosis skin test, but not interferon-gamma-releasing assays is affected by BCG vaccination in HIV patients. *J.Infect.* 2012.

66. **Falkinham,J.O., III,** Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. *J.Appl.Microbiol.* 2009. **107**: 356-367.

67. **Weiss,C.H. and Glassroth,J.,** Pulmonary disease caused by nontuberculous mycobacteria. *Expert.Rev.Respir.Med.* 2012. **6**: 597-612.

68. **Feazel,L.M., Baumgartner,L.K., Peterson,K.L., Frank,D.N., Harris,J.K., and Pace,N.R.,** Opportunistic pathogens enriched in showerhead biofilms. *Proc.Natl.Acad.Sci.U.S.A* 2009. **106**: 16393-16399.

69. **Mack,U., Migliori,G.B., Sester,M., Rieder,H.L., Ehlers,S., Goletti,D., Bossink,A., Magdorf,K., Holscher,C., Kampmann,B., Arend,S.M., Detjen,A., Bothamley,G., Zellweger,J.P., Milburn,H., Diel,R., Ravn,P., Cobelens,F., Cardona,P.J., Kan,B., Solovic,I., Duarte,R., and Cirillo,D.M.,** LTBI: latent tuberculosis infection or lasting immune responses to M. tuberculosis? A TBNET consensus statement. *Eur.Respir.J.* 2009. **33**: 956-973.

70. **Brock,I., Weldingh,K., Lillebaek,T., Follmann,F., and Andersen,P.,** Comparison of tuberculin skin test and new specific blood test in tuberculosis contacts. *Am.J.Respir.Crit Care Med.* 2004. **170**: 65-69.

71. **Diel,R., Goletti,D., Ferrara,G., Bothamley,G., Cirillo,D., Kampmann,B., Lange,C., Losi,M., Markova,R., Migliori,G.B., Nienhaus,A., Ruhwald,M., Wagner,D., Zellweger,J.P., Huitric,E., Sandgren,A., and Manissero,D.,** Interferon-gamma release assays for the diagnosis of latent Mycobacterium tuberculosis infection: a systematic review and meta-analysis. *Eur.Respir.J.* 2011. **37**: 88-99.

72. **Diel,R., Loddenkemper,R., and Nienhaus,A.,** Predictive value of interferon-gamma release assays and tuberculin skin testing for progression from latent TB infection to disease state: a meta-analysis. *Chest* 2012. **142**: 63-75.

73. **Trajman,A., Steffen,R.E., and Menzies,D.,** Interferon-Gamma Release Assays versus Tuberculin Skin Testing for Diagnosis of Latent Tuberculosis Infection: An Overview of the Evidence. *Pulmonary Medicine* 2013. **2013**.

74. **World Health Organization**. Use of Tuberculosis Interferon-Gamma Release Assays (IGRAs) in Low- and Middle-Income Countries: Policy Statement. 2011. World Health Organization, Geneva, Switzerland.

75. **Dheda,K., van Zyl,S.R., Badri,M., and Pai,M.,** T-cell interferon-gamma release assays for the rapid immunodiagnosis of tuberculosis: clinical utility in high-burden vs. low-burden settings. *Curr. Opin.Pulm.Med.* 2009. **15**: 188-200.

76. **Sharma,S.K., Mohanan,S., and Sharma,A.,** Relevance of latent TB infection in areas of high TB prevalence. *Chest* 2012. **142**: 761-773.

77. **Machado,A., Jr., Emodi,K., Takenami,I., Finkmoore,B.C., Barbosa,T., Carvalho,J., Cavalcanti,L., Santos,G., Tavares,M., Mota,M., Barreto,F., Reis,M.G., Arruda,S., and Riley,L.W.,** Analysis of discordance between the tuberculin skin test and the interferon-gamma release assay. *Int.J.Tuberc.Lung Dis.* 2009. **13**: 446-453.

78. **Goletti,D., Butera,O., Vanini,V., Lauria,F.N., Lange,C., Franken,K.L., Angeletti,C., Ottenhoff,T.H., and Girardi,E.,** Response to Rv2628 latency antigen associates with cured tuberculosis and remote infection. *Eur.Respir.J.* 2010. **36**: 135-142.

79. **Geluk,A., van Meijgaarden,K.E., Franken,K.L., Subronto,Y.W., Wieles,B., Arend,S.M., Sampaio,E.P., de,B.T., Faber,W.R., Naafs,B., and Ottenhoff,T.H.,** Identification and characterization of the ESAT-6 homologue of Mycobacterium leprae and T-cell cross-reactivity with Mycobacterium tuberculosis. *Infect.Immun.* 2002. **70**: 2544-2548.

80. **Geluk,A., van Meijgaarden,K.E., Franken,K.L., Wieles,B., Arend,S.M., Faber,W.R., Naafs,B., and Ottenhoff,T.H.,** Immunological crossreactivity of the Mycobacterium leprae CFP-10 with its homologue in Mycobacterium tuberculosis. *Scand.J.Immunol.* 2004. **59**: 66-70.

81. **Lee,S.W., Oh,D.K., Lee,S.H., Kang,H.Y., Lee,C.T., and Yim,J.J.,** Time interval to conversion of interferon-gamma release assay after exposure to tuberculosis. *Eur.Respir.J.* 2011. **37**: 1447-1452.

82. **Phillips,L.,** Infectious disease: TB's revenge. *Nature* 2013. **493**: 14-16.

83. **Mahairas,G.G., Sabo,P.J., Hickey,M.J., Singh,D.C., and Stover,C.K.,** Molecular analysis of genetic differences between Mycobacterium bovis BCG and virulent M. bovis. *J.Bacteriol.* 1996. **178**: 1274-1282.

84. **Hawgood,B.J.,** Albert Calmette (1863-1933) and Camille Guerin (1872-1961): the C and G of BCG vaccine. *J.Med.Biogr.* 2007. **15**: 139-146.

85. **Trunz,B.B., Fine,P., and Dye,C.,** Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *Lancet* 2006. **367**: 1173-1180.

86. **Setia,M.S., Steinmaus,C., Ho,C.S., and Rutherford,G.W.,** The role of BCG in prevention of leprosy: a meta-analysis. *Lancet Infect.Dis.* 2006. **6**: 162-170.

87. **Zodpey,S.P.,** Protective effect of bacillus Calmette Guerin (BCG) vaccine in the prevention of leprosy: a meta-analysis. *Indian J.Dermatol.Venereol.Leprol.* 2007. **73**: 86-93.

88. **Fine,P.E.,** Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* 1995. **346**: 1339-1345.

89. **Elias,D., Britton,S., Aseffa,A., Engers,H., and Akuffo,H.,** Poor immunogenicity of BCG in helminth infected population is associated with increased in vitro TGF-beta production. *Vaccine* 2008. **26**: 3897-3902.

90. **Rafi,W., Ribeiro-Rodrigues,R., Ellner,J.J., and Salgame,P.,** 'Coinfection-helminthes and tuberculosis'. *Curr. Opin.HIV.AIDS* 2012. **7**: 239-244.

91. **Brandt,L., Feino,C.J., Weinreich,O.A., Chilima,B., Hirsch,P., Appelberg,R., and Andersen,P.,** Failure

of the Mycobacterium bovis BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infect.Immun.* 2002. **70**: 672-678.

92. **Hesseling,A.C., Marais,B.J., Gie,R.P., Schaaf,H.S., Fine,P.E., Godfrey-Faussett,P., and Beyers,N.,** The risk of disseminated Bacille Calmette-Guerin (BCG) disease in HIV-infected children. *Vaccine* 2007. **25**: 14-18.

93. **Grode,L., Seiler,P., Baumann,S., Hess,J., Brinkmann,V., Nasser,E.A., Mann,P., Goosmann,C., Bandermann,S., Smith,D., Bancroft,G.J., Reyrat,J.M., van,S.D., Raupach,B., and Kaufmann,S.H.,** Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guerin mutants that secrete listeriolysin. *J.Clin.Invest* 2005. **115**: 2472-2479.

94. **Farinacci,M., Weber,S., and Kaufmann,S.H.,** The recombinant tuberculosis vaccine rBCG DeltaureC::hly(+) induces apoptotic vesicles for improved priming of CD4(+) and CD8(+) T cells. *Vaccine* 2012. **30**: 7608-7614.

95. **Hoft,D.F., Blazevic,A., Abate,G., Hanekom,W.A., Kaplan,G., Soler,J.H., Weichold,F., Geiter,L., Sadoff,J.C., and Horwitz,M.A.,** A new recombinant bacille Calmette-Guerin vaccine safely induces significantly enhanced tuberculosis-specific immunity in human volunteers. *J.Infect.Dis.* 2008. **198**: 1491-1501.

96. **Pym,A.S., Brodin,P., Majlessi,L., Brosch,R., Demangel,C., Williams,A., Griffiths,K.E., Marchal,G., Leclerc,C., and Cole,S.T.,** Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nat.Med.* 2003. **9**: 533-539.

97. **Sun,R., Skeiky,Y.A., Izzo,A., Dheenadhayalan,V., Imam,Z., Penn,E., Stagliano,K., Haddock,S., Mueller,S., Fulkerson,J., Scanga,C., Grover,A., Derrick,S.C., Morris,S., Hone,D.M., Horwitz,M.A., Kaufmann,S.H., and Sadoff,J.C.,** Novel recombinant BCG expressing perfringolysin O and the over-expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with Mycobacterium tuberculosis. *Vaccine* 2009. **27**: 4412-4423.

98. **Kaufmann,S.H. and Gengenbacher,M.,** Recombinant live vaccine candidates against tuberculosis. *Curr.Opin.Biotechnol.* 2012. **23**: 900-907.

99. **Kupferschmidt,K.,** Infectious disease. Taking a new shot at a TB vaccine. *Science* 2011. **334**: 1488-1490.

100. **Walker,K.B., Brennan,M.J., Ho,M.M., Eskola,J., Thiry,G., Sadoff,J., Dobbelaer,R., Grode,L., Liu,M.A., Fruth,U., and Lambert,P.H.,** The second Geneva Consensus: Recommendations for novel live TB vaccines. *Vaccine* 2010. **28**: 2259-2270.

101. **Kamath,A.T., Fruth,U., Brennan,M.J., Dobbelaer,R., Hubrechts,P., Ho,M.M., Mayner,R.E., Thole,J., Walker,K.B., Liu,M., and Lambert,P.H.,** New live mycobacterial vaccines: the Geneva consensus on essential steps towards clinical development. *Vaccine* 2005. **23**: 3753-3761.

102. **Hinchey,J., Jeon,B.Y., Alley,H., Chen,B., Goldberg,M., Derrick,S., Morris,S., Jacobs,W.R., Jr., Porcelli,S.A., and Lee,S.,** Lysine auxotrophy combined with deletion of the SecA2 gene results in a safe and highly immunogenic candidate live attenuated vaccine for tuberculosis. *PLoS.One.* 2011. **6**: e15857.

103. **Saikolappan,S., Estrella,J., Sasindran,S.J., Khan,A., Armitige,L.Y., Jagannath,C., and Dhandayuthapani,S.,** The fbpA/sapM double knock out strain of Mycobacterium tuberculosis is highly attenuated and immunogenic in macrophages. *PLoS.One.* 2012. **7**: e36198.

104. **Sambandamurthy,V.K., Derrick,S.C., Hsu,T., Chen,B., Larsen,M.H., Jalapathy,K.V., Chen,M., Kim,J., Porcelli,S.A., Chan,J., Morris,S.L., and Jacobs,W.R., Jr.,** Mycobacterium tuberculosis DeltaRD1 DeltapanCD: a safe and limited replicating mutant strain that protects immunocompetent and immuno-

compromised mice against experimental tuberculosis. *Vaccine* 2006. **24**: 6309-6320.

105. **Cardona,P.J., Asensio,J.G., Arbues,A., Otal,I., Lafoz,C., Gil,O., Caceres,N., Ausina,V., Gicquel,B., and Martin,C.,** Extended safety studies of the attenuated live tuberculosis vaccine SO2 based on phoP mutant. *Vaccine* 2009. **27**: 2499-2505.

106. **Jensen,K., Ranganathan,U.D., Van Rompay,K.K., Canfield,D.R., Khan,I., Ravindran,R., Luciw,P.A., Jacobs,W.R., Jr., Fennelly,G., Larsen,M.H., and Abel,K.,** A recombinant attenuated Mycobacterium tuberculosis vaccine strain is safe in immunosuppressed simian immunodeficiency virus-infected infant macaques. *Clin.Vaccine Immunol.* 2012. **19**: 1170-1181.

107. **Geluk,A., van den Eeden,S.J., van Meijgaarden,K.E., Dijkman,K., Franken,K.L., and Ottenhoff,T.H.,** A multistage-polyepitope vaccine protects against Mycobacterium tuberculosis infection in HLA-DR3 transgenic mice. *Vaccine* 2012. **30**: 7513-7521.

108. **McShane,H., Pathan,A.A., Sander,C.R., Keating,S.M., Gilbert,S.C., Huygen,K., Fletcher,H.A., and Hill,A.V.,** Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nat.Med.* 2004. **10**: 1240-1244.

109. **Scriba,T.J., Tameris,M., Mansoor,N., Smit,E., van der Merwe,L., Isaacs,F., Keyser,A., Moyo,S., Brittain,N., Lawrie,A., Gelderbloem,S., Veldsman,A., Hatherill,M., Hawkridge,A., Hill,A.V., Hussey,G.D., Mahomed,H., McShane,H., and Hanekom,W.A.,** Modified vaccinia Ankara-expressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional CD4+ T cells. *Eur.J.Immunol.* 2010. **40**: 279-290.

110. **Brookes,R.H., Hill,P.C., Owiafe,P.K., Ibanga,H.B., Jeffries,D.J., Donkor,S.A., Fletcher,H.A., Hammond,A.S., Lienhardt,C., Adegbola,R.A., McShane,H., and Hill,A.V.,** Safety and immunogenicity of the candidate tuberculosis vaccine MVA85A in West Africa. *PLoS.One.* 2008. **3**: e2921.

111. **Scriba,T.J., Tameris,M., Mansoor,N., Smit,E., van der Merwe,L., Mauff,K., Hughes,E.J., Moyo,S., Brittain,N., Lawrie,A., Mulenga,H., de,K.M., Gelderbloem,S., Veldsman,A., Hatherill,M., Geldenhuys,H., Hill,A.V., Hussey,G.D., Mahomed,H., Hanekom,W.A., and McShane,H.,** Dose-finding study of the novel tuberculosis vaccine, MVA85A, in healthy BCG-vaccinated infants. *J.Infect.Dis.* 2011. **203**: 1832-1843.

112. **Tameris,M.D., Hatherill,M., Landry,B.S., Scriba,T.J., Snowden,M.A., Lockhart,S., Shea,J.E., McClain,J.B., Hussey,G.D., Hanekom,W.A., Mahomed,H., and McShane,H.,** Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet* 2013.

113. **Beverley,P., Ronan,E., Lee,L., Arnold,I., Bolinger,B., Powrie,F., and Tchilian,E.,** Environmental effects on protection against Mycobacterium tuberculosis after immunization with Ad85A. *Vaccine* 2013. **31**: 1086-1093.

114. **Santosuosso,M., McCormick,S., Zhang,X., Zganiacz,A., and Xing,Z.,** Intranasal boosting with an adenovirus-vectored vaccine markedly enhances protection by parenteral Mycobacterium bovis BCG immunization against pulmonary tuberculosis. *Infect.Immun.* 2006. **74**: 4634-4643.

115. **Hoft,D.F., Blazevic,A., Stanley,J., Landry,B., Sizemore,D., Kpamegan,E., Gearhart,J., Scott,A., Kik,S., Pau,M.G., Goudsmit,J., McClain,J.B., and Sadoff,J.,** A recombinant adenovirus expressing immunodominant TB antigens can significantly enhance BCG-induced human immunity. *Vaccine* 2012. **30**: 2098-2108.

116. **Abel,B., Tameris,M., Mansoor,N., Gelderbloem,S., Hughes,J., Abrahams,D., Makhethe,L., Erasmus,M., de,K.M., van der Merwe,L.,**

Hawkridge,A., Veldsman,A., Hatherill,M., Schirru,G., Pau,M.G., Hendriks,J., Weverling,G.J., Goudsmit,J., Sizemore,D., McClain,J.B., Goetz,M., Gearhart,J., Mahomed,H., Hussey,G.D., Sadoff,J.C., and Hanekom,W.A., The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults. *Am.J.Respir. Crit Care Med.* 2010. **181**: 1407-1417.

117. **Pitt,J.M., Blankley,S., McShane,H., and O'Garra,A.,** Vaccination against tuberculosis: How can we better BCG? *Microb.Pathog.* 2012.

118. **Lasaro,M.O. and Ertl,H.C.,** New insights on adenovirus as vaccine vectors. *Mol.Ther.* 2009. **17**: 1333-1339.

119. **Madhan,S., Prabakaran,M., and Kwang,J.,** Baculovirus as vaccine vectors. *Curr.Gene Ther.* 2010. **10**: 201-213.

120. **Flatz,L., Hegazy,A.N., Bergthaler,A., Verschoor,A., Claus,C., Fernandez,M., Gattinoni,L., Johnson,S., Kreppel,F., Kochanek,S., Broek,M., Radbruch,A., Levy,F., Lambert,P.H., Siegrist,C.A., Restifo,N.P., Lohning,M., Ochsenbein,A.F., Nabel,G.J., and Pinschewer,D.D.,** Development of replication-defective lymphocytic choriomeningitis virus vectors for the induction of potent CD8+ T cell immunity. *Nat.Med.* 2010. **16**: 339-345.

121. **Day,C.L., Tameris,M., Mansoor,N., van,R.M., de,K.M., Geldenhuys,H., Erasmus,M., Makhethe,L., Hughes,E.J., Gelderbloem,S., Bollaerts,A., Bourguignon,P., Cohen,J., Demoitie,M.A., Mettens,P., Moris,P., Sadoff,J.C., Hawkridge,A., Hussey,G.D., Mahomed,H., Ofori-Anyinam,O., and Hanekom,W.A.,** Induction and Regulation of T Cell Immunity by the Novel TB Vaccine M72/AS01 in South African Adults. *Am.J.Respir.Crit Care Med.* 2013.

122. **Leroux-Roels,I., Forgus,S., De,B.F., Clement,F., Demoitie,M.A., Mettens,P., Moris,P., Ledent,E., Leroux-Roels,G., and Ofori-Anyinam,O.,** Improved CD4(+) T cell responses to Mycobacterium tuberculosis in PPD-negative adults by M72/AS01 as compared to the M72/AS02 and Mtb72F/AS02 tuberculosis candidate vaccine formulations: A randomized trial. *Vaccine* 2012.

123. **van Dissel,J.T., Arend,S.M., Prins,C., Bang,P., Tingskov,P.N., Lingnau,K., Nouta,J., Klein,M.R., Rosenkrands,I., Ottenhoff,T.H., Kromann,I., Doherty,T.M., and Andersen,P.,** Ag85B-ESAT-6 adjuvanted with IC31 promotes strong and long-lived Mycobacterium tuberculosis specific T cell responses in naive human volunteers. *Vaccine* 2010. **28**: 3571-3581.

124. **van Dissel,J.T., Soonawala,D., Joosten,S.A., Prins,C., Arend,S.M., Bang,P., Tingskov,P.N., Lingnau,K., Nouta,J., Hoff,S.T., Rosenkrands,I., Kromann,I., Ottenhoff,T.H., Doherty,T.M., and Andersen,P.,** Ag85B-ESAT-6 adjuvanted with IC31(R) promotes strong and long-lived Mycobacterium tuberculosis specific T cell responses in volunteers with previous BCG vaccination or tuberculosis infection. *Vaccine* 2011. **29**: 2100-2109.

125. **Billeskov,R., Elvang,T.T., Andersen,P.L., and Dietrich,J.,** The HyVac4 subunit vaccine efficiently boosts BCG-primed anti-mycobacterial protective immunity. *PLoS.One.* 2012. **7**: e39909.

126. **Aagaard,C., Hoang,T., Dietrich,J., Cardona,P.J., Izzo,A., Dolganov,G., Schoolnik,G.K., Cassidy,J.P., Billeskov,R., and Andersen,P.,** A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nat.Med.* 2011. **17**: 189-194.

127. **Niu,H., Hu,L., Li,Q., Da,Z., Wang,B., Tang,K., Xin,Q., Yu,H., Zhang,Y., Wang,Y., Ma,X., and Zhu,B.,** Construction and evaluation of a multistage Mycobacterium tuberculosis subunit vaccine candidate Mtb10.4-HspX. *Vaccine* 2011. **29**: 9451-9458.

128. **Windish,H.P., Duthie,M.S., Misquith,A., Ireton,G., Lucas,E., Laurance,J.D., Bailor,R.H., Coler,R.N., and Reed,S.G.,** Protection of mice from Mycobacterium tuberculosis by ID87/GLA-SE, a novel tuberculosis subunit vaccine candidate. *Vaccine* 2011. **29**: 7842-7848.

129. **Andersen,P.,** Host responses and antigens involved in protective immunity to Mycobacterium tuberculosis. *Scand.J.Immunol.* 1997. **45**: 115-131.

130. **Ottenhoff,T.H., Doherty,T.M., van Dissel,J.T., Bang,P., Lingnau,K., Kromann,I., and Andersen,P.,** First in humans: a new molecularly defined vaccine shows excellent safety and strong induction of long-lived Mycobacterium tuberculosis-specific Th1-cell like responses. *Hum.Vaccin.* 2010. **6**: 1007-1015.

131. **Boon,C. and Dick,T.,** How Mycobacterium tuberculosis goes to sleep: the dormancy survival regulator DosR a decade later. *Future Microbiol.* 2012. **7**: 513-518.

132. **Shleeva,M.O., Salina,E.G., and Kaprel'iants,A.S.,** [Dormant form of Mycobacterium tuberculosis]. *Mikrobiologiia* 2010. **79**: 3-15.

133. **Gill,W.P., Harik,N.S., Whiddon,M.R., Liao,R.P., Mittler,J.E., and Sherman,D.R.,** A replication clock for Mycobacterium tuberculosis. *Nat.Med.* 2009. **15**: 211-214.

134. **Sherman,D.R., Voskuil,M., Schnappinger,D., Liao,R., Harrell,M.I., and Schoolnik,G.K.,** Regulation of the Mycobacterium tuberculosis hypoxic response gene encoding alpha -crystallin. *Proc.Natl.Acad.Sci.U.S.A* 2001. **98**: 7534-7539.

135. **Ohno,H., Zhu,G., Mohan,V.P., Chu,D., Kohno,S., Jacobs,W.R., Jr., and Chan,J.,** The effects of reactive nitrogen intermediates on gene expression in Mycobacterium tuberculosis. *Cell Microbiol.* 2003. **5**: 637-648.

136. **Fisher,M.A., Plikaytis,B.B., and Shinnick,T.M.,** Microarray analysis of the Mycobacterium tuberculosis transcriptional response to the acidic conditions found in phagosomes. *J.Bacteriol.* 2002. **184**: 4025-4032.

137. **Rustad,T.R., Sherrid,A.M., Minch,K.J., and Sherman,D.R.,** Hypoxia: a window into Mycobacterium tuberculosis latency. *Cell Microbiol.* 2009. **11**: 1151-1159.

138. **Voskuil,M.I., Schnappinger,D., Visconti,K.C., Harrell,M.I., Dolganov,G.M., Sherman,D.R., and Schoolnik,G.K.,** Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. *J.Exp.Med.* 2003. **198**: 705-713.

139. **Wayne,L.G. and Sohaskey,C.D.,** Nonreplicating persistence of mycobacterium tuberculosis. *Annu.Rev.Microbiol.* 2001. **55**: 139-163.

140. **Schnappinger,D., Ehrt,S., Voskuil,M.I., Liu,Y., Mangan,J.A., Monahan,I.M., Dolganov,G., Efron,B., Butcher,P.D., Nathan,C., and Schoolnik,G.K.,** Transcriptional Adaptation of Mycobacterium tuberculosis within Macrophages: Insights into the Phagosomal Environment. *J.Exp.Med.* 2003. **198**: 693-704.

141. **Kumar,A., Deshane,J.S., Crossman,D.K., Bolisetty,S., Yan,B.S., Kramnik,I., Agarwal,A., and Steyn,A.J.,** Heme oxygenase-1-derived carbon monoxide induces the Mycobacterium tuberculosis dormancy regulon. *J.Biol.Chem.* 2008. **283**: 18032-18039.

142. **Shiloh,M.U., Manzanillo,P., and Cox,J.S.,** Mycobacterium tuberculosis senses host-derived carbon monoxide during macrophage infection. *Cell Host.Microbe* 2008. **3**: 323-330.

143. **Via,L.E., Lin,P.L., Ray,S.M., Carrillo,J., Allen,S.S., Eum,S.Y., Taylor,K., Klein,E., Manjunatha,U., Gonzales,J., Lee,E.G., Park,S.K., Raleigh,J.A., Cho,S.N., McMurray,D.N., Flynn,J.L., and Barry,C.E., III,** Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. *Infect.Immun.* 2008. **76**: 2333-2340.

144. **Lin,M.Y. and Ottenhoff,T.H.,** Not to wake a sleeping giant: new insights into host-pathogen interactions identify new targets for vaccination against latent Mycobacterium tuberculosis infection. *Biol.Chem.* 2008. **389**: 497-511.

145. **Park,H.D., Guinn,K.M., Harrell,M.I., Liao,R., Voskuil,M.I., Tompa,M., Schoolnik,G.K., and Sherman,D.R.,** Rv3133c/dosR is a transcription factor that mediates the hypoxic response of Mycobacterium tuberculosis. *Mol.Microbiol.* 2003. **48**: 833-843.

146. **Rustad,T.R., Harrell,M.I., Liao,R., and Sherman,D.R.,** The enduring hypoxic response of Mycobacterium tuberculosis. *PLoS.One.* 2008. **3**: e1502.

147. **Betts,J.C., Lukey,P.T., Robb,L.C., McAdam,R.A., and Duncan,K.,** Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol.Microbiol.* 2002. **43**: 717-731.

148. **Schaible,U.E. and Kaufmann,S.H.,** A nutritive view on the host-pathogen interplay. *Trends Microbiol.* 2005. **13**: 373-380.

149. **Deb,C., Lee,C.M., Dubey,V.S., Daniel,J., Abomoelak,B., Sirakova,T.D., Pawar,S., Rogers,L., and Kolattukudy,P.E.,** A novel in vitro multiple-stress dormancy model for Mycobacterium tuberculosis generates a lipid-loaded, drug-tolerant, dormant pathogen. *PLoS.One.* 2009. **4**: e6077.

150. **Munoz-Elias,E.J. and McKinney,J.D.,** Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat.Med.* 2005. **11**: 638-644.

151. **Timm,J., Post,F.A., Bekker,L.G., Walther,G.B., Wainwright,H.C., Manganelli,R., Chan,W.T., Tsenova,L., Gold,B., Smith,I., Kaplan,G., and McKinney,J.D.,** Differential expression of iron-, carbon-, and oxygen-responsive mycobacterial genes in the lungs of chronically infected mice and tuberculosis patients. *Proc.Natl.Acad. Sci.U.S.A* 2003. **100**: 14321-14326.

152. **McKinney,J.D., Honer zu,B.K., Munoz-Elias,E.J., Miczak,A., Chen,B., Chan,W.T., Swenson,D., Sacchettini,J.C., Jacobs,W.R., Jr., and Russell,D.G.,** Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* 2000. **406**: 735-738.

153. **Waddell,S.J. and Butcher,P.D.,** Microarray analysis of whole genome expression of intracellular Mycobacterium tuberculosis. *Curr.Mol.Med.* 2007. **7**: 287-296.

154. **Reece,S.T., Loddenkemper,C., Askew,D.J., Zedler,U., Schommer-Leitner,S., Stein,M., Mir,F.A., Dorhoi,A., Mollenkopf,H.J., Silverman,G.A., and Kaufmann,S.H.,** Serine protease activity contributes to control of Mycobacterium tuberculosis in hypoxic lung granulomas in mice. *J.Clin.Invest* 2010. **120**: 3365-3376.

155. **Pichugin,A.V., Yan,B.S., Sloutsky-,A., Kobzik,L., and Kramnik,I.,** Dominant role of the sst1 locus in pathogenesis of necrotizing lung granulomas during chronic tuberculosis infection and reactivation in genetically resistant hosts. *Am.J.Pathol.* 2009. **174**: 2190-2201.

156. **Daniel,J., Maamar,H., Deb,C., Sirakova,T.D., and Kolattukudy,P.E.,** Mycobacterium tuberculosis uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. *PLoS.Pathog.* 2011. **7**: e1002093.

157. **Gengenbacher,M. and Kaufmann,S.H.,** Mycobacterium tuberculosis: success through dormancy. *FEMS Microbiol.Rev.* 2012. **36**: 514-532.

158. **Gideon,H.P., Wilkinson,K.A., Rustad,T.R., Oni,T., Guio,H., Sherman,D.R., Vordermeier,H.M., Robertson,B.D., Young,D.B., and Wilkinson,R.J.,** Bioinformatic and empirical analysis of novel hypoxia-inducible targets of the human antituberculosis T cell response. *J.Immunol.* 2012. **189**: 5867-5876.

159. **Romano,M., Aryan,E., Korf,H., Bruffaerts,N., Franken,C.L., Ottenhoff,T.H., and Huygen,K.,** Potential of Mycobacterium tuberculosis resuscitation-promoting factors as antigens in novel tuberculosis sub-unit vaccines. *Microbes. Infect.* 2012. **14**: 86-95.

160. **Mukamolova,G.V., Kaprelyants,A.S., Young,D.I., Young,M., and Kell,D.B.,** A bacterial cytokine. *Proc.Natl.Acad.Sci.U.S.A* 1998. **95**: 8916-8921.

161. **Mukamolova,G.V., Turapov,O.A., Young,D.I., Kaprelyants,A.S., Kell,D.B., and Young,M.,** A family of autocrine growth factors in Mycobacteri-

um tuberculosis. *Mol.Microbiol.* 2002. **46**: 623-635.

162. **Thakur,A., Pedersen,L.E., and Jungersen,G.,** Immune markers and correlates of protection for vaccine induced immune responses. *Vaccine* 2012. **30**: 4907-4920.

163. **Maglione,P.J. and Chan,J.,** How B cells shape the immune response against Mycobacterium tuberculosis. *Eur.J.Immunol.* 2009. **39**: 676-686.

164. **Walzl,G., Ronacher,K., Hanekom,W., Scriba,T.J., and Zumla,A.,** Immunological biomarkers of tuberculosis. *Nat.Rev.Immunol.* 2011. **11**: 343-354.

165. **Winthrop,K.L.,** Risk and prevention of tuberculosis and other serious opportunistic infections associated with the inhibition of tumor necrosis factor. *Nat.Clin.Pract. Rheumatol.* 2006. **2**: 602-610.

166. **Jacobs,M., Togbe,D., Fremond,C., Samarina,A., Allie,N., Botha,T., Carlos,D., Parida,S.K., Grivennikov,S., Nedospasov,S., Monteiro,A., Le,B.M., Quesniaux,V., and Ryffel,B.,** Tumor necrosis factor is critical to control tuberculosis infection. *Microbes.Infect.* 2007. **9**: 623-628.

167. **Gallegos,A.M., van Heijst,J.W., Samstein,M., Su,X., Pamer,E.G., and Glickman,M.S.,** A gamma interferon independent mechanism of CD4 T cell mediated control of M. tuberculosis infection in vivo. *PLoS.Pathog* 2011. **7**: e1002052.

168. **Green,A.M., Difazio,R., and Flynn,J.L.,** IFN-gamma from CD4 T cells is essential for host survival and enhances CD8 T cell function during Mycobacterium tuberculosis infection. *J.Immunol.* 2013. **190**: 270-277.

169. **Ottenhoff,T.H.,** New pathways of protective and pathological host defense to mycobacteria. *Trends Microbiol.* 2012. **20**: 419-428.

170. **Torrado,E. and Cooper,A.M.,** IL-17 and Th17 cells in tuberculosis. *Cytokine Growth Factor Rev.* 2010. **21**: 455-462.

171. **Ottenhoff,T.H.,** The knowns and unknowns of the immunopathogenesis of tuberculosis. *Int.J.Tuberc.Lung Dis.* 2012. **16**: 1424-1432.

172. **Sallusto,F., Zielinski,C.E., and Lanzavecchia,A.,** Human Th17 subsets. *Eur.J.Immunol.* 2012. **42**: 2215-2220.

173. **Khader,S.A., Bell,G.K., Pearl,J.E., Fountain,J.J., Rangel-Moreno,J., Cilley,G.E., Shen,F., Eaton,S.M., Gaffen,S.L., Swain,S.L., Locksley,R.M., Haynes,L., Randall,T.D., and Cooper,A.M.,** IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat.Immunol.* 2007. **8**: 369-377.

174. **Okamoto,Y.Y., Umemura,M., Yahagi,A., O'Brien,R.L., Ikuta,K., Kishihara,K., Hara,H., Nakae,S., Iwakura,Y., and Matsuzaki,G.,** Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. *J.Immunol.* 2010. **184**: 4414-4422.

175. **Cruz,A., Fraga,A.G., Fountain,J.J., Rangel-Moreno,J., Torrado,E., Saraiva,M., Pereira,D.R., Randall,T.D., Pedrosa,J., Cooper,A.M., and Castro,A.G.,** Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with Mycobacterium tuberculosis. *J.Exp.Med.* 2010. **207**: 1609-1616.

176. **Tang,Q. and Bluestone,J.A.,** The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat.Immunol.* 2008. **9**: 239-244.

177. **Joosten,S.A. and Ottenhoff,T.H.,** Human CD4 and CD8 regulatory T cells in infectious diseases and vaccination. *Hum.Immunol.* 2008. **69**: 760-770.

178. **Annunziato,F. and Romagnani,S.,** The transient nature of the Th17 phenotype. *Eur.J.Immunol.* 2010. **40**: 3312-3316.

179. **Murphy,K.M. and Stockinger,B.,** Effector T cell plasticity: flexibility in the face of changing circumstances. *Nat.Immunol.* 2010. **11**: 674-680.

180. **van der Wel,N., Hava,D., Houben,D., Fluitsma,D., van,Z.M., Pierson,J., Brenner,M., and Peters,P.J.,** M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* 2007. **129**: 1287-1298.

181. **Simeone,R., Bobard,A., Lippmann,J., Bitter,W., Majlessi,L., Brosch,R., and Enninga,J.,** Phagosomal rupture by Mycobacterium tuberculosis results in toxicity and host cell death. *PLoS.Pathog.* 2012. **8**: e1002507.

182. **Winau,F., Weber,S., Sad,S., de,D.J., Hoops,S.L., Breiden,B., Sandhoff,K., Brinkmann,V., Kaufmann,S.H., and Schaible,U.E.,** Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. *Immunity.* 2006. **24**: 105-117.

183. **Albert,M.L., Sauter,B., and Bhardwaj,N.,** Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 1998. **392**: 86-89.

184. **Chen,C.Y., Huang,D., Wang,R.C., Shen,L., Zeng,G., Yao,S., Shen,Y., Halliday,L., Fortman,J., McAllister,M., Estep,J., Hunt,R., Vasconcelos,D., Du,G., Porcelli,S.A., Larsen,M.H., Jacobs,W.R., Jr., Haynes,B.F., Letvin,N.L., and Chen,Z.W.,** A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. *PLoS.Pathog.* 2009. **5**: e1000392.

185. **Bruns,H., Meinken,C., Schauenberg,P., Harter,G., Kern,P., Modlin,R.L., Antoni,C., and Stenger,S.,** Anti-TNF immunotherapy reduces CD8+ T cell-mediated antimicrobial activity against Mycobacterium tuberculosis in humans. *J.Clin.Invest* 2009. **119**: 1167-1177.

186. **Lazarevic,V. and Flynn,J.,** CD8+ T cells in tuberculosis. *Am.J.Respir.Crit Care Med.* 2002. **166**: 1116-1121.

187. **Darrah,P.A., Patel,D.T., De Luca,P.M., Lindsay,R.W., Davey,D.F., Flynn,B.J., Hoff,S.T., Andersen,P., Reed,S.G., Morris,S.L., Roederer,M., and Seder,R.A.,** Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat.Med.* 2007. **13**: 843-850.

188. **Lindenstrom,T., Agger,E.M., Korsholm,K.S., Darrah,P.A., Aagaard,C., Seder,R.A., Rosenkrands,I., and Andersen,P.,** Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. *J.Immunol.* 2009. **182**: 8047-8055.

189. **Derrick,S.C., Yabe,I.M., Yang,A., and Morris,S.L.,** Vaccine-induced anti-tuberculosis protective immunity in mice correlates with the magnitude and quality of multifunctional CD4 T cells. *Vaccine* 2011. **29**: 2902-2909.

190. **Forbes,E.K., Sander,C., Ronan,E.O., McShane,H., Hill,A.V., Beverley,P.C., and Tchilian,E.Z.,** Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against Mycobacterium tuberculosis aerosol challenge in mice. *J.Immunol.* 2008. **181**: 4955-4964.

191. **Harari,A., Rozot,V., Enders,F.B., Perreau,M., Stalder,J.M., Nicod,L.P., Cavassini,M., Calandra,T., Blanchet,C.L., Jaton,K., Faouzi,M., Day,C.L., Hanekom,W.A., Bart,P.A., and Pantaleo,G.,** Dominant TNF-alpha+ Mycobacterium tuberculosis-specific CD4+ T cell responses discriminate between latent infection and active disease. *Nat.Med.* 2011. **17**: 372-376.

192. **Day,C.L., Abrahams,D.A., Lerumo,L., Janse van,R.E., Stone,L., O'rie,T., Pienaar,B., de,K.M., Kaplan,G., Mahomed,H., Dheda,K., and Hanekom,W.A.,** Functional capacity of Mycobacterium tuberculosis-specific T cell responses in humans is associated with mycobacterial load. *J.Immunol.* 2011. **187**: 2222-2232.

193. **Caccamo,N., Guggino,G., Joosten,S.A., Gelsomino,G., Di,C.P., Titone,L., Galati,D., Bocchino,M., Matarese,A., Salerno,A., Sanduzzi,A., Franken,W.P., Ottenhoff,T.H., and Dieli,F.,** Multifunctional CD4(+) T cells correlate with active Mycobacterium tuberculosis infection. *Eur.J.Immunol.* 2010. **40**: 2211-2220.

194. **Sutherland,J.S., Adetifa,I.M., Hill,P.C., Adegbola,R.A., and Ota,M.O.,** Pattern and diversity of cytokine production differentiates between Mycobacterium tuberculosis infection and disease. *Eur.J.Immunol.* 2009. **39**: 723-729.

195. **Kagina,B.M., Abel,B., Scriba,T.J., Hughes,E.J., Keyser,A., Soares,A., Gamieldien,H., Sidibana,M., Hatherill,M., Gelderbloem,S., Mahomed,H., Hawkridge,A., Hussey,G., Kaplan,G., and Hanekom,W.A.,** Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guerin vaccination of newborns. *Am.J.Respir.Crit Care Med.* 2010. **182**: 1073-1079.

196. **Smith,S.G., Lalor,M.K., Gorak-Stolinska,P., Blitz,R., Beveridge,N.E., Worth,A., McShane,H., and Dockrell,H.M.,** Mycobacterium tuberculosis PPD-induced immune biomarkers measurable in vitro following BCG vaccination of UK adolescents by multiplex bead array and intracellular cytokine staining. *BMC.Immunol.* 2010. **11**: 35.

197. **Aagaard,C., Hoang,T.T., Izzo,A., Billeskov,R., Troudt,J., Arnett,K., Keyser,A., Elvang,T., Andersen,P., and Dietrich,J.,** Protection and polyfunctional T cells induced by Ag85B-TB10.4/IC31 against Mycobacterium tuberculosis is highly dependent on the antigen dose. *PLoS.One.* 2009. **4**: e5930.

198. **Kaveh,D.A., Whelan,A.O., and Hogarth,P.J.,** The duration of antigen-stimulation significantly alters the diversity of multifunctional CD4 T cells measured by intracellular cytokine staining. *PLoS.One.* 2012. **7**: e38926.

CHAPTER 2

Identification of human T-cell responses to *Mycobacterium tuberculosis* resuscitation-promoting factors in long-term latently infected individuals

Susanna Commandeur[1,*], Krista E. van Meijgaarden[1,*], May Young Lin[1], Kees L. M. C. Franken[1], Annemieke H. Friggen[1], Jan Wouter Drijfhout[2], Fredrik Oftung[3], Gro Ellen Korsvold[3], Annemieke Geluk[1] and Tom H. M. Ottenhoff[1]

[1] Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands; [2] Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands; [3] Department of Bacteriology and Immunology, Division of Infectious Disease Control, Norwegian Institute of Public Health, Oslo, Norway

[*]These authors contributed equally to this study

Clin Vaccine Immunol. 2011. Apr;18(4):676-83.

Abstract

The *Mycobacterium bovis* BCG vaccine is the only tuberculosis (TB) vaccine available, yet it provides limited protection against pulmonary TB in adults and fails to protect against TB reactivation. We hypothesized that immunity against *Mycobacterium tuberculosis (Mtb)* "resuscitation-promoting factors" (Rpfs), which are small bacterial proteins that promote proliferation of dormant mycobacteria, may be relevant in the human immune response to *Mtb*. In previous unpublished work, we found that Rpfs Rv0867c and Rv2389c induced interferon gamma (IFN-γ) production in the blood of TB patients' healthy household contacts in several different African populations. Here we examine these two dominant Rpf antigens in more detail and define the nature of the responding T-cell subsets. Multiparameter cytokine profiling showed that Rv2389c and, to a lesser extent, Rv0867c were recognized by mycobacterium-responsive healthy Dutch individuals; peptide-scanning revealed several epitopes, including a single immunodominant epitope in Rv2389c. Rv0867c and, to a lesser extent, Rv2389c Rpf-specific T-cell responses were maintained for decades in long-term *Mtb* non-progressors. Prominent Rv0867c-specific double- and single-cytokine-producing $CD8^+$ T-cell subset responses were found, including a large population of $CD8^+$ effector memory and effector T-cell subsets. We conclude that *Mtb* Rpf antigens are important targets in the human immune response to *Mtb*, and represent interesting TB vaccine candidate antigens.

Introduction

It is estimated that over 2 billion people are latently infected with *Mycobacterium tuberculosis* (*Mtb*) and that 5 to 10% of these individuals will develop active tuberculosis (TB) at one point in their lifetime whereas the remainder are able to contain infection long term without developing clinical symptoms [1]. During latency, the bacteria are thought to be in a dormant or slowly replicating state [2]. The vast reservoir of individuals with latent infection is a major source of new TB cases due to reactivation and resuscitation of dormant bacilli [3;4].

The term "dormancy" was first introduced by Joseph Warwick Bigger, who discovered that a culture of *Staphylococcus pyogenes* could not be sterilized after penicillin treatment since there was a small group of antibiotic-resistant bacteria that could be re-grown from such cultures. Bigger proposed that these bacteria were dormant, nonreplicating, and thus insensitive to antimicrobials targeting bacterial metabolic pathways [5].

It is assumed that environmental factors are involved in inducing bacterial dormancy [6]. *Mtb* enters a state of nonreplicating or slowly replicating persistence when grown under gradual oxygen depletion, which is thought to be one of the stress factors that *Mtb* encounters upon infection [7]. Not only oxygen deprivation, but also low pH, NO, nutrient deprivation and host immune pressure are stress factors *Mtb* is subjected to in the lung granulomatous lesions. In response to these stress factors, *Mtb* decreases its metabolic activity and alters its gene expression pattern [6;8;9]. This adaptation results in increased resistance to environmental stress, by means of entering the nonreplicating or slowly replicating persisting state [10].

While several studies have addressed bacterial transition from the replicating to the nonreplicating, slowly replicating or dormant state, little is known regarding the cues that induce bacteria to reactivate and resume growth from dormancy. Mukamolova *et al.* were the first to discover a resuscitation promoting factor (Rpf), a hormone-like protein secreted by *Micrococcus luteus* (*M. luteus*). Addition of this Rpf protein to dormant *M. luteus* resulted in resuscitation of *M. luteus* bacteria [11;12]. *Rpf* genes were then found to be conserved throughout high-G+C Gram-positive bacteria, including *Mtb*. Five such genes were identified in the *Mtb* genome, notably *Rv0867c (rpfA), Rv1009 (rpfB), Rv1884c (rpfC), Rv2389c (rpfD)* and *Rv2450c (rpfE)*. Each *Mtb* Rpf protein contains an ~70 amino-acid rpf-like domain similar to the *M. luteus rpf*-encoded protein [13]. *Mtb* Rpfs showed similar properties to *M. luteus* Rpf, including their ability to resuscitate dormant mycobacteria [13;14]. *Mtb* Rpf protein expression was observed *in vitro* in actively growing *Mtb* and in *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG). Importantly, *Mtb rpf* gene expression was detected in infected murine and human tissue. Moreover, the presence of Rpf-like proteins was shown in *Mtb*-infected human tissue [13;15-17]. Recently, *rpf* gene expression was analyzed in *Mtb* cells grown under different physiological stress conditions and growth factors. All five *rpf* genes were expressed during actively replicating early log-phase growth of *Mtb*, confirming previous findings. Of note, *Mtb rpf* genes displayed differential expression patterns when analyzed

in cultures grown under hypoxia, nutrient starvation and acidic conditions and stationary-, nonculturable-, and resuscitation-phase-like conditions. These differential adaptive *rpf* expression profiles indicate that *Mtb* Rpfs likely play different roles [4].

While *M. luteus* Rpf is essential for *M. luteus* growth, individual *Mtb* Rpfs were found to be redundant for growth *in vitro* and *in vivo* in single-gene-knockout mutants [17;18]. However, when multiple *rpf* deletions were introduced simultaneously in *Mtb* (Δ*Rv0867c*/Δ*Rv1009*/Δ*Rv1884c* and Δ*Rv0867c*/Δ*Rv1009*/Δ*Rv2389c*), a significant loss in the ability of *Mtb* to resuscitate was found, accompanied by *in vitro* growth attenuation [19].

BCG vaccination is widely used and affords protection from severe forms of TB in children, but it provides only limited and highly variable protection against pulmonary TB in adults and does not protect against reactivation. Better TB vaccines are clearly needed [20]. As the *Mtb* Rpf proteins are associated with resuscitation of mycobacteria, we hypothesized that immunity directed against these proteins may play a role in sensing actively replicating *Mtb* organisms and possibly play a role in host immune control of reactivating bacteria. Only two reports have investigated the immunogenicity of the *Mtb* Rpf antigens. One study with mice showed that Rv0867c, Rv1009, Rv2389c and Rv2450c were immunogenic [21]. Recently we identified the first human *Mtb* Rpf-specific T-cell responses against a subset of the five *Mtb* Rpfs in a larger antigen T-cell screening [22]. Interferon gamma (IFN-γ) production was detected in tuberculin skin test (TST)-positive individuals in response to Rv1009, Rv1884c and Rv2450c and, to a lesser extent, to Rv0867c, whereas limited to no IFN-γ was found in TST-negative individuals. Rv2389c, however, was not included in this study [22].

These two studies indicate a possible role for T-cell responses in detecting *Mtb* Rpf antigen during *Mtb* infection. We have, therefore, performed a more detailed analysis studying the immunogenicity of the Rv0867c and Rv2389c *Mtb* Rpf proteins in several groups of mycobacteria-exposed individuals, including long-term non-progressors. These two antigens were selected based on highest recognition of all 5 rpfs in a cross-sectional cohort study of HIV-negative, TST- and/or ESAT6/CFP10-positive household contacts from the Gambia, Uganda, and South Africa (BMGF GCGH GC6#74 Biomarkers for TB consortium; http://www.biomarkers-for-tb.net/; unpublished results). Of interest, both Rv0867c and Rv2389c are predicted to be secreted proteins (SignalP and TMMHM server Technical University of Denmark) [23], which may enhance availability of the antigen to the innate immune system. Indeed, Rv0867c protein is present in *Mtb* culture filtrate [24]. *Rv0867c* and *Rv2389c* are both expressed in early log-phase-grown *Mtb*, but *Rv2389c* expression is induced in stationary and noncultivatable phases of *Mtb* and during acidic conditions, whereas *Rv0867c* expression appears to be higher in nutrient-starved *Mtb* culture. Both genes are also highly induced during early resuscitation [4]. In the work reported here, we (i) identify frequent and significant human T-cell responses against Rv0867c and Rv2389c; (ii) identify a series of novel *Mtb* rpf epitopes, including a single dominant peptide epitope in Rv2389c; and (iii) describe *Mtb* rpf-specific polyfunctional memory $CD4^+$ and particularly $CD8^+$

T-cell memory responses to Rv0867c and Rv2389c *Mtb* rpf proteins in long-term naturally protected, *Mtb* nonprogressors. Based on these data, we propose that rpf antigens are potentially interesting new TB vaccine candidates.

Materials and methods

Study subjects. Blood samples were collected by venipuncture from a group of Dutch individuals including 9 tuberculosis (TB) patients, 10 tuberculin skin test (TST)-positive individuals (indurations of ≥ 10 mm), 10 BCG-vaccinated individuals and 10 non-BCG-vaccinated, TST-negative, healthy individuals, as well as 12 Norwegian TST-positive individuals. The Norwegian donor group consisted of elderly people (average 70 years) exposed to TB transmission several decades ago without developing any clinical disease and without receiving any treatment. Previously recorded TST indurations associated with natural conversion ranged from 12 to 60 mm (average 18 mm). Recent Interferon gamma (IFN-γ) release assay (IGRA) testing with the Quantiferon Gold in-tube test showed that 9 of 12 donors in this category were positive. All donors gave written consent before blood donation. The study protocol (P207/99) was approved by the Institutional Review Board of the Leiden University Medical Center and the Regional Committees for Medical and Health Research Ethics in Norway. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Ficoll density gradient centrifugation and stored in liquid nitrogen until further use.

Recombinant proteins. Recombinant proteins were produced as previously described [25]. Briefly, *Mtb* genes were amplified by PCR from genomic H37Rv DNA and cloned by Gateway technology (Invitrogen, Carlsbad, CA, USA) in a bacterial expression vector containing a histidine tag at the N-terminus. Vectors were over-expressed in *Escherichia coli* BL21(DE3) and purified. The size and purity of recombinant proteins were analyzed by gel electrophoresis and Western blotting with an anti-His antibody (Invitrogen, Carlsbad, CA, USA) and an anti-*E. coli* polyclonal antibody (a kind gift from the Statens Serum Institute (SSI)). Endotoxin contents were below 50 IU/mg recombinant protein, as tested using a Limulus Amebocyte Lysate (LAL) assay (Cambrex, East Rutherford, NJ). All proteins were tested in lymphocyte stimulation assays in order to exclude antigen-nonspecific T-cell stimulation and cellular toxicity by using PBMCs of *in vitro* purified protein derivative (PPD)-negative healthy Dutch donors [26]. PPD of *M. tuberculosis* was purchased from SSI, Copenhagen, Denmark.

Synthetic peptides. Peptides (20-mers overlapping 10 amino acids) were produced at the Leiden University Medical Center (LUMC) facility by simultaneous multiple-peptide synthesis as described previously [27]. Homogeneity and purity were confirmed by analytical reversed-phase high-pressure liquid chromatography, and mass spectrometry showed the expected masses. Peptide purity was ≥ 75%.

Lymphocyte stimulation assay. PBMCs (1.5×10^5/ well) were cultured in triplicate in 96-wells round-bottom plates (Nunc, Roskilde, Denmark) and incubated with or without protein (10 µg/ml) in AIM-V medium (Invitrogen, Breda, The Netherlands) at 37°C, 5% CO_2. After 6 days supernatants were harvested and used for cytokine and chemokine profiling.

Cytokine and chemokine profiling. Levels of cytokines (IFN-γ, interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α)) were analyzed using the Lincoplex® kit (Millipore) according to the Milliplex™ Map protocol. Plates were analyzed using a BioPlex array reader with Bio-Plex software (Bio-Rad Laboratories, Veenendaal, The Netherlands).

Generation of antigen-specific T-cell lines. T-cell lines were generated as previously described [28]. PBMCs ($2x10^6$ cells/well) from cured TB patients or TST-positive or BCG-vaccinated individuals were cultured in 24-well plates in the presence of protein (2 to 10 µg/ml) in IMDM medium (Gibco, Paisley, UK) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin (Gibco, Paisley, UK) and 10% pooled human serum at 37 °C, 5% CO_2. At day 6, recombinant-interleukin-2 (rIL-2) (Cetus, Emeryville, CA, USA) was added to the cell cultures in a final concentration of 25 U/ml. Cultures were maintained for an additional 2 to 3 weeks in the presence of rIL-2. T cells were harvested and stored in liquid nitrogen until further use.

T-cell proliferation of antigen-specific T-cell lines. Thawed T-cell lines were cultured in triplicate in 96-wells flat-bottom plates (1.5×10^4/well) together with HLA-DR-matched irradiated (2000 rad) PBMCs (5×10^4/well) with or without protein (10 µg/ml) or peptide (10 µg/ml) at 37 °C 5% CO_2. After 3 days, supernatants were harvested and stored at -20 °C. Cells were pulsed for an additional 18 hours with [^{3}H]thymidine (0.5 µCi/well), harvested, and counted on a Microbetaplate counter (Wallac Turku, Finland) [29]. A stimulation index (SI) of ≥ 3 was considered positive.

IFN-γ ELISA. The concentration IFN-γ in supernatants was measured by enzyme-linked immunosorbent assay (ELISA) (U-CyTech, Utrecht, The Netherlands) according to the manufacturer's instructions. The detection limit of the assay was 20 pg/ml IFN-γ. Samples were tested in duplicate. An IFN-γ response ≥ 100 pg/ml was considered positive.

Flow cytometric analysis. PBMCs were thawed and rested. After 24 hours, PBMCs were stimulated for 16 hours with protein (10 µg/ml) in the presence of co-stimulatory antibodies anti-CD28 and anti-CD49d (Sanquin, Netherlands and BD Biosciences, respectively). Brefeldin A (3 µg/ml; Sigma) was added after the first 4 to 6 hours. Cells were stained for 30 minutes at 4 °C using the following surface markers; anti-CD3-Pacific blue (PB), anti-CD4-peridinin chlorophyll protein (PercP)-Cy5.5, anti-CD8-AmCyan, anti-CD25-allophycocyanin (APC)-Cy5, anti-CD45RA-phycoerythrin (PE)-Cy5 and anti-CCR7-PE/Cy7 and intracellular staining was performed with

anti-IFN-γ-Alexa 700, anti-TNF-α-APC, anti-IL-2-PE and anti-CD69-fluorescein isothiocyanate (FITC) (BD Biosciences) by using the Intrastain kit (Dako Cytomation, Denmark). Samples were acquired on an LSR II flow cytometer and analyzed using SPICE (software provided by Dr. M. Roederer, National Institute of Allergy and Infectious Disease) and FlowJo software (Treestar Inc, Ashland, OR, USA). Cell populations should contain at least 100 events.

Statistical analyses. Differences between groups were analyzed with the non-parametrical Kruskal-Wallis test in GraphPad Prism (version 4). *P* values were corrected for multiple comparisons. The statistical significance level used was $P < 0.05$.

Results

Recognition of *Mtb* Rpf antigens by T cells from *Mtb*-responsive individuals

To determine the immunogenicities of both Rv0867c and Rv2389c, we investigated whether these two *Mtb* Rpf proteins were recognized by PBMCs of mycobacterium-exposed individuals. PBMCs from four groups of Dutch individuals were tested: (i) HIV-negative, treated tuberculosis patients (TB; $n = 9$); (ii) tuberculin skin test (TST)-positive individuals (TST; $n = 10$); (iii) BCG-vaccinated individuals (BCG; $n = 10$); and (iv) non-BCG-vaccinated, TST-negative and *in vitro* purified-protein-derivative (PPD)-negative healthy controls (HCs; $n = 10$). The secretion of four cytokines (IFN-γ, IL1-β, IL-6 and TNF-α) was analyzed as a multiparameter read out of antigen-specific responses.

As expected, IFN-γ production in response to PPD was low in healthy individuals, whereas the TST^+ individuals and TB patients showed high responses to PPD, by both IFN-γ and the other cytokines analyzed. The BCG-vaccinated individuals showed intermediate levels of responses (Figure 1A to D).

While minor IFN-γ levels were detectable in the three mycobacterium-exposed groups in response to Rv0867c, higher levels were found in response to Rv2389c (HCs and BCG; $P < 0.05$). The healthy controls did not respond to Rv0867c and Rv2389c (Figure 1A). No IL-1β was produced upon Rv0867c stimulation in any of the four groups analyzed; however, Rv2389c stimulation induced production of IL-1β in all mycobacterium-exposed groups (HCs and TB; $P < 0.05$), but not in the healthy controls (Figure 1B). Rv0867c stimulation also induced IL-6 in all three mycobacterium-exposed groups, but not in the healthy individuals (HCs, TST, and TB $P < 0.05$). In contrast to Rv0867c, Rv2389c induced high levels of IL-6 in the HC group but much higher levels in the mycobacterium-exposed individuals (HCs and TB; $P < 0.05$) (Figure 1C). Rv2389c stimulation induced also high levels of TNF-α, especially within the mycobacterium-exposed groups (HCs and TB $P < 0.05$), while Rv0867c induced only limited levels of TNF-α in all four groups (Figure 1D).

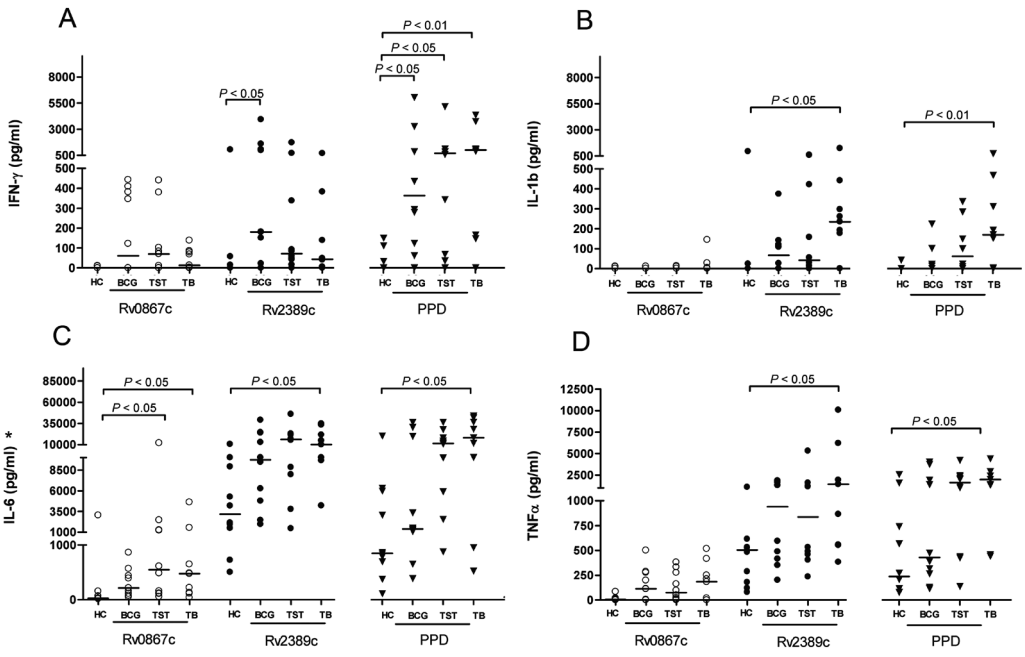


Figure 1. Cytokine and chemokine profiling. PBMCs from healthy individuals (HC; *n* = 10), BCG-vaccinated individuals (BCG; *n* = 10), TST-positive individuals (TST; *n* = 10) and TB patients (TB; *n* = 9) were stimulated with Rv0867c and Rv2389c proteins and PPD for 6 days. Levels of IFN-γ (A), IL-1β (B), IL-6 (C) and TNF-α (D) were measured and corrected for background levels. Horizontal bars represent median cytokine production levels. Antigen stimuli: Rv0867c open circles (○), Rv2389c closed circles (●) and PPD inverted closed triangles (▼) ***** Background not corrected.

Overall, the mycobacterium-exposed individuals produced the highest levels of cytokines upon Rv0867c and Rv2389c stimulation, and TB patients were often the highest responders to Rv2389c (HCs and TB; $P < 0.05$ for IL-1β, IL-6 and TNF-α). Rv2389c was more strongly recognized than Rv0867c, which induced no or low levels of cytokines.

Identification of immunogenic peptides of *Mtb* Rpf proteins

To further characterize the Rpf-specific responses observed above, five $CD4^+$ T-cell lines specific for Rv0867c and five $CD4^+$ T-cell lines specific for Rv2389c were generated. These T-cell lines were tested for recognition of all individual 20-mer peptides (overlapping by 10 amino acids) (see Supplementary Table S1A and B) and both IFN-γ production and T-cell proliferation were measured. Table 1 shows the recognition pattern of the Rpf peptides by the Rv0867c and Rv2389c antigen-specific T-cell lines examined. All T-cell lines responded to the corresponding Rpf protein and to one or more of the proteins' corresponding individual peptides. The Rv0867c-specific T-cell lines recognized many different peptides ranging from 1/5 to 4/5 of the donors. Four out of the five T-cell lines recognized peptides $P5_{41\text{-}60}$ and $P10_{91\text{-}110}$ and three of the five lines recognized

peptide $P7_{61-80}$. In contrast to Rv0867c-specific T cells, all Rv2389c-specific T-cell lines recognized only a single peptide, $P12_{111-130}$.

Table 1. CD4+ antigen-specific T-cell responses to single peptides of *Mtb* Rpf proteins [a]

peptide	rpf protein: Rv0867c	rpf protein: Rv2389c
$p1_{1-20}$		
$p2_{11-30}$		
$p3_{21-40}$		
$p4_{31-50}$		
$p5_{41-60}$		
$p6_{51-70}$		
$p7_{61-80}$		
$p8_{71-90}$		
$p9_{81-100}$		
$p10_{91-110}$		
$p11_{101-120}$		
$p12_{111-130}$		
$p13_{121-140}$		
$p14_{131-150}$		
$p15_{141-160}$		
$p16_{151-170}$		
$p17_{161-180}$		
$p18_{171-190}$		
$p19_{181-200}$		
$p20_{191-210}$		
$p21_{201-220}$		
$p22_{211-230}$		
$p23_{221-240}$		
$p24_{231-250}$		
$p25_{241-260}$		
$p26_{251-270}$		
$p27_{261-280}$		
$p28_{271-290}$		
$p29_{281-300}$		
$p30_{291-310}$		
$p31_{301-320}$		
$p32_{311-330}$		
$p33_{321-340}$		
$p34_{331-350}$		
$p35_{341-360}$		
$p36_{351-370}$		
$p37_{361-380}$		
$p38_{371-390}$		
$p39_{381-400}$		
$p40_{388-407}$		

number of responding T-cell lines

n = 0
n = 1
n = 2
n = 3
n = 4
n = 5

[a] Peptide responses with a proliferation SI of ≥ 3 and IFN-γ responses of ≥ 100 pg/ml were considered positive.

Characterization of *Mtb* Rpf-specific polyfunctional T cells in long-term *Mtb* non-progressors

We next investigated T-cell responses to Rv0867c and Rv2389c antigens in a cohort of long-term *Mtb* non-progressors, which had been infected several decades ago but had never developed any signs of active TB. We analyzed responsive $CD4^+$ and $CD8^+$ T-cell subsets producing IFN-γ, TNF-α and/or IL-2 by multiparameter flow cytometry. Polyfunctional T cells have been associated with protection following vaccination [30], but the situation in human infection is more complex, as polyfunctional T cells are also found in active TB patients [3]. In any case, the memory T-cell subsets responding to Rv0867c or Rv2389c was analyzed using PBMCs from long-term nonprogressors (n = 12) and PPD-negative HCs (n = 11).

Figure 2A shows that the Rv0867c protein induced polyfunctional T cells with different cytokine profiles (triple-positive (IFN-γ^+ TNF-α^+ IL-2^+) or double-positive (IFN-γ^+ TNFα^+, TNFα^+ IL-2^+ and IFN-γ^+ IL-2^+) cells). Such polyfunctional T cells were detected in 9 out of the 12 long-term latently infected individuals. Strikingly, higher frequencies of polyfunctional $CD8^+$ T cells (ranging between 0.20 and 9.72%) were observed, exceeding those of $CD4^+$ T cells (ranging between 0.21 and 1.29%). Only 2 out of 12 donors showed polyfunctional $CD8^+$ T cells recognizing Rv2389c, typically with a lower frequency (ranging between 0.24 and 0.82%) (Figure 2C). Single-cytokine-producing $CD4^+$ and $CD8^+$ T cells were observed for both Rv0867c and Rv2389c, where IFN-γ^+ $CD4^+$ T cells are the most prominent T-cell subset of all single-cytokine-producing T cells. No polyfunctional T cells were induced within the negative healthy control population upon Rv0867c stimulation, although for unknown reasons, low numbers of IL-2^+ single-positive cells were seen (Figure 2B). Some low frequencies of polyfunctional and single-positive T cells were observed upon Rv2389c stimulation within the healthy control group (Figure 2D), but lower than those in the latently infected group.

Figure 2E shows the proportions of polyfunctional and single-positive $CD4^+$ and $CD8^+$ T cells for Rv0867c, as this antigen was the best recognized in this cohort. IFN-γ^+ TNF-α^+ double-cytokine-producing $CD8^+$ polyfunctional T cells were the most prominent polyfunctional T-cell subset identified.

In addition, we analyzed the expression of the T-cell memory markers CCR7 and CD45RA in these IFN-γ^+ TNF-α^+ double-cytokine-producing $CD8^+$ T cells in the Rv0867c responders. After dividing the responding T-cells into central memory (Tcm) and effector memory (Tem) T-cell subsets (according to Seder *et al.* [31;32]), we found that Rv0867c-responding IFN-γ^+ TNFα^+ $CD8^+$ T cells consisted mostly of effector memory (CCR7$^-$ and CD45RA$^-$) and effector (Teff) (CCR7$^-$ and CD45RA$^+$) T-cell subsets (Figure 2F). These data show the presence of Rv0867c-specific $CD8^+$ memory and effector T cells in long-term *Mtb* non-progressors.

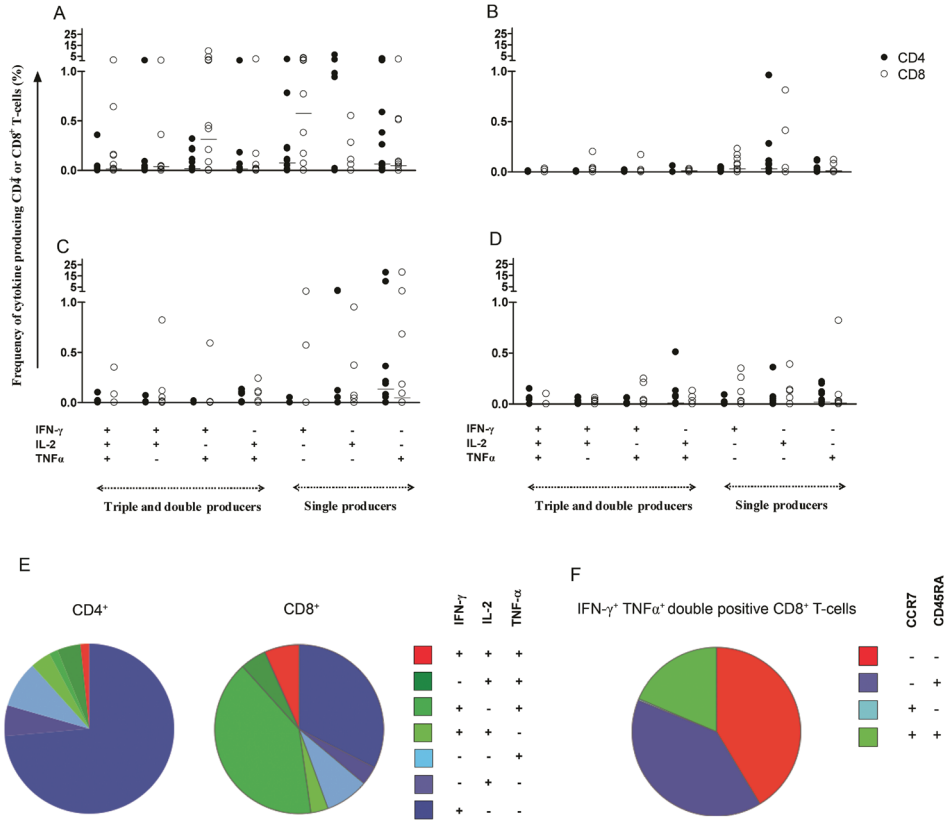


Figure 2. Frequency of antigen-specific polyfunctional T cells in long-term *Mtb* nonprogressors. Shown is the frequency of antigen-specific $CD4^+$ and $CD8^+$ T cells in long-term latently infected elderly subjects (n = 12), producing combinations of IFN-γ, TNF-α and IL-2 after stimulation for 16 hours with Rv0867c (A) or Rv2389c (C) protein. Healthy individuals were also analyzed for their polyfunctional responses to Rv0867c (B) and Rv2389c (D). $CD4^+$ T cells are indicated as closed circles (●) and $CD8^+$ T cells as open circles (○). Horizontal bars represent the median frequency of antigen-specific $CD4^+$ and $CD8^+$ T cells. Slices in pie chart represent the fraction of single (blue)-, double (green)-, or triple (red)-positive $CD4^+$ and $CD8^+$ T cells for Rv0867c (E). Expression of T-cell memory markers CCR7 and CD45RA was analyzed and shown for the largest T-cell population identified: Rv0867c-specific IFN-γ and TNF-α double-positive $CD8^+$ T cells (F). Effector memory T cells are $CCR7^-$ and $CD45RA^-$, central memory T cells are $CCR7^+$ and $CD45RA^-$, naive T cells are $CCR7^+$ and $CD45RA^+$, and effector T cells are $CCR7^-$ and $CD45RA^+$.

Besides the quantity of the T-cell response, the quality of the T-cell response plays an important role in protection. We therefore also measured the median fluorescence intensity (MFI) of each cytokine produced by the Rv0867c-responsive T cells. Multiplication of the frequency by the MFI results in an integrated MFI (iMFI) value, which was introduced previously as a quantitative parameter of the overall functionality of the T-cell response analysed [30]. The MFI values showed that the triple-positive T cells had the highest MFI values, followed by the double-cytokine-producing T cells, whereas single-positive T cells showed only minor MFI values (data

not shown). However, when iMFI values were analyzed, the IFN-γ iMFI was the highest intensity within the IFN-γ^+ TNF-α^+ double-positive $CD8^+$ T-cell population, followed by IFN-γ^+ single-cytokine-producing T cells (Figure 3A). This was also observed for TNF-α iMFI (Figure 3B). Taken together, these results indicate that double- and single-positive T cells contribute quantitatively more to the *Mtb* Rpf Rv0867c antigen response in long-term non-progressors than triple-positive T cells.

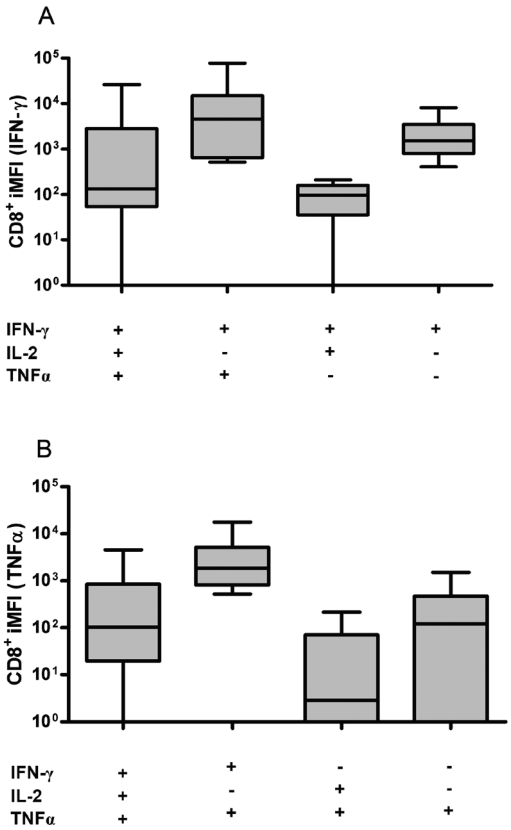


Figure 3. Integrated mean fluorescence intensity (iMFI) of IFN-γ-, TNF-α- and/or IL-2-producing $CD8^+$ T cells. Shown is a box and whisker plot representing the iMFI values of $CD8^+$ T cells from long-term nonprogressors (n = 8) in response to Rv0867c protein. iMFI is the product value of multiplication of the frequency of $CD8^+$ T cells with the indicated (poly- or mono-) cytokine profiles with the MFI of IFN-γ (A) and TNF-α (B) produced by these T-cell subsets. The horizontal line represents the median, the lower boundary of the box represents the 25th percentile, and the upper boundary represents the 75th percentile. Whiskers extend from the box to the highest and lowest values.

Discussion

New TB vaccines are urgently needed to help control the TB pandemic. Both prophylactic and postexposure vaccines are considered important [33], but the antigens that can best be incorporated in such vaccines have been identified incompletely at best. Previously we have shown that dormancy-related or DosR regulon-encoded *Mtb* antigens, which are expressed during hypoxia and nitric oxide stress, were preferentially recognized by TST-positive individuals, suggesting an association with control of infection [22;34;35]. Unexpectedly, BCG vaccination failed to induce responses to these antigens in humans and mice, even though BCG is able to express the DosR regulon under hypoxia conditions *in vitro* [26]. In contrast to the DosR regulon-encoded antigens, *rpf* genes *Rv0867c* and *Rv2389c* are expressed by actively replicating early log-phase-grown *Mtb* [4]. Rv0867c and Rv2389c share the *M. luteus* Rpf protein's property in being able to resuscitate dormant mycobacteria. We therefore hypothesized that immunity directed against these proteins may play a role in sensing and detecting actively resuscitating and replicating *Mtb* organisms and therefore possibly also in host immune control of reactivating bacteria.

We observed significant differences in levels of Rv0867c and Rv2389c recognition between mycobacterium-exposed vs. PPD-negative individuals, yet some Rv2389c and, to a lesser extent, Rv0867c responses were found in the healthy PPD-nonresponding group. We have previously reported a similar recognition pattern for some *Mtb* dosR regulon-encoded antigens by PPD-nonresponding donors. We explained these results by cross-reactive immunity to nontuberculous mycobacteria (NTM) which share large parts of the DosR regulon with *Mtb* and *M. bovis* BCG [36]. Antigen contaminants (*E.coli* products) are less likely to be involved, since our recombinant proteins are standard quality control (QC) tested on cells from 4 to 5 PPD-negative donors and released only when no IFN-γ is produced. A BLAST search (http://www.ncbi.nlm.nih.gov/blast/) of the *Mtb* Rv0867c and Rv2389c Rpf protein sequences indeed showed protein sequence identity in several NTM and nonmycobacterial environmental bacteria (Table 2). Part of the immunogenic peptides identified in our current study are indeed conserved in the identified protein sequences of the species shown in Table 2 ($P5_{41\text{-}60}$ of Rv0867c CE*GGNW*INT and $P12_{111\text{-}130}$ of Rv2389c TQG*GAWP*C), compatible with cross-reactivity at the polyclonal T-cell level.

To more precisely investigate the function and phenotype of Rv0867c- and Rv2389c specific T-cell subsets, we analyzed the presence of mono- and polyfunctional $CD4^+$ and $CD8^+$ T cells in PBMCs of latently infected individuals who had been infected decades ago without developing TB. In the Dutch cohort above, we had identified Rv2389c as the best-recognized antigen, whereas in the long-term *Mtb* nonprogressors Rv0867c induced higher frequencies of polyfunctional and single-positive T cells. This difference might be attributable to the type and/or longevity of the infection as *Mtb* is more likely to be chronic than NTM infections, and *Mtb* might be a stronger immunogen than NTM.

Table 2. *Mtb* rpf protein sequence identity in NTM and nonmycobacterial environmental bacteria

Strain	% protein sequence identity to *Mtb* Rpf antigen [a]	
	Rv0867c	Rv2389c
NTM		
Mycobacterium marinum M	88	55
Mycobacterium ulcerans Agy99	88	54
Mycobacterium avium subsp. Paratuberculosis K10	84	49
Mycobacterium sp. strain MCS	76	55
Mycobacterium vanbaalenii PYR-1	76	52
Mycobacterium smegmatis MC2 155	78	57
Mycobacterium avium 104	83	59
Mycobacterium bovis BCG	100	100
Nonmycobacterial environmental bacteria		
Nocardia farcinica IFM 10152	62	58
Rhodococcus jostii RHA1	61	56
Streptomyces avermitilis MA-4680	56	45
Streptomyces coelicolor A3(2)	70	43
Corynebacterium jeikeium K411	54	52
Corynebacterium diphtheriae NCTC 13129	55	45
Corynebacterium glutamicum ATCC 13032	55	54

[a] H37Rv Rpf protein sequences were compared to nontuberculous mycobacteria and nonmycobacterial environmental bacterial strains by BLAST searches (http://www.ncbi.nlm.nih.gov/blast/). The percentage of protein sequence identity is given per antigen and species. For analysis, a cutoff value of 40% was used.

Among the polyfunctional antigen-specific T cells present in the long-term nonprogressors, predominantly Rv0867c-specific $CD8^+$ were identified, particularly double-producing IFN-γ^+ TNF-α^+, Tem and Teff cells with high iMFI. This suggests that these two Rpf-specific $CD8^+$ T-cell subsets may play a significant role in TB infection, next to triple-positive T cells. Of interest, IFN-γ^+ TNFα^+ double-positive $CD8^+$ T cells were also found following vaccination with AERAS-402 vaccine, containing Ag85A, Ag85B, and TB10.4 antigens as a BCG boost, and these cells persisted over time [37]. Overall, $CD8^+$ T cells are important in controlling *Mtb* infection, and the identification of prominent populations of Rv0867c-specific $CD8^+$ T cells supports this notion. Nevertheless, relatively little is known about these antigen-specific polyfunctional $CD8^+$ T cells and their role in protection [38].

Rv0867c-specific, IFN-γ^+ TNF-α^+ double-cytokine-producing $CD8^+$ T cells were mainly Tem and Teff cells. These high $CD8^+$ Tem en Teff subset responses to Rv0867c in the long-term *Mtb* nonprogressors do not seem to correspond to the hypothesis that after clearance of infection Tem populations gradually wane, resulting mainly in long-lived Tcm cells [39]. A recent study showed that reexposure to antigens increased the number of $CD8^+$ Tem cells. Our latently infected

individuals may be continuously reexposed to Rpf antigens expressed by either endogenous uncleared *Mtb* organisms or by other environmental bacteria expressing cross-reactive Rpf-like antigens, which boost Rv0867c-specific Tem cells [40]. Regardless, Rv0867c *Mtb* Rpf antigen is recognized in long-term *Mtb* nonprogressors, indicating that Rpf-specific T-cell responses are maintained for decades after the initial *Mtb* infection. Of note, the immune system changes with aging. Fewer naïve T-cells will be available during aging as a result of thymic involution. However, $CD8^+$ Tcm, Tem, and Teff cells accumulate with age [41;42]. These data are in agreement with our Tem and Teff findings in our elderly population (average age, 70 years).

Besides $CD8^+$ T cells, also a minor proportion of polyfunctional $CD4^+$ T cells was observed (Figure 2E). Although previous results have indicated that triple-positive polyfunctional $CD4^+$ T cells can be induced by BCG vaccination, they are also clearly present in TB patients, such that they do not necessarily correlate with protection against developing disease [3]. Minor but significant proportions of triple-positive $CD4^+$ polyfunctional T cells were identified in our work reported here.

We conclude that *Mtb* Rv0867c and Rv2389c Rpf antigens are immunogenic in humans, as evidenced by antigen-specific cytokine production and high frequencies and iMFI values. Both single- and double-cytokine-producing Rv0867c-specific $CD4^+$ and $CD8^+$ T cells were identified in long-term latently infected individuals who did not develop TB. By directing T-cell responses to *Mtb* Rpf antigens it may be possible to enhance immune surveillance of reactivating and resuscitating *Mtb* bacilli, and thereby help to control TB reactivation, which is the major complication in latent TB that keeps fuelling the TB pandemic.

Acknowledgements

This work was supported by the FP7 NEWTBVAC project, the Bill and Melinda Gates Foundation Grand Challenges in Global Health (GC6#74), and TI Pharma (project D-101-1). We would like to thank Corine Prins and Sandra Arend for collecting and providing PBMCs used in this study. The text represents the authors' views and does not necessarily represent a position of the FP7 NEWTBVAC Project Commission, who will not be liable for the use made of such information.

References

1. **Ottenhoff,T.H.,** Overcoming the global crisis: "yes, we can", but also for TB ... ? *Eur.J.Immunol.* 2009. **39**: 2014-2020.
2. **Garton,N.J., Waddell,S.J., Sherratt,A.L., Lee,S.M., Smith,R.J., Senner,C., Hinds,J., Rajakumar,K., Adegbola,R.A., Besra,G.S., Butcher,P.D., and Barer,M.R.,** Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. *PLoS.Med.* 2008. **5**: e75.
3. **Caccamo,N., Guggino,G., Joosten,S.A., Gelsomino,G., Di,C.P., Titone,L., Galati,D., Bocchino,M., Matarese,A., Salerno,A., Sanduzzi,A., Franken,W.P., Ottenhoff,T.H., and Dieli,F.,** Multifunctional CD4+T cells correlate with active Mycobacterium tuberculosis infection. *Eur.J.Immunol.* 2010.
4. **Gupta,R.K., Srivastava,B.S., and Srivastava,R.,** Comparative expression analysis of rpf-like genes of Mycobacterium tuberculosis H37Rv under different physiological stress and growth conditions. *Microbiology* 2010.
5. **Bigger, J. W.** Treatment of staphylococcal infections with penicillin. Lancet , 497-500. 1944.
6. **Wayne,L.G. and Sohaskey,C.D.,** Nonreplicating persistence of mycobacterium tuberculosis. *Annu.Rev.Microbiol.* 2001. **55**: 139-163.
7. **Wayne,L.G. and Hayes,L.G.,** An in vitro model for sequential study of shiftdown of Mycobacterium tuberculosis through two stages of nonreplicating persistence. *Infect.Immun.* 1996. **64**: 2062-2069.
8. **Betts,J.C., Lukey,P.T., Robb,L.C., McAdam,R.A., and Duncan,K.,** Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol.Microbiol.* 2002. **43**: 717-731.
9. **Voskuil,M.I., Schnappinger,D., Visconti,K.C., Harrell,M.I., Dolganov,G.M., Sherman,D.R., and Schoolnik,G.K.,** Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. *J.Exp.Med.* 2003. **198**: 705-713.
10. **Shleeva,M.O., Salina,E.G., and Kaprelyants,A.S.,** Dormant forms of mycobacteria. *Microbiology* 2010. **79**: 1-12.
11. **Biketov,S., Mukamolova,G.V., Potapov,V., Gilenkov,E., Vostroknutova,G., Kell,D.B., Young,M., and Kaprelyants,A.S.,** Culturability of Mycobacterium tuberculosis cells isolated from murine macrophages: a bacterial growth factor promotes recovery. *FEMS Immunol.Med.Microbiol.* 2000. **29**: 233-240.
12. **Mukamolova,G.V., Kaprelyants,A.S., Young,D.I., Young,M., and Kell,D.B.,** A bacterial cytokine. *Proc.Natl.Acad.Sci.U.S.A* 1998. **95**: 8916-8921.
13. **Mukamolova,G.V., Turapov,O.A., Young,D.I., Kaprelyants,A.S., Kell,D.B., and Young,M.,** A family of autocrine growth factors in Mycobacterium tuberculosis. *Mol.Microbiol.* 2002. **46**: 623-635.
14. **Kell,D.B. and Young,M.,** Bacterial dormancy and culturability: the role of autocrine growth factors. *Curr.Opin.Microbiol.* 2000. **3**: 238-243.
15. **Davies,A.P., Dhillon,A.P., Young,M., Henderson,B., McHugh,T.D., and Gillespie,S.H.,** Resuscitation-promoting factors are expressed in Mycobacterium tuberculosis-infected human tissue. *Tuberculosis.(Edinb.)* 2008. **88**: 462-468.
16. **Rachman,H., Strong,M., Ulrichs,T., Grode,L., Schuchhardt,J., Mollenkopf,H., Kosmiadi,G.A., Eisenberg,D., and Kaufmann,S.H.,** Unique transcriptome signature of Mycobacterium tuberculosis in pulmonary tuberculosis. *Infect.Immun.* 2006. **74**: 1233-1242.
17. **Tufariello,J.M., Jacobs,W.R., Jr., and Chan,J.,** Individual Mycobacterium tuberculosis resuscitation-promoting factor homologues are dispensable for

growth in vitro and in vivo. *Infect.Immun.* 2004. **72**: 515-526.

18. **Downing,K.J., Betts,J.C., Young,D.I., McAdam,R.A., Kelly,F., Young,M., and Mizrahi,V.,** Global expression profiling of strains harbouring null mutations reveals that the five rpf-like genes of Mycobacterium tuberculosis show functional redundancy. *Tuberculosis. (Edinb.)* 2004. **84**: 167-179.

19. **Downing,K.J., Mischenko,V.V., Shleeva,M.O., Young,D.I., Young,M., Kaprelyants,A.S., Apt,A.S., and Mizrahi,V.,** Mutants of Mycobacterium tuberculosis lacking three of the five rpf-like genes are defective for growth in vivo and for resuscitation in vitro. *Infect.Immun.* 2005. **73**: 3038-3043.

20. **Fine,P.E.,** Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* 1995. **346**: 1339-1345.

21. **Yeremeev,V.V., Kondratieva,T.K., Rubakova,E.I., Petrovskaya,S.N., Kazarian,K.A., Telkov,M.V., Biketov,S.F., Kaprelyants,A.S., and Apt,A.S.,** Proteins of the Rpf family: immune cell reactivity and vaccination efficacy against tuberculosis in mice. *Infect. Immun.* 2003. **71**: 4789-4794.

22. **Schuck,S.D., Mueller,H., Kunitz,F., Neher,A., Hoffmann,H., Franken,K.L., Repsilber,D., Ottenhoff,T.H., Kaufmann,S.H., and Jacobsen,M.,** Identification of T-cell antigens specific for latent mycobacterium tuberculosis infection. *PLoS.One.* 2009. **4**: e5590.

23. **Gomez,M., Johnson,S., and Gennaro,M.L.,** Identification of secreted proteins of Mycobacterium tuberculosis by a bioinformatic approach. *Infect.Immun.* 2000. **68**: 2323-2327.

24. **Malen,H., Berven,F.S., Fladmark,K.E., and Wiker,H.G.,** Comprehensive analysis of exported proteins from Mycobacterium tuberculosis H37Rv. *Proteomics.* 2007. **7**: 1702-1718.

25. **Franken,K.L., Hiemstra,H.S., van Meijgaarden,K.E., Subronto,Y., den,H.J., Ottenhoff,T.H., and Drijfhout,J.W.,** Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. *Protein Expr.Purif.* 2000. **18**: 95-99.

26. **Lin,M.Y., Geluk,A., Smith,S.G., Stewart,A.L., Friggen,A.H., Franken,K.L., Verduyn,M.J., van Meijgaarden,K.E., Voskuil,M.I., Dockrell,H.M., Huygen,K., Ottenhoff,T.H., and Klein,M.R.,** Lack of immune responses to Mycobacterium tuberculosis DosR regulon proteins following Mycobacterium bovis BCG vaccination. *Infect.Immun.* 2007. **75**: 3523-3530.

27. **Hiemstra,H.S., Duinkerken,G., Benckhuijsen,W.E., Amons,R., de Vries,R.R., Roep,B.O., and Drijfhout,J.W.,** The identification of CD4+ T cell epitopes with dedicated synthetic peptide libraries. *Proc.Natl.Acad.Sci.U.S.A* 1997. **94**: 10313-10318.

28. **Ottenhoff,T.H., Elferink,D.G., Hermans,J., and de Vries,R.R.,** HLA class II restriction repertoire of antigen-specific T cells. I. The main restriction determinants for antigen presentation are associated with HLA-D/DR and not with DP and DQ. *Hum.Immunol.* 1985. **13**: 105-116.

29. **Arend,S.M., Geluk,A., van Meijgaarden,K.E., van Dissel,J.T., Theisen,M., Andersen,P., and Ottenhoff,T.H.,** Antigenic equivalence of human T-cell responses to Mycobacterium tuberculosis-specific RD1-encoded protein antigens ESAT-6 and culture filtrate protein 10 and to mixtures of synthetic peptides. *Infect.Immun.* 2000. **68**: 3314-3321.

30. **Darrah,P.A., Patel,D.T., De Luca,P.M., Lindsay,R.W., Davey,D.F., Flynn,B.J., Hoff,S.T., Andersen,P., Reed,S.G., Morris,S.L., Roederer,M., and Seder,R.A.,** Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat.Med.* 2007. **13**: 843-850.

31. **Sallusto,F., Lenig,D., Forster,R., Lipp,M., and Lanzavecchia,A.,** Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999. **401**: 708-712.

32. **Seder,R.A., Darrah,P.A., and Roederer,M.,** T-cell quality in memory and protection: implications for vaccine design. *Nat.Rev.Immunol.* 2008. **8**: 247-258.

33. **Abu-Raddad,L.J., Sabatelli,L., Achterberg,J.T., Sugimoto,J.D., Longini,I.M., Jr., Dye,C., and Halloran,M.E.,** Epidemiological benefits of more-effective tuberculosis vaccines, drugs, and diagnostics. *Proc.Natl.Acad. Sci.U.S.A* 2009. **106**: 13980-13985.

34. **Black,G.F., Thiel,B.A., Ota,M.O., Parida,S.K., Adegbola,R., Boom,W.H., Dockrell,H.M., Franken,K.L., Friggen,A.H., Hill,P.C., Klein,M.R., Lalor,M.K., Mayanja,H., Schoolnik,G., Stanley,K., Weldingh,K., Kaufmann,S.H., Walzl,G., and Ottenhoff,T.H.,** Immunogenicity of novel DosR regulon-encoded candidate antigens of Mycobacterium tuberculosis in three high-burden populations in Africa. *Clin.Vaccine Immunol.* 2009. **16**: 1203-1212.

35. **Leyten,E.M., Lin,M.Y., Franken,K.L., Friggen,A.H., Prins,C., van Meijgaarden,K.E., Voskuil,M.I., Weldingh,K., Andersen,P., Schoolnik,G.K., Arend,S.M., Ottenhoff,T.H., and Klein,M.R.,** Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of Mycobacterium tuberculosis. *Microbes.Infect.* 2006. **8**: 2052-2060.

36. **Lin,M.Y., Reddy,T.B., Arend,S.M., Friggen,A.H., Franken,K.L., van Meijgaarden,K.E., Verduyn,M.J., Schoolnik,G.K., Klein,M.R., and Ottenhoff,T.H.,** Cross-reactive immunity to Mycobacterium tuberculosis DosR regulon-encoded antigens in individuals infected with environmental, nontuberculous mycobacteria. *Infect.Immun.* 2009. **77**: 5071-5079.

37. **Abel,B., Tameris,M., Mansoor,N., Gelderbloem,S., Hughes,J., Abrahams,D., Makhethe,L., Erasmus,M., de,K.M., van der Merwe,L., Hawkridge,A., Veldsman,A., Hatherill,M., Schirru,G., Pau,M.G., Hendriks,J., Weverling,G.J., Goudsmit,J., Sizemore,D., McClain,J.B., Goetz,M., Gearhart,J., Mahomed,H., Hussey,G.D., Sadoff,J.C., and Hanekom,W.A.,** The Novel TB Vaccine, AERAS-402, Induces Robust and Polyfunctional CD4 and CD8 T Cells in Adults. *Am.J.Respir.Crit Care Med.* 2010.

38. **Caccamo,N., Guggino,G., Meraviglia,S., Gelsomino,G., Di,C.P., Titone,L., Bocchino,M., Galati,D., Matarese,A., Nouta,J., Klein,M.R., Salerno,A., Sanduzzi,A., Dieli,F., and Ottenhoff,T.H.,** Analysis of Mycobacterium tuberculosis-specific CD8 T-cells in patients with active tuberculosis and in individuals with latent infection. *PLoS.One.* 2009. **4**: e5528.

39. **Woodland,D.L. and Kohlmeier,J.E.,** Migration, maintenance and recall of memory T cells in peripheral tissues. *Nat. Rev.Immunol.* 2009. **9**: 153-161.

40. **Vezys,V., Yates,A., Casey,K.A., Lanier,G., Ahmed,R., Antia,R., and Masopust,D.,** Memory CD8 T-cell compartment grows in size with immunological experience. *Nature* 2009. **457**: 196-199.

41. **Naylor,K., Li,G., Vallejo,A.N., Lee,W.W., Koetz,K., Bryl,E., Witkowski,J., Fulbright,J., Weyand,C.M., and Goronzy,J.J.,** The influence of age on T cell generation and TCR diversity. *J.Immunol.* 2005. **174**: 7446-7452.

42. **Saule,P., Trauet,J., Dutriez,V., Lekeux,V., Dessaint,J.P., and Labalette,M.,** Accumulation of memory T cells from childhood to old age: central and effector memory cells in CD4(+) versus effector memory and terminally differentiated memory cells in CD8(+) compartment. *Mech.Ageing Dev.* 2006. **127**: 274-281.

Supplementary data

Supplementary Table 1. Overlapping peptide sets of *Mtb* Rpf antigens.

S1A Overlapping peptide set of Rv0867c			S1B Overlapping peptide set of Rv2389c		
Peptide Number	Rv0867c aa sequence	Position start-end	Peptide Number	Rv2389c aa sequence	Position start-end
1	MSGRHRKPTTSNVSVAKIAF	1-20	1	MTPGLLTTAGAGRPRDRCAR	1-20
2	SNVSVAKIAFTGAVLGGGGI	11-30	2	AGRPRDRCARIVCTVFIETA	11-30
3	TGAVLGGGGIAMAAQATAAT	21-40	3	IVCTVFIETAVVATMFVALL	21-40
4	AMAAQATAATDGEWDQVARC	31-50	4	VVATMFVALLGLSTISSKAD	31-50
5	DGEWDQVARCESGGNWSINT	41-60	5	GLSTISSKADDIDWDAIAQC	41-60
6	ESGGNWSINTGNGYLGGLQF	51-70	6	DIDWDAIAQCESGGNWAANT	51-70
7	GNGYLGGLQFTQSTWAAHGG	61-80	7	ESGGNWAANTGNGLYGGLQI	61-80
8	TQSTWAAHGGGEFAPSAQLA	71-90	8	GNGLYGGLQISQATWDSNGG	71-90
9	GEFAPSAQLASREQQIAVGE	81-100	9	SQATWDSNGGVGSPAAASPQ	81-100
10	SREQQIAVGERVLATQGRGA	91-110	10	VGSPAAASPQQQIEVADNIM	91-110
11	RVLATQGRGAWPVCGRGLSN	101-120	11	QQIEVADNIMKTQGPGAWPK	101-120
12	WPVCGRGLSNATPREVLPAS	111-130	12	KTQGPGAWPKCSSCSQGDAP	111-130
13	ATPREVLPASAAMDAPLDAA	121-140	13	CSSCSQGDAPLGSLTHILTF	121-140
14	AAMDAPLDAAAVNGEPAPLA	131-150	14	LGSLTHILTFLAAETGGCSG	131-150
15	AVNGEPAPLAPPPADPAPPV	141-160	15	THILTFLAAETGGCSGSRDD	141-160
16	PPPADPAPPVELAANDLPAP	151-170			
17	ELAANDLPAPLGEPLPAAPA	161-180			
18	LGEPLPAAPADPAPPADLAP	171-190			
19	DPAPPADLAPPAPADVAPPV	181-200			
20	PAPADVAPPVELAVNDLPAP	191-210			
21	ELAVNDLPAPLGEPLPAAPA	201-220			
22	LGEPLPAAPADPAPPADLAP	211-230			
23	DPAPPADLAPPAPADLAPPA	221-240			
24	PAPADLAPPAPADLAPPAPA	231-250			
25	PADLAPPAPADLAPPVELAV	241-260			
26	DLAPPVELAVNDLPAPLGEP	251-270			
27	NDLPAPLGEPLPAAPAELAP	261-280			
28	LPAAPAELAPPADLAPASAD	271-290			
29	PADLAPASADLAPPAPADLA	281-300			
30	LAPPAPADLAPPAPAELAPP	291-310			
31	PPAPAELAPPAPADLAPPAA	301-320			
32	APADLAPPAAVNEQTAPGDQ	311-330			
33	VNEQTAPGDQPATAPGGPVG	321-340			
34	PATAPGGPVGLATDLELPEP	331-350			
35	LATDLELPEPDPQPADAPPP	341-360			
36	DPQPADAPPPGDVTEAPAET	351-370			
37	GDVTEAPAETPQVSNIAYTK	361-380			
38	PQVSNIAYTKKLWQAIRAQD	371-390			
39	KLWQAIRAQDVCGNDALDSL	381-400			
40	AQDVCGNDALDSLAQPYVIG	388-407			

CHAPTER 3

Double- and monofunctional CD4$^+$ and CD8$^+$ T-cell responses to *Mycobacterium tuberculosis* DosR antigens and peptides in long-term latently infected individuals

Susanna Commandeur[1,*], May Young Lin[1,*], Krista E. van Meijgaarden[1,*], Annemieke H. Friggen[1], Kees L. M. C. Franken[1], Jan W. Drijfhout[2], Gro Ellen Korsvold[3], Fredrik Oftung[3], Annemieke Geluk[1] and Tom H. M. Ottenhoff[1]

[1] Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands; [2] Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands; [3] Department of Bacteriology and Immunology, Division of Infectious Disease Control, Norwegian Institute of Public Health, Oslo, Norway

[*] These authors contributed equally to the study

Eur J Immunol. 2011. Oct;41(10):2925-36.

Abstract

More than 2 billion individuals are latently infected with *Mycobacterium tuberculosis (Mtb)*. Knowledge of the key *Mtb* antigens and responding T-cell subsets mediating protection against *Mtb* is critical for developing improved tuberculosis (TB) vaccines. We previously reported that *Mtb* DosR-regulon-encoded antigens are recognized well by human T cells in association with control of *Mtb* infection. The characteristics of the responding T-cell subsets, however, remained unidentified. We have therefore studied the cytokine production and memory phenotypes of *Mtb* DosR-regulon encoded antigen-specific T cells from individuals who had been infected with *Mtb* decades ago, yet never developed TB (long-term latent *Mtb*-infected individuals). Using multi-parameter flow cytometry and intracellular cytokine staining for IFN-γ, TNF-α and IL-2, we found double and single cytokine producing $CD4^+$ as well as $CD8^+$ T cells to be the most prominent subsets, particularly IFN-γ^+ TNF-α^+ $CD8^+$ T cells. The majority of these T cells comprised effector memory and effector T cells. Furthermore, CFSE labeling revealed strong $CD4^+$ and $CD8^+$ T-cell proliferative responses induced by several "immunodominant" *Mtb* DosR antigens and their specific peptide epitopes. These findings demonstrate the prominent presence of double- and monofunctional $CD4^+$ and $CD8^+$ T-cell responses in naturally protected individuals and support the possibility of designing *Mtb* DosR antigen-based TB vaccines.

Introduction

Host defense against mycobacteria critically depends on effective innate and adaptive immunity, culminating in the activity of *Mycobacterium tuberculosis* (*Mtb)*- specific T cells and in the formation of granulomas that contain *Mtb* bacilli. Both $CD4^+$ and $CD8^+$ T-cell responses are involved, and it is undisputed that Th1- and Th17-like cytokines (IL-12, IFN-γ, TNF-α and IL-17) are crucial for optimal host immunity [1;2]. Tuberculosis (TB) continues to claim almost 2 million lives each year, and causes active (infectious) TB disease in over 9 million new cases per annum. Control of TB is further impeded by the strong increase in TB morbidity and mortality due to HIV co-infection, and the rise of multi-drug resistant and extensively drug-resistant *Mtb* strains [3]. At least 2 billion people are latently infected with *Mtb*, representing a huge reservoir of latently infected individuals from which most new TB cases arise. While 90-98% of all *Mtb*-infected individuals are able to contain infection asymptomatically in a latent state, 2-10% of these *Mtb*-infected individuals will progress towards developing TB during their lifetime.

Despite strong international efforts in TB vaccine development, *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) continues to be the only available TB vaccine. BCG vaccination induces effective protection against severe TB in young children and protects against leprosy, but does not provide sufficient protection against the severe and contagious form of TB; pulmonary TB in adults [4;5]. Moreover, BCG does not protect against TB reactivation later in life. Ideally, not only improved preventive vaccines with pre-exposure activity but also therapeutic vaccines with post-exposure activity during late-phase infection are urgently required [2;6]. Such vaccines should prevent reactivation of TB from latency by inducing and maintaining robust immunity to *Mtb* antigens that are expressed by persisting *Mtb* bacilli during latent infection. Such immune responses may not only help controlling but perhaps also eradicating persisting bacilli.

Work from our group has shown that several genes from the recently identified *Mtb* DosR (Rv3133c) regulon encode particular antigens that induce significant T-cell responses in *Mtb*-infected individuals (latently infected or active TB disease) [7]. This 48-gene DosR-regulon is expressed by *Mtb* during *in vitro* exposure to hypoxia, low-dose nitric oxide and carbon monoxide, conditions thought to be encountered by *Mtb in vivo* when persisting in immunocompetent hosts [8]. Approximately half of the *Mtb* dosR-regulon genes are also expressed over prolonged periods of time in a related stress model, the enduring hypoxia response model [9]. Of note, immunity to *Mtb* DosR-regulon-encoded antigens is associated with control of latent *Mtb* infection, as several DosR-regulon-encoded antigens are preferentially recognized by individuals with latent *Mtb* infection [7;10;11]. Thus, enhancing immune responses to these antigens might contribute towards controlling persistent *Mtb* infection with the potential to help preventing reactivation TB disease.

The precise nature of the human T-cell response to *Mtb* DosR-regulon-encoded antigens has not been studied in detail thus far. Most studies have documented IFN-γ production in response

to *Mtb* DosR antigens, but the major cellular source(s) of the produced IFN-γ have not been identified, neither was concomitant production of other cytokines assessed [7;12-14].

In this study we show that *Mtb* DosR-regulon-encoded antigens induce antigen and peptide specific, double and single cytokine producing $CD4^+$ and $CD8^+$ T cells in elderly persons who had been infected with *Mtb* decades ago in the pre-antibiotic era, yet never developed TB (designated here as long-term latent *Mtb*-infected individuals, (ltLTBIs)). Among the responding cells, IFN-γ^+TNF-α^+ $CD8^+$ T cells were highly prevalent, the majority being effector memory ($CCR7^-$ $CD45RA^-$) or effector ($CCR7^-CD45RA^+$) T cells. Furthermore, a register of peptide epitopes recognized by both $CD4^+$ and $CD8^+$ T cells was identified for several *Mtb* DosR-regulon-encoded antigens, which are potently recognized in humans [7]. Collectively, these results underscore the importance of *Mtb* DosR antigens and their association with control of latent *Mtb* infection.

Materials and methods

Study subjects. We studied PBMCs derived from a Norwegian group that had been exposed to *Mtb* decades ago, but had never developed TB despite lack of any treatment. This population was designed as long-term LTBI (ltLTBIs) (n = 13). Their ages ranged from 62 to 74 years (average 70 years) with tuberculin skin test indurations ranging from 12 to 60 mm (average 18 mm). About 77% (10/13) of the Norwegian donors tested positive for Quantiferon® TB Gold (Cellestis Carnegie, Victoria, Australia). PBMCs of healthy PPD negative (PPD^-) blood bank donors were used as negative controls. Donors were considered PPD^- when IFN-γ responses to PPD was < 100 pg/ml. For the second study, buffy coats from 21 *in vitro* PPD responsive (PPD^+) healthy anonymous, HLA-typed blood bank donors were included. PPD responding donors were considered positive when IFN-γ responses (corrected for background values) to PPD exceeded 100 pg/ml, in line with our previous studies [7;15;16]. Buffy coats were used since the number of cells derived from that source allowed us to perform experiments in which the *Mtb* DosR antigen and all single peptides could be tested simultaneously. All donors were HIV-negative and written informed consent was obtained prior venipuncture. The study protocol (P207/99) was approved by the Regional Committees for Medical and Health Research Ethics in Norway and the Institutional Review Board of the Leiden University Medical Center. PBMCs were isolated by standard Ficoll density gradient centrifugation using Leucosep® tubes (Greiner Bio-one, Alphen aan den Rijn, The Netherlands). PBMCs were collected and stored in liquid nitrogen until use.

Mtb *recombinant antigens and synthetic peptides.* Recombinant proteins were produced as described previously [17]. In short, PCR was used to amplify the selected *Mtb* H37Rv genes from genomic H37Rv DNA. The PCR products were cloned using Gateway Technology (Invitrogen, San Diego, CA, USA) and were subsequently sequenced. *Escherichia coli* strain BL21 (DE3) was used to over-express *Mtb* proteins. Recombinant proteins were further purified as described

previously [17]. All recombinant proteins were tested in quality control assays including, size and purity check, determination of residual endotoxin levels as well as non-specific T-cell stimulation and cellular toxicity in lymphocyte stimulation assays [16]. PPD (batch RT49) was purchased from Statens Serum Institute (Copenhagen, Denmark).

Synthetic peptides were synthesized as previously described [18]. Peptides from *Mtb* DosR antigens Rv1733c, Rv2029c, Rv2031c and control antigen Ag85B were 20-mers peptides with 10 aa overlap, except peptides 20-22 of Ag85B which were 15-mers with 10 aa overlap (Supplementary Table S1A-D). The 20-mer peptides of Rv1733c and Rv2029c were elongated with two lysine (K) residues at the C-terminal to improve solubility. The HLA-A*0201-restricted, HIV-1 p17 $Gag_{77–85}$ epitope (SLYNTVATL) was used as control peptide [19].

Functional T-cell analysis. T-cell phenotype analysis was performed as previously described [20]. In brief, PBMCs were stimulated for 16 hours with protein (10 μg/ml) or peptide pools (5 μg/ml) in the presence of co-stimulatory antibodies anti-CD28/anti-CD49d (Sanquin, The Netherlands and BD Biosciences respectively). After 4-6 hours, Brefeldin A (3 μg/ml; Sigma) was added to the culture. Cell surface staining was performed for the following markers; CD3-PB, CD4-PercP/Cy5.5, CD8-AmCyan, CD45RA-PE/Cy5, CD25-APC/Cy7 and CCR7-PE/Cy7. Subsequently, intracellular markers were stained with IFN-γ-Alexa700, TNF-α-APC, IL-2-PE and CD69-FITC (BD Biosciences) using Intrastain kit (Dako Cytomation, Denmark). Samples were acquired on an LSRII. $CD4^+$ and $CD8^+$ populations of ≥ 2 x 10^5 events were analyzed using FlowJo (Treestar, Ashland, OR, USA) and SPICE software (provided by Dr. Mario Roederer, Vaccine Research Center, NIAID, NIH, USA). Boolean gate analysis was used to study the different single, double and polyfunctional $CD4^+$ and $CD8^+$ T cells.

T-cell proliferation. Proliferation was measured using carboxy-fluorescein diacetate, succinimidyl ester (CFSE) dilution and flow cytometry. PBMCs from study subjects were thawed, washed and labeled with CFSE (Molecular Probes, Leiden, The Netherlands) at a final concentration of 5 μM for 10 minutes at 37 °C. Washed, counted and viable cells were seeded in triplicates in 96-well round-bottom plates at a concentration of 1.5 x 10^5 cells/well in the presence of control antigens (PPD 5 μg/ml, PHA 2 μg/ml (Remel, Oxoid, Haarlem, The Netherlands)) or test antigens and peptides (all at final concentrations of 10 μg/ml). Cells were cultured in IMDM supplemented with glutamax, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Breda, The Netherlands), and 10% human serum at 37°C and 5% CO_2. After 7 days of incubation, cells were stained for further analysis on the flow-cytometer.

Cells were stained for the following surface markers; CD8-APC (DakoCytomation, Heverlee, Belgium), CD3-PerCP and CD4-PE (BD Biosciences), washed in PBS 0.1% BSA (Sigma Aldrich, Zwijndrecht, The Netherlands), fixed in 1% paraformaldehyde (Pharmacy LUMC, The Netherlands) and acquired on an LSRII with HTS plate loader (BD Biosciences). Analysis was

performed using FACS DIVA software (BD Biosciences). Live lymphocyte gated cells combined with gating of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells were analyzed for proliferation using CFSE dye dilution. The Δ geometric mean was used as a measure of proliferation and calculated as follows: Δ geometric mean = geometric mean (non-proliferated cells) - geometric mean (total cells). The Δ geometric mean was then used to calculate the 'relative proliferation', which is the percentage of maximal proliferation (PHA) corrected for spontaneous proliferation (HIV-1 $p17_{Gag77-85}$) ((Δ geometric mean sample - Δ geometric mean control medium) / (Δ geometric mean PHA - Δ geometric mean control medium)) x 100% = % of maximal proliferation. The cut-off value for a positive proliferative response was arbitrarily set at 10% relative proliferation in order to limit the number of candidate epitopes to be evaluated in subsequent experiments [21].

IFN-γ ELISA. IFN-γ concentration in cellular supernatants was detected using ELISA (U-CyTech, Utrecht, The Netherlands) as previously described [20].

Results

Selection of recombinant *Mtb* DosR antigens

Our previous work showed that *Mtb* DosR-regulon-encoded antigens are efficiently recognized by *Mtb*-exposed individuals, particularly asymptomatic TST positive individuals [7;12;13]. To study the nature of the response against these antigens in more detail, we selected Rv1733c and Rv2029c as two *Mtb* DosR proteins consistently ranking among the top ten most frequently recognized *Mtb* DosR antigens in *Mtb* exposed individuals across different ethnic populations [7;12;13]. The secreted protein Ag85B and the *Mtb* DosR antigen Rv2031c (HspX, hsp16, α-crystallin) were included as control antigens [22-24]. Besides recombinant proteins (Table 1) overlapping sets of synthetic peptides of all four antigens were produced and tested as well (Supporting Information Table S1A-D).

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	[7;11-13]
	Rv2029c	*pfkB*	35,4	phosphofructokinase PfkB	[7;11;12]
	Rv2031c	*hspX*	16,3	heat shock protein HspX (alpha-crystallin)	[7;10;22-24]
Reference gene	Rv1886c	*fbpB*	34,6	secreted antigen 85-B fbpB (mycolyltransferase 85B, Ag85B)	[37]

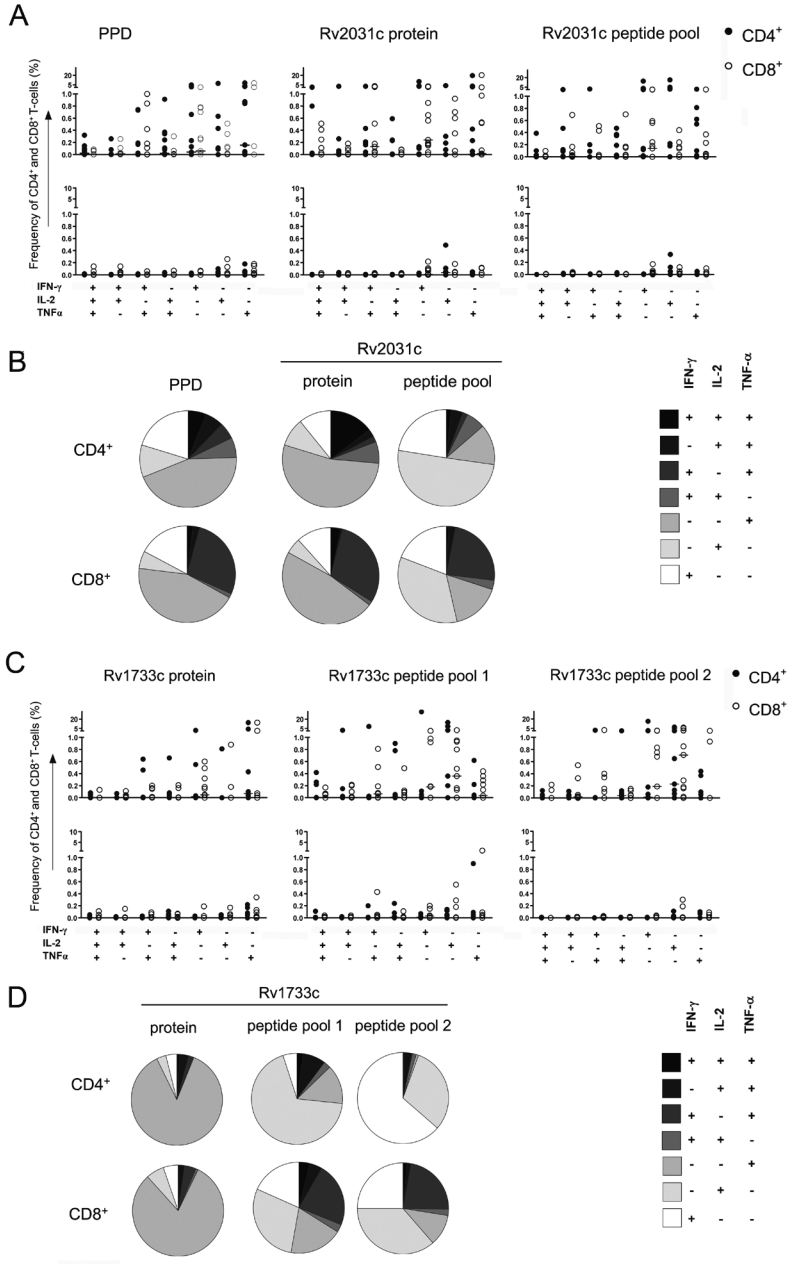
[a] Annotations are from www.tbdb.org and http://genolist.pasteur.fr/TubercuList/

Single, double and polyfunctional T-cell responses against *Mtb* DosR antigens in ltLTBIs

Mtb DosR antigen-specific T-cell recognition described previously in *Mtb*-exposed donors was based mostly on the production of IFN-γ. Previously, polyfunctional T cells producing IFN-γ, TNF-α and IL-2 have been suggested as possible markers of protective immunity, based on observations that vaccine-induced triple positive T cells correlated well with protection [25-31]. However, other studies reported that such T cells were associated with active TB disease [32-35].

The nature of *Mtb* DosR antigen-responsive $CD4^+$ and $CD8^+$ T-cell subsets in untreated *Mtb*-exposed donors who had been infected several decades ago, yet never developed any signs or symptoms of active TB (ltLTBIs), was studied here. *In vitro* purified protein derivative of *Mtb* (PPD) negative (PPD^-) donors were included as uninfected controls. PBMCs of ltLTBIs and PPD^- donors were stimulated with *Mtb* DosR-regulon-encoded antigens or corresponding peptide pools and the responses were analyzed using multi-parameter flow cytometry (Supplementary Figure S1A and S1B). Donors were considered positive when the frequency of a double or polyfunctional T-cell subset population was ≥ 0.2 %, which is equivalent to ≥ 200 events.

In ltLTBIs high percentages of IFN-γ, TNF-α and/or IL-2 cytokine producing $CD4^+$ and $CD8^+$ T cells were found in response to PPD (0.23-7.91% and 0.25-7.55%, respectively), Rv2031c protein (0.21-19.71% and 0.25-20.35%, respectively) and the Rv2031c peptide pool (0.2-16.28% and 0.23-32.92%, respectively), whereas no such responses were observed in PPD^- controls (Figure 1A). The highest frequencies were consistently found within the single cytokine producing $CD4^+$ and $CD8^+$ T-cell populations. Interestingly, many double producing T cells were identified within the $CD8^+$ T-cell population, as shown by Figure 1B, which depicts the proportions of polyfunctional as well as double and single cytokine-producing T cells. For *Mtb* DosR antigen Rv1733c, two peptide pools were tested (Figure 1C). Again high $CD4^+$ and $CD8^+$ T-cell responses were observed (0.43-14.41% and 0.2-14.25%, respectively), with single positive cells being the most frequent. In addition, substantial numbers of double cytokine-producing $CD4^+$ and $CD8^+$ T cells were present in both peptide pool responsive $CD4^+$ and $CD8^+$ T-cells populations, IFN-γ^+ TNF-α^+ $CD8^+$ T cells being the most frequent (Figure 1D). Low to no Rv1733c-specific responses were identified within the PPD^- controls (Figure 1C). A comparable pattern was observed for Rv2029c (0.29-8.41% $CD4^+$ T cells and 0.36-9.55% $CD8^+$ T cells). Unlike Rv1733c, the Rv2029c protein induced a considerable fraction of IFN-γ^+ TNF-α^+ $CD8^+$ T cells. Some responses to Rv2029c peptide pool 1 were also observed in the PPD^- group, but no responses were seen to peptide pools 2 and 3 (Figure 1E and 1F). Of note, stimulation of PBMCs with Staphylococcus enterotoxin B induced high percentages of $CD4^+$ and $CD8^+$ T cells producing single (0.3-26.44% $CD4^+$ T cells and 0.29-12.6% $CD8^+$ T cells), double (0.23-22.26% $CD4^+$ T cells and 0.24-20.17% $CD8^+$ T cells) and triple (0.29-5.37% $CD4^+$ T cell and 0.54-6.91% $CD8^+$ T cells) cytokines in both ltLTBIs and PPD^- donors (data not shown).


A
PPD
Rv2031c protein
Rv2031c peptide pool
CD4+
CD8+
Frequency of CD4+ and CD8+ T-cells (%)
IFN-γ
IL-2
TNFα
B
Rv2031c
PPD
protein
peptide pool
CD4+
CD8+
IFN-γ
IL-2
TNF-α
C
Rv1733c protein
Rv1733c peptide pool 1
Rv1733c peptide pool 2
CD4+
CD8+
Frequency of CD4+ and CD8+ T-cells (%)
IFN-γ
IL-2
TNFα
D
Rv1733c
protein
peptide pool 1
peptide pool 2
CD4+
CD8+
IFN-γ
IL-2
TNF-α

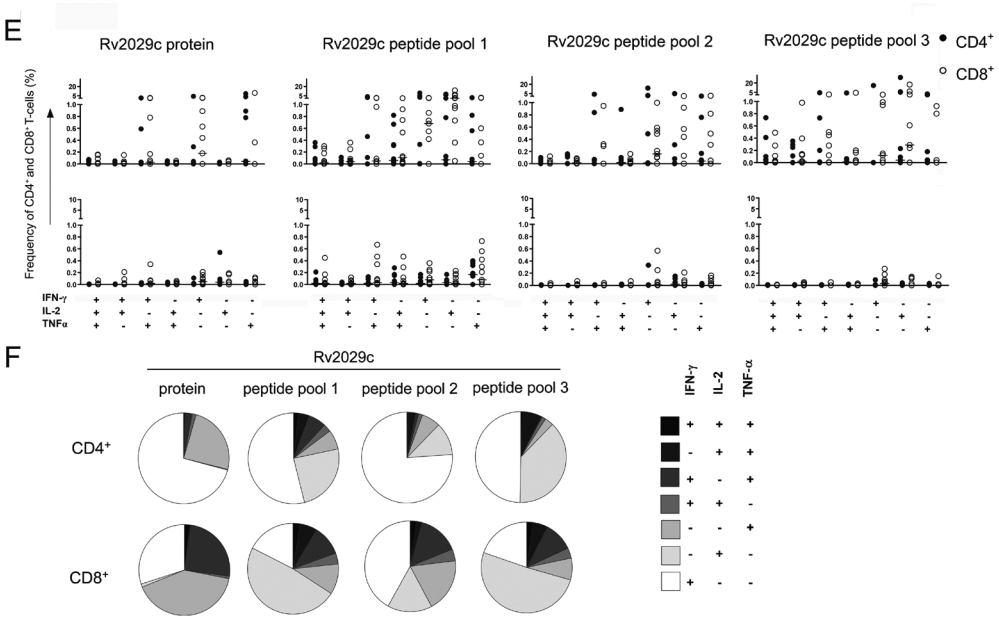


Figure 1. Frequency of antigen-specific polyfunctional T cells in ltLTBIs. Frequency of antigen-specific $CD4^+$ and $CD8^+$ T cells in ltLTBIs (upper panel) and PPD^- control donors (lower panel), producing combinations of IFN-γ, TNF-α or IL-2 after stimulation for 16 hours with control antigens as determined by flow cytometry. (A) Stimulation with PPD, Rv2031c protein and Rv2031c peptide pool 1, $n = 13$ and $n = 11$, (C) stimulation with Rv1733c protein and corresponding peptide pools 1 and 2, $n = 11$ and (E) stimulation with Rv2029c protein and corresponding peptide pools 1-3, $n = 11$. $CD4^+$ T cells are indicated as closed circles (●) and $CD8^+$ T cells as open circles (○). Horizontal bars represent the median frequency of antigen-specific $CD4^+$ and $CD8^+$ T cells. (B, D and F) Pie chart representation of the proportion of single-, double- or triple-positive $CD4^+$ and $CD8^+$ T cells for each antigen. Only $CD4^+$ and $CD8^+$ populations of ≥ 2 x 10^5 events were analyzed. Donors were considered positive when the frequency of a double or polyfunctional T-cell subset population was ≥ 0.2 %, which corresponds to ≥ 200 events. Range total $CD4^+$ T cells: 0-3694 events and total $CD8^+$ T cells: 0-3937 events.

Interestingly, the IFN-γ^+ TNF-α^+ $CD8^+$ T-cell population consistently was the most frequent multiple cytokine-producing T-cell subset identified (Figure 1B, D and F). To assess the memory phenotype of these cells, we measured expression of memory markers CCR7 and CD45RA by *Mtb* antigen or peptide responsive cells from the ltLTBI population (Figure 2A and B). T-cell subsets were classified according to the model described by Seder *et al.* [36]. Only a minor fraction of the IFN-γ^+ TNF-α^+ $CD8^+$ T cells appeared to be "naïve" ($CCR7^+CD45RA^+$) or central memory T cells ($CCR7^+CD45RA^-$), while most were found to be effector memory ($CCR7^-CD45RA^-$) T cells, followed by effector ($CCR7^-CD45RA^+$) T cells (percentages ranged between 36 and 62% (SD ± 0-35) for effector memory T cells and 22-51% (SD± 2.8-32) for effector T cells).

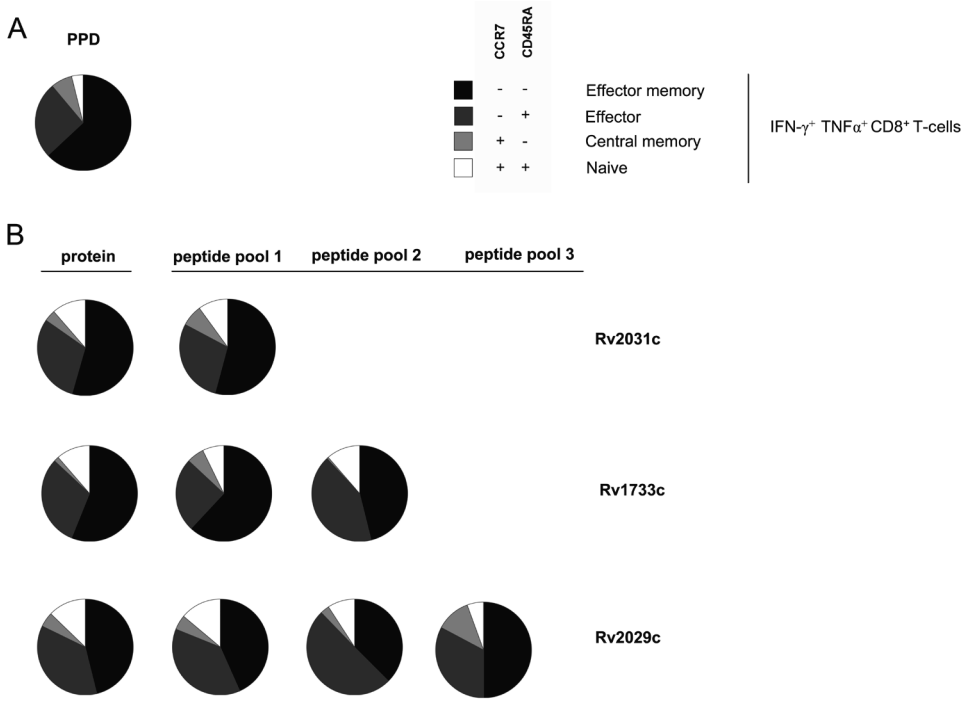


Figure 2. T-cell memory subset distribution of IFN-γ$^+$ TNF-α$^+$ CD8$^+$ T cells. Expression of T-cell memory markers CCR7 and CD45RA was analyzed by flow cytometry in IFN-γ$^+$ TNF-α$^+$ CD8$^+$ T cells of (A) PPD responders (n = 4) and (B) *Mtb* antigen or peptide pool responders from the ltLTBI donors. Rv2031c protein (n = 3), Rv2031 peptide pool 1 (n = 2), Rv1733c protein (n = 2), Rv1733c peptide pool 1 (n = 2), Rv1733c peptide pool 2 (n = 3), Rv2029c protein (n = 3), Rv2029c peptide pool 1 (n = 4), Rv2029c peptide pool 2 (n = 3) and Rv2029c peptide pool 3 (n = 3). CD8$^+$ populations with ≥ 2 x 10^5 events were analyzed. Effector memory T cells are CCR7$^-$ and CD45RA$^-$, central memory T cells are CCR7$^+$ and CD45RA$^-$, naive T cells are CCR7$^+$ and CD45RA$^+$ and effector T cells are CCR7$^-$ and CD45RA$^+$.

Taken together, our results show the presence of *Mtb* DosR-regulon-encoded antigen-specific double- and monofunctional CD4$^+$ and CD8$^+$ T-cell responses in ltLTBIs. IFN-γ$^+$ TNF-α$^+$ CD8$^+$ T cells were the most prominently present multiple cytokine-producing T cells, and comprised mainly of effector memory and effector T cells.

Peptide epitope mapping of *Mtb* DosR antigens by CFSE proliferation and flow cytometry

Next, we analyzed single peptide-induced responses in PPD positive (PPD^+) individuals in order to identify immunogenic *Mtb* DosR antigen epitopes. In view of the number of cells required for these analyses, we used buffy coat derived PBMCs. PBMCs of PPD^+ individuals were incubated with each single peptide of *Mtb* DosR Rv1733c, Rv2029c and Rv2031c and the control protein Ag85B. Proliferative responses were measured using CFSE labeling, an assay that we have described previously [21;34]. Figure 3 demonstrates typical proliferation profiles of $CD4^+$ and $CD8^+$ T cells in response to *Mtb* antigens and control conditions in one PPD^+ donor. Following stimulation of PBMCs with PPD, Rv1733c or its corresponding peptides, significant $CD4^+$ and to a lesser extent $CD8^+$ T-cell proliferation were observed (Figure 3A and B, respectively). No proliferation was observed to the irrelevant control peptide HIV-gag_{77-85} or for medium only (data not shown). A relative proliferation (see Material and methods for calculation) of 10% was considered positive in this assay, in line with previous studies [21;34]. Responses to previously published HLA class I and class II restricted epitopes of Ag85B [37] and Rv2031c [24;35;38-40] could be confirmed, validating this approach (Figure 3A and B).

Results for CFSE-labeled PBMCs from all 15 PPD^+ donors in response to PPD, *Mtb* DosR-regulon encoded proteins Rv1733c, Rv2029c and Rv2031c and Ag85B protein and all respective single peptides from each of the four antigens are given in Figure 4A and B, showing comprehensive epitope maps for $CD4^+$ (Figure 4A) or $CD8^+$ (Figure 4B) T cells. Notwithstanding the expected inter-individual differences in antigen and peptide recognition patterns, all 15 PPD^+ donors responded to at least one of the recombinant proteins and/or peptides investigated. Recombinant antigens Rv1733c, Rv2029c and Rv1886c (Ag85B) were recognized efficiently: 7/15 PPD^+ donors recognized Rv2029c ($CD4^+$: 15-97.2%, $CD8^+$: 10.6-66.6%), 5/15 recognized Rv1733c ($CD4^+$: 20.3-40%, $CD8^+$: 12.2-31.1) and 4/15 recognized Ag85B ($CD4^+$: 13.8-53.4%, $CD8^+$: 12.6-97.7%). Corresponding to our previous observations, Rv2031c/hspX/*acr* was recognized by a minority of the donors ($CD4^+$: 10.9-16.4%, $CD8^+$: 42.7%) [7;12].

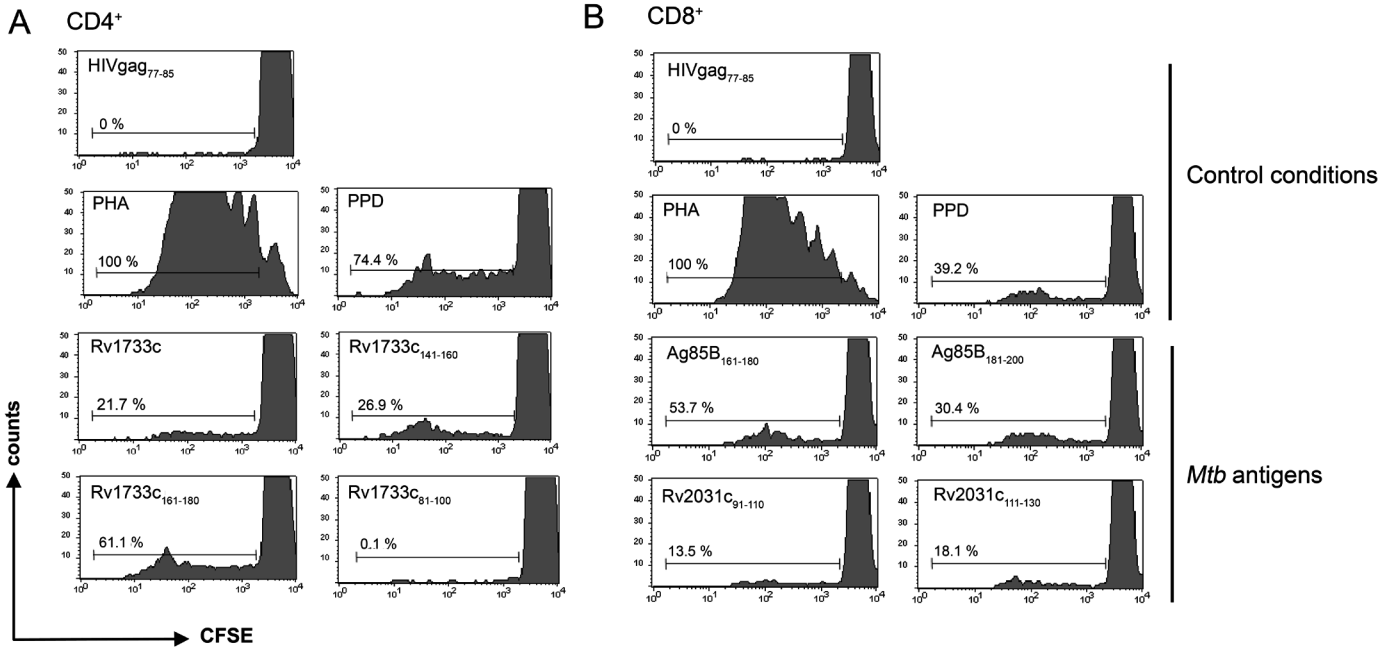


Figure 3. T-cell proliferation in response to *Mtb* antigens, peptides and control conditions. PBMCs from PPD responsive donor 9 were stimulated with PHA or $HIVgag_{77\text{-}85}$ as positive and negative controls, respectively, or with recombinant *Mtb* antigens. After 6 days, cellular proliferation as measured by CFSE dye dilution was measured in (A) $CD4^+$ or (B) $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cells were gated from a $CD3^+$ T-cell gate together with a live lymphocyte gate. Individual histogram plots show the percentage relative proliferation. PBMCs were stimulated with (A) Rv1733c protein and corresponding peptides and (B) peptides of Ag85B and Rv2031c, containing HLA-A2 restricted epitopes.

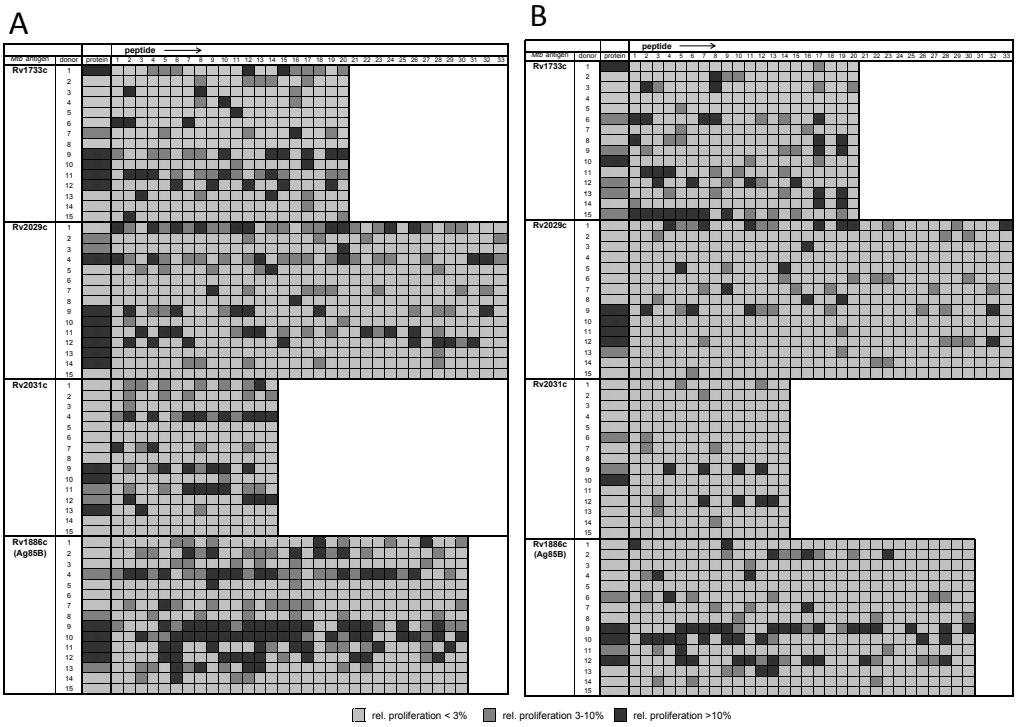


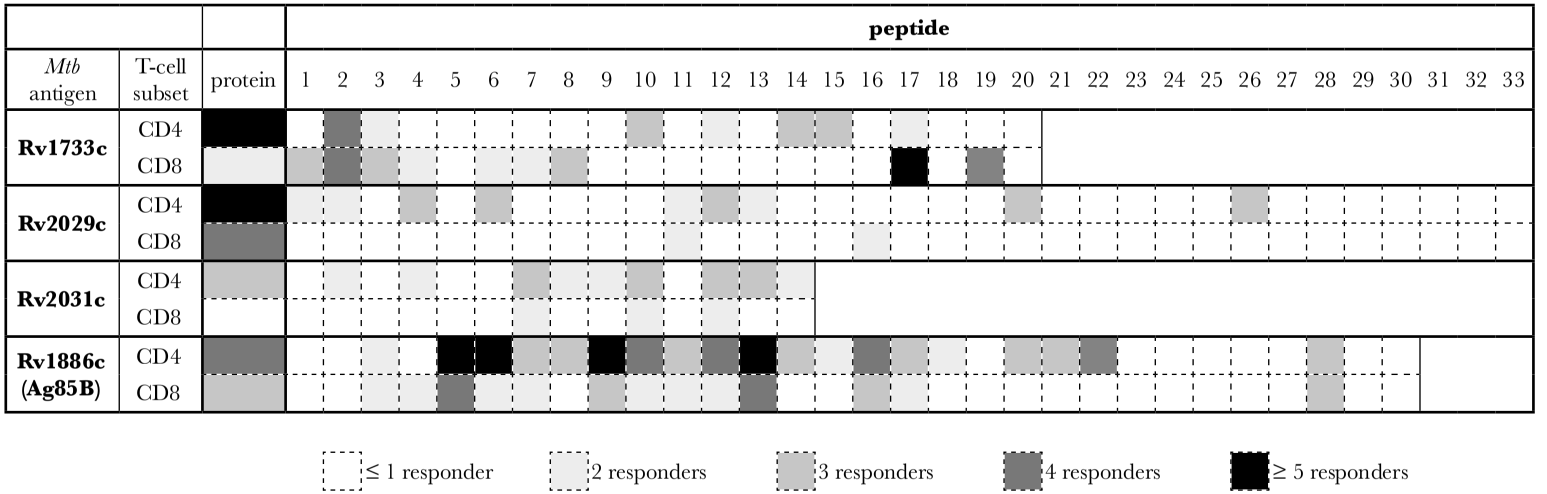
Figure 4. Proliferative CD4$^+$ and CD8$^+$ T-cell responses to *Mtb* DosR antigen epitopes among PPD$^+$ individuals. PBMCs of 15 PPD$^+$ donors were stimulated with either *Mtb* DosR antigen protein, corresponding single peptides or control antigen and peptides of Ag85B. Relative proliferation of (A) CD4$^+$ and (B) CD8$^+$ T cells is indicated in grey tones. Relative proliferation was calculated as follows; ((Δ geometric mean sample - Δ geometric mean control medium) / (Δ geometric mean PHA - Δ geometric mean control medium)) x 100% = % of maximal proliferation. Light grey; less than 3% proliferation, grey; between 3 and 10% proliferation, dark grey; over 10% proliferation. A relative proliferation of ≥ 10% was considered positive.

A substantial number of peptides was recognized by CD4$^+$ and CD8$^+$ T cells for Rv1733c (CD4$^+$: 17/20 (10.1-76.9%) CD8$^+$: 12/20 (10.4-100%)), Rv2029c (CD4$^+$: 25/33 (10.4-100%) CD8$^+$: 14/33 (10.3-66.6%)), Rv2031c (CD4$^+$: 12/14 (10.2-53.8%) CD8$^+$: 5/14 (11.3-42.7%)) and Ag85B (CD4$^+$: 28/30 (10.1-75.3%) CD8$^+$: 25/30 (10.9-97.7%)). Some peptides were recognized by CD4$^+$ T cells from more than one third of the donors (e.g. 6/15 donors in case of Ag85B peptides 9 and 13, and 5/15 for Ag85B peptides 5, 6), whereas other peptides were recognized by CD4$^+$ T cells in 4/15 donors, such as Rv1733c peptide 2 and Ag85B peptides 10, 12, 16 and 22. CD8$^+$ T-cell responses were particularly observed against Rv1733c and Ag85B, these responses were found in four to five donors; Rv1733c peptides 17 (5/15), 2 and 19 (4/15), and Ag85B peptides 5 and 13 (4/15). Notably, some peptides were recognized by both CD4$^+$ and CD8$^+$ T cells (Rv1733c peptide 2, Ag85B peptides 5 and 13). Table 2 shows the cumulative epitope recognition map for both CD4$^+$ and CD8$^+$ T cells in response to all tested proteins and peptides for all donors

tested. Interestingly, the results suggest enrichment of epitopes in certain immunogenic regions, for example $Rv1733c_{(1-40)}$, $Rv1733c_{(161-200)}$ and $Ag85B_{(81-180)}$, which harbour Rv1733c peptides 1-3, 17-19 and Ag85B peptides 5-14.

The above-described *Mtb* DosR antigen-encoded peptide epitopes were recognized by donors with varying HLA-genotypes. Many of the *in vitro* responses given in Figure 4A and B matched with *in silico* epitope motif searches for the relevant HLA-genotypes (data not shown) [41]. This suggests that responses to *Mtb* dosR-regulon-encoded antigens occur in a wide range of HLA backgrounds. In order to better characterize the molecular interactions of *Mtb* DosR antigenic epitope presentation, we examined peptide recognition in the context of the highly frequent HLA-A*0201 genotype (New allele frequency database: http://www.allelefrequencies.net [42]) and found that $Rv1733c_{p181-189}$ specific $CD8^+$ T cells were able to lyse peptide loaded and endogenously processed Rv1733c-antigen loaded target cells in the context of HLA-A*0201 molecules (Supplementary Figure S2A and S2B).

Table 2. Cumulative epitope recognition of CD4⁺ and CD8⁺ T cells to *Mtb* antigens and corresponding peptides



Discussion

We have proposed that *Mtb* DosR-regulon-encoded antigens [7] that are expressed by *Mtb* during *in vitro* conditions mimicking intracellular infection represent rational targets for TB vaccination. Immune responses to *Mtb* DosR-regulon-encoded antigens are prominently found in latently infected individuals, and are associated with latent *Mtb* infection (LTBI) in several ethnically and geographically distinct populations [7;11;13]. Additional work showed that the *Mtb* DosR-regulon-encoded antigen Rv2628 was strongly recognized by individuals with remote *Mtb* infection [13;14]. Thus far, the precise mechanisms and T-cell subsets responsible for the responses against *Mtb* DosR-regulon-encoded antigens have not been studied in detail; and virtually all studies have relied on measuring IFN-γ production by polyclonal cells. Here we report peptide reactivity and memory phenotypes of *Mtb* DosR-regulon-encoded antigen-specific T cells in long-term LTBI, and moreover, document a large series of specific peptide epitopes recognized by specific $CD4^+$ and $CD8^+$ T cells.

Three *Mtb* DosR antigens, Rv1733c, Rv2029c and Rv2031c (HspX, α-crystallin) were tested in this study. Strong *Mtb* DosR antigen-specific $CD4^+$ and $CD8^+$ polyfunctional T-cell responses were detected in ltLTBIs. The highest responses were observed among single cytokine-producing $CD4^+$ and $CD8^+$ T-cell subsets (either TNF-α^+, IL-2^+ or IFN-γ^+, depending on the stimulus) followed by double producing $CD4^+$ and particularly $CD8^+$ T cells. Of interest, the most frequent multiple cytokine-producing T cells were IFN-γ^+ TNF-α^+ $CD8^+$ T cells. These cells were further characterized as effector memory ($CCR7^-$ and $CD45RA^-$) or effector ($CCR7^-$ and $CD45RA^+$) T cells, which have the ability to perform immediate effector functions. This is compatible with an important role for $CD8^+$ T cells in *Mtb* infection [43;44].

Mtb antigen specific polyfunctional T cells have been studied intensely the last few years, both in vaccination and in observational studies in *Mtb* infected individuals [25-36;45]. There is currently no consensus whether polyfunctional T cells represent a marker of protective immunity or of disease activity. The vaccine MVA85A (recombinant replication-deficient vaccinia Ankara, expressing Ag85A) induced polyfunctional $CD4^+$ and $CD8^+$ T cells producing IFN-γ, TNF-α and IL-2 as well as IFN-γ and TNF-α in mice, which correlated with TB protection [26]. This vaccine also induced increased $CD4^+$ T cells expressing IFN-γ, TNF-α and IL-2 in humans when given as a booster to previous BCG vaccination [27;28]. Similar results were reported following human vaccination with the BCG booster AERAS-402 (recombinant replication-deficient Adenovirus (Ad35) virus, expressing a polyprotein of Ag85A, Ag85B and TB10.4) [29]. Finally, mice vaccinated with hybrid subunit vaccines H1 (Ag85-ESAT6) and H56 (H1+Rv2660) also had high numbers of triple cytokine-producing $CD4^+$ T cells [30;31]. However, observational studies in humans have associated polyfunctional $CD4^+$ T cells with TB disease [32;33]. Although limited information is available on *Mtb*-specific polyfunctional $CD8^+$ T cells, two of our recent studies showed that double- and single-producing $CD8^+$ T cells are prominent in latently *Mtb*-infected individuals and

cured TB patients and that IFN-γ^+/IL-2^+ $CD8^+$ T cells are a biomarker of protective host defense against *Mtb* [34;35]. Moreover, no difference in polyfunctional $CD4^+$ T-cell profiles could be identified between BCG-vaccinated children from a high endemic area that either developed TB or did not, indicating that polyfunctional T cells are not a biomarker of BCG-induced protection against TB [45]. In our study here we show the presence of mostly single and double positive T cells, the latter mainly present in $CD8^+$ T cells, supporting previous findings that single and double positive T cells are prominent in LTBI [32;35]. This suggests that these double and single cytokine-producing T cells play a significant role in *Mtb* immunity, although their precise nature and mechanisms of action requires more detailed studies.

While most studies on polyfunctional T cells have focused on highly expressed *Mtb* early phase proteins such as ESAT6 and Ag85B, instead, we here have analyzed *Mtb* antigens that are expressed during dormancy. It remains possible that antigens expressed during different phases of infection may preferentially induce different patterns of single, double and polyfunctional T cells.

A striking observation was the wealth of epitopes that could be identified in *Mtb* DosR-regulon-encoded antigens, in accordance with the significant immunogenicity of *Mtb* DosR-regulon-encoded antigens in a wide variety of HLA backgrounds [46]. The donors used to detect single peptide responses were anonymous Dutch blood bank donors. Although we have no precise information about their mycobacterial exposure status, we have shown previously that over 50% of blood bank donors respond to PPD; furthermore, responses to *Mtb* DosR antigens were also observed in nontuberculous mycobacteria-exposed donors, probably due to the high conservation of these antigens [47].

Within several *Mtb* DosR-regulon-encoded antigens highly immunogenic regions could be identified and a substantial number of peptides elicited both $CD4^+$ and $CD8^+$ T-cell responses. Although HLA-class I presented peptides are typically 8-11 amino acids long, whereas HLA-class II ligands can be between 10-25 amino acids [41;48;49], we nevertheless found efficient $CD8^+$ T-cell responses using 20-mers and confirmed Rv1733c specific lysis of target cells by $Rv1733c_{p181-189}$ specific $CD8^+$ T cells. It has been suggested that apoptosis, induced by the cytotoxic activity of CTL, can inhibit *Mtb* growth or even kill *Mtb* bacteria [50-52]. Granulysin may play a role in this mycobactericidal activity [53]. In addition, vaccine-induced $CD8^+$ T cells in mice indeed can reduce bacterial load *in vivo* [54]. Again, this suggests a protective role for $CD8^+$ T cells in *Mtb* infection.

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 [55;56]. Peptides appeared to be more sensitive tools for detecting $CD4^+$ or $CD8^+$ T-cell responses compared to recombinant proteins; this is likely due to the preprocessed nature of peptides, facilitating highly efficient antigen presentation. Second, peptides were present at much higher molar concentrations since proteins and peptides were tested at 10 µg/ml, regardless of their molecular mass. The lack of competition for processing, with otherwise dominant epitopes

in recombinant proteins, may also have permitted identification of subdominant epitopes using peptides. Thus, peptide-based epitope mapping also offers the potential to elucidate subdominant epitopes, which 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 [57;58]. Of note, previous work has shown the efficacy of vaccines containing subdominant epitopes in protection against infection with *Mtb* [59] .

In conclusion, we report the presence of *Mtb* DosR-regulon-encoded peptide antigen-specific single and double functional $CD4^+$ and $CD8^+$ T-cell responses in ltLTBIs. We show that the majority of multiple cytokine-producing T cells comprise IFN-γ^+ TNF-α^+ $CD8^+$ T cells; these cells were characterized as mainly effector memory or effector T cells. Furthermore, we describe a large series of new peptide epitopes expressed by *Mtb* DosR-regulon-encoded antigens, which are recognized by $CD4^+$ and/or $CD8^+$ T cells of PPD^+ donors. These results significantly enhance our understanding of the human immune response to *Mtb* phase-dependent antigens in long-term control of infection, and pave the way for designing *Mtb* DosR antigen and/or peptide-based vaccination approaches to TB.

Acknowledgements

This work was supported by a grant from the Foundation Microbiology Leiden, the European Commission within the sixth Framework Program (FP6), the Bill and Melinda Gates Foundation, TI Pharma (project D-101-1), Grand Challenges in Global Health (GC6#74, GC12#82), ISA Pharmaceuticals and TBVAC contract no. LSHP-CT-2003-503367 (The text represents the authors' views and does not necessarily represent a position of the Commission who will not be liable for the use made of such information). We thank Corine Prins, Sandra Arend, Michèl R. Klein, Willem Verduijn and his colleagues for their support.

Conflict of interest. The authors declare no financial or commercial conflict of interest.

References

1. **Kaufmann,S.H., Hussey,G., and Lambert,P.H.,** New vaccines for tuberculosis. *Lancet* 2010. **375**: 2110-2119.
2. **Ottenhoff,T.H.,** Overcoming the global crisis: "yes, we can", but also for TB ... ? *Eur.J.Immunol.* 2009. **39**: 2014-2020.
3. **WHO**. Global tuberculosis control : surveillance, planning, financing : WHO report 2008. "WHO/HTM/TB/2008.393". 2010.
4. **Crampin,A.C., Glynn,J.R., and Fine,P.E.,** What has Karonga taught us? Tuberculosis studied over three decades. *Int.J.Tuberc.Lung Dis.* 2009. **13**: 153-164.
5. **Fine,P.E.,** Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* 1995. **346**: 1339-1345.
6. **Young,D. and Dye,C.,** The development and impact of tuberculosis vaccines. *Cell* 2006. **124**: 683-687.
7. **Leyten,E.M., Lin,M.Y., Franken,K.L., Friggen,A.H., Prins,C., van Meijgaarden,K.E., Voskuil,M.I., Weldingh,K., Andersen,P., Schoolnik,G.K., Arend,S.M., Ottenhoff,T.H., and Klein,M.R.,** Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of Mycobacterium tuberculosis. *Microbes.Infect.* 2006. **8**: 2052-2060.
8. **Voskuil,M.I., Schnappinger,D., Visconti,K.C., Harrell,M.I., Dolganov,G.M., Sherman,D.R., and Schoolnik,G.K.,** Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. *J.Exp.Med.* 2003. **198**: 705-713.
9. **Rustad,T.R., Harrell,M.I., Liao,R., and Sherman,D.R.,** The enduring hypoxic response of Mycobacterium tuberculosis. *PLoS.One.* 2008. **3**: e1502.
10. **Demissie,A., Leyten,E.M., Abebe,M., Wassie,L., Aseffa,A., Abate,G., Fletcher,H., Owiafe,P., Hill,P.C., Brookes,R., Rook,G., Zumla,A., Arend,S.M., Klein,M., Ottenhoff,T.H., Andersen,P., and Doherty,T.M.,** Recognition of stage-specific mycobacterial antigens differentiates between acute and latent infections with Mycobacterium tuberculosis. *Clin.Vaccine Immunol.* 2006. **13**: 179-186.
11. **Roupie,V., Romano,M., Zhang,L., Korf,H., Lin,M.Y., Franken,K.L., Ottenhoff,T.H., Klein,M.R., and Huygen,K.,** Immunogenicity of eight dormancy regulon-encoded proteins of Mycobacterium tuberculosis in DNA-vaccinated and tuberculosis-infected mice. *Infect.Immun.* 2007. **75**: 941-949.
12. **Black,G.F., Thiel,B.A., Ota,M.O., Parida,S.K., Adegbola,R., Boom,W.H., Dockrell,H.M., Franken,K.L., Friggen,A.H., Hill,P.C., Klein,M.R., Lalor,M.K., Mayanja,H., Schoolnik,G., Stanley,K., Weldingh,K., Kaufmann,S.H., Walzl,G., and Ottenhoff,T.H.,** Immunogenicity of novel DosR regulon-encoded candidate antigens of Mycobacterium tuberculosis in three high-burden populations in Africa. *Clin.Vaccine Immunol.* 2009. **16**: 1203-1212.
13. **Schuck,S.D., Mueller,H., Kunitz,F., Neher,A., Hoffmann,H., Franken,K.L., Repsilber,D., Ottenhoff,T.H., Kaufmann,S.H., and Jacobsen,M.,** Identification of T-cell antigens specific for latent mycobacterium tuberculosis infection. *PLoS.One.* 2009. **4**: e5590.
14. **Goletti,D., Butera,O., Vanini,V., Lauria,F.N., Lange,C., Franken,K.L., Angeletti,C., Ottenhoff,T.H., and Girardi,E.,** Response to Rv2628 latency antigen associates with cured tuberculosis and remote infection. *Eur.Respir.J.* 2010. **36**: 135-142.
15. **Arend,S.M., Geluk,A., van Meijgaarden,K.E., van Dissel,J.T., Theisen,M., Andersen,P., and Ottenhoff,T.H.,** Antigenic equivalence of human T-cell responses to Mycobacterium tuberculosis-specific RD1-encoded protein antigens ESAT-6 and culture filtrate protein 10 and to mixtures of synthetic

peptides. *Infect.Immun.* 2000. **68**: 3314-3321.

16. **Lin,M.Y., Geluk,A., Smith,S.G., Stewart,A.L., Friggen,A.H., Franken,K.L., Verduyn,M.J., van Meijgaarden,K.E., Voskuil,M.I., Dockrell,H.M., Huygen,K., Ottenhoff,T.H., and Klein,M.R.,** Lack of immune responses to Mycobacterium tuberculosis DosR regulon proteins following Mycobacterium bovis BCG vaccination. *Infect.Immun.* 2007. **75**: 3523-3530.

17. **Franken,K.L., Hiemstra,H.S., van Meijgaarden,K.E., Subronto,Y., den,H.J., Ottenhoff,T.H., and Drijfhout,J.W.,** Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. *Protein Expr.Purif.* 2000. **18**: 95-99.

18. **Hiemstra,H.S., Duinkerken,G., Benckhuijsen,W.E., Amons,R., de Vries,R.R., Roep,B.O., and Drijfhout,J.W.,** The identification of CD4+ T cell epitopes with dedicated synthetic peptide libraries. *Proc.Natl.Acad.Sci.U.S.A* 1997. **94**: 10313-10318.

19. **van der Burg,S.H., Klein,M.R., van de Velde,C.J., Kast,W.M., Miedema,F., and Melief,C.J.,** Induction of a primary human cytotoxic T-lymphocyte response against a novel conserved epitope in a functional sequence of HIV-1 reverse transcriptase. *AIDS* 1995. **9**: 121-127.

20. **Commandeur,S., van Meijgaarden,K.E., Lin,M.Y., Franken,K.L., Friggen,A.H., Drijfhout,J.W., Oftung,F., Korsvold,G.E., Geluk,A., and Ottenhoff,T.H.,** Identification of human T-cell responses to Mycobacterium tuberculosis resuscitation-promoting factors in long-term latently infected individuals. *Clin.Vaccine Immunol.* 2011. **18**: 676-683.

21. **Joosten,S.A., van Meijgaarden,K.E., van Weeren,P.C., Kazi,F., Geluk,A., Savage,N.D., Drijfhout,J.W., Flower,D.R., Hanekom,W.A., Klein,M.R., and Ottenhoff,T.H.,** Mycobacterium tuberculosis peptides presented by HLA-E molecules are targets for human CD8 T-cells with cytotoxic as well as regulatory activity. *PLoS.Pathog.* 2010. **6**: e1000782.

22. **Wilkinson,R.J., Wilkinson,K.A., De Smet,K.A., Haslov,K., Pasvol,G., Singh,M., Svarcova,I., and Ivanyi,J.,** Human T- and B-cell reactivity to the 16kDa alpha-crystallin protein of Mycobacterium tuberculosis. *Scand.J.Immunol.* 1998. **48**: 403-409.

23. **Yuan,Y., Crane,D.D., and Barry,C.E., III,** Stationary phase-associated protein expression in Mycobacterium tuberculosis: function of the mycobacterial alpha-crystallin homolog. *J.Bacteriol.* 1996. **178**: 4484-4492.

24. **Geluk,A., Lin,M.Y., van Meijgaarden,K.E., Leyten,E.M., Franken,K.L., Ottenhoff,T.H., and Klein,M.R.,** T-cell recognition of the HspX protein of Mycobacterium tuberculosis correlates with latent M. tuberculosis infection but not with M. bovis BCG vaccination. *Infect.Immun.* 2007. **75**: 2914-2921.

25. **Darrah,P.A., Patel,D.T., De Luca,P.M., Lindsay,R.W., Davey,D.F., Flynn,B.J., Hoff,S.T., Andersen,P., Reed,S.G., Morris,S.L., Roederer,M., and Seder,R.A.,** Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat.Med.* 2007. **13**: 843-850.

26. **Forbes,E.K., Sander,C., Ronan,E.O., McShane,H., Hill,A.V., Beverley,P.C., and Tchilian,E.Z.,** Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against Mycobacterium tuberculosis aerosol challenge in mice. *J.Immunol.* 2008. **181**: 4955-4964.

27. **Scriba,T.J., Tameris,M., Mansoor,N., Smit,E., van der Merwe,L., Isaacs,F., Keyser,A., Moyo,S., Brittain,N., Lawrie,A., Gelderbloem,S., Veldsman,A., Hatherill,M., Hawkridge,A., Hill,A.V., Hussey,G.D., Mahomed,H., McShane,H., and Hanekom,W.A.,** Modified vaccinia Ankara-expressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional CD4+ T cells. *Eur.J.Immunol.* 2010. **40**: 279-290.

28. **Hawkridge,T., Scriba,T.J., Gelderbloem,S., Smit,E., Tameris,M., Moyo,S., Lang,T., Veldsman,A., Hatherill,M., Merwe,L., Fletcher,H.A., Mahomed,H., Hill,A.V., Hanekom,W.A., Hussey,G.D., and McShane,H.,** Safety and immunogenicity of a new tuberculosis vaccine, MVA85A, in healthy adults in South Africa. *J.Infect.Dis.* 2008. **198**: 544-552.

29. **Abel,B., Tameris,M., Mansoor,N., Gelderbloem,S., Hughes,J., Abrahams,D., Makhethe,L., Erasmus,M., de,K.M., van der Merwe,L., Hawkridge,A., Veldsman,A., Hatherill,M., Schirru,G., Pau,M.G., Hendriks,J., Weverling,G.J., Goudsmit,J., Sizemore,D., McClain,J.B., Goetz,M., Gearhart,J., Mahomed,H., Hussey,G.D., Sadoff,J.C., and Hanekom,W.A.,** The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults. *Am.J.Respir. Crit Care Med.* 2010. **181**: 1407-1417.

30. **Lindenstrom,T., Agger,E.M., Korsholm,K.S., Darrah,P.A., Aagaard,C., Seder,R.A., Rosenkrands,I., and Andersen,P.,** Tuberculosis Subunit Vaccination Provides Long-Term Protective Immunity Characterized by Multifunctional CD4 Memory T Cells. *Journal of Immunology* 2009. **182**: 8047-8055.

31. **Aagaard,C., Hoang,T., Dietrich,J., Cardona,P.J., Izzo,A., Dolganov,G., Schoolnik,G.K., Cassidy,J.P., Billeskov,R., and Andersen,P.,** A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nat.Med.* 2011. **17**: 189-194.

32. **Caccamo,N., Guggino,G., Joosten,S.A., Gelsomino,G., Di,C.P., Titone,L., Galati,D., Bocchino,M., Matarese,A., Salerno,A., Sanduzzi,A., Franken,W.P., Ottenhoff,T.H., and Dieli,F.,** Multifunctional CD4+T cells correlate with active Mycobacterium tuberculosis infection. *Eur.J.Immunol.* 2010.

33. **Sutherland,J.S., Adetifa,I.M., Hill,P.C., Adegbola,R.A., and Ota,M.O.,** Pattern and diversity of cytokine production differentiates between Mycobacterium tuberculosis infection and disease. *Eur.J.Immunol.* 2009. **39**: 723-729.

34. **Tang,S.T., van Meijgaarden,K.E., Caccamo,N., Guggino,G., Klein,M.R., van,W.P., Kazi,F., Stryhn,A., Zaigler,A., Sahin,U., Buus,S., Dieli,F., Lund,O., and Ottenhoff,T.H.,** Genome-Based In Silico Identification of New Mycobacterium tuberculosis Antigens Activating Polyfunctional CD8+ T Cells in Human Tuberculosis. *J.Immunol.* 2010.

35. **Caccamo,N., Guggino,G., Meraviglia,S., Gelsomino,G., Di,C.P., Titone,L., Bocchino,M., Galati,D., Matarese,A., Nouta,J., Klein,M.R., Salerno,A., Sanduzzi,A., Dieli,F., and Ottenhoff,T.H.,** Analysis of Mycobacterium tuberculosis-specific CD8 T-cells in patients with active tuberculosis and in individuals with latent infection. *PLoS.One.* 2009. **4**: e5528.

36. **Seder,R.A., Darrah,P.A., and Roederer,M.,** T-cell quality in memory and protection: implications for vaccine design. *Nat.Rev.Immunol.* 2008. **8**: 247-258.

37. **Geluk,A., van Meijgaarden,K.E., Franken,K.L., Drijfhout,J.W., D'Souza,S., Necker,A., Huygen,K., and Ottenhoff,T.H.,** Identification of major epitopes of Mycobacterium tuberculosis AG85B that are recognized by HLA-A*0201-restricted CD8+ T cells in HLA-transgenic mice and humans. *J.Immunol.* 2000. **165**: 6463-6471.

38. **Caccamo,N., Milano,S., Di,S.C., Cigna,D., Ivanyi,J., Krensky,A.M., Dieli,F., and Salerno,A.,** Identification of epitopes of Mycobacterium tuberculosis 16-kDa protein recognized by human leukocyte antigen-A*0201 CD8(+) T lymphocytes. *J.Infect.Dis.* 2002. **186**: 991-998.

39. **De Groot,A.S., McMurry,J., Marcon,L., Franco,J., Rivera,D., Kutzler,M., Weiner,D., and Martin,B.,** Developing an epitope-driven tuberculosis (TB) vaccine. *Vaccine* 2005. **23**: 2121-2131.

40. **McMurry,J.A., Kimball,S., Lee,J.H., Rivera,D., Martin,W., Weiner,D.B., Kutzler,M., Sherman,D.R., Korn-**

feld,H., and De Groot,A.S., Epitope-driven TB vaccine development: a streamlined approach using immuno-informatics, ELISpot assays, and HLA transgenic mice. *Curr.Mol.Med.* 2007. **7**: 351-368.

41. **Rammensee,H., Bachmann,J., Emmerich,N.P., Bachor,O.A., and Stevanovic,S.,** SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 1999. **50**: 213-219.

42. **Middleton,D., Menchaca,L., Rood,H., and Komerofsky,R.,** New allele frequency database: http://www.allelefrequencies.net. *Tissue Antigens* 2003. **61**: 403-407.

43. **Bruns,H., Meinken,C., Schauenberg,P., Harter,G., Kern,P., Modlin,R.L., Antoni,C., and Stenger,S.,** Anti-TNF immunotherapy reduces CD8+ T cell-mediated antimicrobial activity against Mycobacterium tuberculosis in humans. *J.Clin.Invest* 2009. **119**: 1167-1177.

44. **Flynn,J.L., Goldstein,M.M., Triebold,K.J., Koller,B., and Bloom,B.R.,** Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection. *Proc.Natl.Acad.Sci.U.S.A* 1992. **89**: 12013-12017.

45. **Kagina,B.M., Abel,B., Scriba,T.J., Hughes,E.J., Keyser,A., Soares,A., Gamieldien,H., Sidibana,M., Hatherill,M., Gelderbloem,S., Mahomed,H., Hawkridge,A., Hussey,G., Kaplan,G., and Hanekom,W.A.,** Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guerin vaccination of newborns. *Am.J.Respir.Crit Care Med.* 2010. **182**: 1073-1079.

46. **Ottenhoff,T.H. and de Vries,R.R.,** HLA class II immune response and suppression genes in leprosy. *Int.J.Lepr.Other Mycobact.Dis.* 1987. **55**: 521-534.

47. **Lin,M.Y., Reddy,T.B., Arend,S.M., Friggen,A.H., Franken,K.L., van Meijgaarden,K.E., Verduyn,M.J., Schoolnik,G.K., Klein,M.R., and Ottenhoff,T.H.,** Cross-reactive immunity to Mycobacterium tuberculosis DosR regulon-encoded antigens in individuals infected with environmental, nontuberculous mycobacteria. *Infect.Immun.* 2009. **77**: 5071-5079.

48. **Falk,K., Rotzschke,O., Stevanovic,S., Jung,G., and Rammensee,H.G.,** Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. 1991. *J.Immunol.* 2006. **177**: 2741-2747.

49. **Rammensee,H.G., Friede,T., and Stevanoviic,S.,** MHC ligands and peptide motifs: first listing. *Immunogenetics* 1995. **41**: 178-228.

50. **Molloy,A., Laochumroonvorapong,P., and Kaplan,G.,** Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin. *J.Exp.Med.* 1994. **180**: 1499-1509.

51. **Fratazzi,C., Arbeit,R.D., Carini,C., Balcewicz-Sablinska,M.K., Keane,J., Kornfeld,H., and Remold,H.G.,** Macrophage apoptosis in mycobacterial infections. *J.Leukoc.Biol.* 1999. **66**: 763-764.

52. **Grotzke,J.E. and Lewinsohn,D.M.,** Role of CD8+ T lymphocytes in control of Mycobacterium tuberculosis infection. *Microbes.Infect.* 2005. **7**: 776-788.

53. **Stegelmann,F., Bastian,M., Swoboda,K., Bhat,R., Kiessler,V., Krensky,A.M., Roellinghoff,M., Modlin,R.L., and Stenger,S.,** Coordinate expression of CC chemokine ligand 5, granulysin, and perforin in CD8+ T cells provides a host defense mechanism against Mycobacterium tuberculosis. *J.Immunol.* 2005. **175**: 7474-7483.

54. **Wu,Y., Woodworth,J.S., Shin,D.S., Morris,S., and Behar,S.M.,** Vaccine-elicited 10-kilodalton culture filtrate protein-specific CD8+ T cells are sufficient to mediate protection against Mycobacterium tuberculosis infection. *Infect.Immun.* 2008. **76**: 2249-2255.

55. **Brode,S. and Macary,P.A.,** Cross-presentation: dendritic cells and macrophages bite off more than they can chew! *Immunology* 2004. **112**: 345-351.

56. **Vyas,J.M., Van der Veen,A.G., and Ploegh,H.L.,** The known unknowns of antigen processing and presentation. *Nat. Rev.Immunol.* 2008. **8**: 607-618.

57. **Melief,C.J. and van der Burg,S.H.,** Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. *Nat.Rev.Cancer* 2008. **8**: 351-360.

58. **Moudgil,K.D. and Sercarz,E.E.,** Dominant determinants in hen eggwhite lysozyme correspond to the cryptic determinants within its self-homologue, mouse lysozyme: implications in shaping of the T cell repertoire and autoimmunity. *J.Exp. Med.* 1993. **178**: 2131-2138.

59. **Aagaard,C.S., Hoang,T.T., Vingsbo-Lundberg,C., Dietrich,J., and Andersen,P.,** Quality and vaccine efficacy of CD4+ T cell responses directed to dominant and subdominant epitopes in ESAT-6 from Mycobacterium tuberculosis. *J.Immunol.* 2009. **183**: 2659-2668.

60. **Charo,J., Geluk,A., Sundback,M., Mirzai,B., Diehl,A.D., Malmberg,K.J., Achour,A., Huriguchi,S., van Meijgaarden,K.E., Drijfhout,J.W., Beekman,N., van,V.P., Ossendorp,F., Ottenhoff,T.H., and Kiessling,R.,** The identification of a common pathogen-specific HLA class I A*0201-restricted cytotoxic T cell epitope encoded within the heat shock protein 65. *Eur.J.Immunol.* 2001. **31**: 3602-3611.

61. **Spaenij-Dekking,E.H., Van,D.J., Van Der Meijden,E., Hiemstra,H.S., Falkenburg,J.H., Koning,F., Drijfhout,J.W., and Kluin-Nelemans,J.C.,** Synaptojanin 2 is recognized by HLA class II-restricted hairy cell leukemia-specific T cells. *Leukemia* 2003. **17**: 2467-2473.

62. **Swift,S., Lorens,J., Achacoso,P., and Nolan,G.P.,** Rapid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems. *Curr.Protoc.Immunol.* 2001. **Chapter 10**: Unit.

Supplementary data

Results

The peptides recognized by HLA-A2 positive donors were subjected to further *in silico* minimal epitope motif searches in the context of HLA-A2 to select the precise epitopes embedded in the recognized 20-mer peptides. 25 candidate epitopes were identified and subsequently synthesized as 9-mer peptides. Their ability to bind HLA-A2 was measured using an HLA-A2 binding assay which resulted in a set of epitopes that had an intermediate or high binding affinity (IC50 < 10 μM) for HLA-A*0201 and epitopes that had low (IC50 > 10 μM) or no measurable (IC50 > 100 μM) affinity, this despite high prediction scores and *in vitro* $CD8^+$ T-cell recognition of the corresponding 20-mer peptide (Table S2). In addition to the binding assay, all 25 candidate HLA-A2 epitopes were tested in a lymphocyte stimulation test to examine their recognition in the context of HLA-A2 molecules. Five HLA-A2 candidate peptides were recognized solely by $HLA\text{-}A2^+$ but not $HLA\text{-}A2^-$ donors as assessed by CFSE proliferation (Table S2).

A polyclonal $CD8^+$ T-cell line directed against $Rv1733c_{p181\text{-}189}$ and $Rv2031c_{p96\text{-}104}$ was generated from an HLA-A2 positive donor that responded to both $Rv1733c_{p181\text{-}189}$ and $Rv2031c_{p96\text{-}104}$ epitopes. Effector $CD8^+$ T cells showed specific peptide dependent lysis of allogenic EBV-BLCL JY target-cells (HLA-A*0201) when pulsed with peptide $Rv1733c_{p181\text{-}189}$ (but not with $Rv2031c_{p96\text{-}104}$). Killing was observed at different effector-to-target ratios (E: T) (Figure S2A). $CD8^+$ 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 S2A). Specific peptide dependent lysis of EBV-BLCL JY target cells loaded with HLA-candidate epitopes $Rv1733c_{p181\text{-}189}$ 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 S2B). 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. Collectively these results show that $CD8^+$ T cells can (i) recognize *Mtb* dosR antigen peptide $Rv1733c_{p181\text{-}189}$ presented by HLA- A*0201 restricted target cells and (ii) lyse 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 *Mtb* dosR regulon encoded epitopes for human $CD8^+$ T cells in the context of HLA class I molecules.

Materials and methods

Generation of CD8$^+$ T-cell lines. CD8$^+$ T-cell line was generated from PBMC from an HLA-A2$^+$ TB patient (HLA-A2, A3, and DR11) that was directed against Rv1733c$_{p181-189}$ and Rv2031c$_{p96-104}$ peptides. Lines were generated using (autologous) dendritic cells (DC) as antigen presenting cell (APC) as previously described [60].

Chromium release assay. EBV-BLCL JY (HLA-A*0201, -B7, -Cw7), EBV-BLCL JY-Rv1733c-GFP (EBV-BLCL JY transduced with an expression vector encoding the *Mtb dosR* gene Rv1733c and the GFP marker [61;62] and EBV-BLCL JY-GFP (EBV-BLCL JY transduced with an empty control vector encoding GFP only) were used as target cells and incubated at 37°C for 1 hr with 0.1 μCi $Na_2{}^{51}CrO_4$ (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 or without pan HLA-class I or HLA-class II antibodies. After five hours the supernatants were harvested, and the percentage lysis was calculated as follows: [(release - spontaneous release)/(maximum release- spontaneous release)] * 100%.

Table S1A. Overlapping peptide set of *Mtb* dosR antigen Rv1733c

peptide number	dosR antigen Rv1733c aa sequence	position 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 S1B. Overlapping peptide set of *Mtb* 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 S1C. Overlapping peptide set of *Mtb* dosR antigen Rv2031c

peptide number	dosR antigen Rv2031c aa sequence	position 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 S1D. Overlapping peptide set of *Mtb* early secreted antigen Ag85B (Rv1886c)

peptide number	antigen Ag85B aa sequence	position 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

Table S2. List of *Mtb* dosR candidate HLA-A2 epitopes

Mtb dosR antigen	epitope sequence[a]	start aa	end aa	score *in silico* prediction[a]	K_D <IC50> μM[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	+	-

[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 IFN-γ response to candidate epitope: +, positive responses exclusively found in HLA-A2$^+$ (heterozygous) donors (n=11); -, positive responses found in donors with different HLA-A alleles (n=5).
[d], CD8$^+$ T-cell proliferation following stimulation with candidate epitope (% proliferating CD8$^+$ cells): +, postive responses exclusively found in HLA-A2$^+$ (heterozygous)donors. When % proliferating CD8+ cells was > [medium + 3*SD] it was considered positive.

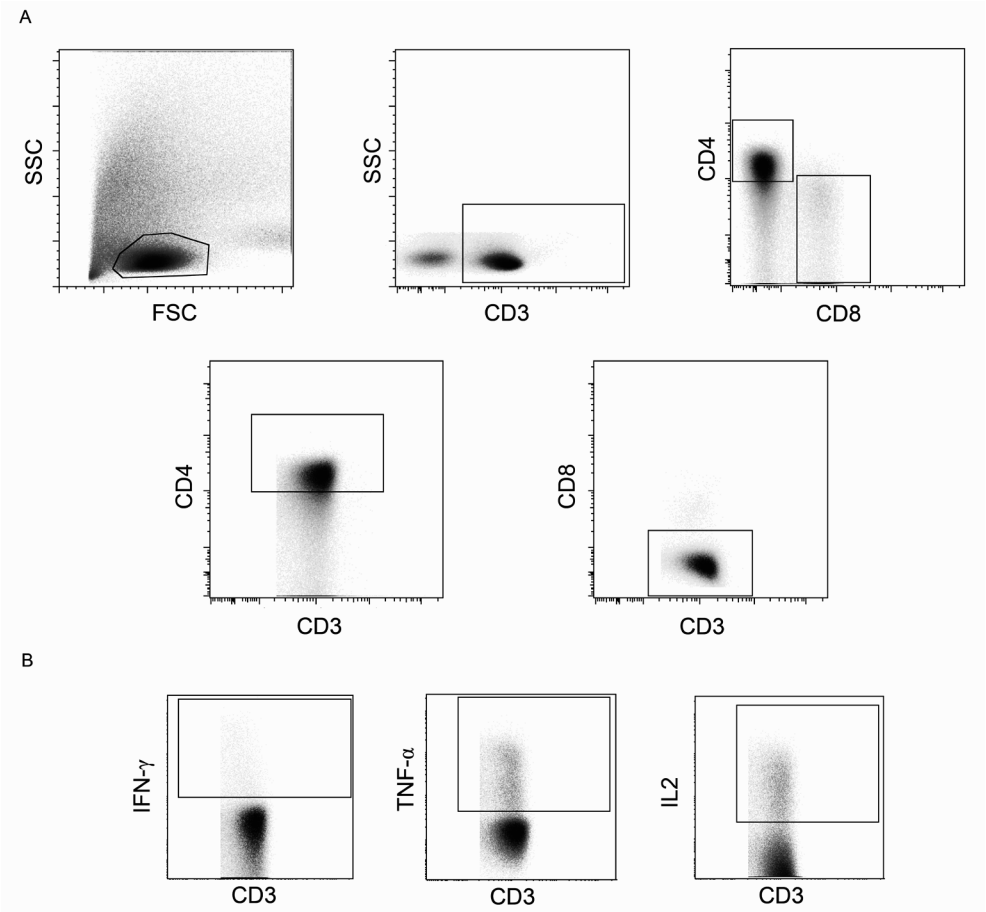


Figure S1. Multi-parameter flow cytometry analysis. The gating strategy of $CD4^+$ T cells from one donor in response to PPD as a representative of all analyzed $CD4^+$ and $CD8^+$ T-cell populations. Lymphocytes were gated using the forward scatter (FSC) and side scatter (SSC) profile. CD3 positive lymphocytes were then plotted against CD4 and CD8. $CD3^+CD4^+$ T-cell populations were plotted against CD8 to obtain the $CD3^+CD4^+CD8^-$ T-cell population ($CD4^+$ T cells) (A). The same approach was used for the selection of $CD3^+CD8^+CD4^-$ T cells ($CD8^+$ T cells). Subsequently, IFN-γ, TNF-α and IL-2 producing T cells were gated (B). Cytokine and surface marker combinations produced and expressed by single, double and polyfunctional $CD4^+$ and $CD8^+$ T cells were further analyzed using Boolean gating.

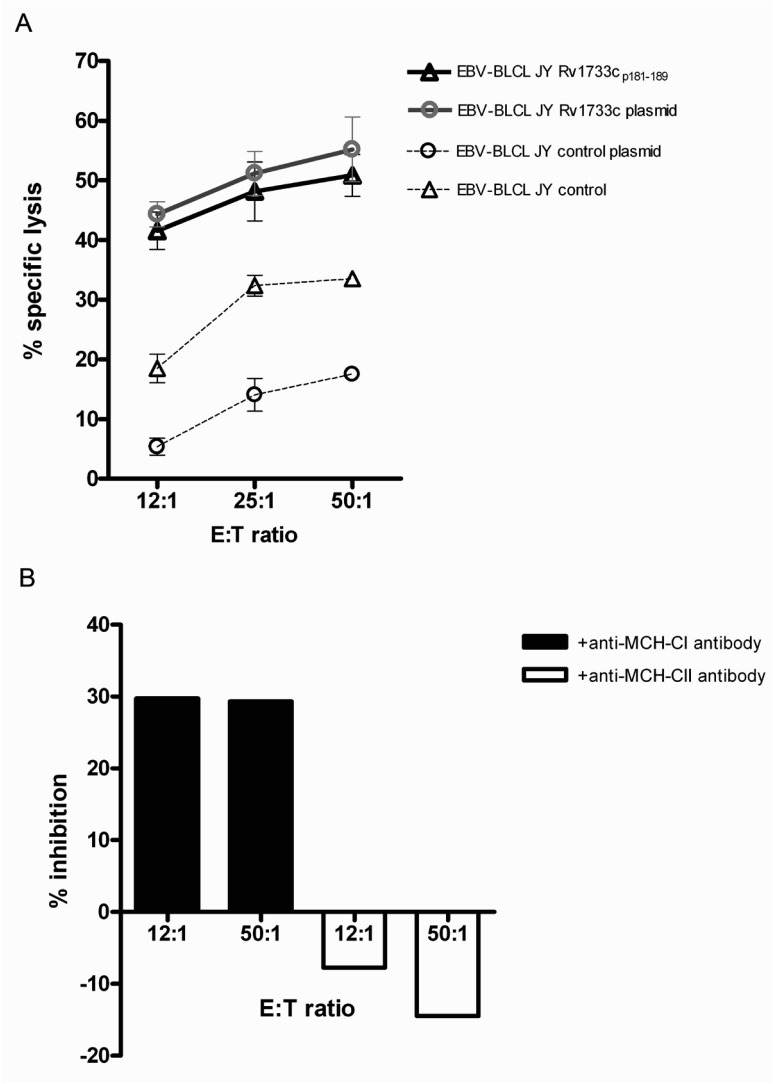


Figure S2. Cytotolytic activity of a CD8$^+$ polyclonal T-cell line against the candidate HLA-A2 restricted *Mtb* epitope Rv1733c$_{p181-189}$. The results show specific recognition of peptide epitope Rv1733c$_{p181-189}$ by CD8$^+$ T cells when peptide was loaded onto EBV-BLCL JY target cells (compared 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 not efficiently killed. Specific lysis of EBV-BLCL JY target cells loaded with Rv1733c$_{p181-189}$ could be inhibited partly by addition of anti-HLA-Class-I antibody but not by addition of anti-HLA-Class-II antibody (B). The % specific lysis was calculated as follows [(release-spontaneous release)/(maximum release-spontaneous release)] x 100%.

CHAPTER 4

An unbiased genome-wide *Mycobacterium tuberculosis* gene expression approach to discover antigens targeted by human T cells expressed during pulmonary infection

Susanna Commandeur[1], Krista E. van Meijgaarden[1], Corine Prins[1], Alexander V. Pichugin[2], Karin Dijkman[1], Annemieke H. Friggen[1], Kees L. M. C. Franken[1], Gregory Dolganov[3], Igor Kramnik[2], Gary K. Schoolnik[3], Fredrik Oftung[4], Gro Ellen Korsvold[4], Annemieke Geluk[1] and Tom H. M. Ottenhoff[1]

[1]Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands; [2] Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, USA; [3] Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, USA; [4] Department of Bacteriology and Immunology, Division of Infectious Disease Control, Norwegian Institute of Public health, Oslo, Norway

J Immunol. 2013 Feb 15;190(4):1659-71.

Abstract

Mycobacterium tuberculosis (*Mtb*) is responsible for almost 2 million deaths annually. *Mycobacterium bovis* bacillus Calmette-Guérin, the only vaccine available against tuberculosis (TB), induces highly variable protection against TB, and better TB vaccines are urgently needed. A prerequisite for candidate vaccine antigens is that they are immunogenic and expressed by *Mtb* during infection of the primary target organ, that is, the lungs of susceptible individuals. In search of new TB vaccine candidate antigens, we have used a genome-wide, unbiased antigen discovery approach to investigate the *in vivo* expression of 2170 *Mtb* genes during *Mtb* infection in the lungs of mice. Four genetically related but distinct mouse strains were studied, representing a spectrum of TB susceptibility controlled by the *supersusceptibility to TB 1* locus. We used stringent selection approaches to select *in vivo*-expressed *Mtb* (IVE-TB) genes and analyzed their expression patterns in distinct disease phenotypes such as necrosis and granuloma formation. To study the vaccine potential of these proteins, we analyzed their immunogenicity. Several *Mtb* proteins were recognized by immune cells from tuberculin skin test-positive, ESAT6/CFP10-responsive individuals, indicating that these antigens are presented during natural *Mtb* infection. Furthermore, TB patients also showed responses towards IVE-TB antigens, albeit lower than tuberculin skin test-positive, ESAT6/CFP10-responsive individuals. Finally, IVE-TB antigens induced strong IFN-γ^+/TNF-α^+ $CD8^+$ and TNF-α^+/IL-2^+ $CD154^+/CD4^+$ T-cell responses in PBMC from long-term latently *Mtb* infected individuals. In conclusion, these IVE-TB antigens are expressed during pulmonary infection *in vivo*, are immunogenic, induce strong-T cell responses in long-term latently *Mtb*-infected individuals and may therefore represent attractive antigens for new TB vaccines.

Introduction

Tuberculosis (TB) remains a leading cause of death, particularly in low and middle income countries [1]. One third of the world population is estimated to be latently infected with *Mycobacterium tuberculosis (Mtb)*, and 3-10% of these will develop active TB during their lifetime. In HIV-infected individuals this proportion increases to 7-10% per life year. The emergence of multidrug-resistant, extensively drug-resistant and more recently also totally drug-resistant *Mtb* strains is further aggravating the TB epidemic. Currently, *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) is the only available vaccine against TB. Although BCG vaccination can prevent severe childhood TB [2], it induces highly variable and inconsistent protection against pulmonary TB, the contagious form of TB in adults [3]. A more recently identified drawback of live BCG vaccination is the occurrence of disseminating BCG infections in HIV infected children [4], similar to severe BCG infections in individuals with genetic defects in the IL-12/IL-23/IFN-γ axis [5;6]. Thus, new TB vaccines are needed that are more effective and safer than BCG.

Understanding the intracellular behavior of *Mtb* during *in vivo* infection is important not only for understanding its infection biology, but it is also essential for the identification of possible novel TB vaccine candidate antigens. Infection stage and site-related differences in *in vivo Mtb* gene expression patterns can clearly affect the repertoire of potential *Mtb* antigens that is available for immune recognition in the primary infected organ, the lung. Antigens expressed in the lungs of *Mtb* infected, susceptible individuals could represent interesting new candidate antigens for TB vaccination, because they would induce responses capable of recognizing in situ *Mtb* infected cells.

Mtb has a remarkable ability to adapt to environmental changes by altering its metabolic state. A major environmental stress factor that *Mtb* is thought to encounter during host infection is the deprivation of oxygen and nutrients. *In vitro* hypoxia induces the expression of the *Mtb* dormancy regulon [7], which is controlled by the master regulator DosR *(Rv3133c)*. The expression of the *Mtb* DosR regulon is also induced by low-dose NO, carbon monoxide exposure, and during infection in IFN-γ-activated macrophages [7;8]. Previously, we have reported broad human T-cell responses to *Mtb* DosR regulon-encoded antigens, and showed that responses to these antigens were prominent and associated with latent tuberculosis infection (LTBI) in ethnically and geographically distinct populations [9-13]. Other work has shown that nutrient limitation can induce the expression of specific *Mtb* genes such as *Rv2660c* [14]. This gene was found to encode a "starvation" antigen with promising long-term vaccine efficacy in preclinical TB infection models, both in mice [15] and in nonhuman primates [16]. The more recently described enduring hypoxic response (EHR) genes represent an alternative hypoxia-induced response model, which includes most of the DosR regulon-encoded genes complemented with an additional number (> 200) of *Mtb* stress response genes [17]. This model has also helped to identify new *Mtb* antigens [18].

A limitation of the models discussed above is that the identification of differentially regulated *Mtb* genes relies on *in vitro* models supposed to recapitulate relevant environmental stress conditions

that *Mtb* encounters upon host infection. First, however, many of these environmental stress factors may not be known as yet, limiting the value of such hypothesis-driven studies. Second, there may be additive or synergistic effects between multiple stress factors *in vivo* which may easily be missed when studied in isolation *in vitro*. Thirdly, and perhaps more importantly, certain key features of host response-induced stress cannot readily be recapitulated *in vitro*, including granuloma formation and TB necrosis, both being cardinal features of TB. To overcome these limitations several laboratories have started to analyze the gene expression profiles of intracellular *Mtb,* either in infected human or murine macrophages [8;19], in the infected tissue of different mouse strains (BALB/c, SCID [20]) or in artificial granuloma mouse models [21]. However, none of these mouse models developed granulomatous necrotic TB lesions [22]. We therefore have studied *Mtb* genome-wide gene expression patterns in mice strains carrying different genotypes of the *supersusceptibility to TB 1* (*sst1*) locus. This genetic locus is located on chromosome 1 and controls the progression of *Mtb* infection to severe and necrotic lesions in a lung-specific manner: C3HeB/FeJ (C3H) mice carrying the susceptible *sst1* allele develop TB pneumonia with strong inflammatory responses with exudation throughout the lung and early onset of massive necrosis, whereas C57BL/6J (B6) mice carrying the resistant *sst1* allele develop smaller, interstitial granulomas without necrotic lesions that control bacterial multiplication. C3H.B6-*sst1* congenic mice carrying the (B6-derived) resistant *sst1* locus on the C3H background showed increased survival after *Mtb* infection compared with the susceptible C3H mice, but less prominently than did the resistant B6 mice. Finally, *Mtb*-infected B6.C3H-*sst1* mice, carrying the susceptible C3H-*sst1* locus on the B6 background, develop robust granulomas that are fenced from the healthy tissue where lesions contain foamy macrophages and develop late-onset necrosis, resembling pulmonary TB in human adults. In contrast, the B6 strain does not display this phenotype, confirming the specific role for *sst1* in the control of cell death [23].

The *sst1* locus carries 22 genes, 1 of which was highly expressed in *Mtb* infected lungs of C3H.B6-*sst1* but not of hypersusceptible C3H mice. Interestingly, the expression of this gene, termed *intracellular pathogen resistance 1* (*Ipr1*), decreased *Mtb* multiplication in susceptible macrophages and induced a switch from necrotic to apoptotic cell death [24]. The lack of *Ipr1* expression in C3H-susceptible *sst1* locus is therefore responsible for the development of lung-specific necrosis upon *Mtb* infection [25]. The closest human homologue of *Ipr1* is *SP110b*. The expression of both *Ipr1* and *SP110b* is regulated by IFNs, indicating a role in immunity [26-28]. Genetic association studies performed in West Africa identified three polymorphisms in the *SP110b* gene that were associated with genetic susceptibility to TB [29]. However, a number of other studies performed in Ghana, Russia, South Africa and Indonesia did not replicate this finding [30-33]. A *SP110b* homologue was also identified in cattle, which correlated to susceptibility to *Mycobacterium avium ssp. paratuberculosis* [34].

These four (congenic) mouse models we have studied here show a spectrum of TB susceptibility which ranges from highly susceptible (C3H) to resistant (B6) mice, with the development of

necrotic lesions depending on the *sst1* locus and the modifying genetic background in which the locus is expressed. This mouse model replicates key features of human *Mtb* infection. In this study, we have taken advantage of this disease spectrum and 1) analyzed quantitative real-time expression patterns of all *Mtb* genes predicted to be the first gene in each operon, in the lungs of *Mtb*-infected mice, aiming to identify the *Mtb* genes that are highly or differentially expressed in the lung during *in vivo* infection (*in vivo*-expressed *Mtb* (IVE-TB) genes); 2) compared these *Mtb* gene expression patterns between susceptible (B6.C3H-*sst1* and C3H) and resistant (C3H.B6-*sst1* and B6) mouse strains in an attempt to correlate expression patterns to infection phenotype, and 3) selected a set of the most consistently expressed *Mtb* genes, produced these as recombinant proteins, and analyzed their immunogenicity in tuberculin skin test $(TST)^+$ healthy, TB-affected individuals as well as long-term LTBI (ltLTBI) as a first step towards their validation as new TB vaccine candidate antigens.

Materials and methods

Mouse strains. C3HeB/FeJ (C3H) and C57BL/6J (B6), mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Congenic C3H.B6-*sst1* and B6.C3H-*sst1* mouse strains carrying the resistant and susceptible alleles of the *sst1* locus, respectively, were generated as previously described [24;35]. Briefly, an ~ 20-cM segment of chromosome 1, containing the *sst1* locus, was introgressed in the opposite background strain via > 10 backcrosses. Mice were bred and housed under specific pathogen-free conditions at the Harvard Medical School of Public Health.

Bacterial strains. *Mtb* suspensions were used as previously described [36]. In short, *Mtb (Erdman* strain; Trudeau Institute, Saranac Lake, NY) cultures were grown to midlog phase in Middlebrook 7H9 medium (BD Biosciences, Franklin Lakes, NJ) ((10% oleic acid/albumin/dextrose/catalase (OADC; Difco), 0.05% Tween 80 (Sigma-Aldrich), and 0.5% glycerol (Sigma-Aldrich)). Bacteria were washed and stored at -80 ºC. Prior to infection, bacteria were thawed, sonicated, and diluted in PBS to 10^6 CFU/ml.

Infection of mice. Mice were infected by aerosol with 25 - 50 CFU *Mtb* using a Madison chamber (College of engineering shops at the University of Wisconsin, Madison, WI) with $n = 2$ per time point [24]. B6 and C3H mice were sacrificed both 6 and 9 weeks postinfection, whereas B6.C3H-*sst1* and C3H.B6-*sst1* mice were sacrificed 9 and 6 weeks postinfection, respectively. For the reactivation model, B6 and B6.C3H-*sst1* mice were infected i.v. via the tail vein with 5×10^4 CFU *Mtb* per mouse as previously described [23].Twelve weeks after challenge the mice were given isoniazid (INH) supplied via the drinking water (10 mg/100 ml) for 90 days. Mice were sacrificed 8 weeks after INH treatment withdrawal.

Genome-Wide* Mtb *transcription profiling via a Two-Step Multiplex Real-Time RT-PCR. Quantification of *Mtb* mRNA gene expression was performed as previously described [15;37;38]. The protocol is based on first-strand cDNA synthesis and controlled multiplex amplification of cDNAs, which is followed by individual real-time PCR (Taqman) quantification of Amplified cDNAs in a 384-well format using a LightCycler 480.

Total *Mtb* RNA was isolated from the infected mouse lung tissue by homogenization in TRIzol and bacillary disruption by bead beating (MP Biomedicals, Solon, OH). Total RNA was isolated using RNeasy columns (Qiagen, Valencia, CA). RNA was precipitated, cleaned with two consecutive off-column RQ1 DNase digestions (Promega, Madison, WI) and resuspended in 50 µl of RNase-free water (Applied Biosystems/Ambion, Austin, TX).

cDNA synthesis was performed using 50 ng total RNA, which was separated in reverse transcriptase $(RT)^+$ and RT^- reactions to control for DNA contamination. Exo-resistant random primer (0.5 µl), 1 µl 10 mM dNTPs, and nuclease-free water was added and incubated for 3 minutes at 70 °C in a thermal cycler. Subsequently, 4 µl 5X Maxima RT Buffer, 0.5 µl RiboLock RNase inhibitor, 0.5 µl Maxima RT enzyme (replaced by water for RT^- control samples) (all Fermentas, Gen Burnie, MD), and nuclease-free water were added and incubated at 50 °C for 1 hour, 95 °C for 2 minutes to inactivate, and then kept at 4 °C.

The generated cDNAs were further amplified via controlled multiplex preamplification with a mix of 2179 *Mtb* gene-specific primers (23.8 µl primer mix: ~50 nM per amplification reaction), 2 µl cDNA, 3 µl 10X Advantage 2 buffer (Clontech, Mountain View, CA), 0.6 µl 1X Advantage 2 Polymerase Mix (Clontech, CA), and 0.6 µl 10 mM dNTPs (Fermentas) to a volume of 30 µl (ftp://smd-ftp.stanford.edu/tbdb/rtpcr/taqman_oligos.fa) [15]. Sequences and design of PCR primer/probe sets are available at http://genes.stanford.edu/technology.php and http://www.tbdb.org/rtpcrData.shtml. A comparative control of 100 pg (2×10^4 gene copy number) genomic H37Rv DNA was also included. As an additional control, 25 *Mtb* reference genes were used to control for variation across amplification mixes. The gene primer sets were designed using Primer Express software (Perkin Elmer, Foster City, CA) to cover at least one gene of each predicted *Mtb* operon. Each reaction was heated at 95 °C for 5 minutes, followed by 15 cycles with 95 °C for 30 seconds, 60 °C for 20 seconds, and 68 °C for 1 minute. Previously we have validated conditions for multiplex PCR preamplification via linearity of amplification assay using all the genes used in the assay. We also validated all individual TaqMan assays from our collection for sensitivity and linearity before we started using them in gene expression profiling in this study.

Individual gene transcript quantification was carried out using TaqMan primer/probe sets (Biosearch Technologies). Quantitative real-time PCR mix contained 0.07 µl preamplified cDNA, 2 µl TaqMan primer/probe mix, 5 µl 2X LightCycler 480 Probes Master Mix, and 2.93 µl Probes Master PCR-grade water (Roche) to a final volume of 10 µl. Reactions were heated at 95 °C for 5 minutes, followed by 40 cycles at 95 °C for 30 seconds and 60 °C for 20 seconds. A cool down step of 40 °C for 30 seconds was run for one cycle. Cycle Threshold values were converted to relative

gene copy numbers (RGCN) based on logarithmic transformation/linear regression equations devised from calibration curves. The dataset is available at http://www.tbdb.org/pubdata/tbdb/publications/Raw-Data-Harvard-Mice.xls.

Correction for some biological heterogeneity between the different mice and mouse strains such as differences in bacterial load was not possible because these were inherent to the extensive differences in genetic TB susceptibility.

In vivo *expressed* Mtb *(IVE-TB) gene selection procedure.* First, genes were selected that were expressed in one data set (>1000 RGCN) but not in the other data set (+/-), thus selecting *Mtb* genes that are differentially expressed owing to genetic host susceptibility and/or infection phenotype variations.

The second approach was to select genes that were highly expressed in both data sets from two different mouse strains in the chosen comparison (+/+), selecting for *Mtb* genes that are expressed independent of the genetic makeup of the host. For this selection, the RGCN data were ranked from the highest to the lowest value and overlapping genes were selected from the top 100 highest expressed genes of both data sets. This number of 100 genes was arbitrarily chosen to limit the number of candidates to be analyzed further.

The third and last approach included genes that were expressed in data set 2 (>1000 RGCN) but not in data set 1 (-/+), following the same rationale as approach 1. Hence, approach 1 and 3 included differentially expressed genes. There was no number restriction limit (because fewer genes were identified compared with the second approach (+/+)) (Figure 1 and 2).

Recombinant proteins. Recombinant proteins were produced from the selected *Mtb* genes as described previously [39]. Briefly, *Mtb* genes were amplified by PCR from genomic H37Rv DNA and cloned by Gateway technology (Invitrogen, Carlsbad, CA) in a bacterial expression vector containing a histidine tag at the N-terminus. Vectors were overexpressed in *Escherichia coli* BL21 (DE3) and purified. Size and purity of recombinant proteins were analyzed by gel electrophoresis and Western blotting with an anti-His Ab (Invitrogen) and an anti-*E. coli* polyclonal Ab (gift of Statens Serum Institute, Copenhagen, Denmark). Rv2380c, Rv2435c and Rv2737c proteins were prepared as two or three recombinant protein fragments owing to their large sizes (C, Middle (M), and N termini). Endotoxin contents were < 50 IU/ mg as tested using a *Limulus* Amebocyte Lysate assay (Cambrex, East Rutherford, NJ). All recombinant proteins were tested in lymphocyte stimulation assays to exclude antigen nonspecific T-cell stimulation and cellular toxicity using PBMC of *in vitro*-purified protein derivative of *Mtb*-negative healthy Dutch donors (Sanquin Blood Bank, Leiden, The Netherlands) [12;40-42]. Purified protein derivative of *Mtb* was purchased from Statens Serum Institute.

Mtb *lysate*. *Mtb* H37Rv organisms were grown to a late log phase in a culture flask at 37 °C and collected in a V-bottom shaped tube. The pellet was washed twice with PBS and heat killed for 30 minutes at 80 °C. The cell suspension was subsequently collected in a BioSpec tube containing 0.1-mm glass beads. The bacteria were disrupted using a Mini-BeadBeater (BioSpec Products). Concentrations of lysates were measured using the BCA assay (Thermo scientific Pierce).

Study subjects. One hundred and thirty-three donors were selected that responded to *Mtb*-purified protein derivative by TST (weak positive 5-11 mm (n = 26); positive ≥ 12 (n = 90); not determined (n = 17), ranging from 6 to 32 mm (average 16 mm)), and that had documented exposure to a TB index case (n = 63) and/or had traveled to high endemic countries (n = 76). TST^+ individuals entered a follow-up study and at recruitment a QuantiFERON-TB Gold In-Tube test (QFT-GIT) was performed (Cellestis, Carnegie, VIC, Australia). The test was considered positive when there were ≥ 0.3 IU/ml. Blood samples were collected by venipuncture and PBMC isolated by standard Ficoll density gradient centrifugation using Leucosep® tubes (Greiner Bio-one, Alphen aan den Rijn, The Netherlands). *Mtb*-unexposed donors were included as healthy control (HC) donors (n = 11). PBMC from TST^+ donors and treated TB patients (n = 7) were used in lymphocyte stimulation assays. PBMC from ltLTBI [41;42] (n = 6) were used for polychromatic flow cytometry assays. Informed consent was obtained prior to venipuncture. The study protocols (P07.048 and P207/99) were approved by the Institutional Review Board of the Leiden University Medical Center and the Regional Committees for Medical and Health Research Ethics in Norway.

Whole blood assay (WBA). Blood was diluted 1:10 in AIM-V medium (Invitrogen, Breda, The Netherlands), incubated in 48-wells plates (Nunc, Roskilde, Denmark) and cultured with or without recombinant protein (10 µg/ml), PHA (2 µg/ml), or *Mtb* lysate (5 µg/ml) at 37°C, 5% CO_2. After 6 days supernatant was harvested and stored at -20 °C for use at a later stage.

Lymphocyte stimulation test. PBMC (1.5 × 10^5/well) were cultured in triplicate in 96-well round-bottom plates (Nunc) and incubated with or without protein (10 µg/ml) in IMDM (Life Technologies/Invitrogen) containing 10% pooled human serum (Invitrogen) at 37 °C in 5% CO_2. After 6 days, supernatants were harvested, pooled, and stored at -20 °C for future use in IFN-γ ELISAs.

IFN-γ ELISA. IFN-γ concentrations in supernatants were measured with a standard ELISA technique (U-CyTech, Utrecht, The Netherlands). ELISA samples were tested in duplicate and the assay was performed according to the manufacturer's guidelines. Detection limit of the assay was set arbitrary at 20 pg/ml for whole blood assay (WBA) and 100 pg/ml for a lymphocyte stimulation test.

Flow cytometric analysis. PBMC (1-2 × 10^6 per tube) were thawed and rested overnight and subsequently stimulated for 16 h with protein (10 µg/ml) in the presence of costimulatory Abs anti-CD28 and anti-CD49d (Sanquin and BD Biosciences, respectively). Brefeldin A (3 µg/ml; Sigma-Aldrich) was added after the first 4-6 h. Cells were stained with Live/Dead fixable violet dead cell stain (ViViD; Invitrogen) to discriminate between live and dead cells according to manufacturer's instructions. Cells were stained for 1 h at 4 °C with the following surface markers: anti-CD3 PE-Cy5 (BD Biosciences), anti-CD4 Texas Red (Caltag) and anti-CD8 V500 (BD Biosciences). Additionally, anti-CD14 Pacific Blue and anti-CD19 Pacific Blue (both Invitrogen) were included to select for $CD14^-$ and $CD19^-$ live cells. Intracellular staining was performed with anti-IFN-γ Alexa 700 (BD Pharmingen), anti-TNF-α PE-Cy7 (BD Biosciences), anti-IL-2 PE (BD Pharmingen), and CD154 APC-Alexa 780 (eBioscience) using the ADG Fix&Perm kit (An Der Grub Bio Research, Vienna, Austria). Data were acquired on a BD LSRFortessa (BD Biosciences) and analyzed using FlowJo version 7.6.5 (Tree Star, Ashland, OR). Single $CD14^-/CD19^-/CD3^+$ live cells were gated to analyze $CD4^+$ and $CD8^+$ cytokine responses. Final antigen-specific $CD4^+$ and $CD8^+$ T-cell subset populations all contained at least 200 events. For comparative purposes, medium background values were subtracted for each response in each donor.

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 5.1). A Mann-Whitney *U* test was used to analyze 1) the difference between cumulative IFN-γ responses for the ESAT6/CFP10 hybrid $(E/C)^+$, E/C^- and HC individuals and 2) the difference between PHA-induced IFN-γ responses measured in E/C^+ and E/C^- donors. A p value ≤ 0.05 was considered significant.

Results

Identification of IVE-TB genes in the lungs of genetically resistant and/or susceptible mice

To start identifying novel candidate *Mtb* antigens in an unbiased and *Mtb* genome-wide fashion, we analyzed the gene expression patterns of 2170 *Mtb* genes, most of which represent the first gene of each predicted *Mtb* operon, in the lungs of four different *Mtb* infected mouse strains (B6, C3H, C3H.B6-*sst1* and B6.C3H-*sst1*) that show a spectrum of TB susceptibility (Table I). The RGCN were determined using quantitative PCR [15]. This allows for absolute quantization of the level of transcripts per sample, because the data are normalized against a standard reference gene number copy (as described in *Materials and Methods*).

Table I. Mouse strains, genetic background and TB infection phenotypes

Mouse strain[a]	Genetic background	*Sst1* allele	Inflammation	Lung necrosis	Clinical TB correlate
C3H	C3H	Susceptible	+++	+ (early)	Caseous pneumonia
C3H.B6-*sst1*	C3H	Resistant	+++	-	Progressive interstitial granuloma without necrosis
B6.C3H-*sst1*	B6	Susceptible	++	+ (late)	Caseous granuloma
B6	B6	Resistant	+	-	Chronic persistent interstitial granuloma without necrosis

+, ++, and +++ indicate intensity of inflammation
[a] Pichugin *et al.* (23) and Pan *et al.* (24)

In this IVE-TB gene selection screen we used the following criteria to select candidate genes for further analysis: first, we used the most strongly upregulated *Mtb* genes in all four analyzed mouse models. This group of genes includes genes that are expressed independently of the host susceptibility background. Second, we used *Mtb* genes specifically upregulated in either B6, C3H, B6.C3H-*sst1* or C3H.B6-*sst1*. The expression of these genes is influenced by the host genetic background and may therefore include genes whose expression is associated to particular TB disease characteristics such as granuloma formation and necrosis. The obtained RGCN of all four mouse models were independently compared with each other as visualized in Figure 1A. All four mouse models were infected with a low-dose aerosol *Mtb* inoculum. Additionally, a subset of B6.C3H-*sst1* was infected using a previously described TB relapse model to study features of gene expression during *Mtb* reactivation [23].

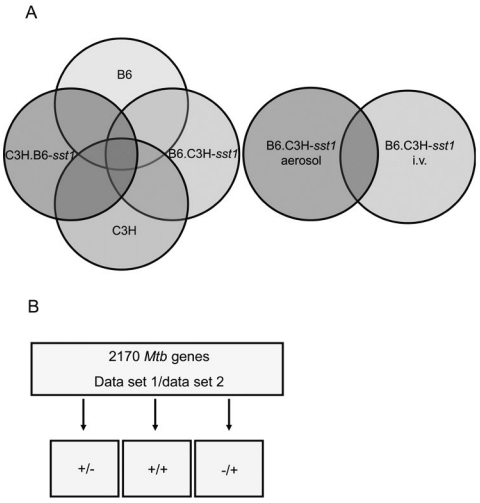


Figure 1. Overview of IVE-TB gene selection procedure. (A) The *Mtb* RGCN profiles of each mouse model were independently compared with each other. (B) Three gene selection procedures were used to select genes for each comparison: genes that were expressed in data set 1 (>1000 RGCN) but not in data set 2 (+/-); genes highly expressed in both data sets (top 100 highest expressed genes in both models and select overlapping genes) (+/+); and genes not expressed in data set 1 but expressed in data set 2 (>1000 RGCN) (-/+)(see also *Materials and Methods*).

Every individual *Mtb* gene's expression data (e.g., B6 versus C3H, B6 versus B6.C3H-*sst1*) was subjected to three different selection approaches as indicated in Figure 1B and described in detail in *Materials and Methods*. The gene selection results for each comparison are visualized in Figure 2 and Supplementary Table I (available online at http://www.jimmunol.org/content/190/4/1659/suppl/DC1). The results shown in Figure 2 were then used to select IVE-TB genes whose expression was associated with particular disease characteristics as indicated in Figure 3. These included 1) *Mtb* genes highly expressed independently of host genetic background (expressed in all four mouse models; these *Mtb* genes included *esxA* encoding ESAT6 and other esx genes); 2) *Mtb* genes expressed in association with necrosis (expressed in both C3H and B6.C3H-*sst1*, but not in B6 or C3H.B6-*sst1*); 3) *Mtb* genes expressed in association with severe necrotic infection (sni) or susceptibility (expressed in C3H, but not in B6, C3H.B6-*sst1* or B6.C3H-*sst1*); 4) *Mtb* genes expressed in association with dense granuloma development (only expressed in B6.C3H-*sst1* but not in C3H, B6 or C3H.B6-*sst1*); 5) *Mtb* genes expressed in association with diffuse granuloma development (expressed in C3H but not in B6.C3H-*sst1*, B6 or C3H.B6-*sst1*); 6) *Mtb* genes expressed in association with resistance (expressed in every more resistant mouse strain per comparison); 7) *Mtb* genes expressed in association with low inflammation (expressed in B6, but not in C3H.B6-*sst1*); 8) inflammation (expressed in C3H.B6-*sst1*, but not in B6) and 9) relapse (expressed in i.v. *Mtb* infected, INH treated B6.C3H-*sst1*, but not low-dose aerosol-infected B6.C3H-*sst1*). An overview of the resulting IVE-TB genes is presented in Supplementary Table II (available online at http://www.jimmunol.org/content/190/4/1659/suppl/DC1).

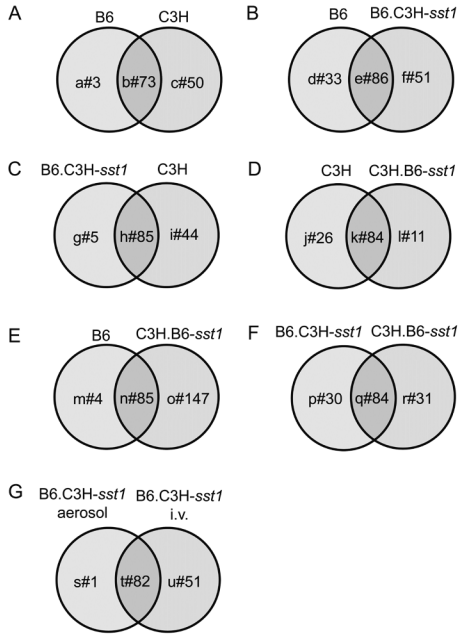


Figure 2. Selection of IVE-TB genes. Numbers of *Mtb* genes obtained after each comparison of two different mouse strains for every approach described in Figure 1 are shown in (A)-(G).

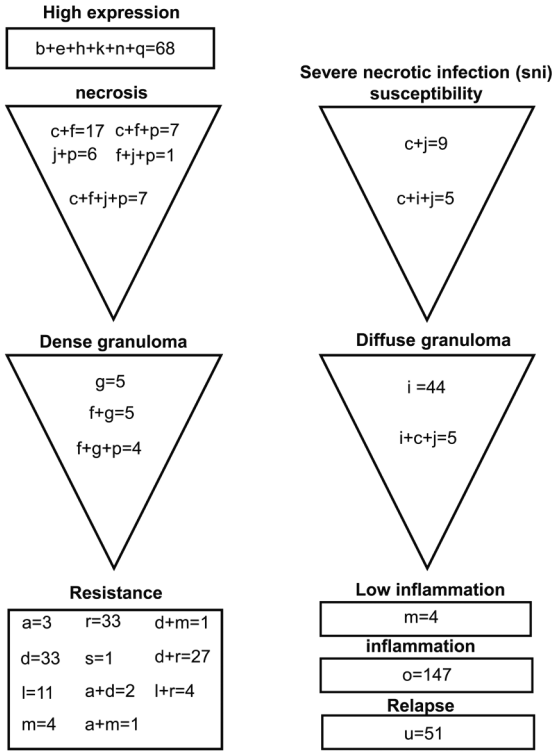


Figure 3. Selection of IVE-TB genes associated with particular TB disease characteristics. Flowchart of analysis to identify IVE-TB genes related to TB disease phenotypes is shown. Letters in graphs refer to the specific selections indicated in Figure 2. *Mtb* genes highly expressed independent of host genetic background are presented in Figure 2A(b), 2B(e), 2C(h), 2D(k), 2E(n) and 2F(q). *Mtb* genes highly expressed in association with necrosis are presented in; Figure 2A(c), 2B(f), 2D(j), and/or 2F(p). *Mtb* genes highly expressed in association with severe necrotic infection or susceptibility are presented in; Figure 2A(c), 2D(j) and/or Figure 2C(i). *Mtb* genes highly expressed in association with dense granuloma are presented in Figure 2C(g), Figure 2B(f) and/or Figure 2F(p). *Mtb* genes highly expressed in association with diffuse granuloma are presented in Figure 2C(i), but also 2A(c) and 2D(j). *Mtb* genes highly expressed in association with resistance are presented in one or more of the following selections: Figure 2A(a), 2B(d), 2D(l), 2E(m) (although not related to the susceptible *sst1* locus), 2F(r) and/or 2G(s). *Mtb* genes highly expressed in association with low inflammation are presented in Figure 2E(m). *Mtb* genes highly expressed in association with inflammation are presented in Figure 2E(o). *Mtb* genes highly expressed in association with relapse are presented in Figure 2G(u).

Table II. Predicted function and classification of selected IVE-TB genes

Gene	Name	IVE-TB selection [a]	Selections [a]	function [b]	Category [c]	classification	Reference
Rv1284	Rv1284	high expression	b + e + h + k + n + q + t	Conserved hypothetical protein (carbonic anhydrase)	7	EHR/starvation	14,17,58
Rv2380c	mbtE	high expression	b + e + h + k + n + q + t	Peptide synthetase mbtE	1		21
Rv3515c	fadD19	high expressed sst1[s]	h + q + t	Pprobable fatty acid-CoA ligase fadD19 (involved in lipid degradation)	1	EHR	17,21
Rv0079	Rv0079	necrosis and sni	c + f + j + p	Hypothetical protein	10	dosR	7,9,12,20,21
Rv2324	Rv2324	sni and relapse	c + j + u	Probable transcriptional regulator, asnC-family	9	EHR	17
Rv2737c	recA	necrosis and sni	c + f + j + p	Recombination protein recombinase A (recA; Mtb recA intein)	2		
Rv2838c	rbfA	sni, diffuse granuloma, and relapse	c + I + j + u	Probable ribosome-binding factor A (P15B protein)	2		
Rv3420c	rimI	sni, diffuse granuloma, and relapse	c + I + j + u	Ribosomal-protein-alanine acetyltransferase rimI	2		
Rv2034	Rv2034	inflammation	o	arsR-type repressor protein	9	EHR/starvation	14,17,20
Rv1956	Rv1956	resistance, diffuse granuloma, low inflammation, and relapse	d + I + j + m + u	Transcriptional regulator (possible antitoxin; TA operon with Rv1955)	0	EHR/starvation	14,17,21
Rv2225	panB	dense granuloma	f + g + p	Probable 3-methyl-2-oxobutanoate hydroxymethyltransferase (panB)	7		21
Rv2465c	rpiB	dense granuloma	f + g + p	Isomerase (ribose 5-phosphate isomerase)	7	EHR	17,2
Rv2982c	gpsA	resistance, inflammation, and relapse	l + o + r + u	Probable glycerol-3-phosphate dehydrogenase (gpdA2)	1		
Rv3353c	Rv3353c	relapse	u	Conserved hypothetical protein	10		
Rv1363c	Rv1363c	resistance	s	Possible membrane protein	3		
Rv2435c	Rv2435c	resistance and inflammation	l + o	Probable cyclase (adenylate or guanylate cyclase)	7		

[a] In reference to Figure 2 and 3

[b] Available at: http://genolist.pasteur.fr/TubercuList/ and www.tbdb.org

[c] TubercuList functional classification codes are available at http://genolist.pasteur.fr/tuberculistsni, Severe Necrotic Infection

Immunogenicity of newly identified IVE-TB antigens

Further down selection of IVE-TB genes. The goal of the above selection of IVE-TB genes was to identify potentially interesting new vaccine candidate antigens. Thus, we next determined their immunogenicity. To this end, a number of *Mtb* genes were further selected that were either present in more than one group or were among the top number of genes in the IVE-TB selections performed (Table II). Some *Mtb* genes identified using our unbiased genome-wide approach were also identified in previous studies as environmental stress induced proteins [7;14;17].

A subsequent literature search revealed that almost all further selected IVE-TB proteins (14 of the 16) have been identified previously in *Mtb* proteomic studies, confirming the protein expression of the *Mtb* genes identified in our study [43-56] (Table III). Indeed, we observed that *Mtb*-infected C3H mice recognized most of the selected IVE-TB antigens as measured by antigen-specific IFN-γ production by splenocytes (Supplementary Figure 1). Furthermore, we analyzed the conservation of these IVE-TB proteins using protein BLAST searches on different *Mtb* strains as well as other mycobacterial species. This showed that the *Mtb* IVE-TB protein sequences are strongly conserved among all tested *Mtb, M. bovis* and *M. africanum* strains. Strong conservation was observed also for other mycobacterial strains (Table IV).

Table III. Identification of IVE-TB proteins in *Mtb*

Gene	Protein Name	Protein Identification	Protein Location				Essential *In Vitro*	Essential *In Vivo*
			Membrane/ Lipid Associated	Cytosol	CF	WCL		
Rv0079	Rv0079	48, 50, 51	x				no (52)	yes (56)
Rv1284	Rv1284	44-47, 50	x		x	x	yes (52)	yes (53)
Rv1363c	Rv1363c	44, 45	x			x	no (52)	
Rv1956	Rv1956	45				x	no (52, 54)	
Rv2034	Rv2034	44, 45	x				no (52)	
Rv2225	panB	45, 51	x			x	yes (52)	
Rv2324	Rv2324	44, 45	x			x	no (52)	
Rv2380c	mbtE	50, 51	x	x			probably yes (54)	
Rv2435c	Rv2435c						no (52)	
Rv2465c	rpiB	43-47	x		x	x	no (52)	
Rv2737c	recA	51		x			slow grow mutant (52)	
Rv2838c	rbfA	45				x	NT	
Rv2982c	gpsA	44, 45, 49, 50	x			x	no (52)	
Rv3353c	Rv3353c						no (52)	
Rv3420c	rimI	51	x				no (52)	
Rv3515c	fadD19	45, 48, 49	x			x	no (52, 54) yes (55)	

Note that locations of the proteins indicated may not be exclusive given limitations and difficulties in annotating exact protein localization (45)
CF, culture filtrate; NT, not tested; WCL, whole cell lysate

Table IV. IVE-TB protein sequence identity among mycobacterial strains

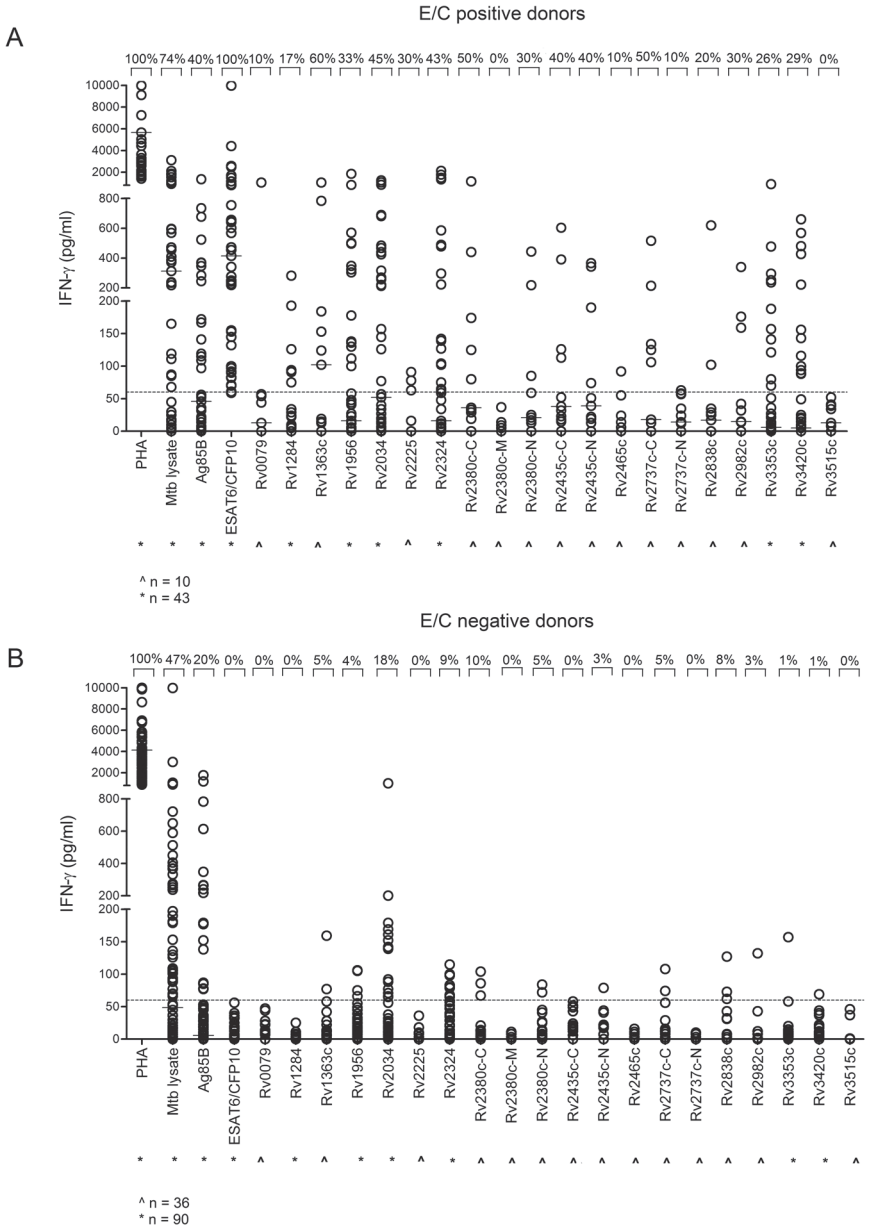
Mycobacterial species	Strain	Rv 0079	Rv 1284	Rv 1363c	Rv 1956	Rv 2034	Rv 2225	Rv 2324	Rv 2380c	Rv 2435c	Rv 2465c	Rv 2737c	Rv 2838c	Rv 2982c	Rv 3353c	Rv 3420c	Rv 3515c
tuberculosis	Erdmann	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
tuberculosis	H37Rv	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
tuberculosis	C	100	100	100	100	99	100	100	100	100	100	99	100	99	100	100	94
tuberculosis	Haarlem	100	100	100	100	99	ND	100	100	100	100	100	100	99	100	100	99
tuberculosis	F11	100	100	100	100	99	100	100	100	100	100	100	100	99	100	100	100
tuberculosis	CDC1551	100	100	100	100	99	100	100	100	100	100	100	100	99	100	100	100
africanum	GM041182	100	100	100	100	100	100	100	100	99	100	99	100	99	100	100	99
bovis	AF2122/97	100	100	99	100	100	100	100	100	100	100	100	100	99	100	100	100
bovis	BCG	100	100	100	100	100	100	100	100	100	100	100	100	99	100	100	100
avium	paratuberculosis K-10	ND	80	74	ND	41	88	91	65	65	91	96	77	84	50	82	90
avium	104	ND	80	74	ND	41	87	89	65	65	91	96	77	40	51	82	90
ulcerans	Agy99	62	85	70	ND	39	86	83	43	60	90	96	81	86	65	82	90
smegmatis	Mc^2 155	33	79	58	ND	39	78	81	66	59	84	93	76	75	ND	67	83
marinum	M	63	85	72	ND	39	86	84	43	81	91	96	81	87	64	82	90
leprae	TN	ND	ND	ND	ND	ND	82	ND	ND	32	89	61	80	77	ND	72	ND

Local BLAST protein sequence identities are available at: www.tbdb.org and www.ncbi.nlm.nih.gov/blast/

Recognition of IVE-TB proteins by TST⁺ positive donors. To assess the immunogenicity of the 16 selected IVE-TB proteins, recombinant proteins of the IVE-TB genes were generated and analyzed for the induction of IFN-γ responses in 133 TST^+ individuals. Approximately one third of the TST^+ individuals responded to *Mtb* specific E/C protein (32%, 61-9980 pg/ml). ESAT6 and CFP10 *Mtb*-specific proteins are frequently used as immunodiagnostic antigens to identify *Mtb* exposure [12]. One third of our population also responded to these diagnostic antigens (ESAT6, CFP10 complemented with TB7.7 (p4) peptides) using the commercial QFT-GIT. The responses in the QFT-GIT correlated very well with responses to our E/C protein measured in WBA (84%). Division of the group into WBA E/C^+ and WBA E/C^- donors showed that 74% of the E/C^+ donors responded to *Mtb* lysate (87-3112 pg/ml) and 40% to Ag85B antigen (97-735 pg/ml) (Figure 4A). Importantly, high levels of recognition of the newly identified *Mtb* proteins were seen within this population, with responses ranging from 10 to 60% of the donors. To identify the antigens that are well recognized, we first examined which ones were recognized by ≥ 25% of the E/C^+ donors. From all 20 proteins and protein fragments (derived from the 16 selected antigens) tested, 13 were recognized by ≥ 25% ($n \geq 3$ of 10 and ≥ 11 of 43) of the donors. Six of 13 antigens induced only intermediate levels of IFN-γ (60-600 pg/ml) after stimulation, whereas high levels of IFN-γ were produced after stimulation with Rv1363c (102-1053 pg/ml), Rv1956 (100-1861 pg/ml), Rv2034 (77-1256 pg/ml), Rv2324 (63-2130 pg/ml), Rv2380c-C (80-1159 pg/ml), Rv3353c (70-899 pg/ml) and Rv3420c (88-660 pg/ml), representing the seven best recognized proteins.

Of the TST^+ E/C^- donors still 47% responded to *Mtb* lysate and 20% to Ag85B (Figure 4B). Additionally, HC were analyzed for possible IVE-TB responses (Figure 4C). Most importantly, limited to no responses against the IVE-TB proteins were detected in the TST^+ E/C^- and HC groups, demonstrating clear specificity of recognition, presumably linked to *Mtb* exposure.

To further verify that the IVE-TB antigens are specifically recognized by E/C^+ donors, the cumulative IFN-γ response to the 20 tested antigens per individual was calculated, as described before [9]. A highly significant difference between the E/C^+ and E/C^- population was observed ($p < 0.0001$). As expected, a significant difference was also observed between E/C^+ and HC donors ($p = 0.049$) (Figure 5), confirming the association between antigen recognition and E/C test positivity. Interestingly, of the seven best recognized proteins, Rv3420c was the most discriminatory between the E/C^+ (≥25%) and E/C^- group (≤ 1%) ($p < 0.0001$) and was not recognized by HC donors ($p = 0.016$), suggesting a possible role as *Mtb*-specific biomarker antigen in addition to ESAT6, CFP10, and TB7.7.


A
E/C positive donors
100% 74% 40% 100% 10% 17% 60% 33% 45% 30% 43% 50% 0% 30% 40% 40% 10% 50% 10% 20% 30% 26% 29% 0%
IFN-γ (pg/ml)
PHA
Mtb lysate
Ag85B
ESAT6/CFP10
Rv0079
Rv1284
Rv1363c
Rv1956
Rv2034
Rv2225
Rv2324
Rv2380c-C
Rv2380c-M
Rv2380c-N
Rv2435c-C
Rv2435c-N
Rv2465c
Rv2737c-C
Rv2737c-N
Rv2838c
Rv2982c
Rv3353c
Rv3420c
Rv3515c
^ n = 10
* n = 43
B
E/C negative donors
100% 47% 20% 0% 0% 0% 5% 4% 18% 0% 9% 10% 0% 5% 0% 3% 0% 5% 0% 8% 3% 1% 1% 0%
IFN-γ (pg/ml)
PHA
Mtb lysate
Ag85B
ESAT6/CFP10
Rv0079
Rv1284
Rv1363c
Rv1956
Rv2034
Rv2225
Rv2324
Rv2380c-C
Rv2380c-M
Rv2380c-N
Rv2435c-C
Rv2435c-N
Rv2465c
Rv2737c-C
Rv2737c-N
Rv2838c
Rv2982c
Rv3353c
Rv3420c
Rv3515c
^ n = 36
* n = 90

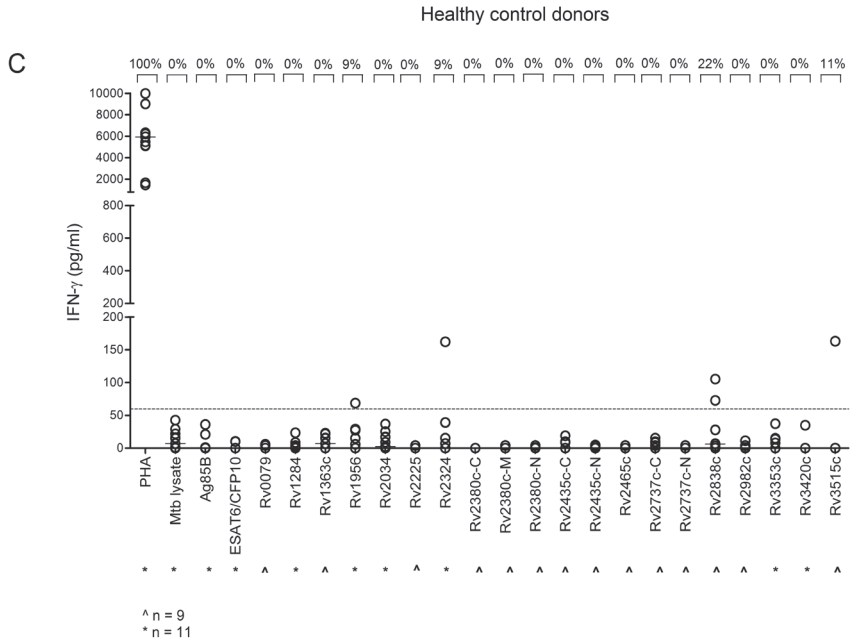


Figure 4. IFN-γ responses to IVE-TB antigens in E/C$^+$ (A), E/C$^-$ (B), and HC donors (C). A total of 43 E/C$^+$ donors (A), 90 E/C$^-$ donors (B) and 11 HC donors (C) were analyzed for their IFN-γ WBA responses to antigens and controls; the antigens were *Mtb* Rv1284, Rv1956, Rv2034, Rv2324, Rv3353c and Rv3420c. Ten E/C$^+$ donors (A), 36 E/C$^-$ donors (B) and 9 HC donors (C) were also analyzed for responses to *Mtb* antigens Rv0079, Rv1363c, Rv2225, Rv2380c, Rv2435c, Rv2465c, Rv2737c, Rv2828c, Rv2982c and Rv3515c IFN-γ. The proportion of responders for each antigen is indicated at the *top* of the graph. For comparative purposes, medium background values were subtracted for each response in each donor. Horizontal bars represent the median IFN-γ responses. The dotted line indicates the cut-off value for positivity, arbitrarily calculated as 3 x medium value.

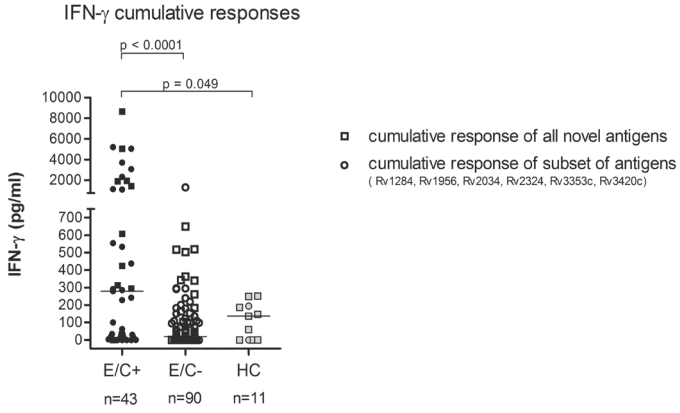


Figure 5. Cumulative IFN-γ responses induced by IVE-TB antigens, calculated per individual in the E/C$^+$, E/C$^-$ and HC groups. Cumulative IFN-γ responses to all 20 IVE-TB protein and protein fragments in E/C$^+$ (n = 43), E/C$^-$ (n = 90), and HC donors (n = 11). Squares indicate cumulative IFN-γ response of all 20 IVE-TB antigens, and circles indicate cumulative IFN-γ response of Rv1284, Rv1956, Rv2034, Rv2324, Rv3353c and Rv3420c antigens. Horizontal bars represent the median cumulative IFN-γ responses.

Recognition of IVE-TB proteins by PBMC from TB patients. We next investigated whether TB patients could also recognize these antigens. PBMC from WBA E/C$^+$ TST$^+$ donors (Figure 6A) and TB patients (Figure 6B) were therefore stimulated with the 20 proteins and protein fragments and IFN-γ production was measured. High IFN-γ responses (123-3391 pg/ml) to the IVE-TB antigens were observed in the PBMC cultures from the WBA E/C$^+$ TST$^+$ population, confirming the results obtained in the WBA assay above. Nine of the tested antigens were recognized by ≥50% of the donors and eight antigens by 38% of the donors. Only one protein fragment was not recognized in this assay (Rv2380c-M). In contrast, seven of the tested IVE-TB antigens did not induce detectable IFN-γ production in PBMC from TB patients, whereas most of the IVE-TB antigens induced only low levels of IFN-γ compared with the WBA E/C$^+$ TST$^+$ individuals (107-1825 pg/ml). Only two antigens induced high levels of IFN-γ in the TB patients. Additionally, only one antigen was recognized by 50% of the TB patients, whereas the remainder of the antigens were recognized by relatively fewer TB patients (14-43%). Thus, IVE-TB antigens seem to be less immunogenic in TB patients than in TST$^+$ individuals.

T-cell responses towards IVE-TB antigens in long-term latent Mtb *infected individuals.* Because the IVE-TB antigens were strongly recognized by TST$^+$ individuals, we subsequently analyzed the immune responses toward the seven best recognized antigens in more detail using PBMC from donors that had been exposed to *Mtb* decades ago, but had never developed TB despite the lack of any preventive treatment, designated ltLTBI [41;42]. Of additional importance, the availability of several vials of PBMC also allowed more detailed cell subset analysis.

Interestingly, high frequencies of TNF-α- and IL-2-producing CD4$^+$ T cells were observed after stimulation with the IVE-TB antigens, whereas only intermediate frequencies of IFN-γ-producing CD4$^+$ T cells were detected (Figure 7A). In contrast, high frequencies of IFN-γ-producing as well as TNF-α$^+$ CD8$^+$ T cells were present in these donors, whereas fewer IL-2$^+$ T cells were detected compared with CD4$^+$ T cells. Besides IFN-γ, TNF-α and IL-2, also the antigen-induced CD4$^+$ T-cell activation marker CD154 [57] was expressed.

More detailed analysis of the multifunctional Th1 responses among CD4$^+$ and CD8$^+$ T cells showed that CD154$^+$CD4$^+$ T cells were mostly TNF-α$^+$/IL-2$^+$ and TNF-α$^+$ (Figure 7B). Furthermore, intermediate frequencies of IFN-γ$^+$/TNF-α$^+$/IL-2$^+$ CD154$^+$CD4$^+$ T cells were detected. Finally, a CD154$^+$ population was detected, producing none of the IFN-γ, TNF-α and IL-2 cytokines. Intriguingly, the same pattern was observed for every IVE-TB antigen or E/C control antigen. Furthermore, interindividual variation of antigen recognition was observed. Remarkably, few TNF-α$^+$/IL-2$^+$ CD8$^+$ T cells were detected compared with TNF-α$^+$/IL-2$^+$ CD4$^+$ T cells. IFN-γ$^+$/TNF-α$^+$ CD8$^+$ T cells were the most prominent population present, followed by TNF-α$^+$ CD8$^+$ T cells. Also intermediate IFN-γ$^+$/TNF-α$^+$/IL-2$^+$ and IFN-γ$^+$ CD8$^+$ T cells were observed. Again, as mentioned for CD4$^+$ T cells, the same patterns were observed for every antigen within the CD8$^+$ T-cell population as well as interindividual variation of antigen recognition.

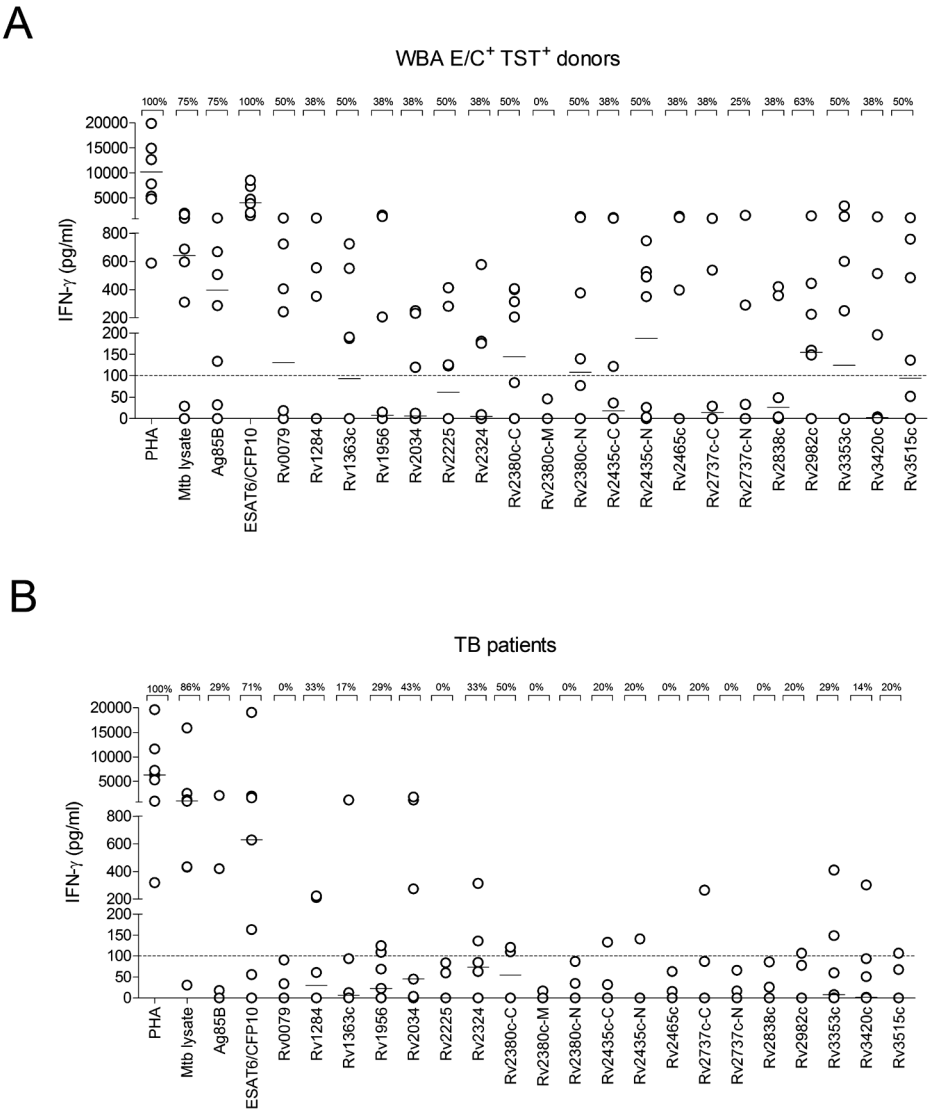


Figure 6. PBMC IFN-γ responses towards IVE-TB antigens in TB patients and WBA E/C$^+$ TST$^+$ donors. PBMC of WBA E/C$^+$ TST$^+$ donors (n = 8) (A) and TB patients (n = 7) (B) were stimulated with IVE-TB antigens and control conditions for 6 days. Levels of IFN-γ were measured and medium background values were subtracted for each response in each donor for comparative purposes. The proportion of responders for each antigen is indicated at the *top* of the graph. Horizontal bars represent the median IFN-γ responses. The dotted line indicates the cut-off value for positivity, arbitrarily set at 100 pg/ml.

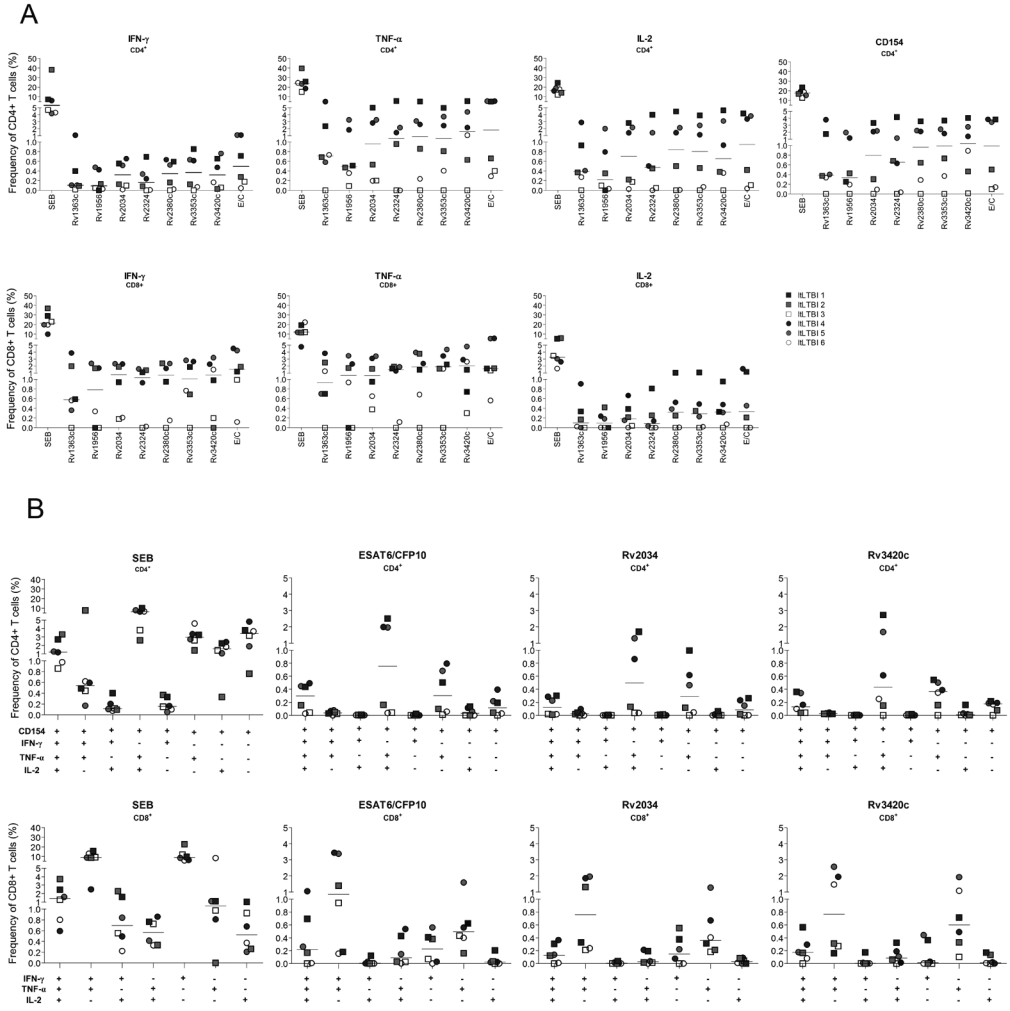


Figure 7. Polychromatic flow cytometric analysis of IVE-TB-specific T-cell responses in long-term latent *Mtb* infected individuals. PBMC from ltLTBI (n = 6) were stimulated for 16 hours with the seven best recognized antigens as determined in Figure 4. Frequencies of IFN-γ-, TNF-α-, IL-2- and CD154-producing $CD4^+$ and $CD8^+$ T cells were determined (A). Subsequently, "multifunctional" responses were determined by analyzing combinations of IFN-γ, TNF-α, IL-2 and CD154 responses for $CD4^+$ T cells and IFN-γ, TNF-α and IL-2 responses for $CD8^+$ T cells. Results for two representative IVE-TB antigens are shown (Rv2034 and Rv3420c) (B). For comparative purposes, medium background values were subtracted for each response in each donor. Horizontal bars represent the median frequency of cytokine-producing $CD4^+$ or $CD8^+$ T cells.

The integrated median fluorescence intensity (iMFI) was calculated to determine the quantitative contribution of cytokines produced by the different multiple and single cytokine producing $CD154^+/CD4^+$ and $CD8^+$ T cells (Figure 8). IFN-γ^+/TNF-α^+/IL-2^+ $CD154^+/CD4^+$ T cells had the highest iMFI, which gradually declined for double producing and single IFN-γ^+ $CD154^+/CD4^+$ T cells. IFN-γ^+/TNF-α^+ $CD8^+$ T cells contributed the most to IFN-γ production, directly followed by the IFN-γ^+/TNF-α^+/IL-2^+ $CD8^+$ T cells. IFN-γ^+/TNF-α^+ $CD8^+$ T cells are also the main contributors for TNF-α, whereas IFN-γ^+/TNF-α^+/IL-2^+ $CD8^+$ T cells showed a higher IL-2 iMFI. TNF-α and IL-2 iMFI were also the highest for TNF-α^+/IL-2^+ $CD154^+/CD4^+$ T cells, followed by the IFN-γ^+/TNF-α^+/IL-2^+ $CD154^+/CD4^+$ T cells. Thus the TNF-α^+/IL-2^+ CD4 and IFN-γ^+/TNF-α^+ CD8 T cells contribute strongly to the production of Th1 cytokines, followed by the triple positive T cells. Single cytokine producing cells only showed a relatively minor contribution.

In conclusion, seven of the identified IVE-TB antigens are strongly immunogenic, triggering specific and high cellular immune responses in E/C^+ TST^+ individuals and ltLTBI individuals, but not in E/C^- TST^+ individuals, healthy mycobacterial naive individuals, and TB patients. The strong IVE-TB responses that were measured in the ltLTBI group were identified as IFN-γ^+/TNF-α^+ $CD8^+$ T cells and TNF-α^+/IL-2^+ $CD4^+$ T cells, which were the most prominent contributors to the produced cytokines, followed by triple positive T cells.

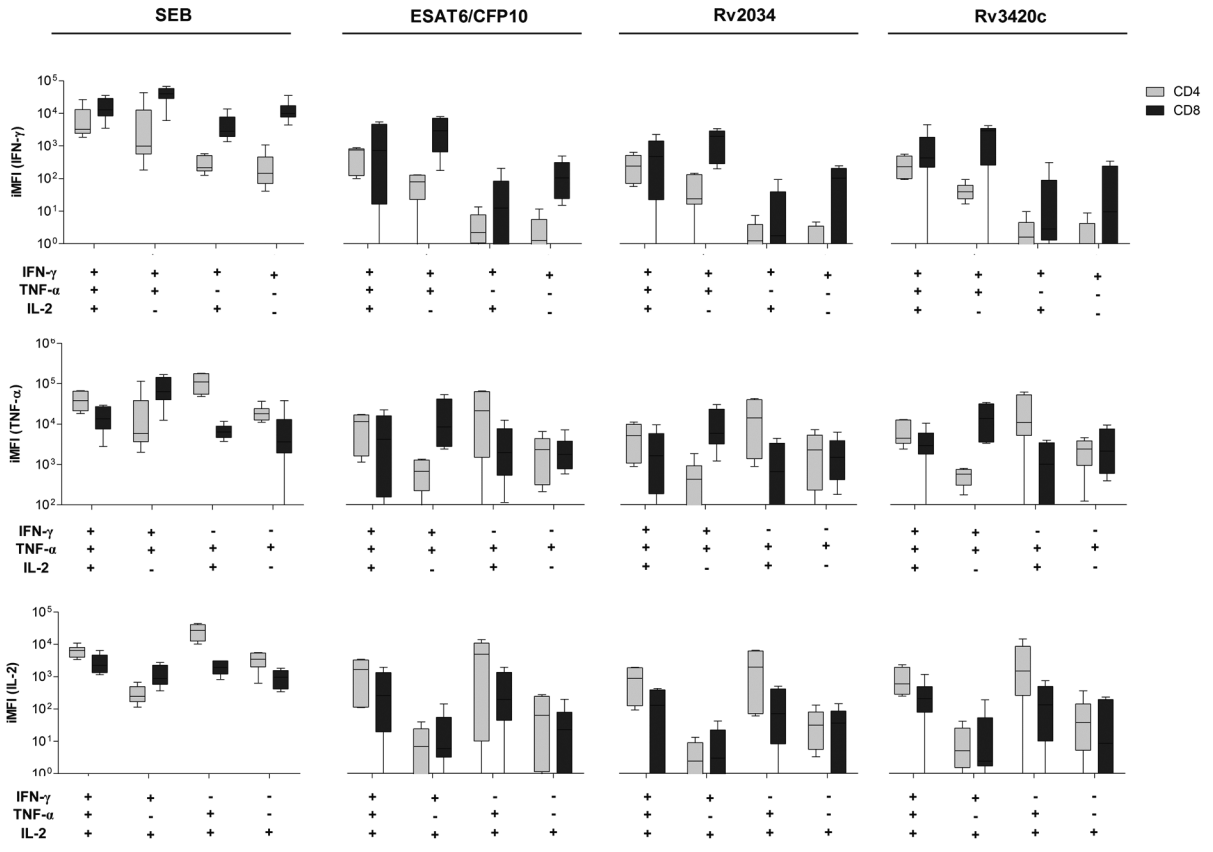


Figure 8. iMFI of IVE-TB-specific CD154$^+$CD4$^+$ and CD8$^+$ T-cell subsets in long-term latent *Mtb* infected individuals. iMFI values for IFN-γ, TNF-α and/or IL-2 were calculated via multiplication of CD154$^+$CD4$^+$ and CD8$^+$ T-cell subset frequency by their MFI. Six ltLTBI donors were analyzed. For comparative purposes, medium background iMFI values were subtracted for each response in each donor. Light grey boxes represent CD154$^+$CD4$^+$ T-cell responses and dark grey boxes CD8$^+$ T-cell responses. Lines within boxes represent the medians. The lower boundary of the box represents the 25th percentile and upper boundary the 75th percentile. Whiskers extend to the lowest and highest values.

Discussion

Using quantitative genome-wide *Mtb* transcriptional profiling we have identified a series of *Mtb* genes that are expressed during *in vivo Mtb* infection in the lungs of resistant and susceptible mice, which we term IVE-TB. Most of the genes identified have previously been found to be expressed in the *Mtb* proteome, and thus encode bona fide *Mtb* proteins. This is further supported by their immunogenicity profiles, as many of these proteins triggered IFN-γ production in human WBA and lymphocyte stimulating assays in *Mtb* ESAT6/CFP10-responsive patients, but not in ESAT6/CFP10$^-$ TST$^+$ individuals, HC donors, or TB patients. This is particularly relevant in the case of *Rv2435c* and *Rv3353c* as their protein products have not been identified yet; however IFN-γ responses were demonstrated in E/C$^+$ TST$^+$ individuals, indirectly showing that these *Mtb* proteins are presented to the human immune system during mycobacterial infection.

Many of the IVE-TB genes we identified have been described previously in relationship to the adaptive response of *Mtb* to environmental stress conditions, especially those that *Mtb* likely encounters during host infection. We identified one *Mtb* DosR regulon-encoded gene (*Rv0079*) [7] as well as six genes that are part of the EHR regulon (*Rv1284, Rv1956, Rv2034, Rv2324, Rv2465* and *Rv3515*) [17]. Three of these have also been described as starvation/nutritional stress genes (*Rv1284, Rv1956* and *Rv2034*) [14]. This function of the IVE-TB genes in responding to host-induced stress conditions during *in vivo* pulmonary infection enhances the biological plausibility of our findings and lends validity to our approach.

Of further interest, nine of the *Mtb* genes identified in this study have not been described previously in relation to *Mtb* host infection, although some of their functions have been linked to possible adaptation to *in vitro* host-induced stress conditions (Supplementary Table III). Several of these genes have a role in metal transport, metallo-regulatory transcriptional regulation, or represent metalloenzymes. Furthermore genes were identified that play a role in lipid metabolism. This is in agreement with the documented shift towards using fatty acids as an alternative carbon source instead of carbohydrates under nutrient-limiting conditions. Altogether, many of the IVE-TB genes we have identified appear to be related to the adaptation of *Mtb* to environmental stress conditions encountered in the host. Of additional importance, the identification of these genes in our *in vivo* model supports previous findings mostly obtained in *in vitro* models, by showing that they are induced during pulmonary *Mtb* infection *in vivo*. On a cautionary note, however, our data do not allow us to discriminate whether the observed differential *Mtb* gene expression patterns are cause or consequence of variations in host susceptibility (background and/or *sst1* locus).

As already mentioned, *Mtb* gene expression profiling has been performed in the past, mostly focusing on *in vitro*-cultured bacteria grown under a variety of different conditions. Subsequent work assessed *Mtb* gene expression profiles following infection of host cells [8;19;58] and some recent studies have analyzed *Mtb* gene expression patterns also *in vivo* [20;59]. Ward *et al.* [59] showed that there was little overlap in the *Mtb* genes reported to be expressed in different

studies reporting on *Mtb* intracellular infection, likely as a result of methodological differences. Nonetheless, the two studies Ward *et al.* described [8;60] indicate that similar functional categories of *Mtb* genes are expressed during intracellular infection. In line with this notion, when comparing our data to previous reports there are few overlapping individual *Mtb* genes, but we nevertheless do identify genes with previous described functional categories. These differences are probably due to differences in selection criteria, in experimental settings such as infection route, and the specific mouse models we have used, which have not been analyzed previously.

Despite these differences, several of our selected IVE-TB genes do overlap with *Mtb* genes identified in other studies as indicated in Table II. The *Mtb* gene *Rv2225* whose expression was TB granuloma associated was also significantly expressed in the artificial granuloma model of Karakousis *et al.* [21]. This strengthens their association with host granuloma formation. Our *in vivo* pulmonary TB granuloma-associated *Mtb*-expressed genes did not overlap with the granuloma associated genes or macrophage associated genes described by Ramakrishnan and colleagues [61;62] for *M. marinum*, which might be due to differences between the mycobacterial species studied. Moreover, several of the IVE-TB genes we identified to be highly expressed have also been described previously, including *Rv0467 (icl)*, which encodes an enzyme in the glyoxylate pathway which is important for *Mtb* persistence of *Mtb* [63;64], and *Rv0991c,* which is part of the so-called *in vivo* expressed genomic island [20].

The new *Mtb* antigens we have identified here may represent interesting targets for vaccination, as they are expressed during *Mtb* infection in the (genetically susceptible) lung, which we consider a critical parameter for appropriate antigen selection. Moreover, successful vaccine antigens should be conserved between multiple *Mtb* strains. All protein sequences examined were conserved among the tested *Mtb* strains. Additionally, for almost all IVE-TB genes multiple proteome studies have documented their expression as proteins in *Mtb* (Table III). A subset of the analyzed IVE-TB proteins was shown to be strongly immunogenic as judged by Th1 responses in WBA, lymphocyte stimulation assays, and polychromatic flow cytometry. Indeed, the highest IFN-γ responses were identified within the E/C^+ population of our TST^+ cohort, whereas no differences in mitogen-induced responses were seen. No responses were seen in *Mtb* nonresponder healthy individuals, suggesting that T-cell recognition of IVE-TB antigens is indeed antigen specific and is correlated with *Mtb* exposure based on TST and QFT-GIT conversions. Interestingly, TB patients showed relatively low recognition of the IVE-TB antigens, suggesting that they did not develop strong Th1 immunity against these antigens.

Importantly, IVE-TB antigen specific responses could be detected in ltLTBI, which have been exposed to *Mtb* many years ago and never developed TB symptoms despite not having had preventive treatment. The most prominent T-cell subsets with activity against IVE-TB antigens included IFN-γ^+/TNF-α^+ $CD8^+$ T cells and TNF-α^+/IL-2^+ $CD154^+CD4^+$ T cells. Thus, $CD8^+$ T cells were the major contributors of IFN-γ production. Interestingly, also a population of antigen-specific-activated $CD154^+CD4^+$ T cells was observed that did not produce IFN-γ, TNF-α or IL-

2. These cells may exert alternative functions, which could include IL-17 production, immune regulation, or yet other functions, which need further study. Finally, we previously reported multifunctional $CD4^+$ and $CD8^+$ T-cell responses towards resuscitation promoting factor and DosR proteins and showed that IFN-γ^+/TNF-α^+ $CD8^+$ T cells were also the most prominent subset in the response to these antigens, suggesting that the development of specific differential T-cell subsets may be unrelated to the nature of the specific protein antigen involved.

$CD8^+$ T cells are activated upon recognition of epitopes presented via MHC-I molecules, indicating that the antigens are present and processed via the canonical cytosolic pathway or via alternative (e.g. cross priming) pathways [65]. Both $CD4^+$ and $CD8^+$ T cells are important in *Mtb* control, and $CD4^+$ and $CD8^+$ T cell-deficient mice, for example, have increased susceptibility to *Mtb* [66]. $CD4^+$ T cells were recently shown to play an important (IFN-γ-independent) role in the indirect activation of IFN-γ^+ $CD8^+$ T cells [67]. In any case, our data obtained in the ltLTBI individuals show that the *Mtb* antigen-specific $CD4^+$ and $CD8^+$ T cells recognizing IVE-TB antigens must be long lived.

The immunogenicity of some of the IVE-TB antigens has been analyzed previously. The immunogenicity of the DosR Rv0079 protein was analyzed in TST^+ (endemic) individuals as well as (cured) TB patients [9;12;68]. In these studies Rv0079 protein was recognized by a minority of individuals, in agreement with our results in this study. The immunogenicity of EHR and starvation antigens Rv1284 and Rv1956 was previously analyzed in *M. bovis*-exposed cattle [17] [69]. Rv1284 was one of the five best recognized antigens, whereas Rv1956 was also highly recognized. In contrast to the responses observed in *M. bovis*-exposed cattle, Rv1284 was moderately recognized in our study, whereas Rv1956 was better recognized.

In conclusion, by combining *Mtb* genome-wide transcriptional profiling in the lungs of infected mice with strikingly differing host susceptibility backgrounds, we have identified *Mtb* genes that are specifically expressed in resistant or susceptible animals during pulmonary infection. These genes reveal a signature of the *Mtb* stress response *in vivo* depending on the genetic host background and host susceptibility. From these genes we selected 16 proteins, of which seven proved to be highly immunogenic in E/C^+ TST^+ donors and ltLTBI and therefore represent interesting TB vaccine candidate and possibly TB biomarker antigens [70].

Acknowledgements

This study was supported by the following organizations: the European Commission (FP7 NEWTBVAC project; FP7 VACTRAIN project) (the text represents the authors' views and does not necessarily represent a position of the Commission who will not be liable for the use made of such information); Top Institute Pharma (project D-101-1); and EDCTP (AE-TBC project). The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank Louis Wilson for the production of the *Mtb* lysates, as well as all blood donors who participated in this study.

Conflict of interest. TO is co-inventor of an *Mtb* latency antigen patent, which is owned by LUMC.

References

1. **WHO**. The global plan to stop TB 2011-2015. 2011.
2. **Trunz,B.B., Fine,P., and Dye,C.,** Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *Lancet* 2006. **367**: 1173-1180.
3. **Fine,P.E.,** Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* 1995. **346**: 1339-1345.
4. **Hesseling,A.C., Marais,B.J., Gie,R.P., Schaaf,H.S., Fine,P.E., Godfrey-Faussett,P., and Beyers,N.,** The risk of disseminated Bacille Calmette-Guerin (BCG) disease in HIV-infected children. *Vaccine* 2007. **25**: 14-18.
5. **Ottenhoff,T.H., Verreck,F.A., Lichtenauer-Kaligis,E.G., Hoeve,M.A., Sanal,O., and van Dissel,J.T.,** Genetics, cytokines and human infectious disease: lessons from weakly pathogenic mycobacteria and salmonellae. *Nat.Genet.* 2002. **32**: 97-105.
6. **van de Vosse,E., Hoeve,M.A., and Ottenhoff,T.H.,** Human genetics of intracellular infectious diseases: molecular and cellular immunity against mycobacteria and salmonellae. *Lancet Infect.Dis.* 2004. **4**: 739-749.
7. **Voskuil,M.I., Schnappinger,D., Visconti,K.C., Harrell,M.I., Dolganov,G.M., Sherman,D.R., and Schoolnik,G.K.,** Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. *J.Exp.Med.* 2003. **198**: 705-713.
8. **Schnappinger,D., Ehrt,S., Voskuil,M.I., Liu,Y., Mangan,J.A., Monahan,I.M., Dolganov,G., Efron,B., Butcher,P.D., Nathan,C., and Schoolnik,G.K.,** Transcriptional Adaptation of Mycobacterium tuberculosis within Macrophages: Insights into the Phagosomal Environment. *J.Exp.Med.* 2003. **198**: 693-704.
9. **Leyten,E.M., Lin,M.Y., Franken,K.L., Friggen,A.H., Prins,C., van Meijgaarden,K.E., Voskuil,M.I., Weldingh,K., Andersen,P., Schoolnik,G.K., Arend,S.M., Ottenhoff,T.H., and Klein,M.R.,** Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of Mycobacterium tuberculosis. *Microbes.Infect.* 2006. **8**: 2052-2060.
10. **Roupie,V., Romano,M., Zhang,L., Korf,H., Lin,M.Y., Franken,K.L., Ottenhoff,T.H., Klein,M.R., and Huygen,K.,** Immunogenicity of eight dormancy regulon-encoded proteins of Mycobacterium tuberculosis in DNA-vaccinated and tuberculosis-infected mice. *Infect.Immun.* 2007. **75**: 941-949.
11. **Schuck,S.D., Mueller,H., Kunitz,F., Neher,A., Hoffmann,H., Franken,K.L., Repsilber,D., Ottenhoff,T.H., Kaufmann,S.H., and Jacobsen,M.,** Identification of T-cell antigens specific for latent mycobacterium tuberculosis infection. *PLoS.One.* 2009. **4**: e5590.
12. **Black,G.F., Thiel,B.A., Ota,M.O., Parida,S.K., Adegbola,R., Boom,W.H., Dockrell,H.M., Franken,K.L., Friggen,A.H., Hill,P.C., Klein,M.R., Lalor,M.K., Mayanja,H., Schoolnik,G., Stanley,K., Weldingh,K., Kaufmann,S.H., Walzl,G., and Ottenhoff,T.H.,** Immunogenicity of novel DosR regulon-encoded candidate antigens of Mycobacterium tuberculosis in three high-burden populations in Africa. *Clin.Vaccine Immunol.* 2009. **16**: 1203-1212.
13. **Goletti,D., Butera,O., Vanini,V., Lauria,F.N., Lange,C., Franken,K.L., Angeletti,C., Ottenhoff,T.H., and Girardi,E.,** Response to Rv2628 latency antigen associates with cured tuberculosis and remote infection. *Eur.Respir.J.* 2010. **36**: 135-142.
14. **Betts,J.C., Lukey,P.T., Robb,L.C., McAdam,R.A., and Duncan,K.,** Evaluation of a nutrient starvation model of Mycobacterium tuberculosis per-

sistence by gene and protein expression profiling. *Mol.Microbiol.* 2002. **43**: 717-731.

15. **Aagaard,C., Hoang,T., Dietrich,J., Cardona,P.J., Izzo,A., Dolganov,G., Schoolnik,G.K., Cassidy,J.P., Billeskov,R., and Andersen,P.,** A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nat.Med.* 2011. **17**: 189-194.

16. **Lin,P.L., Dietrich,J., Tan,E., Abalos,R.M., Burgos,J., Bigbee,C., Bigbee,M., Milk,L., Gideon,H.P., Rodgers,M., Cochran,C., Guinn,K.M., Sherman,D.R., Klein,E., Janssen,C., Flynn,J.L., and Andersen,P.,** The multistage vaccine H56 boosts the effects of BCG to protect cynomolgus macaques against active tuberculosis and reactivation of latent Mycobacterium tuberculosis infection. *J.Clin.Invest* 2012. **122**: 303-314.

17. **Rustad,T.R., Harrell,M.I., Liao,R., and Sherman,D.R.,** The enduring hypoxic response of Mycobacterium tuberculosis. *PLoS.One.* 2008. **3**: e1502.

18. **Gideon,H.P., Wilkinson,K.A., Rustad,T.R., Oni,T., Guio,H., Kozak,R.A., Sherman,D.R., Meintjes,G., Behr,M.A., Vordermeier,H.M., Young,D.B., and Wilkinson,R.J.,** Hypoxia induces an immunodominant target of tuberculosis specific T cells absent from common BCG vaccines. *PLoS.Pathog* 2010. **6**: e1001237.

19. **Cappelli,G., Volpe,E., Grassi,M., Liseo,B., Colizzi,V., and Mariani,F.,** Profiling of Mycobacterium tuberculosis gene expression during human macrophage infection: upregulation of the alternative sigma factor G, a group of transcriptional regulators, and proteins with unknown function. *Res.Microbiol.* 2006. **157**: 445-455.

20. **Talaat,A.M., Lyons,R., Howard,S.T., and Johnston,S.A.,** The temporal expression profile of Mycobacterium tuberculosis infection in mice. *Proc. Natl.Acad.Sci.U.S.A* 2004. **101**: 4602-4607.

21. **Karakousis,P.C., Yoshimatsu,T., Lamichhane,G., Woolwine,S.C., Nuermberger,E.L., Grosset,J., and Bishai,W.R.,** Dormancy phenotype displayed by extracellular Mycobacterium tuberculosis within artificial granulomas in mice. *J.Exp.Med.* 2004. **200**: 647-657.

22. **Young,D.,** Animal models of tuberculosis. *Eur.J.Immunol.* 2009. **39**: 2011-2014.

23. **Pichugin,A.V., Yan,B.S., Sloutsky-,A., Kobzik,L., and Kramnik,I.,** Dominant role of the sst1 locus in pathogenesis of necrotizing lung granulomas during chronic tuberculosis infection and reactivation in genetically resistant hosts. *Am.J.Pathol.* 2009. **174**: 2190-2201.

24. **Pan,H., Yan,B.S., Rojas,M., Shebzukhov,Y.V., Zhou,H., Kobzik,L., Higgins,D.E., Daly,M.J., Bloom,B.R., and Kramnik,I.,** Ipr1 gene mediates innate immunity to tuberculosis. *Nature* 2005. **434**: 767-772.

25. **Kramnik,I.,** Genetic dissection of host resistance to Mycobacterium tuberculosis: the sst1 locus and the Ipr1 gene. *Curr.Top. Microbiol.Immunol.* 2008. **321**: 123-148.

26. **Grotzinger,T., Jensen,K., and Will,H.,** The interferon (IFN)-stimulated gene Sp100 promoter contains an IFN-gamma activation site and an imperfect IFN-stimulated response element which mediate type I IFN inducibility. *J.Biol.Chem.* 1996. **271**: 25253-25260.

27. **Kadereit,S., Gewert,D.R., Galabru,J., Hovanessian,A.G., and Meurs,E.F.,** Molecular cloning of two new interferon-induced, highly related nuclear phosphoproteins. *J.Biol.Chem.* 1993. **268**: 24432-24441.

28. **Bloch,D.B., Nakajima,A., Gulick,T., Chiche,J.D., Orth,D., de La Monte,S.M., and Bloch,K.D.,** Sp110 localizes to the PML-Sp100 nuclear body and may function as a nuclear hormone receptor transcriptional coactivator. *Mol. Cell Biol.* 2000. **20**: 6138-6146.

29. **Tosh,K., Campbell,S.J., Fielding,K., Sillah,J., Bah,B., Gustafson,P., Manneh,K., Lisse,I., Sirugo,G., Bennett,S., Aaby,P., McAdam,K.P., Bah-Sow,O., Lienhardt,C., Kramnik,I., and Hill,A.V.,** Variants in the SP110 gene are associated with genetic susceptibility to tuberculosis in West Africa. *Proc.Natl.Acad.Sci.U.S.A* 2006. **103**: 10364-10368.

30. **Thye,T., Browne,E.N., Chinbuah,M.A., Gyapong,J., Osei,I., Owusu-Dabo,E., Niemann,S., Rusch-Gerdes,S., Horstmann,R.D., and Meyer,C.G.,** No associations of human pulmonary tuberculosis with Sp110 variants. *J.Med.Genet.* 2006. **43**: e32.

31. **Szeszko,J.S., Healy,B., Stevens,H., Balabanova,Y., Drobniewski,F., Todd,J.A., and Nejentsev,S.,** Resequencing and association analysis of the SP110 gene in adult pulmonary tuberculosis. *Hum.Genet.* 2007. **121**: 155-160.

32. **Babb,C., Keet,E.H., van Helden,P.D., and Hoal,E.G.,** SP110 polymorphisms are not associated with pulmonary tuberculosis in a South African population. *Hum.Genet.* 2007. **121**: 521-522.

33. **Png,E., Alisjahbana,B., Sahiratmadja,E., Marzuki,S., Nelwan,R., Adnan,I., van,d., V, Hibberd,M., van,C.R., Ottenhoff,T.H., and Seielstad,M.,** Polymorphisms in SP110 are not associated with pulmonary tuberculosis in Indonesians. *Infect.Genet.Evol.* 2012.

34. **Ruiz-Larranaga,O., Garrido,J.M., Iriondo,M., Manzano,C., Molina,E., Montes,I., Vazquez,P., Koets,A.P., Rutten,V.P., Juste,R.A., and Estonba,A.,** SP110 as a novel susceptibility gene for Mycobacterium avium subspecies paratuberculosis infection in cattle. *J.Dairy Sci.* 2010. **93**: 5950-5958.

35. **Yan,B.S., Kirby,A., Shebzukhov,Y.V., Daly,M.J., and Kramnik,I.,** Genetic architecture of tuberculosis resistance in a mouse model of infection. *Genes Immun.* 2006. **7**: 201-210.

36. **Yan,B.S., Pichugin,A.V., Jobe,O., Helming,L., Eruslanov,E.B., Gutierrez-Pabello,J.A., Rojas,M., Shebzukhov,Y.V., Kobzik,L., and Kramnik,I.,** Progression of pulmonary tuberculosis and efficiency of bacillus Calmette-Guerin vaccination are genetically controlled via a common sst1-mediated mechanism of innate immunity. *J.Immunol.* 2007. **179**: 6919-6932.

37. **Dolganov,G.M., Woodruff,P.G., Novikov,A.A., Zhang,Y., Ferrando,R.E., Szubin,R., and Fahy,J.V.,** A novel method of gene transcript profiling in airway biopsy homogenates reveals increased expression of a Na+-K+-Cl-cotransporter (NKCC1) in asthmatic subjects. *Genome Res.* 2001. **11**: 1473-1483.

38. **Woodruff,P.G., Boushey,H.A., Dolganov,G.M., Barker,C.S., Yang,Y.H., Donnelly,S., Ellwanger,A., Sidhu,S.S., Dao-Pick,T.P., Pantoja,C., Erle,D.J., Yamamoto,K.R., and Fahy,J.V.,** Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. *Proc.Natl.Acad.Sci.U.S.A* 2007. **104**: 15858-15863.

39. **Franken,K.L., Hiemstra,H.S., van Meijgaarden,K.E., Subronto,Y., den,H.J., Ottenhoff,T.H., and Drijfhout,J.W.,** Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. *Protein Expr.Purif.* 2000. **18**: 95-99.

40. **Lin,M.Y., Geluk,A., Smith,S.G., Stewart,A.L., Friggen,A.H., Franken,K.L., Verduyn,M.J., van Meijgaarden,K.E., Voskuil,M.I., Dockrell,H.M., Huygen,K., Ottenhoff,T.H., and Klein,M.R.,** Lack of immune responses to Mycobacterium tuberculosis DosR regulon proteins following Mycobacterium bovis BCG vaccination. *Infect.Immun.* 2007. **75**: 3523-3530.

41. **Commandeur,S., van Meijgaarden,K.E., Lin,M.Y., Franken,K.L., Friggen,A.H., Drijfhout,J.W., Oftung,F., Korsvold,G.E., Geluk,A., and Ottenhoff,T.H.,** Identification of human T-cell responses to Mycobacterium tuberculosis resuscitation-promoting factors in long-term latently infected individuals. *Clin.Vaccine Immunol.* 2011. **18**: 676-683.

42. **Commandeur,S., Lin,M.Y., van Meijgaarden,K.E., Friggen,A.H., Franken,K.L., Drijfhout,J.W., Korsvold,G.E., Oftung,F., Geluk,A., and Ottenhoff,T.H.,** Double- and monofunctional CD4 and CD8 T-cell responses to Mycobacterium tuberculosis DosR antigens and peptides in long-term latently infected individuals. *Eur.J.Immunol.* 2011. **41**: 2925-2936.

43. **Malen,H., Berven,F.S., Fladmark,K.E., and Wiker,H.G.,** Com-

prehensive analysis of exported proteins from Mycobacterium tuberculosis H37Rv. *Proteomics.* 2007. **7**: 1702-1718.

44. **Malen,H., Pathak,S., Softeland,T., de Souza,G.A., and Wiker,H.G.,** Definition of novel cell envelope associated proteins in Triton X-114 extracts of Mycobacterium tuberculosis H37Rv. *BMC.Microbiol.* 2010. **10**: 132.

45. **de Souza,G.A., Leversen,N.A., Malen,H., and Wiker,H.G.,** Bacterial proteins with cleaved or uncleaved signal peptides of the general secretory pathway. *J.Proteomics.* 2011. **75**: 502-510.

46. **Rosenkrands,I., King,A., Weldingh,K., Moniatte,M., Moertz,E., and Andersen,P.,** Towards the proteome of Mycobacterium tuberculosis. *Electrophoresis* 2000. **21**: 3740-3756.

47. **Mattow,J., Schaible,U.E., Schmidt,F., Hagens,K., Siejak,F., Brestrich,G., Haeselbarth,G., Muller,E.C., Jungblut,P.R., and Kaufmann,S.H.,** Comparative proteome analysis of culture supernatant proteins from virulent Mycobacterium tuberculosis H37Rv and attenuated M. bovis BCG Copenhagen. *Electrophoresis* 2003. **24**: 3405-3420.

48. **Wolfe,L.M., Mahaffey,S.B., Kruh,N.A., and Dobos,K.M.,** Proteomic definition of the cell wall of Mycobacterium tuberculosis. *J.Proteome.Res.* 2010. **9**: 5816-5826.

49. **Kruh,N.A., Troudt,J., Izzo,A., Prenni,J., and Dobos,K.M.,** Portrait of a pathogen: the Mycobacterium tuberculosis proteome in vivo. *PLoS.One.* 2010. **5**: e13938.

50. **Gu,S., Chen,J., Dobos,K.M., Bradbury,E.M., Belisle,J.T., and Chen,X.,** Comprehensive proteomic profiling of the membrane constituents of a Mycobacterium tuberculosis strain. *Mol. Cell Proteomics.* 2003. **2**: 1284-1296.

51. **Mawuenyega,K.G., Forst,C.V., Dobos,K.M., Belisle,J.T., Chen,J., Bradbury,E.M., Bradbury,A.R., and Chen,X.,** Mycobacterium tuberculosis functional network analysis by global subcellular protein profiling. *Mol.Biol.Cell* 2005. **16**: 396-404.

52. **Sassetti,C.M., Boyd,D.H., and Rubin,E.J.,** Genes required for mycobacterial growth defined by high density mutagenesis. *Mol.Microbiol.* 2003. **48**: 77-84.

53. **Sassetti,C.M. and Rubin,E.J.,** Genetic requirements for mycobacterial survival during infection. *Proc.Natl.Acad. Sci.U.S.A* 2003. **100**: 12989-12994.

54. **Lamichhane,G., Zignol,M., Blades,N.J., Geiman,D.E., Dougherty,A., Grosset,J., Broman,K.W., and Bishai,W.R.,** A postgenomic method for predicting essential genes at subsaturation levels of mutagenesis: application to Mycobacterium tuberculosis. *Proc.Natl. Acad.Sci.U.S.A* 2003. **100**: 7213-7218.

55. **Griffin,J.E., Gawronski,J.D., Dejesus,M.A., Ioerger,T.R., Akerley,B.J., and Sassetti,C.M.,** High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. *PLoS.Pathog.* 2011. **7**: e1002251.

56. **Dutta,N.K., Mehra,S., Didier,P.J., Roy,C.J., Doyle,L.A., Alvarez,X., Ratterree,M., Be,N.A., Lamichhane,G., Jain,S.K., Lacey,M.R., Lackner,A.A., and Kaushal,D.,** Genetic requirements for the survival of tubercle bacilli in primates. *J.Infect.Dis.* 2010. **201**: 1743-1752.

57. **Frentsch,M., Arbach,O., Kirchhoff,D., Moewes,B., Worm,M., Rothe,M., Scheffold,A., and Thiel,A.,** Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. *Nat.Med.* 2005. **11**: 1118-1124.

58. **Fontan,P., Aris,V., Ghanny,S., Soteropoulos,P., and Smith,I.,** Global transcriptional profile of Mycobacterium tuberculosis during THP-1 human macrophage infection. *Infect.Immun.* 2008. **76**: 717-725.

59. **Ward,S.K., Abomoelak,B., Marcus,S.A., and Talaat,A.M.,** Transcriptional profiling of mycobacterium tuberculosis during infection: lessons learned. *Front Microbiol.* 2010. **1**: 121.

60. **Rachman,H., Strong,M., Schaible,U., Schuchhardt,J., Hagens,K., Mollenkopf,H., Eisenberg,D., and Kaufmann,S.H.,** Mycobacterium tu-

berculosis gene expression profiling within the context of protein networks. *Microbes. Infect.* 2006. **8**: 747-757.

61. **Ramakrishnan,L., Federspiel,N.A., and Falkow,S.,** Granuloma-specific expression of Mycobacterium virulence proteins from the glycine-rich PE-PGRS family. *Science* 2000. **288**: 1436-1439.

62. **Chan,K., Knaak,T., Satkamp,L., Humbert,O., Falkow,S., and Ramakrishnan,L.,** Complex pattern of Mycobacterium marinum gene expression during long-term granulomatous infection. *Proc.Natl.Acad.Sci.U.S.A* 2002. **99**: 3920-3925.

63. **McKinney,J.D., Honer zu,B.K., Munoz-Elias,E.J., Miczak,A., Chen,B., Chan,W.T., Swenson,D., Sacchettini,J.C., Jacobs,W.R., Jr., and Russell,D.G.,** Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* 2000. **406**: 735-738.

64. **Munoz-Elias,E.J. and McKinney,J.D.,** Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat.Med.* 2005. **11**: 638-644.

65. **Harriff,M.J., Purdy,G.E., and Lewinsohn,D.M.,** Escape from the Phagosome: The Explanation for MHC-I Processing of Mycobacterial Antigens? *Front Immunol.* 2012. **3**: 40.

66. **Mogues,T., Goodrich,M.E., Ryan,L., LaCourse,R., and North,R.J.,** The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice. *J.Exp.Med.* 2001. **193**: 271-280.

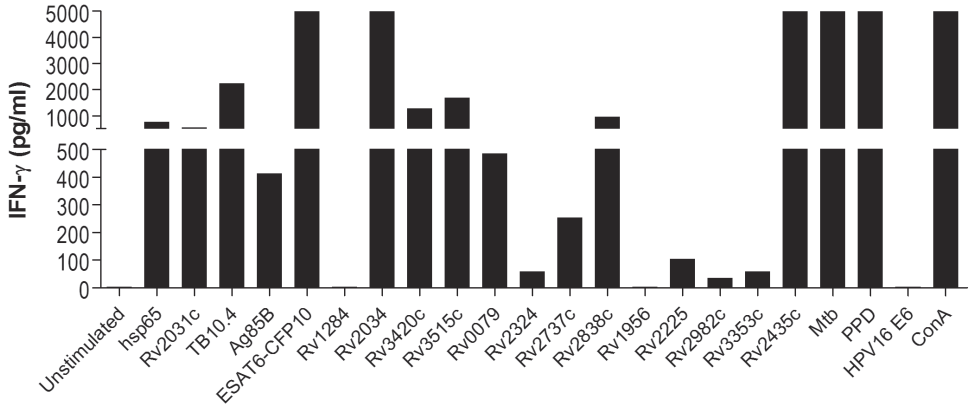
67. **Bold,T.D. and Ernst,J.D.,** CD4+ T cell-dependent IFN-gamma production by CD8+ effector T cells in Mycobacterium tuberculosis infection. *J.Immunol.* 2012. **189**: 2530-2536.

68. **Chegou,N.N., Black,G.F., Loxton,A.G., Stanley,K., Essone,P.N., Klein,M.R., Parida,S.K., Kaufmann,S.H., Doherty,T.M., Friggen,A.H., Franken,K.L., Ottenhoff,T.H., and Walzl,G.,** Potential of novel Mycobacterium tuberculosis infection phase-dependent antigens in the diagnosis of TB disease in a high burden setting. *BMC.Infect. Dis.* 2012. **12**: 10.

69. **Jones,G.J., Pirson,C., Gideon,H.P., Wilkinson,K.A., Sherman,D.R., Wilkinson,R.J., Hewinson,R.G., and Vordermeier,H.M.,** Immune Responses to the Enduring Hypoxic Response Antigen Rv0188 Are Preferentially Detected in Mycobacterium bovis Infected Cattle with Low Pathology. *PLoS.One.* 2011. **6**: e21371.

70. **Ottenhoff,T.H. and Kaufmann,S.H.,** Vaccines against Tuberculosis: Where Are We and Where Do We Need to Go? *PLoS. Pathog.* 2012. **8**: e1002607.

Supplementary data



Supplementary Figure 1. IFN-γ production in response to IVE-TB proteins by splenocytes of *Mtb* infected C3H mice. C3H mice were infected intranasally with *Mtb* (10^4 CFU), and after 6 weeks their splenocytes were harvested and stimulated with control antigens (hsp65, Rv2031c, TB10.4, Ag85B, ESAT-6/CFP10, *Mtb* lysate and PPD), IVE-TB antigens (Rv1284, Rv2034, Rv4320c, Rv3515c, Rv0079, Rv2324, Rv2737c, Rv2838c, Rv1956, Rv2225, Rv2982c, Rv3353c and Rv2435c all in final concentration of 10μg/ml), mitogen (conA; 1 μg/ml; positive control), or HPV16E6 recombinant protein (10 μg/ml; negative protein control). Rv2737c and Rv2435c protein fragments were pooled. All groups included three mice. All mice were separately analyzed. Results are shown for one animal, representative for each test group.

Supplementary Table III. Putative IVE-TB protein functions in association with *Mtb* infection

Gene	Protein	Described (predicted) function	Possible role in *Mtb* host infection	References
Rv1284	Metalloenzyme β-carbonic anhydrase	Catalyzes the hydrolysis of carbon dioxide to bicarbonate and protons	Biocarbonate and protons essential in many processes including fatty acid metabolism and regulation of pH homeostasis	[1-3]
Rv2380c	Peptide synthetase (MbtE)	Involved in assembly of mycobactin (lipophylic siderophore)	Mycobactins acquire iron from the (host) environment	[4-12]
Rv3515c	Fatty acyl-CoA ligase (FadD19)	Possibly involved in degradation of sterol side chains	Enzyme involved in lipid degradation (alternative use of fatty acids instead of carbohydrates as energy source *in vivo*)	[13-18]
Rv0079	Unknown	Cytoplasmic translation factor properties	Possible inhibitory effect on protein synthesis	[19;20]
Rv2324	Asparagine synthase C gene family protein (AsnC)	Transcriptional regulator, plays a role in asparagine biosynthesis	Lrp/AsnC gene members associated to adaptation to environmental conditions (feast/famine regulatory proteins)	[21-23]
Rv2737c	Recombinase A (RecA)	Repairs DNA double-strand breaks via homologous recombination	DNA repair induced by e.g. host cell induced oxidative stress or chemotherapy	[1;24-32]
Rv2838c	Ribosome binding factor A (RbfA)	Cold shock protein, binds to ribosomal 30S subunit	Allows protein translation by ribosomes at low temperatures, which *Mtb* may encounter during air-borne transmission	[33]
Rv3420c	Ribosomal protein alanine acetyltransferase (rimI)	Catalyzes transfer of acetyl group present on acyl-CoA to amines	Amines are present in many different molecules; prokaryotic protein acetylation particularly of enzymes involved in metabolic pathways such as fatty acid metabolism and gluconeogenesis	[34-37]
Rv2034	Arsenic repressor (ArsR) protein (SmtB/ArsR family)	Metalloregulatory transcriptional repressor	Positively regulates *PhoP*, an essential virulence regulatory system of *Mtb* and also binds to hypoxia and fatty acid metabolism related genes	[15;38-44]
Rv2982c	Glycerol-3-phosphate dehydrogenase (gpdA2)	Involved in phospholipid biosynthesis	Unknown	
Rv2435c	Membrane-bound adenylyl cyclase	Cyclases necessary for production of the secondary messenger cAMP	Secretion of bacterial cAMP plays a major role in survival of intracellular *Mtb*	[45-49]
Rv1956	Antitoxin (HigA)	Part of toxin-antitoxin (TA) operon, together with Rv1955 (HigB)	Under normal growth conditions toxin and antitoxin form inactive complexes, but during stress antitoxin is degraded allowing toxin targeted regulation	[23;44;50-54]
Rv1363c	Predicted membrane protein	Unknown, possibly mammalian cell entry (Mce) associated gene (Mas)	Possibly involved in host cell invasion	[55;56]
Rv2225	PanB	Initiates first step in biosynthesis of panthothenate (vitamin B5, precursor of CoA)	Crucial for fatty acid metabolism	[18;57;58]
Rv2465c	Ribose-5-phosphate isomerase (rpiB)	Catalyzing step in one of the reactional steps in arabinose metabolism	Structures that are generated are major components in the mycobacterial cell wall	[59]
Rv3353c	Conserved hypothetical protein	Unknown	Unknown	

References

1. **Sassetti,C.M., Boyd,D.H., and Rubin,E.J.,** Genes required for mycobacterial growth defined by high density mutagenesis. *Mol.Microbiol.* 2003. **48**: 77-84.
2. **Suarez,C.A., Larsson,A.M., Hogbom,M., Lindberg,J., Bergfors,T., Bjorkelid,C., Mowbray,S.L., Unge,T., and Jones,T.A.,** Structure and function of carbonic anhydrases from Mycobacterium tuberculosis. *J.Biol.Chem.* 2005. **280**: 18782-18789.
3. **Minakuchi,T., Nishimori,I., Vullo,D., Scozzafava,A., and Supuran,C.T.,** Molecular cloning, characterization, and inhibition studies of the Rv1284 beta-carbonic anhydrase from Mycobacterium tuberculosis with sulfonamides and a sulfamate. *J.Med.Chem.* 2009. **52**: 2226-2232.
4. **Mukhopadhyay,S., Nair,S., and Ghosh,S.,** Pathogenesis in tuberculosis: transcriptomic approaches to unraveling virulence mechanisms and finding new drug targets. *FEMS Microbiol.Rev.* 2012. **36**: 463-485.
5. **Boshoff,H.I., Myers,T.G., Copp,B.R., McNeil,M.R., Wilson,M.A., and Barry,C.E., III,** The transcriptional responses of Mycobacterium tuberculosis to inhibitors of metabolism: novel insights into drug mechanisms of action. *J.Biol.Chem.* 2004. **279**: 40174-40184.
6. **Banerjee,S., Farhana,A., Ehtesham,N.Z., and Hasnain,S.E.,** Iron acquisition, assimilation and regulation in mycobacteria. *Infect.Genet.Evol.* 2011. **11**: 825-838.
7. **Quadri,L.E., Sello,J., Keating,T.A., Weinreb,P.H., and Walsh,C.T.,** Identification of a Mycobacterium tuberculosis gene cluster encoding the biosynthetic enzymes for assembly of the virulence-conferring siderophore mycobactin. *Chem.Biol.* 1998. **5**: 631-645.
8. **Siegrist,M.S., Unnikrishnan,M., McConnell,M.J., Borowsky,M., Cheng,T.Y., Siddiqi,N., Fortune,S.M., Moody,D.B., and Rubin,E.J.,** Mycobacterial Esx-3 is required for mycobactin-mediated iron acquisition. *Proc.Natl.Acad.Sci.U.S.A* 2009. **106**: 18792-18797.
9. **Serafini,A., Boldrin,F., Palu,G., and Manganelli,R.,** Characterization of a Mycobacterium tuberculosis ESX-3 conditional mutant: essentiality and rescue by iron and zinc. *J.Bacteriol.* 2009. **191**: 6340-6344.
10. **Olakanmi,O., Schlesinger,L.S., Ahmed,A., and Britigan,B.E.,** Intraphagosomal Mycobacterium tuberculosis acquires iron from both extracellular transferrin and intracellular iron pools. Impact of interferon-gamma and hemochromatosis. *J.Biol.Chem.* 2002. **277**: 49727-49734.
11. **Luo,M., Fadeev,E.A., and Groves,J.T.,** Mycobactin-mediated iron acquisition within macrophages. *Nat.Chem.Biol.* 2005. **1**: 149-153.
12. **Rodriguez,G.M., Voskuil,M.I., Gold,B., Schoolnik,G.K., and Smith,I.,** ideR, An essential gene in mycobacterium tuberculosis: role of IdeR in iron-dependent gene expression, iron metabolism, and oxidative stress response. *Infect.Immun.* 2002. **70**: 3371-3381.
13. **BLOCH,H. and Segal,W.,** Biochemical differentiation of Mycobacterium tuberculosis grown in vivo and in vitro. *J.Bacteriol.* 1956. **72**: 132-141.
14. **Wilbrink,M.H., Petrusma,M., Dijkhuizen,L., and van der Geize,R.,** FadD19 of Rhodococcus rhodochrous DSM43269, a steroid-coenzyme A ligase essential for degradation of C-24 branched sterol side chains. *Appl.Environ.Microbiol.* 2011. **77**: 4455-4464.
15. **McKinney,J.D., Honer zu,B.K., Munoz-Elias,E.J., Miczak,A., Chen,B., Chan,W.T., Swenson,D., Sacchettini,J.C., Jacobs,W.R., Jr., and Russell,D.G.,** Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* 2000. **406**: 735-738.

16. **Munoz-Elias,E.J. and McKinney,J.D.,** Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat.Med.* 2005. **11**: 638-644.

17. **Schnappinger,D., Ehrt,S., Voskuil,M.I., Liu,Y., Mangan,J.A., Monahan,I.M., Dolganov,G., Efron,B., Butcher,P.D., Nathan,C., and Schoolnik,G.K.,** Transcriptional Adaptation of Mycobacterium tuberculosis within Macrophages: Insights into the Phagosomal Environment. *J.Exp.Med.* 2003. **198**: 693-704.

18. **Karakousis,P.C., Yoshimatsu,T., Lamichhane,G., Woolwine,S.C., Nuermberger,E.L., Grosset,J., and Bishai,W.R.,** Dormancy phenotype displayed by extracellular Mycobacterium tuberculosis within artificial granulomas in mice. *J.Exp.Med.* 2004. **200**: 647-657.

19. **Mishra,S.,** Function prediction of Rv0079, a hypothetical Mycobacterium tuberculosis DosR regulon protein. *J.Biomol.Struct.Dyn.* 2009. **27**: 283-292.

20. **Kumar,A., Majid,M., Kunisch,R., Rani,P.S., Qureshi,I.A., Lewin,A., Hasnain,S.E., and Ahmed,N.,** Mycobacterium tuberculosis DosR regulon gene Rv0079 encodes a putative, 'dormancy associated translation inhibitor (DATIN)'. *PLoS.One.* 2012. **7**: e38709.

21. **Deng,W., Wang,H., and Xie,J.,** Regulatory and pathogenesis roles of Mycobacterium Lrp/AsnC family transcriptional factors. *J.Cell Biochem.* 2011. **112**: 2655-2662.

22. **Reddy,M.C., Gokulan,K., Jacobs,W.R., Jr., Ioerger,T.R., and Sacchettini,J.C.,** Crystal structure of Mycobacterium tuberculosis LrpA, a leucine-responsive global regulator associated with starvation response. *Protein Sci.* 2008. **17**: 159-170.

23. **Betts,J.C., Lukey,P.T., Robb,L.C., McAdam,R.A., and Duncan,K.,** Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol.Microbiol.* 2002. **43**: 717-731.

24. **Zhang,L., Zheng,Y., Xi,Z., Luo,Z., Xu,X., Wang,C., and Liu,Y.,** Metal ions binding to recA inteins from Mycobacterium tuberculosis. *Mol.Biosyst.* 2009. **5**: 644-650.

25. **Gupta,R., Barkan,D., Redelman-Sidi,G., Shuman,S., and Glickman,M.S.,** Mycobacteria exploit three genetically distinct DNA double-strand break repair pathways. *Mol. Microbiol.* 2011. **79**: 316-330.

26. **Davis,E.O., Jenner,P.J., Brooks,P.C., Colston,M.J., and Sedgwick,S.G.,** Protein splicing in the maturation of M. tuberculosis recA protein: a mechanism for tolerating a novel class of intervening sequence. *Cell* 1992. **71**: 201-210.

27. **Papavinasasundaram,K.G., Colston,M.J., and Davis,E.O.,** Construction and complementation of a recA deletion mutant of Mycobacterium smegmatis reveals that the intein in Mycobacterium tuberculosis recA does not affect RecA function. *Mol.Microbiol.* 1998. **30**: 525-534.

28. **Zhang,L., Xiao,N., Pan,Y., Zheng,Y., Pan,Z., Luo,Z., Xu,X., and Liu,Y.,** Binding and inhibition of copper ions to RecA inteins from Mycobacterium tuberculosis. *Chemistry.* 2010. **16**: 4297-4306.

29. **Zhang,L., Zheng,Y., Callahan,B., Belfort,M., and Liu,Y.,** Cisplatin inhibits protein splicing, suggesting inteins as therapeutic targets in mycobacteria. *J.Biol.Chem.* 2011. **286**: 1277-1282.

30. **Paulus,H.,** Protein splicing inhibitors as a new class of anti mycobacterial agents. *Drugs of the Future* 2007. **32**: 973-984.

31. **Raman,K. and Chandra,N.,** Mycobacterium tuberculosis interactome analysis unravels potential pathways to drug resistance. *BMC.Microbiol.* 2008. **8**: 234.

32. **Sander,P., Papavinasasundaram,K.G., Dick,T., Stavropoulos,E., Ellrott,K., Springer,B., Colston,M.J., and Bottger,E.C.,** Mycobacterium bovis BCG recA deletion mutant shows increased susceptibility to DNA-damaging agents but wild-type survival in a mouse infection model. *Infect. Immun.* 2001. **69**: 3562-3568.

33. **Golovlev,E.L.,** [Bacterial cold shock response at the level of DNA transcription, translation and chromosome dynamics]. *Mikrobiologiia* 2003. **72**: 5-13.

34. **Vetting,M.W., Bareich,D.C., Yu,M., and Blanchard,J.S.,** Crystal structure of RimI from Salmonella typhimurium LT2, the GNAT responsible for N(alpha)-acetylation of ribosomal protein S18. *Protein Sci.* 2008. **17**: 1781-1790.

35. **Vetting,M.W., Errey,J.C., and Blanchard,J.S.,** Rv0802c from Mycobacterium tuberculosis: the first structure of a succinyltransferase with the GNAT fold. *Acta Crystallogr.Sect.F.Struct.Biol.Cryst. Commun.* 2008. **64**: 978-985.

36. **Yoshikawa,A., Isono,S., Sheback,A., and Isono,K.,** Cloning and nucleotide sequencing of the genes rimI and rimJ which encode enzymes acetylating ribosomal proteins S18 and S5 of Escherichia coli K12. *Mol.Gen.Genet.* 1987. **209**: 481-488.

37. **Xu,H., Hegde,S.S., and Blanchard,J.S.,** Reversible acetylation and inactivation of Mycobacterium tuberculosis acetyl-CoA synthetase is dependent on cAMP. *Biochemistry* 2011. **50**: 5883-5892.

38. **Gao,C.H., Yang,M., and He,Z.G.,** Characterization of a Novel ArsR-Like Regulator Encoded by Rv2034 in Mycobacterium tuberculosis. *PLoS.One.* 2012. **7**: e36255.

39. **Osman,D. and Cavet,J.S.,** Bacterial metal-sensing proteins exemplified by ArsR-SmtB family repressors. *Nat.Prod.Rep.* 2010. **27**: 668-680.

40. **Guo,M., Feng,H., Zhang,J., Wang,W., Wang,Y., Li,Y., Gao,C., Chen,H., Feng,Y., and He,Z.G.,** Dissecting transcription regulatory pathways through a new bacterial one-hybrid reporter system. *Genome Res.* 2009. **19**: 1301-1308.

41. **Eisenreich,W., Dandekar,T., Heesemann,J., and Goebel,W.,** Carbon metabolism of intracellular bacterial pathogens and possible links to virulence. *Nat. Rev.Microbiol.* 2010. **8**: 401-412.

42. **Gao,C.H., Yang,M., and He,Z.G.,** An ArsR-like transcriptional factor recognizes a conserved sequence motif and positively regulates the expression of phoP in mycobacteria. *Biochem.Biophys.Res.Commun.* 2011. **411**: 726-731.

43. **Makarova,K.S., Wolf,Y.I., and Koonin,E.V.,** Comprehensive comparative-genomic analysis of type 2 toxin-antitoxin systems and related mobile stress response systems in prokaryotes. *Biol.Direct.* 2009. **4**: 19.

44. **Keren,I., Minami,S., Rubin,E., and Lewis,K.,** Characterization and transcriptome analysis of Mycobacterium tuberculosis persisters. *MBio.* 2011. **2**: e00100-e00111.

45. **Agarwal,N., Lamichhane,G., Gupta,R., Nolan,S., and Bishai,W.R.,** Cyclic AMP intoxication of macrophages by a Mycobacterium tuberculosis adenylate cyclase. *Nature* 2009. **460**: 98-102.

46. **Bai,G., Knapp,G.S., and McDonough,K.A.,** Cyclic AMP signalling in mycobacteria: redirecting the conversation with a common currency. *Cell Microbiol.* 2011. **13**: 349-358.

47. **Shenoy,A.R., Sivakumar,K., Krupa,A., Srinivasan,N., and Visweswariah,S.S.,** A survey of nucleotide cyclases in actinobacteria: unique domain organization and expansion of the class III cyclase family in Mycobacterium tuberculosis. *Comp Funct.Genomics* 2004. **5**: 17-38.

48. **Bai,G., Schaak,D.D., and McDonough,K.A.,** cAMP levels within Mycobacterium tuberculosis and Mycobacterium bovis BCG increase upon infection of macrophages. *FEMS Immunol. Med.Microbiol.* 2009. **55**: 68-73.

49. **Lowrie,D.B., Jackett,P.S., and Ratcliffe,N.A.,** Mycobacterium microti may protect itself from intracellular destruction by releasing cyclic AMP into phagosomes. *Nature* 1975. **254**: 600-602.

50. **Smollett,K.L., Fivian-Hughes,A.S., Smith,J.E., Chang,A., Rao,T., and Davis,E.O.,** Experimental determination of translational start sites resolves uncertainties in genomic open reading frame

predictions - application to Mycobacterium tuberculosis. *Microbiology* 2009. **155**: 186-197.

51. **Fivian-Hughes,A.S. and Davis,E.O.,** Analyzing the regulatory role of the HigA antitoxin within Mycobacterium tuberculosis. *J.Bacteriol.* 2010. **192**: 4348-4356.

52. **Ramage,H.R., Connolly,L.E., and Cox,J.S.,** Comprehensive functional analysis of Mycobacterium tuberculosis toxin-antitoxin systems: implications for pathogenesis, stress responses, and evolution. *PLoS.Genet.* 2009. **5**: e1000767.

53. **Rustad,T.R., Harrell,M.I., Liao,R., and Sherman,D.R.,** The enduring hypoxic response of Mycobacterium tuberculosis. *PLoS.One.* 2008. **3**: e1502.

54. **Bordes,P., Cirinesi,A.M., Ummels,R., Sala,A., Sakr,S., Bitter,W., and Genevaux,P.,** SecB-like chaperone controls a toxin-antitoxin stress-responsive system in Mycobacterium tuberculosis. *Proc.Natl.Acad.Sci.U.S.A* 2011. **108**: 8438-8443.

55. **Zhang,F. and Xie,J.P.,** Mammalian cell entry gene family of Mycobacterium tuberculosis. *Mol.Cell Biochem.* 2011. **352**: 1-10.

56. **Casali,N. and Riley,L.W.,** A phylogenomic analysis of the Actinomycetales mce operons. *BMC.Genomics* 2007. **8**: 60.

57. **Sugantino,M., Zheng,R., Yu,M., and Blanchard,J.S.,** Mycobacterium tuberculosis ketopantoate hydroxymethyltransferase: tetrahydrofolate-independent hydroxymethyltransferase and enolization reactions with alpha-keto acids. *Biochemistry* 2003. **42**: 191-199.

58. **Pearce,M.J., Arora,P., Festa,R.A., Butler-Wu,S.M., Gokhale,R.S., and Darwin,K.H.,** Identification of substrates of the Mycobacterium tuberculosis proteasome. *EMBO J.* 2006. **25**: 5423-5432.

59. **Wolucka,B.A.,** Biosynthesis of D-arabinose in mycobacteria - a novel bacterial pathway with implications for antimycobacterial therapy. *FEBS J.* 2008. **275**: 2691-2711.

CHAPTER 5

The newly identified *Mycobacterium tuberculosis* antigen Rv2034 induces $CD4^+$ T cells that protect against pulmonary infection in HLA-DR transgenic mice

Susanna Commandeur, Susan J.F. van den Eeden, Karin Dijkman, Kees L.M.C. Franken, Krista E. van Meijgaarden, Louis Wilson, Tom H.M. Ottenhoff and Annemieke Geluk

Department of Infectious Diseases, Leiden University Medical Center, The Netherlands

Submitted

Abstract

Tuberculosis (TB) remains one of the most serious infectious diseases worldwide. *Mycobacterium bovis* BCG, the current vaccine against TB, provides insufficient protection against adult pulmonary TB, indicating the need for improved TB vaccines. Besides being immunogenic, *Mycobacterium tuberculosis* (*Mtb*) antigens that represent potential vaccine candidates should also be expressed *in vivo* during *Mtb* infection. We have analyzed the *in vivo* immunogenicity and protective efficacy of Rv2034, a novel potential vaccine candidate that is expressed in lungs of *Mtb*-infected susceptible mice and that was recognized by T cells from tuberculin skin test-positive individuals. The Rv2034 protein was highly immunogenic in HLA-DR3-immunized tg mice, but not in HLA-DR3-negative littermates. Rv2034 protein immunization induced IFN-γ^+/TNF$^+$ and IFN-γ^+ CD4$^+$ T cells specific for an HLA-DR3-restricted epitope, Rv2034 peptide 31-50. CD4$^+$ T-cell responses were optimally induced when using TLR9 and TLR3 ligand-adjuvants, whereas Rv2034-specific antibodies were observed using ligands for TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9. Finally, Rv2034/CpG immunization reduced the number of bacilli in the lungs after *Mtb* challenge specifically in HLA-DR3 mice indicating the potential of Rv2034 in TB vaccines.

Introduction

8.7 million new tuberculosis (TB) cases and 1.4 million deaths due to TB were recorded for 2011, illustrating the enormous global burden of TB on human health [1]. BCG, the only registered TB vaccine, does protect against severe forms of childhood TB but not sufficiently against pulmonary TB in adults, nor does protect against reactivation from latent TB [2;3]. There is wide consensus that better TB vaccines are urgently needed.

In search of new TB vaccine candidate antigens, we have recently used a genome-wide unbiased antigen discovery approach in which we investigated the expression of 2170 *Mycobacterium tuberculosis* (*Mtb*) genes during infection in the lungs of four genetically related but distinct mouse strains, which ranged in TB susceptibility and TB disease manifestations. We identified a series of *in vivo* expressed *Mtb* (IVE-TB) genes and showed that several of these IVE-TB gene encoded proteins were efficiently recognized by human T cells and thus qualify as candidate TB vaccine antigens [4]. One of the IVE-TB genes identified, *Rv2034*, encodes for an arsenic repressor (ArsR) protein that belongs to the *Synechococcus* metallothionein regulator (SmtB)/ArsR family of metallo-regulatory transcriptional repressors [5]. Rv2034 is suggested to be involved in the regulation of lipid metabolism and the hypoxic stress response of *Mtb* [6-8]. This is further supported by studies showing *Rv2034* gene expression by *Mtb* cultures grown under starvation-, [9] hypoxic- [10], and multi-stress conditions [11], as well as expression in *Mtb* persister populations [12].

SmtB/ArsR transcriptional repressors control resistance to metal ions and bind to DNA elements in genes involved in metal-metabolism and -detoxification. Upon metal binding the SmtB/ArsR family repressor protein dissociates from the ligand DNA, thus activating transcription. The SmtB/ArsR family regulator is widely present and at least one SmtB/ArsR member is encoded by most (sequenced) bacterial genomes. Notably, the *Mtb* genome encodes 12 SmtB/ArsR-like repressors, which is highly unusual compared to bacteria other than Actinobacteria [13]. Recent work identified several DNA binding sites for Rv2034, including promoter regions of major stress response genes such as the DosR gene *Rv2031c* (α-crystallin/ hspX), *Rv0350-Rv0353* (DnaK, GrpE), *Rv0440* (GroEL2), *Rv1907c-Rv1912c* (katG, FurA) and *Rv3197A* (WhiB7), as well as genes involved in fatty acid metabolism; *Rv3543c-Rv3545c* (FadE28, FadE29), *Rv3504-Rv350*5 (FadE26, FadE27), *Rv1094* (DesA2) and *Rv0244* (FadE5) [7]. Interestingly, besides displaying self-regulation, the Rv2034 protein, in contrast to its predicted function as a repressor, positively regulates the expression of g*roEL2* and *phoP*, an important virulence gene of *Mtb* [6;8]. Thus, *Rv2034* gene expression and protein function support a role in adaptation to stress environment and regulation of lipid metabolism, both essential in *in vivo Mtb* infection. In view of its potential mechanistic function in *Mtb*'s strategy to survive inside the human host, the induction of immune responses against Rv2034 would be an attractive option to direct immunity towards *Mtb* proteins that are essential to *Mtb* and that are expressed in the primary target organ, the lung.

In this study, we have investigated the vaccine potency of the Rv2034 protein in the context of HLA using the HLA-DR3 transgenic mouse model [14] and describe the *in vivo* immunogenicity of the Rv2034 protein and synthetic peptides thereof, as well as their efficacy to reduce the number of *Mtb* bacteria after intranasal challenge with live *Mtb*.

Materials and methods

Mouse strains. HLA-DRB1*03:01/DRA, murine class II-deficient (designated HLA-DR3) mice were generated as described [14], backcrossed for 10 generations with C57Bl/10 and eventually intercrossed. During breeding, PBMCs of each mouse were typed by flow cytometry for HLA-DR (FITC-conjugated mouse IgG2 κ anti-HLA-DR; BD Biosciences, Franklin Lakes, NJ), murine-CD4 (PE-Cy5-conjugated Rat IgG2a, κ anti-mouse CD4; BD Biosciences) and PE-conjugated mouse (BALB/c) IgG2a κ anti-mouse I-A^b; BD Biosciences). Littermates lacking HLA-DR expression (designated HLA-$DR3^{neg}$) were used as negative controls. HLA-A*02:01/H2-D Enge/J (designated HLA-A2) mice were purchased from Jackson Laboratory (Bar Harbor, ME) [15]. Surface expression of HLA-A*02:01 was confirmed by flow cytometry analysis. HLA-A2. DR3 transgenic mice were generated by mating the HLA-DR3 with HLA-A2 mice. All mice were bred under specific pathogen-free conditions.

Synthetic peptides. Peptides were purchased from Peptide 2.0 Inc. (Chantilly, VA, USA). Homogeneity and purity were confirmed by analytical HPLC and by mass spectrometry. Purity of all peptides was ≥ 80%. All impurities consist of shorter versions of the peptides caused by < 100% coupling efficiency in each round of synthesis.

Recombinant proteins. *Mtb* genes were amplified by PCR from genomic *Mtb* DNA and cloned using Gateway technology (Invitrogen, Carlsbad, CA) [16]. Purified recombinant proteins were produced and analyzed as described [16;17] and contained endotoxin levels below 50 IU per mg recombinant protein as tested using a Limulus Amebocyte Lysate (LAL) assay (Cambrex, East Rutherford, NJ). Recombinant proteins were tested to exclude protein non-specific T-cell stimulation and cellular toxicity in IFN-γ release assays using PBMC of *in vitro* purified protein derivative (PPD)-negative, healthy Dutch donors recruited at the Blood Bank Sanquin, Leiden, The Netherlands. None of these controls had experienced any known prior contact with TB patients.

Immunizations. For protein immunization, mice (4 - 5 animals per group) were injected subcutaneously (s.c.) three times with 25 μg protein generally in combination with 50 μg CpG (ODN1826 5'-TCC ATG ACG TTC CTG ACG TT -3'; InvivoGen, San Diego, CA; TLR9 ligand) in 200 μl PBS in the right flank with two weeks intervals. Control mice were injected s.c. with 10^6 colony forming units (CFU) BCG 1331 (*M. bovis* Bacillus Calmette Guérin; Statens Serum

Institute, Copenhagen, Denmark) from frozen ampoules. Splenocytes were harvested 21 days after final injections. Since ODNs containing unmethylated CpG motifs can activate immune cells to produce cytokines [18], we routinely immunize with CpG alone as a (negative) control to assess the antigen specificity of immunization. For adjuvants comparison experiments identical amounts of Rv2034 protein were administered s.c. with either: 50 µg PAM3CysK4 (N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]- lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine; Invivogen; TLR2 ligand), 50 µg Poly(I:C) HMW (polyinosinic-polycytidylic acid high molecular weight; Invivogen; TLR3 ligand), 10 µg MPL/TDM (monophosphoryl lipid A/ trehalose dicorynomycolate; Sigma-Aldrich, St. Louis, MO; TLR4 ligand), 10 µg Flagellin (Invivogen; TLR5 ligand) or 50 µg R848 (Imidazoquinoline compound, Invivogen; human TLR7/8 and mouse TLR7 ligand).

Ethics statement. The handling of mice was conducted in accordance with the regulations set forward by the animal care committee of the LUMC and in compliance with European Community Directive 86/609 for the care and use of laboratory animals.

Welfare monitoring. All animals included in the experiments were weighed once a week and observed daily in order to ensure ethics requirements and to monitor any adverse effects possibly related to the vaccination.

In vitro *cultures*. Splenocytes were isolated from individual animals by homogenizing spleens through a plastic cellstrainer (BD Biosciences) and splenocytes (3 x 10^6 cells/ml) were resuspended in IMDM (Invitrogen) supplemented with 2 mM L-glutamine (Invitrogen) penicillin (100 U/ml), streptomycin (100 µg/ml) (Invitrogen), 8% heat-inactivated FCS (Greiner Bio-One) and $5x10^{-5}$ M β-mercaptoethanol (Sigma). Cell suspensions (100 µl) were added to 96-well round-bottomed microtiter plates (Costar, Corning Incorporated). Cells were incubated in quadruplicates with 100 µl of medium, recombinant protein (1 or 10 µg/ml), or synthetic peptide (1 or 10 µg/ml). The mitogen concanavalin A (conA; 2 µg/ml; Sigma) was used in all experiments as a positive control for cell viability. After 6 days supernatants were taken from each well, quadruplicates pooled and frozen at -20 ºC until performing IFN-γ ELISA assay.

IFN-γ ELISA. Before ELISAs were performed on supernatants from *Mtb* infected murine material, supernatants or sera were transferred into 0.2 µM filter plates (Corning, NY, USA) and centrifuged for 3 minutes at 1300 rpm. The filtrated material was collected in clean 96-wells plates and transferred out of the MLIII lab for further analyses. Detection of IFN-γ in culture supernatants of *in vitro* cultured splenocytes was performed by ELISA (BD Biosciences) according to the manufacturer's instructions. OD values were converted into concentrations using Microplate Manager software, version 5.2.1 (Bio-Rad Laboratories, Veenendaal, The Netherlands). The cut-

off value to define positive responses was set beforehand at 100 pg/ml. The assay sensitivity level was 20 pg/ml. Values for unstimulated whole blood cultures were typically < 30 pg/ml.

Intracellular cytokine staining. For polychromatic flow cytometry, splenocytes (3 x10^6cells/ml) were cultured *in vitro* with peptide (5 μg/ml). After 7 days, cells were incubated with medium or fresh peptide (5 μg/ml). After 4 hours brefeldin A (Sigma; 2.5 μg/ml) was added for overnight (20 hour) incubation after which cells were permeabilized and fixed using Cytofix/Cytoperm (BD Biosciences) and Perm/Wash (BD Biosciences) according to the manufacturer's instructions and stained using phycoerythrin (PE)-conjugated anti-CD8β_2 (BD Pharmingen), PECy5-conjugated anti-CD4 (BD Pharmingen), eFluor450-conjugated anti-CD19 (eBioscience, San Diego, CA, USA), Vivid (Invitrogen), Allophycocyanin (APC)-conjugated anti-IL-2 (BD Pharmingen), Alexafluor700-conjugated anti-IFN-γ (BD Pharmingen) and PeCy7-conjugated anti–TNF (BD Pharmingen). As positive controls for detection of IL-2, IFN-γ or TNF, MiCK-1 cytokine positive control cells (BD Pharmingen) were used.

Determination of anti-Rv2034 antibodies (Ab). Levels of antibody directed against Rv2034 in serum from immunized mice were determined by ELISA. Briefly, plates were coated overnight at 4 °C with recombinant Rv2034 protein or PBS (0.4% BSA) as a negative control. Plates were blocked for 2 hours using PBS containing 1% BSA and 1% Tween-20. Different sample dilutions (100 μl/well) were added to wells and incubated at 37°C for 2 hours. Plates were washed three times using PBS containing 0.05% Tween-20 and 100 μl/well horse radish peroxide (HRP)-conjugated, rabbit-anti mouse total IgG, IgA and IgM (P0260 Dako, Glostrup, Denmark). After 2 hours at 37°C, plates were washed three times using PBS containing 0.05% Tween-20 and 100 μl/well tetramethylbenzidine substrate (TMB; Sigma) was added for 15 min at RT. The reaction was stopped by addition of H_2SO_4 (1M; 100 μl/well). OD values at 450 nm were determined using BioRad Microplate reader 680 (BioRad Laboratories, Veenendaal, The Netherlands). Mean Ab concentrations were calculated from the linear part of the titration curve.

Infection with live* Mtb *and determination of bacterial burden. Naive and immunized mice (5 animals per group) were infected with live *Mtb* strain H37Rv 3 weeks after the third protein immunization or 12 weeks after BCG immunization. The mice were anesthetized with isofluran (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane; Pharmachemie BV, Haarlem, The Netherlands) and intranasal (i.n.) infected with 10^5 CFU *Mtb* from frozen ampoules. Mice were sacrificed 6 weeks after *Mtb* challenge and lungs were aseptically removed. The organs were homogenized in sterile PBS and the number of bacteria was determined by culturing serial dilutions of the homogenates on 7H11 agar plates (BD Biosciences) supplemented with BD BBL™ Middlebrook OADC enrichment (100 ml per bottle; BD Biosciences), PANTA [BD Biosciences; 1 vial per liter containing Polymyxin B (6.000 units), Amphotericin B (600 μg), Nalidixic acid (2.400

μg), Trimethoprim (600 μg), Azlocillin (600 μg)] and ampicillin (3.4 mg/ml; Vepidan, Denmark). Colonies were counted after 3 weeks incubation at 37 ºC. In case of animals that received *Mtb* infection combined with BCG vaccination, 7H11 agar plates containing 2-thiophene carboxylic acid hydrazide (2 μg/ml; Sigma) were used to distinguish BCG colonies from *Mtb* colonies. Protective efficacies are expressed as log10 bacterial counts in immunized mice compared to BCG immunized mice.

Statistical analysis. Graph Pad Prism (version 5) software was used for statistical analysis. Bacterial titers were analyzed by the Mann-Whitney test. *In vitro* cytokine levels were compared using student's t test. P-values ≤ 0.05 were considered significant.

Results

Identification of Rv2034 T-cell epitopes in HLA-DR3 tg mice

In order to evaluate the *in vivo* immunogenicity of Rv2034 different HLA-transgenic (tg) mouse models were used: HLA-DR3 tg mice lacking murine MHC class II (HLA-DR3.Ab^{0}), which has been shown to be suitable for *in vivo* identification of HLA-DR3 restricted T-cell epitopes [14;19], and HLA-A*0201 mice [20;21] which express endogenous MHC class II in addition to the hybrid class I MHC (α1 and α2 domains of HLA-A2*0201; α3 of H-2D^{d}) [15].

Mice were immunized three times with Rv2034 protein in CpG as adjuvant and IFN-γ levels in splenocytes induced by the Rv2034 protein as well as ten 20-mer peptides spanning the entirety of Rv2034, were analyzed (Figure 1). Immunization with Rv2034 in CpG induced significant levels of IFN-γ in HLA-DR3 and HLA-A2.DR3 transgenic mice against both the protein and peptides p31-50, p41-60 and p51-70. These peptides represent 20-mers which each overlap 10 amino acids with the previous and subsequent peptide (Table 1). Rv2034-immunized HLA-A2 mice responded to a 13-fold lower extent to the whole protein, but not to any of the 20-mer peptides, perhaps due to the low IFN-γ production observed in these mice in response to the protein.

HLA-DR3^{neg}/murine MHC class II^{neg} mice did not respond to any of the stimuli except the conA control. Also HLA-DR3 mice that received CpG alone did not show any IFN-γ response except to conA. None of the Rv2034-immunized mice recognized the control protein (HPV16E6) or peptide (HLA-DR3-restricted peptide hsp65 p3-13) [22], indicating the Rv2034-specificity. Thus, these results indicate that IFN-γ secretion after Rv2034 immunization in the models used, is predominantly HLA-DR3-restricted and that presentation of Rv2034 protein most likely does not occur via endogenous murine MHC class II such as present in HLA-A2 mice.

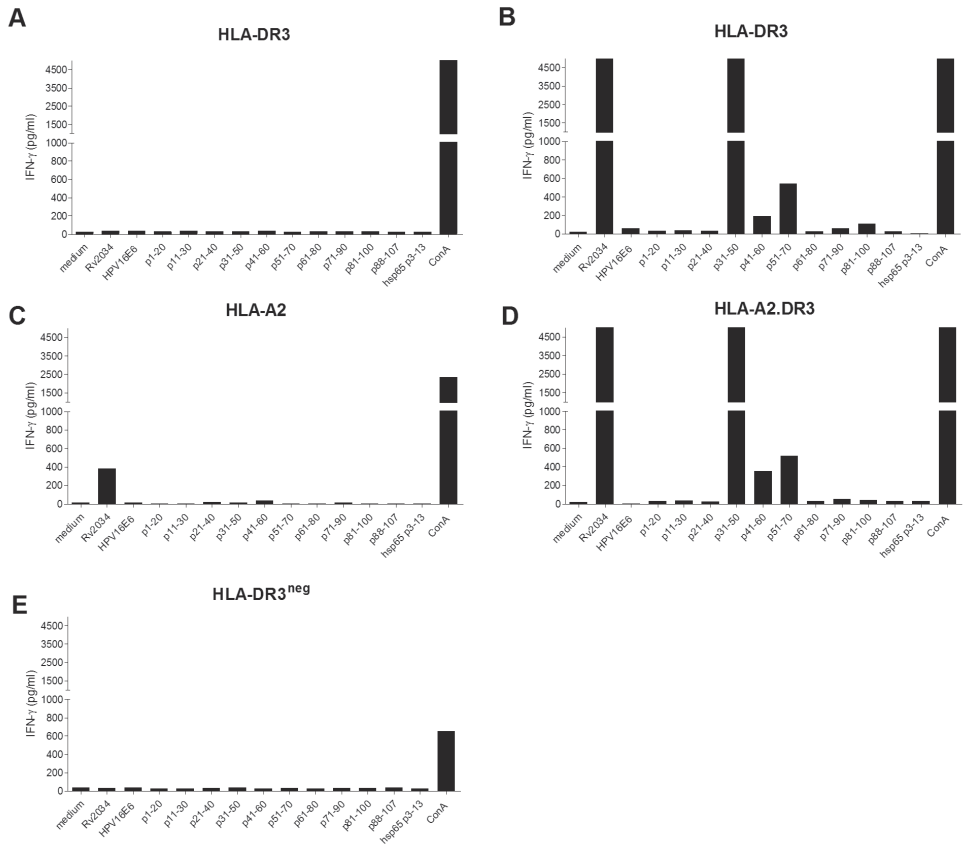


Figure 1. IFN-γ secretion after Rv2034 immunization is HLA-DR3-restricted. HLA-DR3 mice were immunized three times with CpG alone (**A**) or Rv2034 recombinant protein in CpG (**B**). In addition HLA-A2 (**C**), HLA-A2.DR3 (**D**) or HLA-DR3^{neg} (**E**) mice were immunized three times with Rv2034 recombinant protein in CpG. IFN-γ secretion was analyzed by ELISA after 5 days *in vitro* stimulation of splenocytes with Rv2034 protein (10 μg/ml), 10 synthetic Rv2034 peptides (20-mers overlapping 10 amino acids; 10 μg/ml), mitogen (conA; positive control), HPV16E6 recombinant protein (10 μg/ml as negative protein control) and HLA-DR3-restricted epitope hsp65 p3-13 (10 μg/ml as negative peptide control). All groups included five mice. All mice were separately analyzed. Results are shown for one animal, representative for each test group.

Table 1. Rv2034 overlapping 20-mers and 30-mer

peptide	amino acid sequence
p1-20[a]	MSTYRSPDRAWQALADGTRR
p11-30	WQALADGTRRAIVERLAHGP
p21-40	AIVERLAHGPLAVGELARDL
p31-50	LAVGELARDLPVSRPAVSQH
p41-60	PVSRPAVSQHLKVLKTARLV
p51-70	LKVLKTARLVCDRPAGTRRV
p61-80	CDRPAGTRRVYQLDPTGLAA
p71-90	YQLDPTGLAALRTDLDRFWT
p81-100	LRTDLDRFWTRALTGYAQLI
p88-107	FWTRALTGYAQLIDSEGDDT
p31-60	LAVGELARDLPVSRPAVSQHLKVLKTARLV

[a] p: peptide

Rv2034 peptide immunization in HLA-DR3 mice

Synthetic long peptides (SLP) are able to induce $CD4^+$ and $CD8^+$ T cells more efficiently than shorter epitopes [23]. SLP require professional APC such as dendritic cells (DCs) for antigen processing and presentation which increases their vaccine efficacy significantly compared to shorter peptides that are loaded directly onto MHC class II molecules [24]. Therefore, we synthesized the 30-mer sequence Rv2034 p31-60 covering the three immunogenic Rv2034 peptides (p31-50, p41-60, p51-70) and used this to immunize HLA-DR3 mice (Figure 2A). Immunization of HLA-DR3 mice with SLP p31-60 induced efficient responses to Rv2034 p31-50 and p31-60 but also to the Rv2034 protein.

Most current immunization strategies aim at priming immune responses against antigens and epitopes that already are dominant T-cell targets during natural infection. A broader T-cell repertoire including T cells specific for subdominant epitopes not recognized during natural infection, may, when primed sufficiently through immunization, offer additional protection. Peptide epitope-based vaccines can circumvent the propensity of the immune system to focus on immunodominant epitopes by simultaneously targeting the response to dominant as well as subdominant epitopes [25]. Thus, we also used Rv2034 peptide pool composed of ten overlapping peptides with or without the immunodominant p31-50 to immunize HLA-DR3 mice. Rv2034 peptide pool immunization induced responses to the same three core p31-50 related peptides as did the whole Rv2034 protein (Figure 2B). Instead, immunization with a pool of Rv2034 peptides lacking p31-50 induced IFN-γ responses to p41-60, p51-70 and p31-60 but did not result in IFN-γ responses to any of the other peptides, indicating that Rv2034 does not contain any subdominant epitopes in the context of HLA-DR3 (Figure 2C).

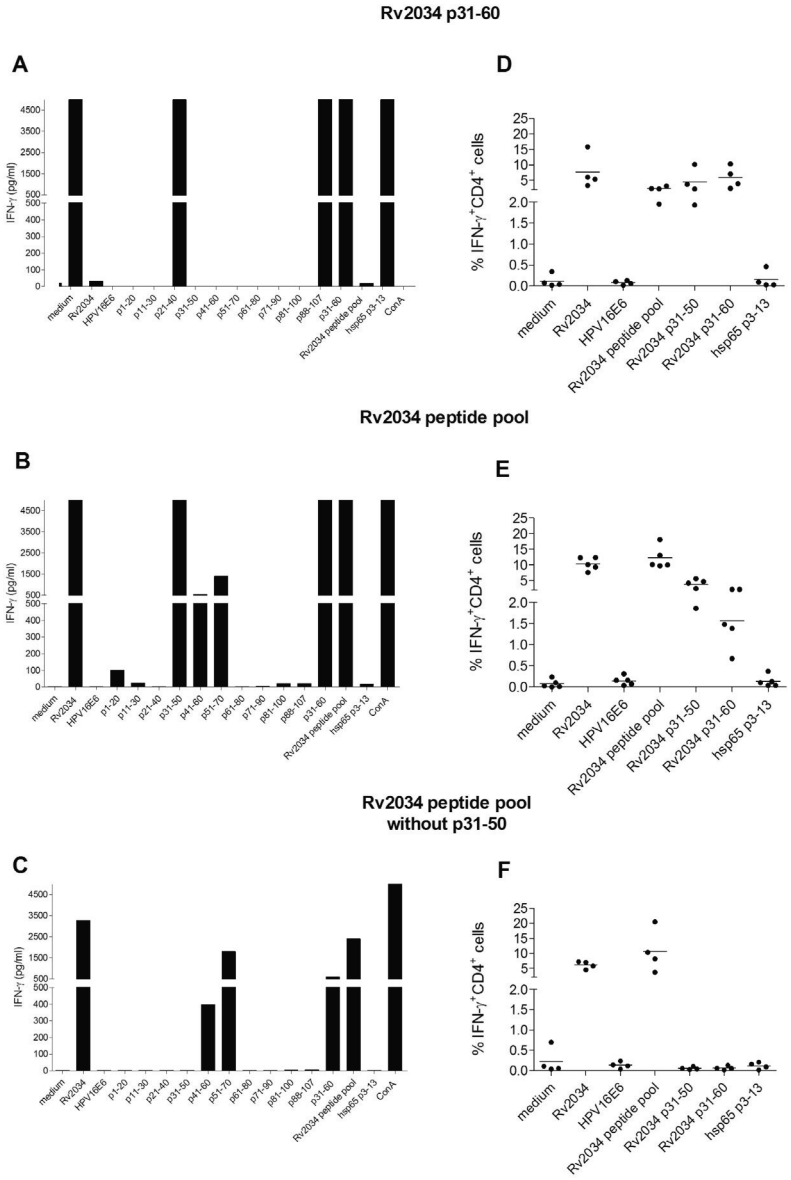


Figure 2. IFN-γ production after Rv2034 peptide immunization. HLA-DR3 mice were immunized three times with Rv2034 30-mer p31-60 in CpG (**A** and **D**), Rv2034 peptide pool composed of ten 20-mers overlapping 10 amino acids (10 μg/ peptide) including p31-50 in CpG (**B** and **E**) or without p31-50 in CpG (**C** and **F**). IFN-γ secretion was analyzed by ELISA (**A**, **B**, **C**) after 5 days *in vitro* stimulation of splenocytes with Rv2034 protein or peptides, control protein HPV16E6, control peptide hsp16 p3-13 (all 10 μg/ml) or control mitogen (1 μg/ml). Intracellular IFN-γ production by $CD4^+$ T cells was measured (**D**, **E**, **F**) after *in vitro* stimulation of splenocytes of immunized mice with the same antigen as used for immunization. After 7 days, cells were incubated with medium or fresh peptides for 1h before addition of brefeldin A and analysis for intracellular IFN-γ production. No IFN-γ was detected in $CD8^+$ T cells (data not shown). All groups included five mice. All mice were separately analyzed.

Since T-cell subtypes differ in their ability to induce protection against *Mtb*, we identified the phenotype of Rv2034-responsive T cells: splenocytes of HLA-DR3 mice immunized with Rv2034 peptide or peptide pools were stimulated *in vitro* for 7 days. Subsequently, intracellular IFN-γ production was assessed by flow cytometry analysis (Figure 2D-F). Rv2034 peptide pool immunization of HLA-DR3 mice induced IFN-γ production by $CD4^+$ T cells after *in vitro* stimulation with Rv2034 protein, Rv2034 peptides and peptide pool, whereas no responses were observed in mice that were injected with CpG alone nor in HLA-DR3-negative littermates and HLA-A2 mice (data not shown). The control protein HPV16E6 and HLA-DR3-restricted peptide hsp65 p3-13 did not induce IFN-γ in any of the immunized mice. As expected, immunization with the peptide pool lacking the dominant peptide Rv2034 p31-50 did not induce any responses to p31-50 or p31-60, whereas immunization with 30-mer p31-60 induced strong responses to the whole protein as well. $CD8^+$ T cells did not produce IFN-γ following *in vitro* peptide re-stimulation (data not shown) confirming $CD4^+$ T cell-specificity for the HLA-DR3-restricted response to Rv2034.

Induction of polyfunctional T cells in response to Rv2034

Antigen-specific IFN-γ production is often used as a proxy biomarker of vaccine-induced and protection-associated Th1 responses. More recently, the induction of polyfunctional $CD4^+$ Th1 cells was found to correlate better with vaccine-induced protection [26] in several model settings, even though the value of polyfunctional T cells as biomarkers of protection in *Mtb* infection remains questionable [27-29]. To estimate the frequency of polyfunctional T cells in the Rv2034-immunized HLA-DR3 mouse vaccine model, intracellular production of TNF, IL-2 and IFN-γ was assessed simultaneously using polychromatic flow cytometry (Figure 3). Splenocytes of immunized HLA-DR3 mice that were *in vitro* restimulated with medium mainly showed TNF^+ (0.5%) single positive and IFN-γ^+ (0.12%) single positive $CD4^+$ T cells. *In vitro* restimulation with Rv2034 protein and Rv2034 p31-60 increased the number of IFN-γ^+ $CD4^+$ T cells to 1.28% and 1.93%, and induced 0.71 % and 0.54 % double (IFN-γ^+/TNF^+) positive $CD4^+$ T cells, respectively. The percentages of triple (IFN-γ^+/IL-2^+/TNF^+) positive $CD4^+$ T cells, however, remained low. These data indicate that immunization of HLA-DR3 mice with Rv2034 or Rv2034 p31-60 induces a significant proportion of single and dual functional T cells specific for Rv2034.

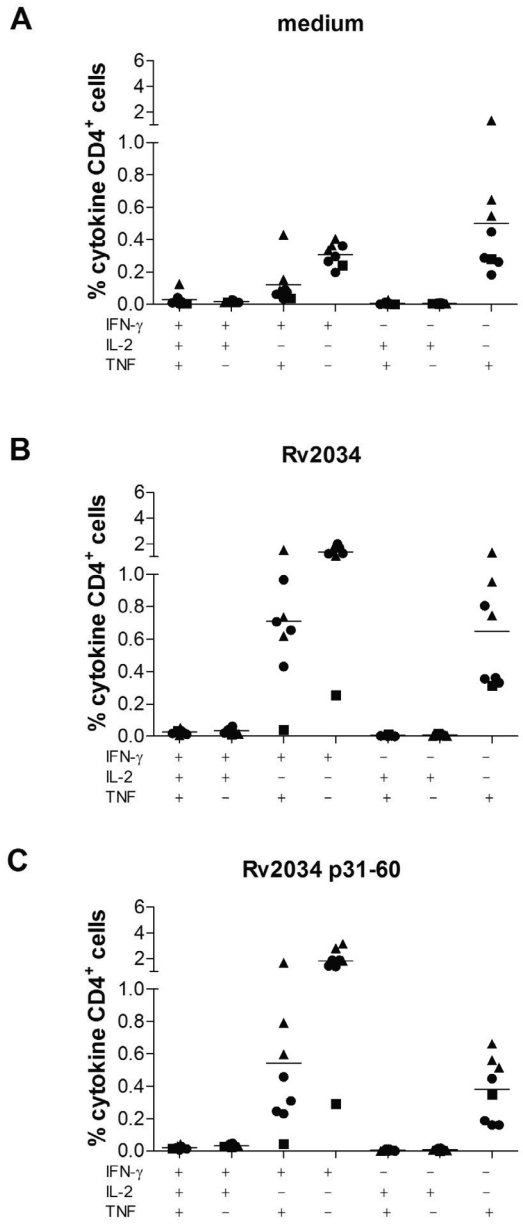


Figure 3. Frequency of polyfunctional CD4⁺ T cells. Percentage of IFN-γ, IL-2 and/or TNF producing CD4⁺ T cells in splenocytes of HLA-DR3 mice immunized with Rv2034 protein or Rv2034 p31-60 in CpG and re-stimulated *in vitro* with medium (**A**), Rv2034 protein (**B**) or Rv2034 p31-60 (**C**). Each symbol represents one mouse immunized with Rv2034 protein (●), Rv2034 p31-60 (▲) or unimmunized (■). The total number of CD4⁺ T cells analyzed in immunized mice was 56,000. Only CD4⁺ populations of > 5 x 10^4 events were analyzed.

Rv2034 immunization induces antibodies directed against *Mtb*

Since we recently showed that even mycobacterial peptides can induce specific antibody responses *in vivo* in HLA-A2 mice [20] and HLA-DR3 [19], we also analyzed the humoral response induced by the Rv2034 protein. A high antibody titer against Rv2034 protein was observed after Rv2034/CpG immunization of HLA-DR3 mice (Figure 4). Mock-immunized mice did not show any antibody reactivity, nor were antibodies detected in Rv2034-immunized mice against an unrelated recombinant protein HPV16 E6. Finally, no antibodies were generated in Rv2034-immunized HLA-A2 mice. These data indicate that Rv2034 protein induces strong cellular as well as humoral immune responses *in vivo*.

Effect of the use of different TLR ligands as adjuvants for Rv2034 immunization

Besides the *Mtb* antigen, the adjuvant used also plays a major role in the induction of an effective Th1/Th2 balance and subsequent protection *in vivo* [30]. In order to estimate the adjuvant-effect on protection-associated Th1 immunity, HLA-DR3 mice were immunized three times with identical amounts of Rv2034 protein adjuvanted with different TLR ligands: PAM3CysK4 (TLR2), Poly(I:C) (TLR3), MPL/TDM (TLR4), Flagellin (TLR5) and R848 (Imidazoquinoline; TLR7). The TLR9 ligand (CpG) as well as the TLR3 ligand, Poly(I:C), induced strong Th1 responses as measured by IFN-γ^+CD4$^+$ T cells, whereas the TLR4 ligand, MPL/TDM, induced detectable but significantly reduced numbers of IFN-γ^+ CD4$^+$ T cells in response to Rv2034 (Figure 5A). TLR2, 5 and 7 ligands did not induce IFN-γ^+ CD4$^+$ T cells at all. In none of the conditions CD8$^+$ IFN-γ^+ T cells were detected (data not shown). In contrast, analyses of humoral responses induced by immunization with various adjuvants showed that all different TLR ligands induced strong, Rv2034-specific antibody responses (Figure 5B), indicating a clear separation of these TLR specific adjuvants on the induction of humoral vs. cellular immune responses at least to *Mtb* antigens.

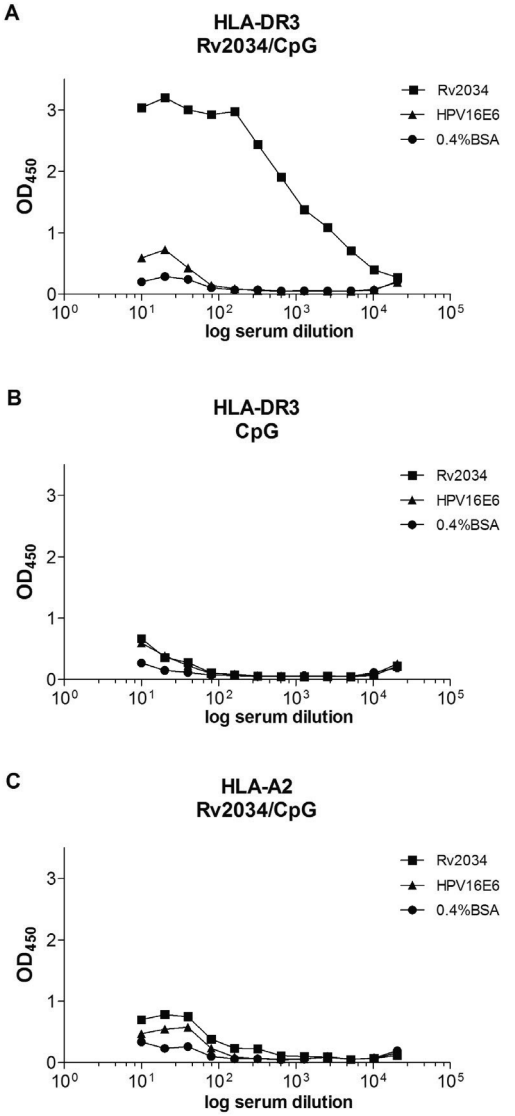


Figure 4. Quantification of serum antibodies to Rv2034. Following immunization of HLA-DR3 mice with Rv2034 in CpG (**A**) or with CpG alone (**B**) antibody titer (OD_{450}) against Rv2034 (■) was determined by ELISA. As a specificity control HPV16E6 (▲) or 0.4% BSA in PBS alone (●) were tested. Rv2034/CpG immunization of HLA-A2 mice were included as negative control (**C**). Serum dilutions are shown on the *x*-axis. All groups included five mice. All mice were separately analyzed.

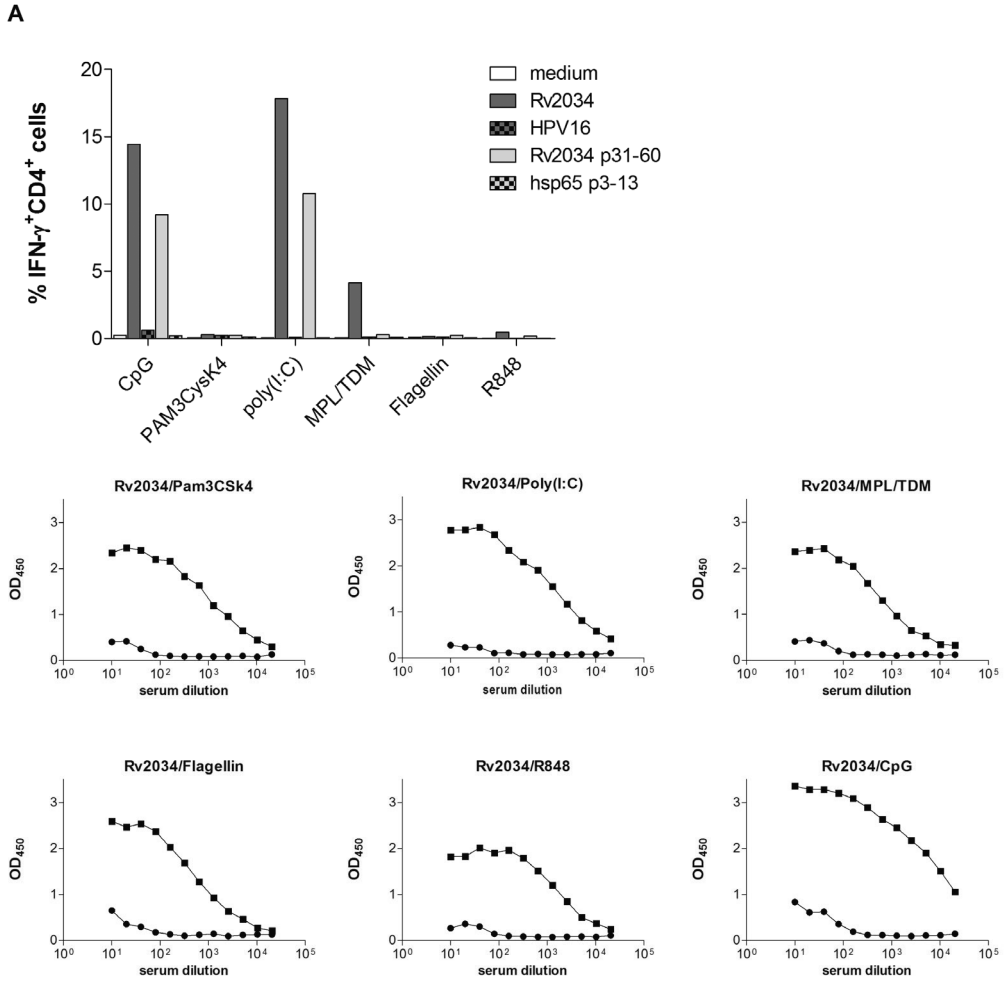


Figure 5. Rv2034 immunization of HLA-DR3 mice using different adjuvants. (**A**) HLA-DR3 mice were immunized three times with Rv2034 protein in either PAM3CysK4, Poly(I:C), MPL/TDM, Flagellin, R848 or CpG. Splenocytes were stimulated *in vitro* with Rv2034 protein, HPV16E6 (control recombinant protein), Rv2034 p31-60 or hsp65 p3-13 (control HLA-DR3-restricted peptide). After 7 days, cells were incubated with medium or fresh antigen for 1h before addition of brefeldin A and analyzed for intracellular IFN-γ production. Adjuvants are indicated on the *x*-axis.
(**B**): Sera of HLA-DR3 mice immunized as above or with CpG only were used to determine antibody titers (OD_{450}) by ELISA against Rv2034 (■) or BSA (0.4% in PBS) alone (●). Adjuvants are indicated above each figure. Serum dilutions are shown on the *x*-axis. Groups included 4-5 mice. Results are shown for one animal, representative for each test group.

Protective efficacy of Rv2034 immunization against live *Mtb* challenge

To assess the vaccine potential of the Rv2034 protein adjuvanted by CpG, its prophylactic protective effect was evaluated in a live *Mtb* challenge model, by enumerating the colony forming units (CFU) in the lungs. Interestingly, Rv2034 immunization reduced the number of CFU in the lungs by almost one log (from $6x10^6$ to $9x10^5$) whereas BCG caused a reduction of over a log to $1.8x10^5$ CFU (Figure 6). As a reference, recombinant hybrid protein Ag85-ESAT6 (H1) [31] adjuvanted in CpG was used as well: immunization with H1/CPG decreased the number of *Mtb* bacteria in the lung to a similar extent as Rv2034 (to $5x10^5$) being slightly less effective than BCG. The combination of BCG followed by Rv2034/CpG immunization 6 weeks afterwards also resulted in a decreased CFU load, but did not lead to an improvement compared to BCG alone. Importantly, in HLA-DR3^{neg} mice Rv2034 vaccination did not reduce the number of CFU, confirming the HLA-DR3-restriction of the T-cell mediated protection.

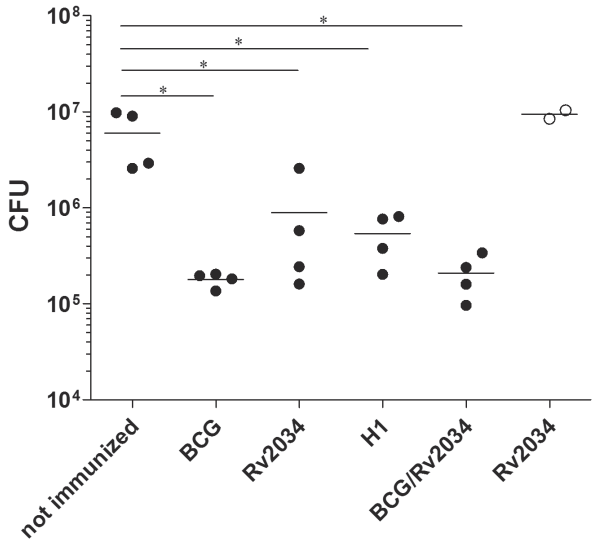


Figure 6. Determination of colony forming units (CFU) in the lungs of *Mtb*- infected mice. CFU were determined in lung homogenates derived from *Mtb* infected HLA-DR3 mice (●) or HLA-DR3^{neg} (○) mice that were left unimmunized or had been immunized with BCG, Rv2034 in CpG, H1 in CpG or immunized with BCG followed by a boost with Rv2034 in CpG as indicated on the *x*-axis. Protective efficacies are expressed as log10 bacterial counts. All groups included 4 mice.

Discussion

New vaccines against TB need to induce immune responses that target *Mtb* at the primary site of infection, which is the lung. Thus, *Mtb* antigens *in vivo* expressed during pulmonary *Mtb* infection (IVE-TB) might represent interesting vaccine candidates. In this study we have analyzed one of our newly identified IVE-TB proteins, the ArsR protein Rv2034, and show that it is strongly immunogenic *in vivo* in HLA-DR transgenic mice. Immunization of HLA-DR3 mice with Rv2034 induced specific cellular as well as humoral immunity against the protein and its dominant HLA-DR3-restricted epitope p31-50. Importantly, prophylactic immunization of HLA-DR3 mice with Rv2034 in CpG induced a significant protective response to *Mtb* which was similar to BCG, as judged by decreased bacterial numbers upon *Mtb* challenge. Importantly, previous work showed that Rv2034-specific T cells were induced by *Mtb* infection in mice and humans [4], supporting a biological role for Rv2034 in *in vivo* infection and rendering it an interesting vaccine target antigen.

Recently, we have shown that immunization of HLA-DR3 mice with an *Mtb* polyepitope in CpG induced strong cellular as well as humoral (antigen-specific Ig) responses [19]. Similar to Rv2034 immunization, this *Mtb* polyepitope immunization caused a significant reduction of bacterial load upon *Mtb* challenge. In contrast to the role of T cells, the role of *Mtb* antigen-specific antibodies in the control of *Mtb* remains largely unknown and has long been debated [32;33]. Antibodies can induce multiple immune-modulatory effects which can play a role in reducing the bacterial load such as mediating effector functions via binding to Fc receptors on immune cells, including activation of T-cell immunity and antibody-dependent cell-mediated cytotoxicity (ADCC) [34]. Indeed, several studies showed that immunoglobulins mediate protection against TB [35-37], whereas the organs of mice that are deficient for humoral immunity [μ-chain knock out (B cell$^{-/-}$ or Ig^{-}) and γ-chain subunit knock out (Fcγ-chain$^{-/-}$)] displayed 3- to 8-fold elevated numbers of viable bacilli compared with normal littermates, while splenic IFN-γ responses to whole antigen were unimpaired [38-40]. Moreover, in *Mtb* infected non-human primates active B cells were present in clusters within granulomas. Furthermore, high levels of *Mtb* antigen specific antibodies were detected within *Mtb* infected tissues, indicating that B cells play a role in *Mtb* infection [41]. Thus, Rv2034 antibody responses may substantially contribute to protection against *Mtb* infection.

Studies on animal models have shown that polyfunctional T cells which secrete IFN-γ, TNF-α and IL-2 simultaneously, are functionally superior in vaccine-induced protection and also often induced in *Mtb* vaccine studies [31;42-44]. In our current study, Rv2034 immunization of HLA-DR3 mice induced strong IFN-γ^{+}/TNF^{+} and IFN-γ^{+} CD4^{+} T cells specific for Rv2034, but no IFN-γ^{+}/TNF^{+}/IL-2^{+} CD4^{+} T cells. Importantly, control cytokine producing cells (MiCK-1) displayed IL-2 production demonstrating that IL-2 was detectable using this assay. Thus, despite the lack of substantial numbers of triple positive (IFN-γ^{+}/TNF^{+}/IL-2^{+}) CD4^{+} T cells, Rv2034 immunization reduced the number of bacteria in HLA-DR3 mice, but not in HLA-DR3^{neg} littermates or HLA-A2 mice. Therefore, it remains uncertain whether the presence of IFN-γ^{+}/TNF^{+}/IL-2^{+} CD4^{+} T cells

is an absolute requirement for vaccine-induced protection against *Mtb*. In view of our data, it is of note that the described *Mtb* vaccine studies also identified double and single cytokine producing $CD4^+$ T cells [31;42-44]. Interestingly, it is uncertain whether polyfunctional T cells correlate with TB protection [27-29]. Multiple factors are suggested to be involved in development or detection of polyfunctional T cells such as antigen dose and time point of T-cell analysis [28;45;46]. Also, previous work showed that the frequency of IFN-γ producing $CD4^+$ T cells does not directly associate to vaccine-induced protection [47] or might even be irrelevant in killing *Mtb* [48;49]. This further indicates that other (yet unknown) mechanisms are involved in controlling *Mtb* infection [50;51].

The use of TLR9 ligand as adjuvant in mouse models has shown efficient induction of both humoral and cellular immunity [19;20;52]. Of note is that the distribution of TLR9 in mice differs from that in humans being either broadly expressed on all types of DC in C57BL/6 mice or restricted to plasmacytoid DC in humans [53]. Out of the TLRs tested in this study that are broadly expressed on several DC types of both species (TLR2, 3, 4, 5 and 7) [54], only poly(I:C) (TLR3) and to a lesser extent MPL/TDM (TLR 4) induced Th1 responses. In contrast, regardless of the TLR ligand used as adjuvant for Rv2034 immunization in this study (TLR2, 3, 4, 5, 7 or 9), strong Ig responses were observed specific for Rv2034. Thus, these findings clearly show the separation of the effect on humoral and cellular responses by these adjuvants. Similar data have been reported in a single long peptide vaccination model in which all different adjuvants activated DC *in vitro* but only MPL and CpG (TLR4 and TLR9) induced a strong functional T-cell response *in vivo* [30]. This effect depended mainly on the capacity of ligands to mature pro-inflammatory DC and the duration of their *in vivo* stimulatory effect.

Besides the choice of adjuvant, the route of administration might play an important role in the development of robust Th1 immunity upon immunization [30;55]. In this study, adjuvanted *Mtb* antigens were administered subcutaneously representing a custom vaccination route in human vaccination programs.

Immunization with Rv2034/CpG resulted in decreased bacterial load in the lung after *Mtb* challenge, which was dependent on induced HLA-DR3-restricted T-cell immunity. Although Rv2034 immunization resulted in reduction of CFU, boosting BCG with Rv2034 did not further improve the protective effect of BCG alone. In addition, immunization with Ag85B/ESAT6 (H1) in CpG decreased the number of *Mtb* bacteria in the lung to a similar extent as Rv2034, slightly less than the BCG induced reduction. Interestingly, fusion protein H1, Ag85B/ESAT6/Rv2660 (H56), Ag85B/TB10.4 (HyVac4) and Mtb10.4/HspX (MH) showed an improved CFU reduction when used as BCG booster [44;56;57]. However, when administered as a subunit vaccine alone, the fusion proteins showed less protection compared to BCG, in line with previous findings [44;57] and this study. Thus, since Rv2034 is highly expressed in the lungs during *Mtb* infection, incorporating Rv2034 in a fusion protein may induce efficient protection when used as BCG booster. Rv2034 may thus have potential prophylactic and therapeutic vaccine properties when combined with early stage and TB latency proteins into a multistage vaccine [44], which should be capable of targeting relevant different stages

of the *Mtb* infection cycle. Additionally, the immunogenic peptide may also be integrated in a poly-epitope vaccine, containing multiple immunogenic epitopes from different *Mtb* proteins [19].

In summary, we have shown that the IVE-TB protein Rv2034 is immunogenic *in vivo* and has protective efficacy as evidenced by its ability to reduce the bacterial load *in vivo* after a live challenge with *Mtb*. Thus Rv2034 represents an interesting new antigen for subunit TB vaccination.

Acknowledgements

This study was supported by FP7 NEWTBVAC project contract no. LSHP-CT-2003-503367 (the text represents the authors' views and does not necessarily represent a position of the Commission who will not be liable for the use made of such information), the Bill and Melinda Gates Foundation Grand Challenges in Global Health (GC6#74), TI Pharma (project D-101-1) and ISA Pharmaceuticals.

Conflict of interest. The authors declare to have no financial/ commercial conflicts of interests. TO is co-inventor of an *Mtb* latency antigen patent, which is owned by LUMC.

References

1. **WHO**. Global Tuberculosis Report 2012. 2012.
2. **Trunz,B.B., Fine,P., and Dye,C.,** Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *Lancet* 2006. **367**: 1173-1180.
3. **Fine,P.E.,** Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* 1995. **346**: 1339-1345.
4. **Commandeur,S., van Meijgaarden,K.E., Prins,C., Pichugin,A.V., Dijkman,K., van den Eeden,S.J., Friggen,A.H., Franken,K.L., Dolganov,G., Kramnik,I., Schoolnik,G.K., Oftung,F., Korsvold,G.E., Geluk,A., and Ottenhoff,T.H.,** An unbiased genome-wide Mycobacterium tuberculosis gene expression approach to discover antigens targeted by human T cells expressed during pulmonary infection. *J.Immunol.* 2013. **190**: 1659-1671.
5. **Busenlehner,L.S., Pennella,M.A., and Giedroc,D.P.,** The SmtB/ArsR family of metalloregulatory transcriptional repressors: Structural insights into prokaryotic metal resistance. *FEMS Microbiol. Rev.* 2003. **27**: 131-143.
6. **Gao,C.H., Yang,M., and He,Z.G.,** Characterization of a novel ArsR-like regulator encoded by Rv2034 in Mycobacterium tuberculosis. *PLoS.One.* 2012. **7**: e36255.
7. **Guo,M., Feng,H., Zhang,J., Wang,W., Wang,Y., Li,Y., Gao,C., Chen,H., Feng,Y., and He,Z.G.,** Dissecting transcription regulatory pathways through a new bacterial one-hybrid reporter system. *Genome Res.* 2009. **19**: 1301-1308.
8. **Gao,C.H., Yang,M., and He,Z.G.,** An ArsR-like transcriptional factor recognizes a conserved sequence motif and positively regulates the expression of phoP in mycobacteria. *Biochem.Biophys.Res.Commun.* 2011. **411**: 726-731.
9. **Betts,J.C., Lukey,P.T., Robb,L.C., McAdam,R.A., and Duncan,K.,** Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol.Microbiol.* 2002. **43**: 717-731.
10. **Rustad,T.R., Harrell,M.I., Liao,R., and Sherman,D.R.,** The enduring hypoxic response of Mycobacterium tuberculosis. *PLoS.One.* 2008. **3**: e1502.
11. **Deb,C., Lee,C.M., Dubey,V.S., Daniel,J., Abomoelak,B., Sirakova,T.D., Pawar,S., Rogers,L., and Kolattukudy,P.E.,** A novel in vitro multiple-stress dormancy model for Mycobacterium tuberculosis generates a lipid-loaded, drug-tolerant, dormant pathogen. *PLoS.One.* 2009. **4**: e6077.
12. **Keren,I., Minami,S., Rubin,E., and Lewis,K.,** Characterization and transcriptome analysis of Mycobacterium tuberculosis persisters. *MBio.* 2011. **2**: e00100-e00111.
13. **Osman,D. and Cavet,J.S.,** Bacterial metal-sensing proteins exemplified by ArsR-SmtB family repressors. *Nat.Prod.Rep.* 2010. **27**: 668-680.
14. **Geluk,A., Taneja,V., van Meijgaarden,K.E., Zanelli,E., Abou-Zeid,C., Thole,J.E., de Vries,R.R., David,C.S., and Ottenhoff,T.H.,** Identification of HLA class II-restricted determinants of Mycobacterium tuberculosis-derived proteins by using HLA-transgenic, class II-deficient mice. *Proc.Natl. Acad.Sci.U.S.A* 1998. **95**: 10797-10802.
15. **Newberg,M.H., Smith,D.H., Haertel,S.B., Vining,D.R., Lacy,E., and Engelhard,V.H.,** Importance of MHC class 1 alpha2 and alpha3 domains in the recognition of self and non-self MHC molecules. *J.Immunol.* 1996. **156**: 2473-2480.
16. **Franken,K.L., Hiemstra,H.S., van Meijgaarden,K.E., Subronto,Y., den,H.J., Ottenhoff,T.H., and Drijfhout,J.W.,** Purification of his-tagged proteins by immobilized chelate affinity

chromatography: the benefits from the use of organic solvent. *Protein Expr.Purif.* 2000. **18**: 95-99.

17. **Commandeur,S., Lin,M.Y., van Meijgaarden,K.E., Friggen,A.H., Franken,K.L., Drijfhout,J.W., Korsvold,G.E., Oftung,F., Geluk,A., and Ottenhoff,T.H.,** Double- and monofunctional CD4(+) and CD8(+) T-cell responses to Mycobacterium tuberculosis DosR antigens and peptides in long-term latently infected individuals. *Eur.J.Immunol.* 2011. **41**: 2925-2936.
18. **Juffermans,N.P., Leemans,J.C., Florquin,S., Verbon,A., Kolk,A.H., Speelman,P., van Deventer,S.J., and van der Poll,T.,** CpG oligodeoxynucleotides enhance host defense during murine tuberculosis. *Infect.Immun.* 2002. **70**: 147-152.
19. **Geluk,A., van den Eeden,S.J., van Meijgaarden,K.E., Dijkman,K., Franken,K.L., and Ottenhoff,T.H.,** A multistage-polyepitope vaccine protects against Mycobacterium tuberculosis infection in HLA-DR3 transgenic mice. *Vaccine* 2012. **30**: 7513-7521.
20. **Geluk,A., van den Eeden,S.J., Dijkman,K., Wilson,L., Kim,H.J., Franken,K.L., Spencer,J.S., Pessolani,M.C., Pereira,G.M., and Ottenhoff,T.H.,** ML1419c peptide immunization induces Mycobacterium leprae-specific HLA-A*0201-restricted CTL in vivo with potential to kill live mycobacteria. *J.Immunol.* 2011. **187**: 1393-1402.
21. **Geluk,A., van Meijgaarden,K.E., Franken,K.L., Drijfhout,J.W., D'Souza,S., Necker,A., Huygen,K., and Ottenhoff,T.H.,** Identification of major epitopes of Mycobacterium tuberculosis AG85B that are recognized by HLA-A*0201-restricted CD8+ T cells in HLA-transgenic mice and humans. *J.Immunol.* 2000. **165**: 6463-6471.
22. **Geluk,A., Bloemhoff,W., de Vries,R.R., and Ottenhoff,T.H.,** Binding of a major T cell epitope of mycobacteria to a specific pocket within HLA-DRw17(DR3) molecules. *Eur.J.Immunol.* 1992. **22**: 107-113.
23. **Quakkelaar,E.D. and Melief,C.J.,** Experience with synthetic vaccines for cancer and persistent virus infections in nonhuman primates and patients. *Adv.Immunol.* 2012. **114**: 77-106.
24. **Melief,C.J. and van der Burg,S.H.,** Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. *Nat.Rev.Cancer* 2008. **8**: 351-360.
25. **Aagaard,C.S., Hoang,T.T., Vingsbo-Lundberg,C., Dietrich,J., and Andersen,P.,** Quality and vaccine efficacy of CD4+ T cell responses directed to dominant and subdominant epitopes in ESAT-6 from Mycobacterium tuberculosis. *J.Immunol.* 2009. **183**: 2659-2668.
26. **Darrah,P.A., Patel,D.T., De Luca,P.M., Lindsay,R.W., Davey,D.F., Flynn,B.J., Hoff,S.T., Andersen,P., Reed,S.G., Morris,S.L., Roederer,M., and Seder,R.A.,** Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat.Med.* 2007. **13**: 843-850.
27. **Harari,A., Rozot,V., Enders,F.B., Perreau,M., Stalder,J.M., Nicod,L.P., Cavassini,M., Calandra,T., Blanchet,C.L., Jaton,K., Faouzi,M., Day,C.L., Hanekom,W.A., Bart,P.A., and Pantaleo,G.,** Dominant TNF-alpha+ Mycobacterium tuberculosis-specific CD4+ T cell responses discriminate between latent infection and active disease. *Nat.Med.* 2011. **17**: 372-376.
28. **Caccamo,N., Guggino,G., Joosten,S.A., Gelsomino,G., Di,C.P., Titone,L., Galati,D., Bocchino,M., Matarese,A., Salerno,A., Sanduzzi,A., Franken,W.P., Ottenhoff,T.H., and Dieli,F.,** Multifunctional CD4(+) T cells correlate with active Mycobacterium tuberculosis infection. *Eur.J.Immunol.* 2010. **40**: 2211-2220.
29. **Sutherland,J.S., Adetifa,I.M., Hill,P.C., Adegbola,R.A., and Ota,M.O.,** Pattern and diversity of cytokine production differentiates between Mycobacterium tuberculosis infection and disease. *Eur.J.Immunol.* 2009. **39**: 723-729.
30. **Welters,M.J., Bijker,M.S., van den Eeden,S.J., Franken,K.L., Melief,C.J., Offringa,R., and van der Burg,S.H.,** Multiple CD4 and CD8 T-cell activation parameters predict vac-

cine efficacy in vivo mediated by individual DC-activating agonists. *Vaccine* 2007. **25**: 1379-1389.

31. **Lindenstrom,T., Agger,E.M., Korsholm,K.S., Darrah,P.A., Aagaard,C., Seder,R.A., Rosenkrands,I., and Andersen,P.,** Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. *J.Immunol.* 2009. **182**: 8047-8055.
32. **Abebe,F. and Bjune,G.,** The protective role of antibody responses during Mycobacterium tuberculosis infection. *Clin.Exp. Immunol.* 2009. **157**: 235-243.
33. **Maglione,P.J. and Chan,J.,** How B cells shape the immune response against Mycobacterium tuberculosis. *Eur.J.Immunol.* 2009. **39**: 676-686.
34. **Joller,N., Weber,S.S., and Oxenius,A.,** Antibody-Fc receptor interactions in protection against intracellular pathogens. *Eur.J.Immunol.* 2011. **41**: 889-897.
35. **Teitelbaum,R., Glatman-Freedman,A., Chen,B., Robbins,J.B., Unanue,E., Casadevall,A., and Bloom,B.R.,** A mAb recognizing a surface antigen of Mycobacterium tuberculosis enhances host survival. *Proc.Natl.Acad. Sci.U.S.A* 1998. **95**: 15688-15693.
36. **Hamasur,B., Haile,M., Pawlowski,A., Schroder,U., Kallenius,G., and Svenson,S.B.,** A mycobacterial lipoarabinomannan specific monoclonal antibody and its F(ab') fragment prolong survival of mice infected with Mycobacterium tuberculosis. *Clin.Exp.Immunol.* 2004. **138**: 30-38.
37. **Balu,S., Reljic,R., Lewis,M.J., Pleass,R.J., McIntosh,R., van,K.C., van,E.M., Challacombe,S., Woof,J.M., and Ivanyi,J.,** A novel human IgA monoclonal antibody protects against tuberculosis. *J.Immunol.* 2011. **186**: 3113-3119.
38. **Vordermeier,H.M., Venkataprasad,N., Harris,D.P., and Ivanyi,J.,** Increase of tuberculous infection in the organs of B cell-deficient mice. *Clin.Exp. Immunol.* 1996. **106**: 312-316.
39. **Maglione,P.J., Xu,J., Casadevall,A., and Chan,J.,** Fc gamma receptors regulate immune activation and susceptibility during Mycobacterium tuberculosis infection. *J.Immunol.* 2008. **180**: 3329-3338.
40. **Maglione,P.J., Xu,J., and Chan,J.,** B cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with Mycobacterium tuberculosis. *J.Immunol.* 2007. **178**: 7222-7234.
41. **Phuah,J.Y., Mattila,J.T., Lin,P.L., and Flynn,J.L.,** Activated B cells in the granulomas of nonhuman primates infected with Mycobacterium tuberculosis. *Am.J.Pathol.* 2012. **181**: 508-514.
42. **Abel,B., Tameris,M., Mansoor,N., Gelderbloem,S., Hughes,J., Abrahams,D., Makhethe,L., Erasmus,M., de,K.M., van der Merwe,L., Hawkridge,A., Veldsman,A., Hatherill,M., Schirru,G., Pau,M.G., Hendriks,J., Weverling,G.J., Goudsmit,J., Sizemore,D., McClain,J.B., Goetz,M., Gearhart,J., Mahomed,H., Hussey,G.D., Sadoff,J.C., and Hanekom,W.A.,** The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults. *Am.J.Respir. Crit Care Med.* 2010. **181**: 1407-1417.
43. **Derrick,S.C., Yabe,I.M., Yang,A., and Morris,S.L.,** Vaccine-induced anti-tuberculosis protective immunity in mice correlates with the magnitude and quality of multifunctional CD4 T cells. *Vaccine* 2011. **29**: 2902-2909.
44. **Aagaard,C., Hoang,T., Dietrich,J., Cardona,P.J., Izzo,A., Dolganov,G., Schoolnik,G.K., Cassidy,J.P., Billeskov,R., and Andersen,P.,** A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nat.Med.* 2011. **17**: 189-194.
45. **Aagaard,C., Hoang,T.T., Izzo,A., Billeskov,R., Troudt,J., Arnett,K., Keyser,A., Elvang,T., Andersen,P., and Dietrich,J.,** Protection and polyfunctional T cells induced by Ag85B-TB10.4/IC31 against Mycobacterium tuberculosis is highly dependent on the antigen dose. *PLoS.One.* 2009. **4**: e5930.

46. **Han,Q., Bagheri,N., Bradshaw,E.M., Hafler,D.A., Lauffenburger,D.A., and Love,J.C.,** Polyfunctional responses by human T cells result from sequential release of cytokines. *Proc. Natl.Acad.Sci.U.S.A* 2012. **109**: 1607-1612.

47. **You,Q., Jiang,C., Wu,Y., Yu,X., Chen,Y., Zhang,X., Wei,W., Wang,Y., Tang,Z., Jiang,D., Wu,Y., Wang,C., Meng,X., Zhao,X., and Kong,W.,** Subcutaneous administration of modified vaccinia virus Ankara expressing an Ag85B-ESAT6 fusion protein, but not an adenovirus-based vaccine, protects mice against intravenous challenge with Mycobacterium tuberculosis. *Scand.J.Immunol.* 2012. **75**: 77-84.

48. **Gallegos,A.M., van Heijst,J.W., Samstein,M., Su,X., Pamer,E.G., and Glickman,M.S.,** A gamma interferon independent mechanism of CD4 T cell mediated control of M. tuberculosis infection in vivo. *PLoS.Pathog.* 2011. **7**: e1002052.

49. **Cowley,S.C. and Elkins,K.L.,** CD4+ T cells mediate IFN-gamma-independent control of Mycobacterium tuberculosis infection both in vitro and in vivo. *J.Immunol.* 2003. **171**: 4689-4699.

50. **Ottenhoff,T.H.,** New pathways of protective and pathological host defense to mycobacteria. *Trends Microbiol.* 2012. **20**: 419-428.

51. **Torrado,E. and Cooper,A.M.,** What do we really know about how CD4 T cells control Mycobacterium tuberculosis? *PLoS.Pathog.* 2011. **7**: e1002196.

52. **Zwaveling,S., Ferreira Mota,S.C., Nouta,J., Johnson,M., Lipford,G.B., Offringa,R., van der Burg,S.H., and Melief,C.J.,** Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. *J.Immunol.* 2002. **169**: 350-358.

53. **Mazzoni,A. and Segal,D.M.,** Controlling the Toll road to dendritic cell polarization. *J.Leukoc.Biol.* 2004. **75**: 721-730.

54. **Kadowaki,N., Ho,S., Antonenko,S., Malefyt,R.W., Kastelein,R.A., Bazan,F., and Liu,Y.J.,** Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J.Exp.Med.* 2001. **194**: 863-869.

55. **Slutter,B., Bal,S.M., Ding,Z., Jiskoot,W., and Bouwstra,J.A.,** Adjuvant effect of cationic liposomes and CpG depends on administration route. *J.Control Release* 2011. **154**: 123-130.

56. **Niu,H., Hu,L., Li,Q., Da,Z., Wang,B., Tang,K., Xin,Q., Yu,H., Zhang,Y., Wang,Y., Ma,X., and Zhu,B.,** Construction and evaluation of a multistage Mycobacterium tuberculosis subunit vaccine candidate Mtb10.4-HspX. *Vaccine* 2011. **29**: 9451-9458.

57. **Billeskov,R., Elvang,T.T., Andersen,P.L., and Dietrich,J.,** The HyVac4 subunit vaccine efficiently boosts BCG-primed anti-mycobacterial protective immunity. *PLoS.One.* 2012. **7**: e39909.

CHAPTER 6

Clonal analysis of the T-cell response to *in vivo* expressed *Mycobacterium tuberculosis* protein Rv2034, using a CD154 expression based T-cell cloning method

Susanna Commandeur, Karin Dijkman, Annemieke H. Friggen, Krista E. van Meijgaarden, Susan J.F. van den Eeden, Louis Wilson, Jolien J. van der Ploeg-van Schip, Kees L.M.C Franken, Annemieke Geluk and Tom H.M. Ottenhoff

Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands

Submitted

Abstract

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), remains a leading cause of death worldwide. A better understanding of the role of $CD4^+$ and $CD8^+$ T cells, which are both important to TB protection, is essential to unravel the mechanisms of protection and to identify the key antigens seen by these T cells. We have recently identified a set of *in vivo* expressed *Mtb* genes (IVE-TB) which is expressed during *in vivo* pulmonary infection in mice, and shown that their encoded antigens are potently recognized by polyclonal T cells from tuberculin skin test-positive, *in vitro* ESAT6/CFP10-responsive individuals. Here we have cloned T cells specific for one of these newly identified *in vivo* expressed *Mtb* (IVE-TB) antigens, Rv2034. T cells were enriched based on the expression of CD154 (CD40L), which represents a new method for selecting antigen-specific (low frequency) T cells independent of their specific function. An Rv2034-specific $CD4^+$ T-cell clone expressed the Th1 markers T-bet, IFN-γ, TNF-α, IL-2 and the cytotoxicity related markers granzyme B and CD107a as measured by flow cytometry. The clone specifically recognized Rv2034 protein, Rv2034 peptide p81-100 and *Mtb* lysate. Remarkably, while the recognition of the dominant p81-100 epitope was HLA-DR restricted, the T-cell clone also recognized a neighboring epitope (p88-107) in an HLA-DR- as well as HLA-DQ1-restricted fashion. Importantly, the T-cell clone was able to inhibit *Mtb* outgrowth from infected monocytes significantly. The characterization of the polyfunctional and *Mtb* inhibitory T-cell response to IVE-TB Rv2034 at the clonal level provides further insights into the potential of IVE-TB antigens as new vaccine candidate antigens in TB.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), accounts for ≥ 1.5 million deaths each year and remains one of the leading causes of death due to infectious disease. There is no efficient vaccine against TB yet, since the only *Mtb* vaccine available, *Mycobacterium bovis* bacille Calmette-Guerin (BCG), induces limited and variable protection against pulmonary TB, the transmissible form of the disease. Novel *Mtb* vaccines, including improved BCG strains, attenuated *Mtb* strains and subunit vaccines, are currently under study [1]. Given the major role of $CD4^+$ and $CD8^+$ T cells during infection with *Mtb*, better understanding of the role and function of these T cells during *Mtb* infection and post (BCG) vaccination is of key importance to developing further improved vaccines [2].

Advanced flow cytometry allows for detailed characterization of specific T-cell subsets. Due to major advances in the development of improved instruments and reagents an increasing number of parameters can be measured simultaneously [3;4]. The measurement of IFN-γ production is generally used to detect *Mtb* antigen-specific T cells. However, this approach is inherently biased towards detecting Th1 cells, and potentially falls short of detecting other antigen specific T-cell subsets. Although in theory the use of T-cell activation markers, such as CD25 and CD69 can circumvent such bias, substantial levels of CD25 and CD69 expression are detected also in unstimulated samples, indicating that their induction is not strictly antigen induced [5;6]. Expression analysis of CD154 (CD40L) may provide a suitable alternative to overcome these issues. CD154 has previously been described as an antigen-specific induced cell surface marker, which is transiently expressed on T cells upon specific antigen recognition via TCR [6;7]. CD154 interacts with CD40, which leads to subsequent activation of APC, driving both humoral and cellular immunity [8;9].

Recently we identified a set of novel *in vivo* expressed *Mtb* (IVE-TB) antigens as possible TB vaccine candidate antigens [10]. One of the IVE-TB encoding genes, *Rv2034*, was found to be expressed during inflammatory pulmonary infection, and its encoded protein was strongly recognized by T cells from mycobacteria exposed individuals [10]. Moreover, vaccination of HLA-transgenic mice with Rv2034 protein significantly reduced *Mtb* load (Commandeur *et al.* unpublished data). Understanding the immunological response to IVE-TB antigens, as well as to early-phase expressed (*e.g.* ESAT-6) or late phase-expressed (*e.g.* DosR regulon and Rpf) proteins [11;12] is important to the development of better TB vaccines and TB biomarkers. Therefore we performed a more detailed analysis of the T-cell response to *Mtb* IVE-TB antigen Rv2034 using an Rv2034 specific $CD4^+$ T-cell clone that was generated using a novel CD154-expression based cell selection method described here, and analyzed both its specificity and phenotype. The T-cell clone was specific for an HLA-DR restricted epitope in Rv2034 p81-100. Surprisingly, this was neighbored (p88-107) by a both HLA-DR and HLA-DQ presentable epitope, and in agreements with this the p81-107 sequence was found to possess multiple promiscuous MHC class II binding

features. Furthermore, the T-cell clone recognized *Mtb* lysate, and expressed the Th1 markers T-bet, IFN-γ, TNF-α and IL-2 and cytotoxic markers granzyme B and CD107a, consistent with a Th1 phenotype that co-expresses cytotoxicity granule markers. Importantly, the T-cell clone was able to inhibit *Mtb* outgrowth from infected monocytes.

Materials and methods

Recombinant proteins. Recombinant (fusion) proteins were produced as previously described [13]. In short, gene amplified PCR products were cloned by Gateway Technology (Invitrogen, San Diego, CA, USA) in a bacterial expression vector containing an N-terminal hexa-histidine (His) tag. Generated vectors were sequenced to confirm correct insertion of the product. Recombinant proteins were overexpressed in *Escherichia coli* strain BL21 (DE3) and further purified. The size and purity of the proteins were analyzed by gel electrophoresis, using Coomassie brilliant blue and Western blotting using an anti-His antibody (Invitrogen, Carlsbad, CA, USA). Endotoxin contents were below 50 EU (endotoxin unit) per mg of recombinant protein as tested using a Limulus Amebocyte Lysate (LAL) QCL-1000 assay (Lonza Inc., Basel, Switzerland). Recombinant proteins were tested to exclude protein non-specific T-cell stimulation and cellular toxicity in IFN-γ release assays using PBMC of *in vitro* PPD-negative, healthy Dutch donors recruited at the Blood Bank Sanquin, Leiden, The Netherlands [14;15]. None of these controls had experienced any known prior contact with TB patients.

Study subjects. Blood samples were collected by venipuncture. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Ficoll density gradient centrifugation and stored in liquid nitrogen until further use.

Ethical Statement. Donors gave written informed consent before blood donation. The study protocol (P207/99) was approved by the Institutional Review Board of the Leiden University Medical Center.

Synthetic peptides. Rv2034 20-mer peptides with 10 aa overlap were synthesized by Peptide 2.0 Inc. (Chantilly, VA, USA) [16] Generated peptides were analyzed by HPLC to determine purity of peptide products (> 75%). Mass-spectrometry was performed to determine the molecular weight of the peptides.

Enzymatic digestion of* Mtb *lysate. *Mtb* lysate was generated as previously described [10]. To generate hypoxic *Mtb* lysate, H37Rv was grown to late log phase under a flow of nitrogen containing oxygen tension for 24 hours in a shaking incubator at 37 °C [17]. To generate starvation *Mtb* lysate, H37Rv was grown to log phase, washed and incubated for an additional 7 days in PBS

at 37 °C [18]. *Mtb* lysates were treated with Proteinase K (Promega, Madison, WI, USA) and Lysozyme (Sigma Aldrich, St. Louis, MO, USA), both at a final concentration of 5 µg/ml for 1 hour at 37 °C in a shaking incubator. Subsequently, the enzymes were inactivated for 90 minutes at 99 °C.

Lymphocyte stimulation test. PBMC (1.5*10^5) were cultured in triplicates in 96-well round-bottom plates (Nunc, Roskilde, Denmark) and incubated with or without protein (10 µg/ml) in a final volume of 200 µl AIM-V medium (Invitrogen, Breda, Netherlands) at 37°C and 5% CO_2. After 6 days, supernatants were harvested and used for IFN-γ determination. Subsequently, cells were pulsed with [^{3}H]-thymidine (0.5 µCi/well) and incubated 16-18 hours at 37°C and 5% CO_2. Incorporation of [^{3}H]-thymidine was measured using a Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter (Wallac, Turku, Finland).

Generation of CD154 specific T-cell clones. PBMC (0.5-1*10^6) were seeded in 96-wells round-bottom plates together with peptide pool mix (1 µg/ml per peptide) in Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Invitrogen, Breda, the Netherlands) containing 10% pooled human serum (HuS) and human IL-2 (Proleukin; Novartis Pharmaceuticals UK Ltd., Horsham, UK) at a final concentration of 100 IU/ml and incubated for 1 week at 37°C and 5% CO_2. After 1 week, the cells were restimulated with peptide pool (1 µg/ml per peptide) and autologous PBMC (0.5-1*10^6) in IMDM containing 10% HuS and 1:100 diluted anti-CD40 antibody (clone HB14, Miltenyi Biotec, Auburn, CA, USA) and incubated 16 hours at 37°C and 5% CO_2. CD154 positive cells were sorted and seeded in 96-wells round-bottom plates (0.3, 1 and multiple cell(s)/well) containing IMDM, irradiated (3000 rad) feeders (5*10^4 cells/well), IL-2 (100 IU/ml), 10% HuS and phytohemagglutinin (PHA) at a final dilution of 4 µg/ml (Remel, Oxoid, Haarlem, the Netherlands). After one week IL-2 was added (100 IU/ml) and cultured for approximately another 1-2 weeks until clonal expansion was established.

Magnetic-activated Cell Sorting (MACS) of CD154 positive cells. Stimulated PBMC were collected and labeled with anti-CD154 phycoerythrin (PE) (Miltenyi Biotec, Auburn, CA, USA). Subsequently cells were washed and incubated with anti-PE MicroBeads (Miltenyi Biotec, Auburn, CA, USA). Labeled and washed cells were applied to a MS Column (Miltenyi Biotec, Auburn, CA, USA) to enrich for CD154 positive T cells. Samples collected prior cell sort, CD154-enriched population and flow through were additionally stained for anti-CD3 Pacific Blue (PB) (Biolegend), anti-CD4-Alexa Fluor® 700 (eBioscience), anti-CD8 HorizonV500 (BD Biosciences) (Supplementary figure 1). MACS buffer (PBS supplemented with 2mM EDTA and 2% FCS) was used during cell sorting procedure.

RNA isolation and cDNA synthesis. RNA was isolated from 5*10^6 T cells using 1 ml Trizol® Reagent (Invitrogen, Life Technologies, Paisley UK) according to the manufacturer's protocol and dissolved in 40 µl RNAse free H_2O (Ambion, Invitrogen, Life Technologies, Paisley, UK). RNA concentration was determined by measuring the OD_{260} with the Nanodrop ND1000 (Thermo Scientific, Waltham, MA). Complementary first strand DNA was synthesized from 0.6 µg RNA, and with 10 pmol oligoDt primer (Life Technologies, Paisley, UK). This mixture was first incubated at 72 °C for 2 minutes to facilitate primer annealing. Superscript III Reverse Transcriptase (1µl), 10 nmol dNTPs, 0.1 mM DTT (all Invitrogen, Life Technologies, Paisley, UK) were added to this mixture and subsequently incubated at 42°C for 60 minutes. Amplification was performed by adding 2 µl of cDNA, 10 nmol dNTPs, 20 pmol oligoDt primer, 20 pmol capswitch primer and 38 µl H_20 in a total volume of 50 µl. The amplification PCR program consisted of an initial denaturation cycle of 1 minute at 95 °C, followed by 20 amplification cycles (5 seconds at 95 °C, 5 seconds at 65 °C and 6 minutes at 68 °C). The quality of the amplified cDNA was analyzed by gel electrophoresis.

Determination of T-cell clonality. Clonality of the clone was assessed by PCR amplification of the Vβ and Vα regions, with a constant primer [19] for the Cα and Cβ region and 26 and 37 V region-specific primers for the Vβ and Vα region, respectively [19]. Mastermix contained 3 (Vβ) or 4 (Vα) µl cDNA, 5 pmol reverse primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.5 unit of GoTaq Flexi DNA polymerase and 1x Green GoTaq Flexi Polymerase Buffer (all Promega, Madison, WI) and 1x bovine serum albumin (BSA, New England Biolabs, Ipswich, MA). 5 pmol of each V-specific primer was added. A total volume of 10 µl was used for PCR amplification. An initial denaturation step at 95 °C for 2 minutes was followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 65 °C (Vα) or 60 °C (Vβ) for 30 seconds and extension at 72 °C for 30 seconds. Finally, a 5 minute extension step was performed at 72 °C. PCR products were analyzed by standard gel electrophoresis. Sequencing templates were amplified using Phusion DNA Polymerase (New England Biolabs, Ipswich, MA) and a constant primer extended with a M13 sequence (TGTAAAACGACGGCCAGT). Amplicons spanning the variable, CDR3 and joining regions were purified using Wizard® SV Gel and PCR clean up system (Promega, Madison, WI) according to manufacturers' protocol. Samples were sequenced at Baseclear (www.baseclear.com) using a separate M13 primer.

Restimulation of T-cell clones. Irradiated (3000 rad) feeders were pulsed with peptide mix (20 µg/ml per peptide) for 2 hours and washed. Clones were seeded in 96 wells at 2*10^4/ well together with the feeders (1*10^5/ well). For $CD4^+$ T cells, clones were incubated in IMDM containing 10% HuS and 1% LeucoA (Sigma Aldrich, St. Louis, MO, USA) at 37°C and 5% CO_2. After three days, IL-2 was added (25 IU/ml). For $CD8^+$ T cells, clones were incubated in IMDM contain 10% HuS, IL-7 and IL-15 both at a final concentration of 5 ng/ml (PeproTech Inc., Rocky Hill, NJ, USA). After three days IL-2 (50-100 IU/ml) was added.

T-cell subset analysis using flow cytometry. Feeders (3000 rad) were seeded to 96 flat-bottom plate (1*10^5) and incubated for 4-6 hours. The plates were washed three times with RPMI. T cells were added, together with antigen (10 µg/ml), peptide or peptide pool (5 µg/ml per peptide). After 2-4 hours 3 µg/ml Brefeldin A (BFA; Sigma Aldrich, St. Louis, MO, USA) was added and incubated overnight (o/n). Cells were harvested and collected in 5 ml round-bottom FACS tubes (BD Biosciences), washed and stained for T-helper markers. For additional flow cytometry, irradiated PBMC (2000 rad) were seeded together with antigen (10 µg/ml) or peptide (5 µg/ml) to 96 flat-bottom plate (1*10^5) and incubated o/n. plates were washed with RPMI and T cells were added (1*10^5). After 2-4 hours 3 µg/ml Brefeldin A (BFA; Sigma Aldrich, St. Louis, MO, USA) was added and incubated overnight (o/n). Cells were harvested and collected in FACS tubes, washed and stained.

Cells were stained with LIVE/DEAD® fixable violet dead cell stain (ViViD; Invitrogen, Carlsbad, CA, USA) prior surface and intracellular staining to discriminate between live and dead cells according to manufacturer's instructions. CD14 and CD19 markers were included to eliminate monocytes and B cells using a DUMP channel [20]. Following ViViD, surface markers were stained in PBS 0.1% BSA for 1/2-1 hour at 4 °C. Subsequently intracellular staining (ICS) was performed using fixation and permeabilization (Intrastain) kit (DakoCytomation, Glostrup, Denmark). Unstimulated and unstained samples were included as negative controls. Different T-cell subset panels were used. All included, *surface staining*; anti-CD3 PE-Cy5 or anti-CD3 PE-Cy7, (both BD Biosciences), anti-CD4 Texas Red (Caltag), anti-CD8 HorizonV500 (BD Biosciences), anti-CD14 PB (Invitrogen) and anti-CD19 PB (Invitrogen), *ICS;* anti-CD154 APC-Alexa eFluor® 780 (eBioscience). In addition, the following markers were used for (i) **T-helper subset panel**, *surface staining*; anti-TCRα/β Fluorescein isothiocyanate (FITC), *ICS*; anti-IFN-γ Alexa Fluor® 700 (BD Pharmingen), anti-TNF-α PE-Cy7 (BD Biosciences) and anti-IL-2 PE (BD Pharmingen) (anti-Granzyme B APC (Caltag)). (ii) **Transcription factor subset panel,** *ICS*; anti-IFN-γ Alexa Fluor® 700 (BD Pharmingen), anti-T-bet eFluor®660 (eBioscience), anti-GATA-3 PerCP-eFluor® 710 (eBioscience), anti-Ror-γt PE (eBioscience) and anti-IL-17 FITC (eBioscience). (iii) **Regulatory T cell subset panel**, *surface staining*; anti-CD25 PE-Cy5 (BD Biosciences) and anti-CD39 PE (Biolegend), *ICS*; anti-Foxp3 Alexa Fluor® 700 (eBioscience) and anti-IL-10 APC (Miltenyi Biotec). (iv) **T cell cytotoxicity subset panel**, *ICS*; anti-Granzyme B APC (Caltag), anti-IFN-γ Alexa 700 (BD Pharmingen), anti-CD107a PE-Cy5 (BD Pharmingen) and anti-Perforin PE (BD Pharmingen). Of note, anti-CD107a PE-Cy5 was already added during incubation of the T cells with APC and antigen. Fluorescence minus one (FMO) controls were included for each T-cell subset panel. Data were acquired on a BD LSRFortessa (BD Biosciences) and analyzed using Flowjo version 7.6.5 (Tree Star Inc., Ashland, OR, USA).

Antigen specificity test. T cells (1*10^4) were seeded in 96 flat-bottom plates in presence of HLA class I and/or class II (mis)matched irradiated (2000 rad) PBMC (5*10^4) [21]. T cells were stimulated with either protein (10 µg/ml), peptide (10 µg/ml), PHA (2 µg/ml) or purified protein derivative (PPD; Statens Serum Institute, Copenhagen, Denmark; 5 µg/ml) and incubated for 3 days at 37°C and 5% CO_2. At day 3, supernatant was harvested and tested for IFN-γ production. Subsequently, cells were pulsed with [^{3}H]-thymidine and incubated 16-18 hours at 37°C and 5% CO_2. Incorporation of [^{3}H]-thymidine was measured using a Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter.

Blocking of HLA-DR and HLA-DQ molecules. Irradiated (2000 rad) PBMC (5*10^4) and monoclonal antibodies (20 µg/ml) directed against HLA-DR (B8.11.2) or HLA-DQ (SPV-L3) molecules (provided by Prof. F. Claas, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands) were seeded in 96 flat-bottom plates and incubated for 2 hours. T cells (1*10^4) and protein antigen, peptide (5 µg/ml) or PHA (2 µg/ml) were added and co-incubated for 3 days at 37°C and 5% CO_2. Both IFN-γ production and proliferation were determined as previously described. $Rp15_{1\text{-}1}$ was included as reference $CD4^+$ T-cell clone [22;23].

Western blot analysis. Recombinant protein (0.4 µg), *Mtb* lysates (10 µg) and ultralow molecular weight marker (Color Marker Ultra Low Range; Sigma Aldrich, St. Louis, MO, USA) were separated under denaturizing conditions using a 17% Tricine sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) at 100-200 V for 2-3 hours [24]. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane by electroblotting (40V; 250 mA). The membranes were blocked using 5% skim milk (Fluka, Biochemika, Sigma Aldrich, 70166), washed (PBS 0.1% Tween20) and incubated with Rv2034-immunized or naive mouse serum. After incubation blots were washed and incubated with secondary horseradish peroxidase (HRP)–conjugated rabbit anti-mouse Ig, detecting total IgG, IgA and IgM (P0260 Dako, Glostrup, Denmark). Protein bands were visualized using chemiluminescence (ECL) HRP substrate (Amersham™ ECL Select™, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Blots were developed using Fuji super RX medical X-ray films.

Generation of monocyte derived DC. PBMC were thawed and monocytes were isolated by positive selection using MACS CD14 MicroBeads (Miltenyi Biotec, Auburn, CA, USA) following manufacturer's instructions. Monocytes were seeded in T75 flasks (Corning) in RPMI medium containing 10% heat-inactivated fetal calf serum (FCS), 50 U/ml penicillin, 50 µg/ml streptomycin (Gibco, Paisley, United Kingdom), IL-4 and GM-CSF (Biosource International, Camarillo, CA, USA) both at 10 ng/ml for 6 days to generate monocyte-derived immature DCs. Mature DCs were generated by stimulating DCs with 10 ng/ml lipopolysaccharide (LPS) for an additional 24 hours.

DC differentiation and maturation was validated by flow cytometry using anti-CD14 PE (BD Pharmingen), anti-HLA-DR FITC (BD Pharmingen), anti-CD1a Alexa Fluor® 700 (Biolegend), anti-CD163 Alexa Fluor® 647 (Biolegend), anti-CD80 PE-Cy7 (Biolegend), anti-CD86 PE-Cy5 (BD Pharmingen) and anti-CD3 PB (Biolegend).

Matured DCs were loaded with Rv2034 protein, Rv2034 p81-100 and different conditions of *Mtb* lysate and incubated for 24 hours. Cells were washed and Rv2034 $CD4^+$ T-cell clone added. After 2 hours BFA was added and culture incubated o/n. Activation of T cells was determined by detection of CD154 and Th1 markers using the T-helper subset panel.

Mtb *inhibition assay*. Autologous PBMC were isolated using Ficoll density centrifugation from venous, heparinized blood and plated in quadruplicate cultures (1*10^6 cells/well; assuming 10% monocytes) in 48-wells plates (Costar Corporation, Cambridge, Mass.) in PBS. After overnight incubation at 37°C, PBS was removed and replaced by IMDM containing 10% human serum of 37°C. The adhering monocytes were infected with *Mtb* H37Rv at an MOI of 10. After overnight incubation at 37°C, T-cell clone were added at effector:target (E:T) ratios of 20:1 and 50:1. After an additional o/n incubation, cells were lysed. Lysates were homogenized in PBS and the number of bacteria was determined by culturing serial dilutions of the homogenates on 7H10 agar plates (BD Biosciences) supplemented with BD BBL™ Middlebrook OADC enrichment (100 ml per bottle; BD Biosciences). Colonies were counted after 2-3 weeks incubation at 37 °C.

Results and discussion

Generation of T-cell clones for subsequent phenotypic and functional analysis

We previously reported that *in vivo* expressed *Mtb* (IVE-TB) antigens have significant vaccine potential [10] [Commandeur et al. unpublished data]. It is thus important to characterize the underlying immune responses to these antigens in more detail, particularly in humans. To this end, clonally expanded antigen specific T cells are powerful tools [25;26]. To generate IVE-TB specific T cells we used a T-cell cloning method based on CD154 expression to enrich for antigen-specific T cells irrespective of function. We selected two *Mtb* antigens; the well-studied TB10.4 protein [27] and the recently described *in vivo* expressed *Mtb* (IVE-TB) antigen Rv2034 [10].

First, PBMC of an *in vitro* PPD^+ donor known to respond to these antigens (Figure 1A and 1B) were stimulated with peptide pools from TB10.4 or Rv2034 for one week in the presence of IL-2, followed by re-stimulation with the peptide pool for 16 hours, in the presence of anti-CD40 antibodies to inhibit CD154-CD40 interactions [6] preventing loss of surface expressed CD154 [28;29]. CD154 positive cells were then sorted using Magnetic-activated Cell Sorting (MACS) and seeded at 0.3 or 1 cell per well densities as described in detail in the materials and methods section. To analyze the antigen specificities of the expanded T-cell clones, cell populations were gated based on live $CD14^-CD19^-CD3^+$ T cells. For TB10.4, eight clones showed responses towards

TB10.4 peptide pool stimulation (Table 1). Out of these, five T-cell clones were $CD154^+CD4^+$ and produced either IFN-γ, TNF-α and IL-2, or TNF-α and IL-2. Two clones appeared to be $CD8^+$ T cells that produced IFN-γ, with one of these producing a relatively low amount of IL-2 as well. CD154 is known to be expressed on activated $CD4^+$ T cells, but can also be upregulated on a fraction of $CD8^+$ T cells [30]. However, none of the $CD8^+$ T cells expressed CD154. Somewhat surprisingly, one T-cell clone was $CD3^{+/}CD4^{-/}CD8^-$, (double negative (DN) T cells) and did not express CD154 either, nevertheless, $CD3^+$ $CD4^{-/}CD8^-$ are able to express CD154 [31]). This DN T-cell clone produced IFN-γ in response to TB10.4 antigen. The remaining expanded T-cell cultures were TB10.4 non-responsive and are indicated in Table 1.

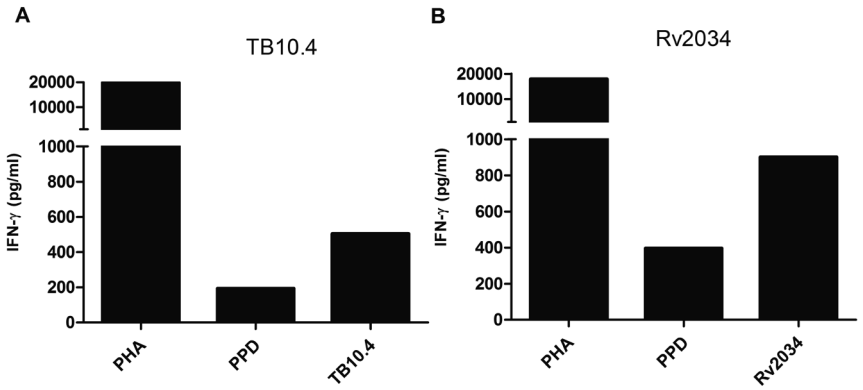


Figure 1. **PBMC recognition of TB10.4 and IVE-TB antigen Rv2034.** PBMC from a PPD^+ donor were stimulated with different stimuli for 6 days and IFN-γ production (pg/ml) was determined in the supernatants. Both TB10.4 protein (10 µg/ml) (A) and Rv2034 protein (10 µg/ml) (B) were analyzed as well as control mitogen PHA and *Mtb* derived PPD (A and B). Medium values (unstimulated PBMC) were subtracted.

Table 1. Analysis of TB10.4 clonal cultures

Clone	Subset [a]	CD154	IFN-γ	TNF-α	IL-2
1	CD4	+	-	+	+
2	CD4	+	+	+	+
3	DN	-	+/-	-	-
4	CD4	+/-	+/-	+/-	+/-
5	CD4	+	+	+	+
6	CD8	-	+	-	+/-
7	CD8	-	+	-	-
8	CD4	+	+/-	+	+

[a] Single live $CD14^-CD19^-CD3^+$ cells (population > 100 cells)
Non-responding clonal cultures included CD4 (n=9), CD8 (n=2) and DN (n=2) cells
DN = Double negative
+ = > 1% positive cells
+/- = <1% positive cells

For Rv2034, eight antigen responsive T-cell clones were generated using the same approach as described for TB10.4, among which three were $CD4^+$ T cells (Table 2). One of these $CD4^+$ T-cell cultures showed specific CD154, IFN-γ, TNF-α and IL-2 expression, whereas the remaining two only produced IL-2 in response to antigen. All three identified $CD8^+$ T cells produced IFN-γ. As for TB10.4, two potential clones appeared to be DN T cells and also showed a potential to produce IFN-γ and/or IL-2. Proliferating Rv2034 non-responsive T-cell clones are also indicated in Table 2. Thus, using CD154 sorting we were able to generate IVE-TB Rv2034 induced T-cell clones which included $CD4^+$, $CD8^+$ and DN T cells. Interestingly, besides $CD4^+$ and $CD8^+$ T cells also DN T cells have been associated with control of intracellular bacteria including *Mtb* [32;33]. Furthermore, DN T cells also show possible regulatory functions [34;35]. Besides classically HLA-class Ia restricted cells, the $CD8^+$ T-cell clones we isolated might also represent circulating alternative T-cell subsets, such as mucosal associated invariant T (MAIT) cells [36;37] or HLA-E restricted CD8 T cells [38].

Table 2. Analysis of Rv2034 clonal cultures

Clone	Subset [a]	CD154	IFN-γ	TNF-α	IL-2
1	DN	-	-	-	+/-
2	CD4	-	-	-	+/-
3	CD8	-	+	-	-
4	CD8	-	+	-	-
5	DN	-	+/-	-	+/-
6	CD4	+	+	+	+
7	CD8	-	+	-	-
8	CD4	-	-	-	+/-

[a] Single live $CD14^-CD19^-CD3^+$ cells (population > 100 cells)
Non-responding clonal cultures included CD4 (n=18), CD8 (n=0) and DN (n=1) cells
DN = Double negative
+ = > 1% positive cells
+/- = <1% positive cells

$CD4^+$ T-cell clone specific for *Mtb* IVE-TB antigen Rv2034

Thus, both TB10.4 and Rv2034 T-cell clones could be generated using the CD154 sorting method. As the main aim of this work was the analysis of IVE-TB specific T-cell responses, we further studied a $CD4^+$ T-cell clone that responded to Rv2034 peptide pool stimulation by CD154, IFN-γ, TNF-α and IL-2 expression (Figure 2). First, its clonality was further confirmed by PCR for both TCRα and TCRβ. The detected variable regions consisted of Vα13, Vα27 and Vβ14, following Arden nomenclature [39] (data not shown). Vα27 was predicted to be an unproductive TCRA rearranged sequence due to an out-of-frame junction, whereas Vα13 was successfully rearranged (the international ImMunoGeneTics database (IMTG)). Thus the clone represents a truly clonal population based on TCR genotyping.

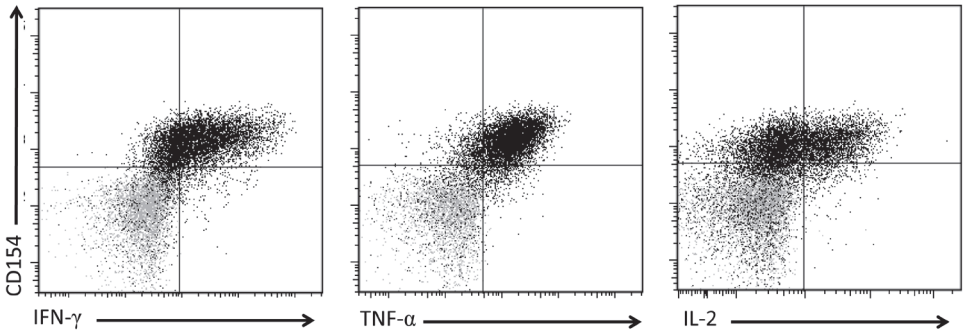


Figure 2. Rv2034 responsive CD4⁺ T-cell clone phenotype. The shown CD4⁺ T-cell clone that had been expanded was restimulated with the Rv2034 peptide pool and analyzed for the expression of CD154 expression, IFN-γ, TNF-α and IL-2 (black dots). CD154 and Th1 cytokine expression of non-activated T cells is indicated in grey dots. Dot blots show single live $CD14^{-}CD19^{-}CD3^{+}CD4^{+}$ T cells.

Identification of immunogenic epitope(s) of Rv2034 recognized by the CD4 T-cell clone

To identify the specific T cell epitope(s) of Rv2034, the CD4⁺ T-cell clone was restimulated with single 20-mer peptides from Rv2034. As expected, the Rv2034 peptide pool was strongly recognized by the clone as measured by IFN-γ production and T-cell proliferation (Figure 3A). Rv2034 p81-100 was identified as the dominant immunogenic epitope, while variable responses were observed to Rv2034 p88-107, which overlapped 13 amino acids with Rv2034 p81-100. In addition, recombinant protein Rv2034 and fusion protein Ag85B/ESAT-6/Rv2034 were both recognized, suggesting that Rv2034 epitope is adequately processed from this protein. The fusion protein Ag85B/ESAT-6/Rv2034 includes two early infection phase expressed *Mtb* proteins (Ag85B and ESAT6; together designated as H1), fused to the *in vivo* expressed Rv2034 protein. Inclusion of multiple infection phase related *Mtb* proteins in a single fusion construct has been shown to improve vaccine efficacy in both mouse and non-human primate models of TB [40;41], especially if the antigens are expressed during different phases of *Mtb* infection. It is therefore relevant that the immunogenic epitope present in Rv2034 p81-100 is efficiently processed and presented from both the Rv2034 protein and the trimeric Ag85B/ESAT6/Rv2034 fusion protein. The negative control proteins HPV16E6, Ag85B and ESAT6/CFP10 fusion protein, and the negative control peptide HIV-GAG were not recognized, in agreement with the strict Rv2034-specificity. As expected, expression of CD154 and Th1 cytokines was detected upon stimulation of the CD4⁺ T-cell clone with Rv2034 p81-100 (Figure 3B) and Rv2034 protein (Figure 3C) whereas no activation was observed upon stimulation with negative control peptide p11-30 of Rv2034 (Figure 3D), further demonstrating the specificity of this CD4⁺ T cell-clone for Rv2034 p81-100.

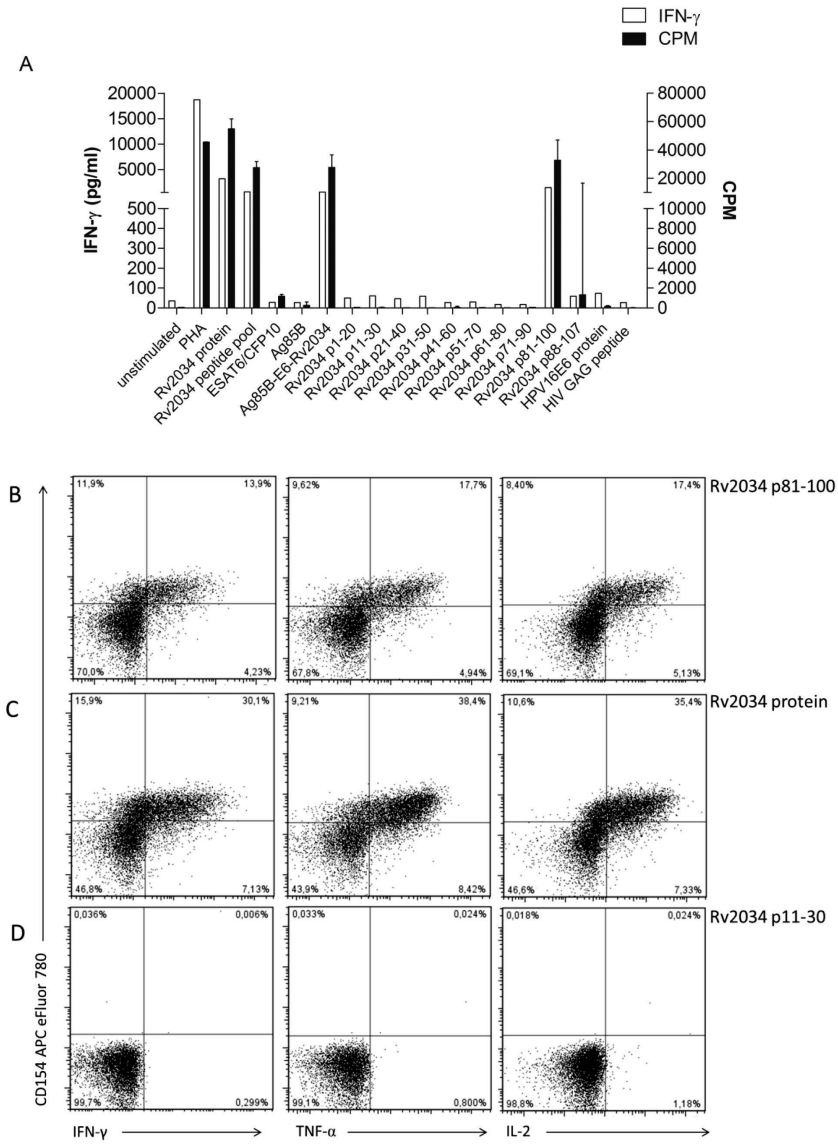


Figure 3. Identification of immunogenic epitope(s) of Rv2034 recognized by CD4 T-cell clone. To identify the immunogenic epitope(s) in Rv2034, the CD4+ T-cell clone was stimulated with all individual Rv2034 20-mer peptides with 10 aa overlap; Rv2034 recombinant protein; Rv2034 peptide pool; control ESAT6/CFP10 fusion protein; an Ag85B/ESAT6/Rv2034 trimeric fusion protein; and negative and positive control conditions. Autologous irradiated PBMC were used as APCs. Both IFN-γ (open bars) and T-cell proliferation (black bars) were determined (A). To determine the Rv2034 p81-100 specific response by flow cytometry, the CD4+ T-cell clone was stimulated with Rv2034 p81-100 (B), Rv2034 protein (C) and Rv2034 p11-30 (D) using autologous irradiated PBMC, in the presence of BFA. Intracellular CD154 and Th1 cytokine expression was determined. Flow cytometry plots show single live $CD14^{-}CD19^{-}CD3^{+}CD4^{+}$ T cells, the frequency of all subsets of $CD3^{+}CD4^{+}$ T cells are indicated in the corners of each plot.

T-cell subset panel analysis

The Rv2034 responding $CD4^+$ T-cell clone was subsequently analyzed for Th17, Th2, regulatory and cytotoxicity T cell (CTL) markers to further specify its functional activities. The Rv2034 p81-100 peptide-induced CD154 expression correlated with IFN-γ expression (Figure 4A) while no IL-17 expression was observed. Transcription factor analysis revealed expression of T-bet (Figure 4B), a transcription factor that regulates Th1 development and controls IFN-γ production [42;43], but there was no expression of GATA-3 (Th2 [44]), RORγt (Th17 [45;46]) or FOXP3 (Treg [47]). T-bet was constitutively expressed in both p81-100 peptide stimulated and unstimulated T cells. Furthermore, the clone did not reveal any specific combination(s) of other reported T-cell regulatory markers (Figure 4C): no IL-10 could be detected although CD25 and CD39 [48] were constitutively expressed, which, however, are also expressed by activated non-Tregs [49;50]. Of note, CD25 expressing non-Treg cells mostly express intermediate levels of CD25 ($CD25^{int}$), whereas Tregs typically express high levels of CD25 ($CD25^{hi}$) [49]. The expansion of our T-cell clone was mediated by IL-2 which theoretically could have enhanced CD25 expression [51]. Finally, the T-cell clone expressed granzyme B and CD107a but no detectable perforin (Figure 4D). The expression of granzyme B and CD107a was antigen-stimulation dependent. Cytotoxic $CD4^+$ T cells have been reported not only in viral but also in mycobacterial infections [52-54]. The expression of the degranulation marker CD107a indicates the release of lysosomal products from granules after antigen-specific activation of the T cell, which likely includes granzyme B. Anti-CD107a antibodies were administered during antigen stimulation of the T-cell clone, because once CD107a is released with its vesicles' granule contents and becomes integrated in the membrane of the cell, the antibodies will bind to CD107a and are subsequently internalized together with CD107, enabling detection of degranulation. Further studies are required to determine whether granulysin and other granzymes are released by the T-cell clone. These data were obtained by ICS using brefeldin A to accumulate intracellular cytokines by preventing their secretion. All subset conditions were also analyzed using monensin, which acts as protein transport inhibitor via disruption of trans-Golgi protein transport whereas BFA inhibits protein transport between endoplasmic reticulum (ER) and the Golgi apparatus. Monensin, however in our hands did not enhance any of the responses (data not shown). Taken together, these observations show that the Rv2034-specific $CD4^+$ T cell-clone is a pure Th1 clone expressing IFN-γ, TNF-α, IL-2 and several cytotoxic granule markers.

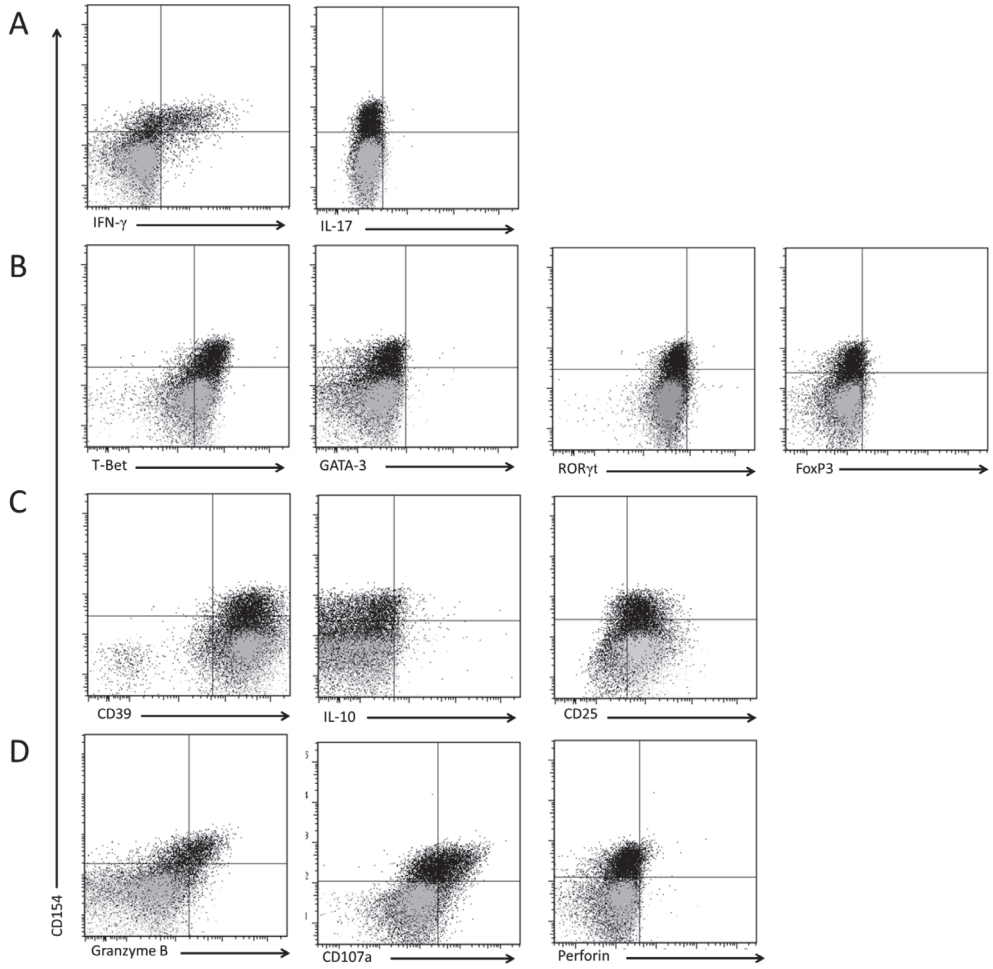


Figure 4. Subset analysis of the CD4 T-cell clone. The $CD4^+$ T-cell clone was stimulated with Rv2034 p81-100 in presence of BFA and analyzed the expression of CD154 (A-D), IFN-γ and IL-17 (A) and additional markers: the transcriptional factor markers T-bet (Th1), GATA-3 (Th2), RORγt (Th17) and FoxP3 (Treg) (B); T-cell regulatory markers: CD39, IL-10 and CD25 (C); T-cell cytotoxicity markers: Granzyme B, CD107a and Perforin (D). Markers were analyzed using the mAb subset panels described in the materials and methods section. Stimulated T cells are indicated in black and unstimulated T cells in grey. Dot blots show single live $CD14^-CD19^-CD3^+CD4^+$ T cells.

Determination of genetic HLA-restriction of CD4$^+$ T-cell clone

To identify the genetic HLA-restriction of T-cell recognition of Rv2034 p81-100, the T-cell clone was cultured with (irradiated) PBMC from donors expressing various matching or mismatching HLA class II alleles in the presence of Rv2034 p81-100 (Figure 5A), Rv2034 protein (Figure 5B) and Ag85B/ESAT-6/Rv2034 fusion protein (Figure 5C). The autologous HLA genotype was HLA-DR2(15),-DR3(17),-DQ1(6),-DQ2,-DR51,-DR52. As expected, completely HLA-DRB1 and HLA-DQ mismatched PBMC (HLA-DR11,13 and HLA-DQ3(7)) failed to present peptide or protein antigens to the T-cell clone, even though these cells were matched for HLA-DRB3 (DR52), indicating that DR52 was not involved in Rv2034 p81-100 presentation. Both HLA-DR2(15)$^+$/DQ1(6)$^+$ and HLA-DR3(17)$^+$/DQ1(5)$^+$ PBMC induced strong T-cell activation. Since HLA-DR2(15) is in strong genetic linkage disequilibrium with HLA-DQ1(6) it was impossible to match for HLA-DR2(15) while mismatching for HLA-DQ1(6). HLA-DQ1(5,6)$^+$ PBMC, which were mismatched for HLA-DR3(17) and HLA-DR2(15), also induced T-cell proliferation and IFN-γ in response to Rv2034 (fusion) protein and p81-100, whereas HLA-DQ2$^+$ PBMC failed to present antigen. While the results were suggestive of HLA-DR3 and HLA-DQ1 presentation, the addition of purified monoclonal antibodies specific for only HLA-DR but not HLA-DQ backbone determinants resulted in a strong reduction of autologous APC presented Rv2034 p81-100 induced T-cell proliferation (Figure 5D). Thus the autologous responses towards Rv2034 p81-100 is predominantly HLA-DR and not HLA-DQ-restricted.

Surprisingly, however, while responses to the Rv2034 peptide p81-100 were HLA-DR restricted, the response to the whole Rv2034 protein was inhibited both by HLA-DR and by HLA-DQ-blocking antibodies (Figure 5D). Thus, while the epitope contained with p81-100 is HLA-DR restricted, the Rv2034 protein also contains an HLA-DQ1(6) restricted epitope sequence that can be recognized by the same T-cell clone. Of note, variable recognition was observed for Rv2034 p88-107 by the T-cell clone (Figure 4A and data not shown). Strong responses to p81-100 were observed at a concentration of 1 μg/ml (Figure 5D), whereas no responses to p88-107 could be detected at this concentration (data not shown). However, at a concentration of 5 μg/ml, p88-107 was able to activate the T-cell clone and, importantly, this could be blocked by both HLA-DR and HLA-DQ antibodies (Figure 5D). Thus, the Rv2034 p81-107 sequence possesses HLA-DR and HLA-DQ binding epitopes that were recognized by a single, apparently promiscuous CD4$^+$ T-cell clone.

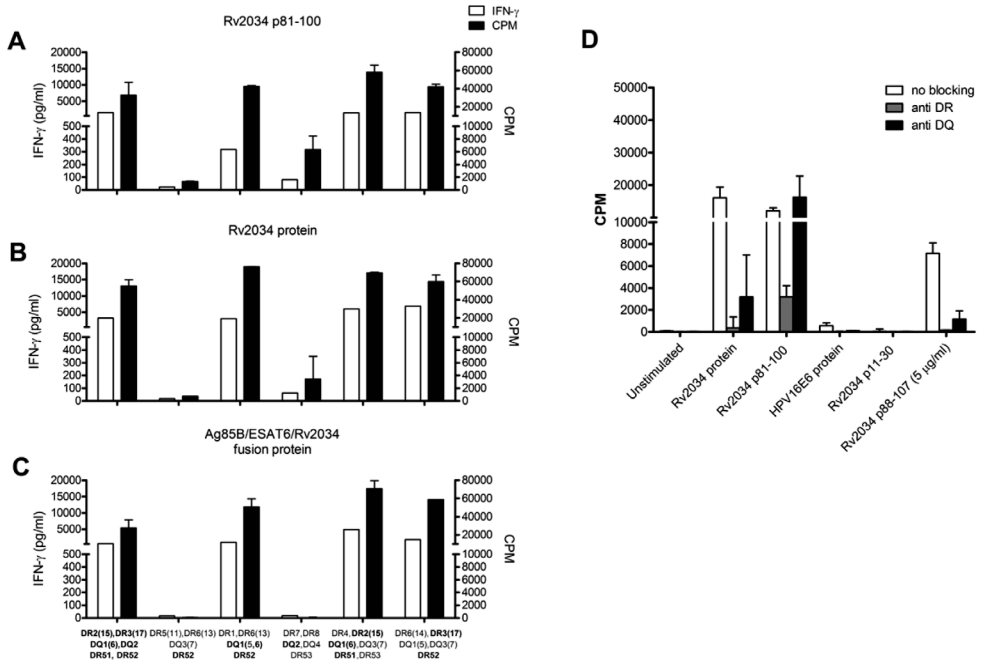


Figure 5. Restriction of Rv2034 p81-100 and p88-107 presentation by HLA-DR and -DQ molecules. To determine the HLA-DR and -DQ restriction of Rv2034 p81-100 responding CD4+ T-cell clone, T cells were incubated with Rv2034 p81-100 (A), Rv2034 protein (B) or Ag85B/ESAT-6/Rv2034 fusion protein (C), in the presence of irradiated PBMC with (mis)matched HLA-DR and/or DQ molecules. Both IFN-γ production (white bars) and proliferation (CPM) (black bars) was determined. CPM bars represent median ranging the highest and lowest value. Matching HLA alleles are indicated in bold on the X-axis. HLA-DR and HLA-DQ molecules of APCs pulsed with either Rv2034 protein, Rv2034 p81-100, Rv2034 p88-107 (5 µg/ml) or control conditions, were blocked using monoclonal antibodies directed against HLA-DR or -DQ [21], and T-cell proliferation was analyzed (CPM) (D).

In silico HLA class II peptide binding predictions (SYFPEITHI [55], NetMHCIIpan 2.1, NetMHCII 2.2 and IEDB) revealed that Rv2034 p81-100 (LRTDLDRFWTRALTGYAQLI) indeed contains sequences with high predicted binding affinity for multiple HLA-DRB1 and HLA-DQ molecules (Supplementary table 1). HLA-DR2(15) and HLA-DR3(17) show binding potential for LDRFWTRAL, which is located at the beginning of the peptide while the TRALTGYAQ sequence has predicted potential to bind to HLA-DQ1(6). Although Rv2034 p88-107 (FWTRALTGYAQLIDSEGDDT) contains a potential epitope (ALTGYAQLI) that could bind to HLA-DR2(15), it contains limited predicted epitopes available for HLA-DR3(17). Nevertheless, predicted HLA-DQ1(6) epitopes were identified (TRALTGYAQ and TGYAQLIDS) (Supplementary table 2). As a control, very few predicted epitopes were observed for Rv2034 p11-30 (Supplementary table 3), indicating that the p81-107 sequence has a rather unique high (predicted) epitope density. These data are best explained by a model in which the T-cell clone

recognizes Rv2034 p81-100 and Rv2034 p88-107 via multiple HLA molecules, possibly including HLA-DR2, -DR3 as well as HLA-DQ1 registers, but that the dominant response to the protein is HLA-DR restricted. Such promiscuous peptides, capable of being presented by multiple HLA molecules, have been shown for several mycobacterial proteins [56], including HspX (16 kDa protein) [57], TB10.4 [58] and Hsp65 [59]. In addition, some (*Mtb* antigen) studies showed possible presentation of a single epitope, specific for one clone, via both HLA-DR and HLA-DQ [57;60]. Other studies also demonstrated promiscuity of viral epitope presentation [61;62]. The presentation of a specific epitope via multiple HLA molecules has been described as HLA cross-restriction [63;64] and this promiscuity phenomenon is suggested to be extensively present [65;66]. Promiscuous *Mtb* peptides are particularly interesting for development of novel *Mtb* vaccines since they would be able to cover many different HLA alleles throughout different populations.

Recognition of Rv2034 in *Mtb* lysate

We next wished to demonstrate recognition of native *Mtb* expressed Rv2034 protein by the T-cell clone in order to better understand its possible role during *Mtb* infection. First we examined whether Rv2034 protein was expressed in *Mtb* lysates by western blot analysis. Using sera of Rv2034-immunized (HLA-DR3 transgenic) mice, expression of Rv2034 was identified in both log phase *Mtb* lysate and *Mtb* grown during hypoxic and starvation conditions (Figure 6A). Although the protein size was predicted to be approximately 12 kDa (http://www.sciencegateway.org/tools/proteinmw.htm), the native Rv2034 present in the lysates was larger than expected. The recombinant Rv2034 protein includes a histidine tag and thus was expected to have a molecular weight of ~14 kDa. Since Rv2034 contains dimerization sites [67], this higher relative molecular mass could be due to formation of multimers. Alternatively but not mutually exclusive, post-translational modifications or binding to other molecules might be responsible for the higher molecular weight. Two bands were clearly visible, the smallest band likely relating to Rv2034 protein monomer, whereas the upper band probably indicates multimer formation. The binding of antibodies present in the sera of Rv2034-immunized mice was specific since no bands were observed using sera from non-immunized HLA-DR3 mice. Indeed, Rv2034 protein was also previously identified in *in vitro* grown *Mtb* cultures [10;68;69], specifically in lipid (membrane) associated fractions, indicating that the protein is indeed expressed by *Mtb*.

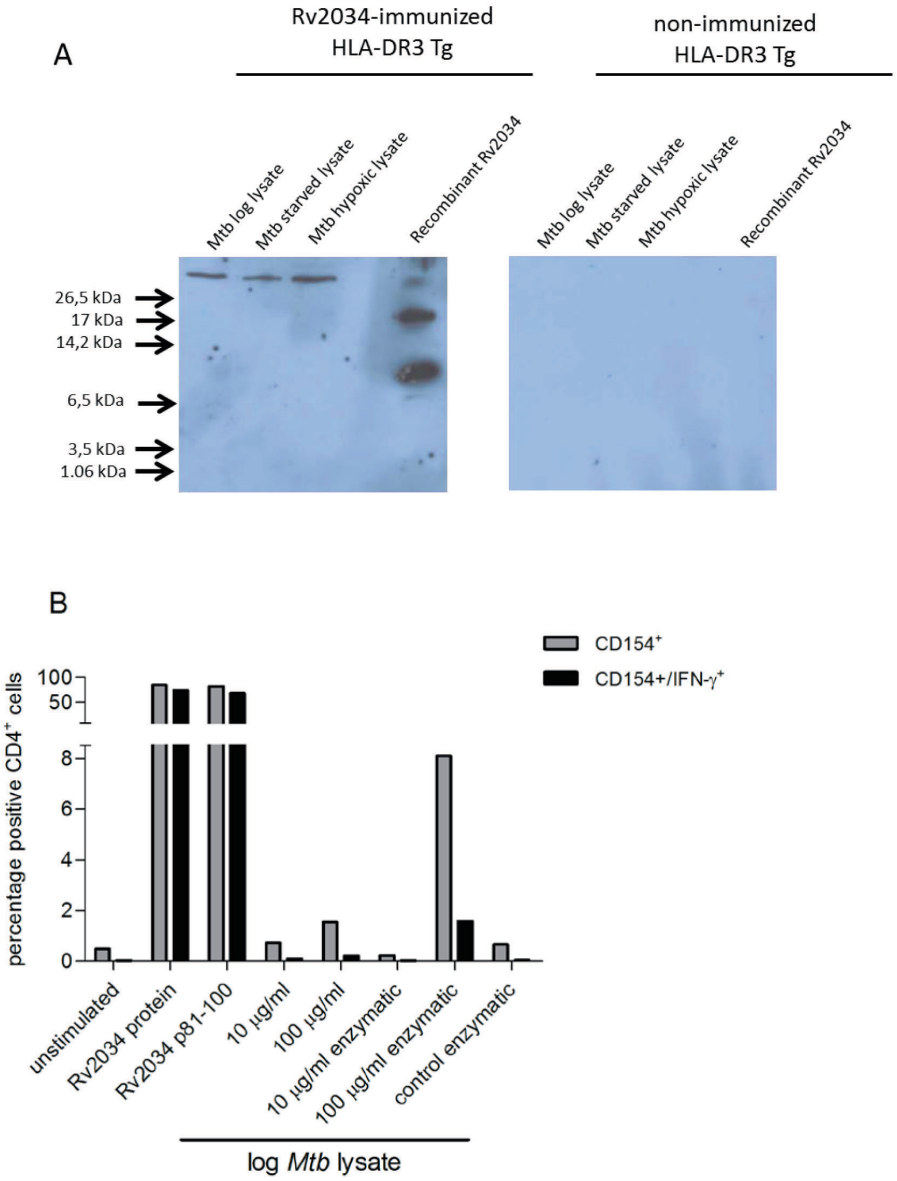


Figure 6. Recognition of native Rv2034 in *Mtb* lysate by CD4⁺ T-cell clone. *Mtb* lysates (10 µg) derived from *Mtb* grown in log-phase-, starvation- and hypoxic- conditions and recombinant protein Rv2034 (0.4 µg) were analyzed by western blotting for their recognition by specific antibodies using sera of Rv2034-immunized or non-immunized HLA-DR3 transgenic mice (A). Monocyte derived matured DCs were loaded with Rv2034 p81-100, Rv2034 protein and different conditions of *Mtb* lysate for 24h. Subsequently, the CD4⁺ T-cell clone was incubated with these antigen-loaded DCs and after 2 hours BFA was added and incubated o/n. CD154 and IFN-γ expression was determined using flow cytometry. Percentage of responding $CD4^+CD154^+$ (grey bars) and $CD4^+CD154^+IFN\text{-}\gamma^+$ (black bars) T cells are given (B).

We next analyzed the T-cell clone's reactivity against *Mtb* lysate to see if the T-cell clone was able to detect the native Rv2034 protein. HLA-matched matured dendritic cells (DC) were used as antigen-presenting cells and activated $CD4^+CD154^+$ and $CD4^+CD154^+IFN\text{-}\gamma^+$ responding T cells analyzed (Figure 6B). Both Rv2034 protein and Rv2034 p81-100 strongly activated the T-cell clone, whereas *Mtb* lysate at 10 µg/ml did not. Increasing the *Mtb* concentration to 100 µg/ml, however, induced CD154 expression. Rv2034 might be difficult for DC to process and present due to possible posttranslational modifications, dimer formation [67], or associations to other molecules (Rv2034 is lipid or membrane associated [68;69]). It should be noted, however, that Rv2034 is efficiently recognized by T cells from tuberculin skin test-positive, *in vitro* ESAT6/CFP10-responsive individuals in whole blood or PBMC assays [10]. To improve processing of the lysate by the APC, the lysate was further digested and homogenized using proteinase K (serine protease) and lysozyme (peptidoglycan degradation) enzymes. Treating the *Mtb* lysate concentration resulted in an increased population of activated $CD154^+$ and $CD154^+/IFN\text{-}\gamma^+$ $CD4^+$ T cells. Importantly, incubating the cells with a control sample containing the same amount of inactivated enzymes as used for the lysate did not show any recognition, indicating that T-cell activation is *Mtb* lysate-specific.

Nonetheless, activation of the T-cell clone using the enzyme treated *Mtb* lysate did not reach the full level of activation as observed for recombinant Rv2034 protein and Rv2034 p81-100. Obviously, *Mtb* lysate is a very crude product containing many different proteins as well as other molecules and fragments, such that only low levels of Rv2034 might be present in the lysate. Furthermore, only activation was observed after enzyme treatment verifying that Rv2034 was associated to certain structures that prevented or hindered correct processing by the matured DC. A lysate concentration dependent effect on T cells has been observed previously [52].

The T-cell clone thus expressed cytotoxic markers (Figure 4) and recognized native Rv2034 in *Mtb* lysate (Figure 6B). To test whether the T-cell clone could directly inhibit *Mtb* outgrowth from infected APCs, autologous monocytes were infected with *Mtb* and the T-cell clone was added in an effector/target ratio of 20:1 and 50:1. Interestingly, addition of the T-cell clone resulted in a significant decrease of CFU in a dose-dependent fashion (Figure 7). Thus the T-cell clone is able to inhibit the outgrowth of *Mtb* from infected cells directly.

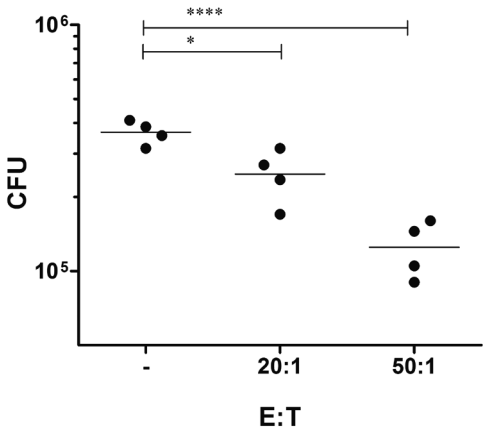


Figure 7. CD4⁺ T-cell clone has *Mtb* inhibitory properties. Autologous PBMC were loaded with *Mtb* (10 MOI) and CD4⁺ T-cell clone was added at different effector/target (E/T) ratios as indicated on the *x*-axis. CFU were determined after o/n incubation. Four replicates were performed for each condition. The horizontal bar indicates the mean value of the four replicates.

Concluding remarks

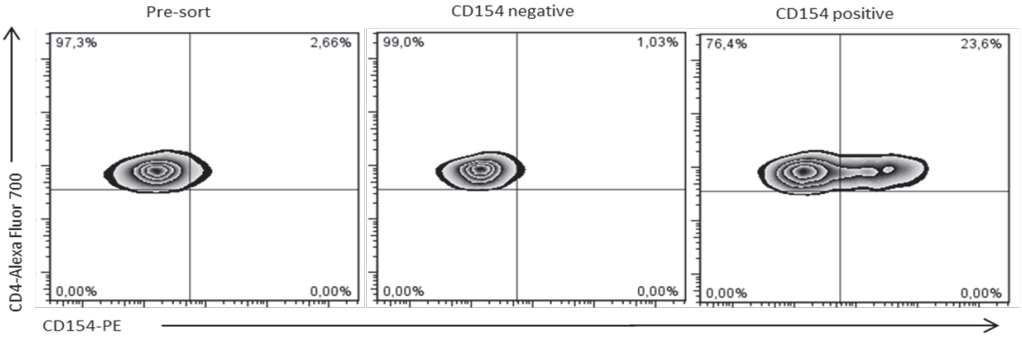
A better understanding of T-cell immunity during infection and following vaccination is important in many diseases, including tuberculosis. Here, we describe an approach that can be used for the rapid generation of antigen specific T-cell clones irrespective of functional properties such as cytokine secretion, in order to study antigen specific T-cell subsets in more detail, even for low frequency responses. Both CD4⁺, CD8⁺ and DN T-cell clones could be generated, which showed responses to the secreted TB10.4 or the IVE-TB Rv2034 *Mtb* antigens. An Rv2034 specific CD4⁺ T-cell clone was further analyzed, its antigen-specificity confirmed for (native) Rv2034 protein, a dominant peptide epitope identified and a promiscuous HLA-DR/DQ restriction pattern elucidated. Furthermore, the T-cell clone expressed Th1 and cytotoxicity related markers, and had significant *Mtb* inhibition activity. Using this approach, the T-cell responses to different *Mtb* specific antigens, including phase-dependent and IVE-TB antigens, can be further analyzed which should help to understand the immune response to *Mtb*. This can eventually be useful for developing novel TB vaccines and improved therapies.

Acknowledgements

This study was supported by: the European Commission FP7 NEWTBVAC project (LSHP-CT-2003-503367); FP7 VACTRAIN project; FP7 IDEA (FP7-HEALTH-2009-241642); FP7 ADITEC (FP7-HEALTH-2010-280873); FP7 EURIPRED (FP7-INFRA-2012-312661); Top Institute Pharma (project D-101-1) and EDCTP (AE-TBC project grant agreement N° IP_09_32040). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We would like to thank Hetty Jolink for her advice on CD154 clone experiments and Arno van der Slik for his help on TCR phenotyping.

Conflict of interest. TO is co-inventor of an *Mtb* latency antigen patent, which is owned by LUMC.

Supplementary Figure 1.



Supplementary Figure 1. MACS sort of CD154 positive T cells from Rv2034 peptide pool stimulated PBMC. PBMC were stimulated for 16 hours and labeled with anti-CD154 PE and anti-PE microbeads and sorted. A small sample was collected to analyze the positive CD154 positive population. Subsequently after sorting CD154 negative and CD154 positive collections were stained as well.

Supplementary Table I. HLA-DR and DQ binding predictions Rv2034 p81-100 (LRTDLDRFWTRALTGYAQLI)

HLA-DR	Allele molecule	SYFPEITHI [a]	NetMHCIIpan 2.1	NetMHCII 2.2	IEDB	IEDB
Sequence	**HLA-DRB1**	**Score**	**Affinity nM**	**Affinity nM**	**arbpython (ARB) Affinity nM**	**SMM Affinity nM**
DRFWTRALTGYAQLI	0101	**25**	**9.71 (SB) WTRALTGYA**	**18.2 (SB) TRALTGYAQ**	**21.7 (SB) WTRALTGYA**	**148.0 (WB) TRALTGYAQ**
RTDLDRFWTRALTGY	0101	23	**308.33 (WB) LDRFWTRAL**	**97.8 (WB) LDRFWTRAL**	**422.5 (WB) FWTRALTGY**	**465.0 (WB) LDRFWTRAL**
RTDLDRFWTRALTGY	0301 (17)	19	1562.32 TDLDRFWTR	523.4 TDLDRFWTR	**397.1 (WB) TDLDRFWTR**	5181.0 TDLDRFWTR
LRTDLDRFWTRALTG	0301 (17)	2	893.68 TDLDRFWTR	**208.0 (WB) TDLDRFWTR**	**189.8 (WB) LRTDLDRFW**	1164.0 TDLDRFWTR
RTDLDRFWTRALTGY	0401	**26**	1230.41 LDRFWTRAL	510.2 RFWTRALTG	1045.4 LDRFWTRAL	824.0 LDRFWTRAL
DRFWTRALTGYAQLI	0401	**28**	**75.81 (WB) WTRALTGYA**	**216.0 (WB) WTRALTGYA**	1500.4 WTRALTGYA	1294.0 TRALTGYAQ
DLDRFWTRALTGYAQ	0401	**0**	**254.70 (WB) WTRALTGYA**	**208.7 (WB) FWTRALTGY**	1045.4 LDRFWTRAL	1022.0 LDRFWTRAL
RTDLDRFWTRALTGY	0701	24	**236.64 (WB) LDRFWTRAL**	**181.2 (WB) RFWTRALTG**	**129.0 (WB) LDRFWTRAL**	**490.0 (WB) LDRFWTRAL**
DRFWTRALTGYAQLI	0701	**28**	**56.19 (WB) WTRALTGYA**	**221.4 (WB) RFWTRALTG**	1364.7 RFWTRALTG	1052.0 WTRALTGYA
LRTDLDRFWTRALTG	0701	**4**	**396.16 (WB) LDRFWTRAL**	**169.1 (WB) RFWTRALTG**	**129.0 (WB) LDRFWTRAL**	503.0 LDRFWTRAL
DRFWTRALTGYAQLI	0801	ND	**45.80 (SB) WTRALTGYA**	ND	ND	ND
DRFWTRALTGYAQLI	0901	ND	**19.99 (SB) WTRALTGYA**	**21.2 (SB) WTRALTGYA**	1575.8 WTRALTGYA	**176.0 (WB) WTRALTGYA**
DRFWTRALTGYAQLI	1001	ND	**36.40 (SB) WTRALTGYA**	ND	ND	ND
DRFWTRALTGYAQLI	1101	18	**47.00 (SB) WTRALTGYA**	**73.9 (WB) WTRALTGYA**	506.2 WTRALTGYA	793.0 WTRALTGYA
DLDRFWTRALTGYAQ	1101	7	**118.48 (WB) WTRALTGYA**	**44.3 (SB) DRFWTRALT**	506.2 WTRALTGYA	542.0 LDRFWTRAL
TDLDRFWTRALTGYA	1101	9	**176.31 (WB) WTRALTGYA**	**47.9 (SB) DRFWTRALT**	506.2 WTRALTGYA	**264.0 (WB) LDRFWTRAL**
DRFWTRALTGYAQLI	1201	ND	**30.49 (SB) WTRALTGYA**	ND	**24.7 (SB) DRFWTRALT**	584.0 FWTRALTGY
TDLDRFWTRALTGYA	1201	ND	**54.33 (WB) LDRFWTRAL**	ND	**24.7 (SB) DRFWTRALT**	598.0 FWTRALTGY
RTDLDRFWTRALTGY	1301	ND	896.73 LDRFWTRAL	ND	ND	ND
LDRFWTRALTGYAQL	1401	ND	518.20 WTRALTGYA	ND	ND	ND
RTDLDRFWTRALTGY	1501	**28**	**160.58 (WB) LDRFWTRAL**	**81.6 (WB) LDRFWTRAL**	**260.8 (WB) LDRFWTRAL**	**292.0 (WB) LDRFWTRAL**
DRFWTRALTGYAQLI	1501	8	**144.92 (WB) WTRALTGYA**	**292.7 (WB) WTRALTGYA**	1164.6 ALTGYAQLI	1208.0 WTRALTGYA
DRFWTRALTGYAQLI	1601	ND	**88.90 (WB) WTRALTGYA**	ND	ND	ND

HLA-DQ	Allele molecule	SYFPEITHI [a]	NetMHCIIpan 2.1	NetMHCII 2.2	IEDB	IEDB
Sequence	**HLA-DQA1-DQB1**	**Score**	**Affinity nM**	**Affinity nM**	**arbpython (ARB) Affinity nM**	**SMM Affinity nM**
RTDLDRFWTRALTGY	0101-0501 (DQ1(5))	ND	ND	3350.1 LDRFWTRAL	17544.9 LDRFWTRAL	2122.0 LDRFWTRAL
LDRFWTRALTGYAQL	0101-0501 (DQ1(5))	ND	ND	3624.5 LDRFWTRAL	6495.7 RALTGYAQL	1964.0 LDRFWTRAL
LDRFWTRALTGYAQL	0102-0602 (DQ1(6))	ND	ND	**110.3 (WB) TRALTGYAQ**	**416.4 (WB) TRALTGYAQ**	863.0 TRALTGYAQ
DRFWTRALTGYAQLI	0301-0302 (DQ3(8))	ND	ND	741.7 TRALTGYAQ	1508.9 RALTGYAQL	2599.0 TRALTGYAQ
DRFWTRALTGYAQLI	0401-0402 (DQ4)	ND	ND	2289.2 ALTGYAQLI	8839.3 TRALTGYAQ	5501.0 TRALTGYAQ
DRFWTRALTGYAQLI	0501-0201 (DQ2)	ND	ND	2258.9 ALTGYAQLI	3126.6 TRALTGYAQ	4043.0 RALTGYAQL
DRFWTRALTGYAQLI	0501-0301 (DQ3(7))	ND	ND	**39.8 (SB) ALTGYAQLI**	**268.7 (WB) RALTGYAQL**	**150.0 (WB) TRALTGYAQ**

[a] SYFPEITHI scores ≥ 25 are considered positive
Affinity nM: Strong binder (SB) = between 1 and 50 nM
Weak binder (WB) = between 50 and 500 nM
Core amino acids sequences are indicated along with Affinity nM values

Supplementary Table II. HLA-DR and DQ binding predictions Rv2034 p88-107 (FWTRALTGYAQLIDSEGDDT)

HLA-DR	Allele molecule	SYFPEITHI [a]	NetMHCIIpan 2.1	NetMHCII 2.2	IEDB	IEDB
Sequence	**HLA-DRB1**	**Score**	**Affinity nM**	**Affinity nM**	**arbpython (ARB) Affinity nM**	**SMM Affinity nM**
LTGYAQLIDSEGDDT	0101	18	**312.24 (WB) YAQLIDSEG**	**461.4 (WB) YAQLIDSEG**	**231.3 (WB) YAQLIDSEG**	**253.0 (WB) YAQLIDSEG**
TRALTGYAQLIDSEG	0101	9	**181.26 (WB) ALTGYAQLI**	**92.6 (WB) ALTGYAQLI**	**24.6 (SB) ALTGYAQLI**	**160.0 (WB) ALTGYAQLI**
FWTRALTGYAQLIDS	0101	10	**19.73 (SB) WTRALTGYA**	**35.8 (SB) TRALTGYAQ**	**21.7 (SB) WTRALTGYA**	**199.0 (WB) TRALTGYAQ**
TRALTGYAQLIDSEG	0301 (17)	18	8678.79 ALTGYAQLI	8615.4 LTGYAQLID	6774.6 LTGYAQLID	12827.0 ALTGYAQLI
WTRALTGYAQLIDSE	0301 (17)	4	7642.93 ALTGYAQLI	7257.2 LTGYAQLID	6774.6 LTGYAQLID	12186.0 ALTGYAQLI
ALTGYAQLIDSEGDD	0301 (17)	1	10489.46 GYAQLIDSE	12849.8 LTGYAQLID	**134.5 (WB) QLIDSEGDD**	11880.0 ALTGYAQLI
LTGYAQLIDSEGDDT	0401	16	845.88 YAQLIDSEG	**298.6 (WB) YAQLIDSEG**	1206.2 YAQLIDSEG	910.0 YAQLIDSEG
FWTRALTGYAQLIDS	0401	6	**235.93 (WB) WTRALTGYA**	623.0 TRALTGYAQ	1500.4 WTRALTGYA	1752.0 TRALTGYAQ
FWTRALTGYAQLIDS	0701	16	**149.85 (WB) WTRALTGYA**	1010.6 ALTGYAQLI	1564.2 FWTRALTGY	1435.0 ALTGYAQLI
FWTRALTGYAQLIDS	0801	ND	**114.75 (WB) WTRALTGYA**	ND	ND	ND
FWTRALTGYAQLIDS	0901	ND	**49.71 (SB) WTRALTGYA**	**44.5 (SB) WTRALTGYA**	1575.8 WTRALTGYA	508.0 WTRALTGYA
FWTRALTGYAQLIDS	1001	ND	**90.27 (WB) WTRALTGYA**	ND	ND	ND
LTGYAQLIDSEGDDT	1101	22	5316.28 YAQLIDSEG	4891.0 YAQLIDSEG	10335.7 YAQLIDSEG	2749.0 YAQLIDSEG
FWTRALTGYAQLIDS	1101	0	**129.36 (WB) WTRALTGYA**	**270.2 (WB) TRALTGYAQ**	506.2 WTRALTGYA	1727.0 TRALTGYAQ
WTRALTGYAQLIDSE	1201	ND	**130.28 (WB) WTRALTGYA**	ND	**76.9 (WB) GYAQLIDSE**	5793.0 ALTGYAQLI
FWTRALTGYAQLIDS	1201	ND	**73.92 (WB) WTRALTGYA**	ND	**226.0 (WB) FWTRALTGY**	1436.0 FWTRALTGY
FWTRALTGYAQLIDS	1301	ND	1301.10 ALTGYAQLI	ND	ND	ND
FWTRALTGYAQLIDS	1401	ND	1119.26 WTRALTGYA	ND	ND	ND
TRALTGYAQLIDSEG	1501	**26**	1920.39 ALTGYAQLI	2419.7 ALTGYAQLI	1164.6 ALTGYAQLI	2869.0 ALTGYAQLI
LTGYAQLIDSEGDDT	1501	4	6484.58 YAQLIDSEG	7948.3 LTGYAQLID	499550.7 LIDSEGDDT	8760.0 LTGYAQLID
FWTRALTGYAQLIDS	1501	4	**423.74 (WB) WTRALTGYA**	**404.7 (WB) WTRALTGYA**	1164.6 ALTGYAQLI	1725.0 TRALTGYAQ
FWTRALTGYAQLIDS	1601	ND	**244.06 (WB) WTRALTGYA**	ND	ND	ND

HLA-DQ	Allele molecule	SYFPEITHI [a]	NetMHCIIpan 2.1	NetMHCII 2.2	IEDB	IEDB
Sequence	**HLA-DQA1-DQB1**	**Score**	**Affinity nM**	**Affinity nM**	**arbpython (ARB) Affinity nM**	**SMM Affinity nM**
WTRALTGYAQLIDSE	0101-0501 (DQ1(5))	ND	ND	932.5 ALTGYAQLI	6495.7 RALTGYAQL	3032.0 LTGYAQLID
FWTRALTGYAQLIDS	0102-0602 (DQ1(6))	ND	ND	**200.7 (WB) TRALTGYAQ**	**416.4 (WB) TRALTGYAQ**	875.0 TRALTGYAQ
WTRALTGYAQLIDSE	0102-0602 (DQ1(6))	ND	ND	**278.2 (WB) TGYAQLIDS**	**416.4 (WB) TRALTGYAQ**	982.0 TGYAQLIDS
WTRALTGYAQLIDSE	0301-0302 (DQ3(8))	ND	ND	791.3 TRALTGYAQ	1508.9 RALTGYAQL	2218.0 ALTGYAQLI
RALTGYAQLIDSEGD	0301-0302 (DQ3(8))	ND	ND	918.5 GYAQLIDSE	1508.9 RALTGYAQL	2038.0 GYAQLIDSE
WTRALTGYAQLIDSE	0401-0402 (DQ4)	ND	ND	720.1 LTGYAQLID	8839.3 TRALTGYAQ	1907.0 LTGYAQLID
TRALTGYAQLIDSEG	0401-0402 (DQ4)	ND	ND	839.6 GYAQLIDSE	6060.8 YAQLIDSEG	1823.0 GYAQLIDSE
LTGYAQLIDSEGDDT	0501-0201 (DQ2)	ND	ND	576.6 GYAQLIDSE	2693.9 GYAQLIDSE	**498.0 (WB) GYAQLIDSE**
WTRALTGYAQLIDSE	0501-0201 (DQ2)	ND	ND	**383.2 (WB) GYAQLIDSE**	2693.9 GYAQLIDSE	1259.0 RALTGYAQL
FWTRALTGYAQLIDS	0501-0301 (DQ3(7))	ND	ND	**31.6 (SB) TGYAQLIDS**	**268.7 (WB) RALTGYAQL**	**116.0 (WB) ALTGYAQLI**

[a] SYFPEITHI scores ≥ 25 are considered positive
Affinity nM: Strong binder (SB) = between 1 and 50 nM
Weak binder (WB) = between 50 and 500 nM
Core amino acids sequences are indicated along with Affinity nM values

Supplementary Table III. HLA-DR and DQ binding predictions Rv2034 p11-30 (WQALADGTRRAIVERLAHGP)

HLA-DR	Allele molecule	SYFPEITHI [a]	NetMHCIIpan 2.1	NetMHCII 2.2	IEDB	IEDB
Sequence	**HLA-DRB1**	**Score**	**Affinity nM**	**Affinity nM**	**arbpython (ARB) Affinity nM**	**SMM Affinity nM**
WQALADGTRRAIVER	0101	17	936.57 WQALADGTR	1338.2 WQALADGTR	**328.2 (WB) WQALADGTR**	1280.0 WQALADGTR
ADGTRRAIVERLAHG	0101	10	548.27 TRRAIVERL	**217.2 (WB) TRRAIVERL**	**162.3 (WB) TRRAIVERL**	966.0 TRRAIVERL
DGTRRAIVERLAHGP	0101	17	649.35 TRRAIVERL	**358.4 (WB) TRRAIVERL**	**162.3 (WB) TRRAIVERL**	704.0 RRAIVERLA
WQALADGTRRAIVER	0301 (17)	17	**382.05(WB) ALADGTRRA**	3657.0 ALADGTRRA	778.5 WQALADGTR	1964.0 ALADGTRRA
LADGTRRAIVERLAH	0301 (17)	2	2573.34 TRRAIVERL	2246.2 TRRAIVERL	7041.1 RRAIVERLA	3947.0 TRRAIVERL
WQALADGTRRAIVER	0401	20	2619.02 ALADGTRRA	1776.5 ALADGTRRA	6144.3 ALADGTRRA	1671.0 ALADGTRRA
QALADGTRRAIVERL	0401	12	3992.11 ALADGTRRA	1705.9 ALADGTRRA	1899.1 TRRAIVERL	2629.0 LADGTRRAI
WQALADGTRRAIVER	0701	16	2050.02 LADGTRRAI	7737.1 LADGTRRAI	7861.3 LADGTRRAI	2323.0 ADGTRRAIV
LADGTRRAIVERLAH	0701	0	1160.30 TRRAIVERL	2146.9 TRRAIVERL	**416.1 (WB) TRRAIVERL**	1726.0 TRRAIVERL
QALADGTRRAIVERL	0701	16	1391.66 TRRAIVERL	1753.3 TRRAIVERL	**416.1 (WB) TRRAIVERL**	1222.0 LADGTRRAI
DGTRRAIVERLAHGP	0801	ND	**102.49 (WB) RAIVERLAH**	ND	ND	ND
LADGTRRAIVERLAH	0901	ND	942.60 TRRAIVERL	1257.0 RRAIVERLA	7453.7 TRRAIVERL	796.0 RRAIVERLA
WQALADGTRRAIVER	0901	ND	1869.34 WQALADGTR	1622.3 LADGTRRAI	4085.1 WQALADGTR	2363.0 LADGTRRAI
ADGTRRAIVERLAHG	1001	ND	1264.06 TRRAIVERL	ND	ND	ND
DGTRRAIVERLAHGP	1101	16	**499.56 (WB) RAIVERLAH**	**90.7 (WB) RAIVERLAH**	**437.9 (WB) TRRAIVERL**	1172.0 RAIVERLAH
QALADGTRRAIVERL	1101	9	1500.09 LADGTRRAI	589.6 LADGTRRAI	**437.9 (WB) TRRAIVERL**	981.0 LADGTRRAI
DGTRRAIVERLAHGP	1201	ND	**209.21 (WB) RAIVERLAH**	ND	**233.7 (WB) AIVERLAHG**	5680.0 AIVERLAHG
ALADGTRRAIVERLA	1201	ND	**450.26 (WB) TRRAIVERL**	ND	**236.4 (WB) RRAIVERLA**	38230.0 ALADGTRRA
DGTRRAIVERLAHGP	1301	ND	1347.99 TRRAIVERL	ND	ND	ND
DGTRRAIVERLAHGP	1401	ND	1333.73 RAIVERLAH	ND	ND	ND
WQALADGTRRAIVER	1501	14	3684.32 ALADGTRRA	7965.6 ADGTRRAIV	39071.2 LADGTRRAI	8956.0 ALADGTRRA
DGTRRAIVERLAHGP	1501	4	2064.34 TRRAIVERL	3040.1 AIVERLAHG	**420.3 (WB) IVERLAHGP**	2426.0 RRAIVERLA
DGTRRAIVERLAHGP	1601	ND	1556.01 RRAIVERLA	ND	ND	ND

HLA-DQ	Allele molecule	SYFPEITHI [a]	NetMHCIIpan 2.1	NetMHCII 2.2	IEDB	IEDB
Sequence	**HLA-DQA1-DQB1**	**Score**	**Affinity nM**	**Affinity nM**	**arbpython (ARB) Affinity nM**	**SMM Affinity nM**
ADGTRRAIVERLAHG	0101-0501 (DQ1(5))	ND	ND	20442.7 TRRAIVERL	39420.9 TRRAIVERL	19682.0 TRRAIVERL
WQALADGTRRAIVER	0101-0501 (DQ1(5))	ND	ND	30725.4 ADGTRRAIV	30620.6 LADGTRRAI	25918.0 WQALADGTR
ADGTRRAIVERLAHG	0102-0602 (DQ1(6))	ND	ND	**342.7 (WB) TRRAIVERL**	2666.1 TRRAIVERL	1450.0 RRAIVERLA
WQALADGTRRAIVER	0102-0602 (DQ1(6))	ND	ND	5621.9 ADGTRRAIV	1049.0 LADGTRRAI	3782.0 ADGTRRAIV
LADGTRRAIVERLAH	0301-0302 (DQ3(8))	ND	ND	1204.6 DGTRRAIVE	9616.6 RAIVERLAH	1551.0 TRRAIVERL
QALADGTRRAIVERL	0401-0402 (DQ4)	ND	ND	1364.5 DGTRRAIVE	10443.7 TRRAIVERL	2530.0 GTRRAIVER
LADGTRRAIVERLAH	0401-0402 (DQ4)	ND	ND	1758.0 DGTRRAIVE	10443.7 TRRAIVERL	2523.0 GTRRAIVER
DGTRRAIVERLAHGP	0501-0201 (DQ2)	ND	ND	1730.8 TRRAIVERL	5997.0 TRRAIVERL	3385.0 TRRAIVERL
QALADGTRRAIVERL	0501-0201 (DQ2)	ND	ND	**484.9 (WB) TRRAIVERL**	5997.0 TRRAIVERL	4564.0 GTRRAIVER
ALADGTRRAIVERLA	0501-0301 (DQ3(7))	ND	ND	**143.7 (WB) TRRAIVERL**	**14.6 (SB) ADGTRRAIV**	**255.0 (WB) ADGTRRAIV**
QALADGTRRAIVERL	0501-0301 (DQ3(7))	ND	ND	**154.4 (WB) ADGTRRAIV**	**14.6 (SB) ADGTRRAIV**	**237.0 (WB) ADGTRRAIV**

[a] SYFPEITHI scores ≥ 25 are considered positive
Affinity nM: Strong binder (SB) = between 1 and 50 nM
Weak binder (WB) = between 50 and 500 nM
Core amino acids sequences are indicated along with Affinity nM values

References

1. **Ottenhoff,T.H. and Kaufmann,S.H.,** Vaccines against tuberculosis: where are we and where do we need to go? *PLoS.Pathog.* 2012. **8**: e1002607.
2. **Ottenhoff,T.H.,** The knowns and unknowns of the immunopathogenesis of tuberculosis. *Int.J.Tuberc.Lung Dis.* 2012. **16**: 1424-1432.
3. **Perfetto,S.P., Chattopadhyay,P.K., and Roederer,M.,** Seventeen-colour flow cytometry: unravelling the immune system. *Nat.Rev.Immunol.* 2004. **4**: 648-655.
4. **Corti,D., Sallusto,F., and Lanzavecchia,A.,** High throughput cellular screens to interrogate the human T and B cell repertoires. *Curr.Opin.Immunol.* 2011. **23**: 430-435.
5. **Wehler,T.C., Karg,M., Distler,E., Konur,A., Nonn,M., Meyer,R.G., Huber,C., Hartwig,U.F., and Herr,W.,** Rapid identification and sorting of viable virus-reactive CD4(+) and CD8(+) T cells based on antigen-triggered CD137 expression. *J.Immunol.Methods* 2008. **339**: 23-37.
6. **Frentsch,M., Arbach,O., Kirchhoff,D., Moewes,B., Worm,M., Rothe,M., Scheffold,A., and Thiel,A.,** Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. *Nat.Med.* 2005. **11**: 1118-1124.
7. **Lindgren,H., Axcrona,K., and Leanderson,T.,** Regulation of transcriptional activity of the murine CD40 ligand promoter in response to signals through TCR and the costimulatory molecules CD28 and CD2. *J.Immunol.* 2001. **166**: 4578-4585.
8. **Banchereau,J., Bazan,F., Blanchard,D., Briere,F., Galizzi,J.P., Van,K.C., Liu,Y.J., Rousset,F., and Saeland,S.,** The CD40 antigen and its ligand. *Annu.Rev.Immunol.* 1994. **12**: 881-922.
9. **Elgueta,R., Benson,M.J., de Vries,V.C., Wasiuk,A., Guo,Y., and Noelle,R.J.,** Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol.Rev.* 2009. **229**: 152-172.
10. **Commandeur,S., van Meijgaarden,K.E., Prins,C., Pichugin,A.V., Dijkman,K., van den Eeden,S.J., Friggen,A.H., Franken,K.L., Dolganov,G., Kramnik,I., Schoolnik,G.K., Oftung,F., Korsvold,G.E., Geluk,A., and Ottenhoff,T.H.,** An Unbiased Genome-Wide Mycobacterium tuberculosis Gene Expression Approach To Discover Antigens Targeted by Human T Cells Expressed during Pulmonary Infection. *J.Immunol.* 2013.
11. **Commandeur,S., van Meijgaarden,K.E., Lin,M.Y., Franken,K.L., Friggen,A.H., Drijfhout,J.W., Oftung,F., Korsvold,G.E., Geluk,A., and Ottenhoff,T.H.,** Identification of human T-cell responses to Mycobacterium tuberculosis resuscitation-promoting factors in long-term latently infected individuals. *Clin.Vaccine Immunol.* 2011. **18**: 676-683.
12. **Commandeur,S., Lin,M.Y., van Meijgaarden,K.E., Friggen,A.H., Franken,K.L., Drijfhout,J.W., Korsvold,G.E., Oftung,F., Geluk,A., and Ottenhoff,T.H.,** Double- and monofunctional CD4 and CD8 T-cell responses to Mycobacterium tuberculosis DosR antigens and peptides in long-term latently infected individuals. *Eur.J.Immunol.* 2011. **41**: 2925-2936.
13. **Franken,K.L., Hiemstra,H.S., van Meijgaarden,K.E., Subronto,Y., den,H.J., Ottenhoff,T.H., and Drijfhout,J.W.,** Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. *Protein Expr.Purif.* 2000. **18**: 95-99.
14. **Leyten,E.M., Lin,M.Y., Franken,K.L., Friggen,A.H., Prins,C., van Meijgaarden,K.E., Voskuil,M.I., Weldingh,K., Andersen,P., Schoolnik,G.K., Arend,S.M., Ottenhoff,T.H., and Klein,M.R.,**

Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of Mycobacterium tuberculosis. *Microbes.Infect.* 2006. **8**: 2052-2060.

15. **Black,G.F., Thiel,B.A., Ota,M.O., Parida,S.K., Adegbola,R., Boom,W.H., Dockrell,H.M., Franken,K.L., Friggen,A.H., Hill,P.C., Klein,M.R., Lalor,M.K., Mayanja,H., Schoolnik,G., Stanley,K., Weldingh,K., Kaufmann,S.H., Walzl,G., and Ottenhoff,T.H.,** Immunogenicity of novel DosR regulon-encoded candidate antigens of Mycobacterium tuberculosis in three high-burden populations in Africa. *Clin.Vaccine Immunol.* 2009. **16**: 1203-1212.

16. **Geluk,A., van den Eeden,S.J., van Meijgaarden,K.E., Dijkman,K., Franken,K.L., and Ottenhoff,T.H.,** A multistage-polyepitope vaccine protects against Mycobacterium tuberculosis infection in HLA-DR3 transgenic mice. *Vaccine* 2012. **30**: 7513-7521.

17. **Rosenkrands,I., Slayden,R.A., Crawford,J., Aagaard,C., Barry,C.E., III, and Andersen,P.,** Hypoxic response of Mycobacterium tuberculosis studied by metabolic labeling and proteome analysis of cellular and extracellular proteins. *J.Bacteriol.* 2002. **184**: 3485-3491.

18. **Betts,J.C., Lukey,P.T., Robb,L.C., McAdam,R.A., and Duncan,K.,** Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol.Microbiol.* 2002. **43**: 717-731.

19. **Unger,W.W., Pearson,T., Abreu,J.R., Laban,S., van der Slik,A.R., der Kracht,S.M., Kester,M.G., Serreze,D.V., Shultz,L.D., Griffioen,M., Drijfhout,J.W., Greiner,D.L., and Roep,B.O.,** Islet-Specific CTL Cloned from a Type 1 Diabetes Patient Cause Beta-Cell Destruction after Engraftment into HLA-A2 Transgenic NOD/SCID/IL2RG Null Mice. *PLoS.One.* 2012. **7**: e49213.

20. **Perfetto,S.P., Chattopadhyay,P.K., Lamoreaux,L., Nguyen,R., Ambrozak,D., Koup,R.A., and Roederer,M.,** Amine-reactive dyes for dead cell discrimination in fixed samples. *Curr.Protoc.Cytom.* 2010. **Chapter 9**: Unit.

21. **Ottenhoff,T.H., Neuteboom,S., Elferink,D.G., and de Vries,R.R.,** Molecular localization and polymorphism of HLA class II restriction determinants defined by Mycobacterium leprae-reactive helper T cell clones from leprosy patients. *J.Exp.Med.* 1986. **164**: 1923-1939.

22. **Geluk,A., van Meijgaarden,K.E., Janson,A.A., Drijfhout,J.W., Meloen,R.H., de Vries,R.R., and Ottenhoff,T.H.,** Functional analysis of DR17(DR3)-restricted mycobacterial T cell epitopes reveals DR17-binding motif and enables the design of allele-specific competitor peptides. *J.Immunol.* 1992. **149**: 2864-2871.

23. **Joosten,S.A., van Meijgaarden,K.E., van Weeren,P.C., Kazi,F., Geluk,A., Savage,N.D., Drijfhout,J.W., Flower,D.R., Hanekom,W.A., Klein,M.R., and Ottenhoff,T.H.,** Mycobacterium tuberculosis peptides presented by HLA-E molecules are targets for human CD8 T-cells with cytotoxic as well as regulatory activity. *PLoS.Pathog.* 2010. **6**: e1000782.

24. **Schagger,H. and Von,J.G.,** Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal.Biochem.* 1987. **166**: 368-379.

25. **Ottenhoff,T.H., Elferink,D.G., Klatser,P.R., and de Vries,R.R.,** Cloned suppressor T cells from a lepromatous leprosy patient suppress Mycobacterium leprae reactive helper T cells. *Nature* 1986. **322**: 462-464.

26. **Ottenhoff,T.H., Klatser,P.R., Ivanyi,J., Elferink,D.G., de Wit,M.Y., and de Vries,R.R.,** Mycobacterium leprae-specific protein antigens defined by cloned human helper T cells. *Nature* 1986. **319**: 66-68.

27. **Billeskov,R., Elvang,T.T., Andersen,P.L., and Dietrich,J.,** The HyVac4 subunit vaccine efficiently boosts BCG-primed anti-mycobacterial protective immunity. *PLoS.One.* 2012. **7**: e39909.

28. **Yellin,M.J., Sippel,K., Inghirami,G., Covey,L.R., Lee,J.J., Sinning,J., Clark,E.A., Chess,L., and Lederman,S.,** CD40 molecules induce down-modulation and endocytosis of T cell surface T cell-B cell activating molecule/CD40-L. Potential role in regulating helper effector function. *J.Immunol.* 1994. **152**: 598-608.

29. **Graf,D., Muller,S., Korthauer,U., Van,K.C., Weise,C., and Kroczek,R.A.,** A soluble form of TRAP (CD40 ligand) is rapidly released after T cell activation. *Eur.J.Immunol.* 1995. **25**: 1749-1754.

30. **Hermann,P., Van-Kooten,C., Gaillard,C., Banchereau,J., and Blanchard,D.,** CD40 ligand-positive CD8+ T cell clones allow B cell growth and differentiation. *Eur.J.Immunol.* 1995. **25**: 2972-2977.

31. **Horner,A.A., Jabara,H., Ramesh,N., and Geha,R.S.,** gamma/delta T lymphocytes express CD40 ligand and induce isotype switching in B lymphocytes. *J.Exp.Med.* 1995. **181**: 1239-1244.

32. **Cowley,S.C., Hamilton,E., Frelinger,J.A., Su,J., Forman,J., and Elkins,K.L.,** CD4-CD8- T cells control intracellular bacterial infections both in vitro and in vivo. *J.Exp.Med.* 2005. **202**: 309-319.

33. **Derrick,S.C., Evering,T.H., Sambandamurthy,V.K., Jalapathy,K.V., Hsu,T., Chen,B., Chen,M., Russell,R.G., Junqueira-Kipnis,A.P., Orme,I.M., Porcelli,S.A., Jacobs,W.R., Jr., and Morris,S.L.,** Characterization of the protective T-cell response generated in CD4-deficient mice by a live attenuated Mycobacterium tuberculosis vaccine. *Immunology* 2007. **120**: 192-206.

34. **Pinheiro,M.B., Antonelli,L.R., Sathler-Avelar,R., Vitelli-Avelar,D.M., Spindola-de-Miranda,S., Guimaraes,T.M., Teixeira-Carvalho,A., Martins-Filho,O.A., and Toledo,V.P.,** CD4-CD8-alphabeta and gammadelta T cells display inflammatory and regulatory potentials during human tuberculosis. *PLoS.One.* 2012. **7**: e50923.

35. **D'Acquisto,F. and Crompton,T.,** CD3+CD4-CD8- (double negative) T cells: saviours or villains of the immune response? *Biochem.Pharmacol.* 2011. **82**: 333-340.

36. **Gold,M.C., Cerri,S., Smyk-Pearson,S., Cansler,M.E., Vogt,T.M., Delepine,J., Winata,E., Swarbrick,G.M., Chua,W.J., Yu,Y.Y., Lantz,O., Cook,M.S., Null,M.D., Jacoby,D.B., Harriff,M.J., Lewinsohn,D.A., Hansen,T.H., and Lewinsohn,D.M.,** Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS.Biol.* 2010. **8**: e1000407.

37. **Martin,E., Treiner,E., Duban,L., Guerri,L., Laude,H., Toly,C., Premel,V., Devys,A., Moura,I.C., Tilloy,F., Cherif,S., Vera,G., Latour,S., Soudais,C., and Lantz,O.,** Stepwise development of MAIT cells in mouse and human. *PLoS.Biol.* 2009. **7**: e54.

38. **Joosten,S.A., van Meijgaarden,K.E., van Weeren,P.C., Kazi,F., Geluk,A., Savage,N.D., Drijfhout,J.W., Flower,D.R., Hanekom,W.A., Klein,M.R., and Ottenhoff,T.H.,** Mycobacterium tuberculosis peptides presented by HLA-E molecules are targets for human CD8 T-cells with cytotoxic as well as regulatory activity. *PLoS.Pathog.* 2010. **6**: e1000782.

39. **Arden,B., Clark,S.P., Kabelitz,D., and Mak,T.W.,** Human T-cell receptor variable gene segment families. *Immunogenetics* 1995. **42**: 455-500.

40. **Aagaard,C., Hoang,T., Dietrich,J., Cardona,P.J., Izzo,A., Dolganov,G., Schoolnik,G.K., Cassidy,J.P., Billeskov,R., and Andersen,P.,** A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nat.Med.* 2011. **17**: 189-194.

41. **Lin,P.L., Dietrich,J., Tan,E., Abalos,R.M., Burgos,J., Bigbee,C., Bigbee,M., Milk,L., Gideon,H.P., Rodgers,M., Cochran,C., Guinn,K.M., Sherman,D.R., Klein,E., Janssen,C., Flynn,J.L., and Andersen,P.,** The multistage vaccine H56 boosts the effects of BCG to protect cynomolgus

macaques against active tuberculosis and reactivation of latent Mycobacterium tuberculosis infection. *J.Clin.Invest* 2012. **122**: 303-314.

42. **Hatton,R.D. and Weaver,C.T.,** Immunology. T-bet or not T-bet. *Science* 2003. **302**: 993-994.

43. **Szabo,S.J., Kim,S.T., Costa,G.L., Zhang,X., Fathman,C.G., and Glimcher,L.H.,** A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000. **100**: 655-669.

44. **Zheng,W. and Flavell,R.A.,** The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 1997. **89**: 587-596.

45. **Ivanov,I.I., McKenzie,B.S., Zhou,L., Tadokoro,C.E., Lepelley,A., Lafaille,J.J., Cua,D.J., and Littman,D.R.,** The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 2006. **126**: 1121-1133.

46. **Manel,N., Unutmaz,D., and Littman,D.R.,** The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat.Immunol.* 2008. **9**: 641-649.

47. **Hori,S., Nomura,T., and Sakaguchi,S.,** Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003. **299**: 1057-1061.

48. **Boer,M.C., van Meijgaarden,K.E., Bastid,J., Ottenhoff,T.H., and Joosten,S.A.,** CD39 is involved in mediating suppression by Mycobacterium bovis BCG-activated human CD8 CD39 regulatory T cells. *Eur.J.Immunol.* 2013.

49. **Triplett,T.A., Curti,B.D., Bonafede,P.R., Miller,W.L., Walker,E.B., and Weinberg,A.D.,** Defining a functionally distinct subset of human memory CD4+ T cells that are CD25POS and FOXP3NEG. *Eur.J.Immunol.* 2012. **42**: 1893-1905.

50. **Moncrieffe,H., Nistala,K., Kamhieh,Y., Evans,J., Eddaoudi,A., Eaton,S., and Wedderburn,L.R.,** High expression of the ectonucleotidase CD39 on T cells from the inflamed site identifies two distinct populations, one regulatory and one memory T cell population. *J.Immunol.* 2010. **185**: 134-143.

51. **Malek,T.R.,** The biology of interleukin-2. *Annu.Rev.Immunol.* 2008. **26**: 453-479.

52. **Mutis,T., Cornelisse,Y.E., and Ottenhoff,T.H.,** Mycobacteria induce CD4+ T cells that are cytotoxic and display Th1-like cytokine secretion profile: heterogeneity in cytotoxic activity and cytokine secretion levels. *Eur.J.Immunol.* 1993. **23**: 2189-2195.

53. **Bastian,M., Braun,T., Bruns,H., Rollinghoff,M., and Stenger,S.,** Mycobacterial lipopeptides elicit CD4+ CTLs in Mycobacterium tuberculosis-infected humans. *J.Immunol.* 2008. **180**: 3436-3446.

54. **Ottenhoff,T.H. and Mutis,T.,** Role of cytotoxic cells in the protective immunity against and immunopathology of intracellular infections. *Eur.J.Clin.Invest* 1995. **25**: 371-377.

55. **Rammensee,H., Bachmann,J., Emmerich,N.P., Bachor,O.A., and Stevanovic,S.,** SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 1999. **50**: 213-219.

56. **Blythe,M.J., Zhang,Q., Vaughan,K., de,C.R., Jr., Salimi,N., Bui,H.H., Lewinsohn,D.M., Ernst,J.D., Peters,B., and Sette,A.,** An analysis of the epitope knowledge related to Mycobacteria. *Immunome.Res.* 2007. **3**: 10.

57. **Caccamo,N., Barera,A., Di,S.C., Meraviglia,S., Ivanyi,J., Hudecz,F., Bosze,S., Dieli,F., and Salerno,A.,** Cytokine profile, HLA restriction and TCR sequence analysis of human CD4+ T clones specific for an immunodominant epitope of Mycobacterium tuberculosis 16-kDa protein. *Clin.Exp.Immunol.* 2003. **133**: 260-266.

58. **Axelsson-Robertson,R., Weichold,F., Sizemore,D., Wulf,M., Skeiky,Y.A., Sadoff,J., and Maeurer,M.J.,** Extensive major histocompatibility complex class I binding promiscuity for Mycobacterium tuberculosis TB10.4

peptides and immune dominance of human leucocyte antigen (HLA)-B*0702 and HLA-B*0801 alleles in TB10.4 CD8 T-cell responses. *Immunology* 2010. **129**: 496-505.

59. **Mustafa,A.S., Lundin,K.E., Meloen,R.H., Shinnick,T.M., and Oftung,F.,** Identification of promiscuous epitopes from the Mycobacterial 65-kilodalton heat shock protein recognized by human CD4(+) T cells of the Mycobacterium leprae memory repertoire. *Infect. Immun.* 1999. **67**: 5683-5689.

60. **Ho,P.C., Mutch,D.A., Winkel,K.D., Saul,A.J., Jones,G.L., Doran,T.J., and Rzepczyk,C.M.,** Identification of two promiscuous T cell epitopes from tetanus toxin. *Eur.J.Immunol.* 1990. **20**: 477-483.

61. **Rao,X., Hoof,I., Costa,A.I., van,B.D., and Kesmir,C.,** HLA class I allele promiscuity revisited. *Immunogenetics* 2011. **63**: 691-701.

62. **Geluk,A., van Meijgaarden,K.E., and Ottenhoff,T.H.,** Flexibility in T-cell receptor ligand repertoires depends on MHC and T-cell receptor clonotype. *Immunology* 1997. **90**: 370-375.

63. **Yousef,S., Planas,R., Chakroun,K., Hoffmeister-Ullerich,S., Binder,T.M., Eiermann,T.H., Martin,R., and Sospedra,M.,** TCR bias and HLA cross-restriction are strategies of human brain-infiltrating JC virus-specific CD4+ T cells during viral infection. *J.Immunol.* 2012. **189**: 3618-3630.

64. **Sospedra,M., Zhao,Y., Giulianotti,M., Simon,R., Pinilla,C., and Martin,R.,** Combining positional scanning peptide libraries, HLA-DR transfectants and bioinformatics to dissect the epitope spectrum of HLA class II cross-restricted CD4+ T cell clones. *J.Immunol. Methods* 2010. **353**: 93-101.

65. **Greenbaum,J., Sidney,J., Chung,J., Brander,C., Peters,B., and Sette,A.,** Functional classification of class II human leukocyte antigen (HLA) molecules reveals seven different supertypes and a surprising degree of repertoire sharing across supertypes. *Immunogenetics* 2011. **63**: 325-335.

66. **McKinney,D.M., Southwood,S., Hinz,D., Oseroff,C., Arlehamn,C.S., Schulten,V., Taplitz,R., Broide,D., Hanekom,W.A., Scriba,T.J., Wood,R., Alam,R., Peters,B., Sidney,J., and Sette,A.,** A strategy to determine HLA class II restriction broadly covering the DR, DP, and DQ allelic variants most commonly expressed in the general population. *Immunogenetics* 2013.

67. **Gao,C.H., Yang,M., and He,Z.G.,** Characterization of a novel ArsR-like regulator encoded by Rv2034 in Mycobacterium tuberculosis. *PLoS.One.* 2012. **7**: e36255.

68. **Malen,H., Pathak,S., Softeland,T., de Souza,G.A., and Wiker,H.G.,** Definition of novel cell envelope associated proteins in Triton X-114 extracts of Mycobacterium tuberculosis H37Rv. *BMC.Microbiol.* 2010. **10**: 132.

69. **de Souza,G.A., Leversen,N.A., Malen,H., and Wiker,H.G.,** Bacterial proteins with cleaved or uncleaved signal peptides of the general secretory pathway. *J.Proteomics.* 2011. **75**: 502-510.

CHAPTER 7

Summary and Discussion

Summary and Discussion

Currently, only one TB vaccine is available: *Mycobacterium bovis* Bacille Calmette-Guérin (BCG). This vaccine induces highly variable protection against pulmonary TB, the most common and contagious form of TB. There is an urgent need for an effective TB vaccine which is safe also in the immunocompromised host.

Multiple subunit vaccines are currently under study and consist mostly of antigens that are expressed by actively replicating *Mtb*. However, *Mtb* has the ability to enter into a less active, latent or dormant state. *Mtb* uses this adaptation strategy to survive harsh and stressful conditions inside human cells. *Mtb* adapts to these conditions by changing its gene expression pattern. A gene cluster specifically upregulated in response to hypoxia is known as the dormancy regulon encoded (DosR) gene cluster. A second set of proteins is involved in the reactivation of dormant bacteria, and is known as resuscitation promoting factors (Rpfs).

DosR regulon encoded proteins and Rpf proteins are associated with certain phases of the *Mtb* infection cycle, and have therefore been coined *Mtb* infection-phase related antigens. It remains unclear how many other proteins are regulated during *Mtb* infection. These phase related proteins could represent antigens which might represent efficient subunit vaccine candidates for prophylactic or therapeutic vaccines during essential steps of the infection cycle. To qualify as an efficient vaccine candidate, an antigen should meet several prerequisites:

(i) The antigen should be immunogenic, that is, the protein should be recognized by antigen-specific T cells from mycobacteria exposed individuals.

(ii) The antigen should be expressed during *in vivo* infection in infected tissue, such that vaccine-induced T cells are able to recognize *Mtb* infected cells.

(iii) The antigen should induce TB protection, that is: the protein should generate an immune response that is able to limit the growth of *Mtb* or eliminate the bacteria, with limited tissue damage.

(iv) The protein sequence should be conserved among multiple *Mtb* strains, such that vaccines based on these antigens can be applied globally against multiple *Mtb* strains.

The work in this thesis has focused on *Mtb* infection-phase related antigens as possible TB subunit vaccine candidates, and will be discussed here with a particular focus on these four requirements.

Immunogenic *Mtb* infection-phase related antigens as new TB vaccine candidates

Understanding *Mtb* specific T-cell responses in clinically relevant human populations is an important step in identifying essential mechanisms of protective immunity against TB, which in turn will facilitate the design of better TB vaccines. We have studied the ability of T cells to recognize three different classes of new candidate *Mtb* antigens, described in this thesis, as new antigen candidates for improved TB vaccines. Rpf and DosR regulon encoded antigens

were identified previously by our group as *Mtb* infection-phase related antigens, whereas *in vivo* expressed *Mtb* (IVE-TB) antigens are described here for the first time.

Vaccine induced, Rpf-specific T cells might be useful in enhancing immune surveillance of reactivating and resuscitating *Mtb* and thereby help to control *Mtb* reactivation. Of the five Rpf homologous proteins encoded by *Mtb* (Rv0867c, Rv1009, Rv1884c, Rv2389c, Rv2450c: RpfA-E) two, Rv0867c and Rv2389c, were screened for their immunogenicity. Both Rpf antigens were immunogenic (**chapter 2**), based on antigen-specific T-cell cytokine production, the presence of immunogenic epitopes and highly specific T-cell frequencies in latent infected individuals. Therefore, these proteins fulfill at least one of the requirements for a potential *Mtb* vaccine candidate, and this was confirmed by other groups studying mycobacteria exposed individuals [1-3]. The immunogenicity of Rpf antigens has also been analyzed in C57BL/6 and BALB/c mice [4]. Rv0867c and Rv2389c did not show any responses either induced by mycobacterial infection (Rv0867c) or DNA vaccination (Rv2389c). This was surprising in view of their immunogenicity in humans, and is most likely by the lack of proper MHC molecules able to present Rv0867c or Rv2389c immunogenic epitopes. In any event, this demonstrates the difficulty of translating results across species. Using humanized HLA-transgenic mice for such analyses could limit these species differences (**chapter 5**) [5].

Mtb DosR regulon encoded antigens Rv1733c, Rv2029c and Rv2031c (HspX) were shown to be immunogenic as measured by proliferation of $CD4^+$ and $CD8^+$ T cells from purified protein derivative positive (PPD^+) donors and the presence of cytokine producing $CD4^+$ and $CD8^+$ T cells in long-term latently infected individuals (ltLTBI) (**chapter 3**), corresponding to previous work [2;6;7]. These ltLTBI donors had been exposed to *Mtb* decades ago yet did not develop any clinical signs of TB despite never having received prophylactic treatment. Thus, they represent a group of "naturally protected" individuals. Multiple immunogenic regions were detected for both $CD4^+$ and $CD8^+$ T cells. A recent study incorporated HLA-DR3 restricted epitopes of Rv1733c ($Rv1733_{63-77}$), Rv2031c ($Rv2031c_{31-50}$) and Ag85B ($Ag85B_{56-46}$) in a poly-epitope vaccine which induced an HLA-DR3-specific reduction of *Mtb* CFU in HLA-DR3 transgenic mice [5], indicating that these antigens are of particular interest as vaccine candidates.

Recognition of the dormancy antigen HspX by mycobacteria exposed individuals is inconsistent (ranging from being well recognized e.g. [8;9] to limited recognition e.g. [6;7;10]) but despite these contrasting findings HspX has been analyzed in multiple vaccination studies [5;11-15], showing protective efficacy against *Mtb*. Importantly, BCG vaccination does not induce antigen-specific T cells against *Mtb* DosR regulon encoded antigens, including HspX [9;16] whereas overexpression of HspX in BCG enhances *Mtb* protection, indicating that HspX may play a role in TB control depending on the precise vaccine background [11]. Interestingly, in one particular study recombinant HspX vaccination did not provide TB protection, whereas the native form of HspX did [13]. It was suggested that the native form chaperones an immunogenic and protective antigen or peptide(s) [13]. Thus, despite the variable immunogenicity results, HspX remains an interesting vaccine candidate.

In **chapter 4** we describe a novel set of antigens, namely *in vivo* expressed *Mtb* antigens (IVE-TB). These were identified from *Mtb* gene expression profiles detected in the lungs of *Mtb* infected mouse strains across a spectrum of TB susceptibility. Recombinant proteins were produced from a selection of these genes and their immunogenicity was analyzed using PBMC from tuberculin skin test positive (TST^+) donors. The IVE-TB antigens were specifically recognized by cells from TST^+ ESAT-6/CFP-10 (E/C^+) individuals. Furthermore, the IVE-TB protein sequences were conserved within *Mtb* strains. The immunogenicity of Rv0079, Rv1284 and Rv1956 has been analyzed previously. Rv0079 was recognized by a minority of mycobacteria exposed individuals [3;6;7], whereas Rv1284 and Rv1956 were strongly recognized in *Mycobacterium bovis* exposed cattle [17]. Indeed, we observed limited recognition of Rv0079 and strong recognition of Rv1284 and Rv1956 (**chapter 4**). Nevertheless Rv1284 could not induce protection against *Mtb* challenge in C57BL/6 mice, which was attributed to the lack of presentation of $H\text{-}2^b$ restricted T-cell epitopes [18]. Importantly, seven of the sixteen analyzed IVE-TB antigens were previously identified in studies analyzing the gene expression profile of *Mtb* grown under *in vitro* conditions that are thought to mimic those encountered by *Mtb in vivo*, including Rv0079, Rv1284 and Rv1956 [19-21]. This suggests that these conditions are also relevant during *in vivo* infection. Finally, immunization of HLA-DR transgenic mice with IVE-TB antigen Rv2034 resulted in a decrease of CFU after *Mtb* challenge. Thus, Rv2034 is able to induce a protective response against *Mtb* (**chapter 5**). Finally, (native) Rv2034 contains an HLA-DR restricted epitope that could be recognized by an Rv2034-specific $CD4^+$ T-cell clone as determined in **chapter 6**. Overall, the analyzed IVE-TB antigens are immunogenic, conserved, expressed *in vivo* and -at least for Rv2034- able to induce responses that reduce *Mtb* burden *in vivo*.

IVE-TB antigens: revealing potential *Mtb* pathways as targets for intervention

Analyzing *Mtb* gene expression patterns during *in vivo* infection can provide insight into microbial stress response pathways during infection, which may facilitate the selection of interesting vaccine candidate antigens. It is of obvious importance to select *Mtb* vaccine candidates that are expressed *in vivo* during infection. These antigens should include proteins that are not only expressed -and often secreted- during the early active replication phase (such as the current vaccine proteins ESAT-6 and Ag85B), but also proteins that are strongly expressed at later time points during infection. Studies reported the rather limited recognition of antigens from the Ag85 complex in mycobacteria exposed individuals, both in active TB patients and LTBI [1;3;22]. This unexpectedly low recognition may be explained by a low expression level of the gene encoding Ag85B (*fibronectin-binding protein B* (*fbpB*)) *in vivo* in the chronic phase of infection, as shown by Bold *et al.* [23], an effect they interpreted as an immune evasion mechanism. A related proposed mechanism is 'immunological decoy', implying that a non-essential (secreted) protein is highly produced initially (in the early phase of infection) and thus generates a pool of antigen-specific T cells, but is subsequently down regulated in the prolonged phase of infection, thus "misdirecting"

the immune system by hampering strong immunity against late infection-phase associated proteins [24]. This hypothesis argues against the use of only early secreted antigens as vaccine candidates since some of these may be down-regulated later in time. Thus (persistently) *in vivo* highly expressed antigens might have interesting potential as vaccine candidates.

In **chapter 4** a group of 68 genes was identified to be highly expressed *in vivo*, independent of genetic differences in host background of the mouse strains studied. Within the group of highly expressed *Mtb* genes, a cluster of three genes, *Rv2380c*, *Rv2381c* and *Rv2382c* was identified which encode for proteins MbtE, MbtD and MbtC. These proteins are involved in the synthesis of mycobactins, which are lipophylic siderophores. Mycobactins, together with carboxymycobactins, are required for iron acquisition from the host environment by hijacking iron from host iron-binding proteins [25], which supports the view that *Mtb* requires iron from the host *in vivo* for survival. Importantly, upon APC activation, the expression of the natural resistance associated macrophage protein (NRAMP1) metal transporter protein's expression is enhanced. This host protein is involved in the reduction of intraphagosomal iron levels [26]. Polymorphisms of the *NRAMP/solute carrier 11A1(SLC11A1))* gene are associated with TB susceptibility [26;27], supporting iron-competition between host and pathogen during *Mtb* infection. ESX-3, a secretion system suggested to be essential for survival, is involved in transport of iron-loaded mycobactins [28;29]. Interestingly, this cluster expresses two Esx-like molecules, EsxG and EsxH (*Rv0287* and *Rv0288*) which were both present in our set of highly expressed genes (**chapter 4**). Thus *Mtb* iron acquisition seems to be important during *in vivo* infection.

Not surprisingly, multiple genes were detected in our highly expressed set of genes that are linked to the virulence associated ESX-1 secretion system (*Rv3615c* (EspC), *Rv3616c* (EspA), *Rv3864* (EspE), *Rv3865* (EspF) and *Rv3875* (ESAT-6/EsxA)).

Lastly, the gene coding for isocitrate lyase (*Rv0467)*, which initiates the first step in the glyoxylate shunt and is required for *in vivo* survival, was also highly expressed during infection in the lung [30-32]. Also other genes were detected in this set that supposedly play a role in *Mtb* cell wall lipid assembly, lipid degradation and gluconeogenesis (e.g. FadD28 (DIM[33]), FadE5 (β-oxidation (cholesterol[34])) and FadB2 (β-oxidation [35]). Thus, these lipid associated metabolic pathways are likely to be important during *in vivo* infection.

Ex vivo and *in vivo* (whole genome) *Mtb* microarrays have been performed to identify genes that are expressed during infection as reviewed by Ward *et al.* [36] and Waddell *et al.* [37]. Limited overlap was observed between *Mtb* genes expressed during *ex vivo* and *in vivo* conditions, although the same categories of genes could be identified [36]. Indeed the expression of iron scavenger related genes, including genes from the above mentioned *mbt* gene cluster, was observed in *Mtb* infected murine and human host cells [38-42] and in *in vivo* infection [43], compatible with low levels of iron within the host cell [44]. Also the expression of genes associated with lipid metabolism, including isocitrate lyase were upregulated both *ex vivo* [37;39-42] and *in vivo* [37;43], further verifying our results. Overall, several interesting *Mtb* metabolic pathways have been identified using these gene

discovery approaches. These findings might further promote the identification of novel vaccine candidates, but also help to understand the behavior of *Mtb in vivo*.

Correlation of IVE-TB antigens and expression with TB disease phenotypes

In addition to the highly expressed genes, a number of different TB disease phenotype related *Mtb* gene expression groups were generated in **chapter 4**. Here, specific *Mtb* gene expression was correlated to particular TB disease key phenotypes such as necrosis and granuloma formation, based on comparisons made between C3H, B6, C3H.B6-*sst1* and B6.C3H-*sst1* mouse models. Unfortunately, limited data is available on the role of *Mtb* in the development of such phenotypes. The environmental host conditions associated with these phenotypes might influence the behavior and adaptation of bacteria and alter the expression of these genes, but it is also possible that *Mtb* shifts its genetic -and subsequently protein- expression to induce or enhance these host TB phenotypes. *M. marinum* mutagenesis studies revealed a role for (RD-1 specific) *Mtb* genes in the development and containment of granulomas [45-48]. However, RD-1 specific proteins were highly expressed in all mouse strains in our system as discussed in the previous section, and therefore were not specifically associated with a granuloma phenotype.

Furthermore, *M. marinum* specific granuloma activated genes (GAPs), were described to be expressed within granulomas of infected frogs [49]. Unfortunately, no overlap between our detected granuloma associated genes and the GAPs was observed, which might be related to differences between animal models and mycobacterial species used. Nevertheless our approach described in **chapter 4** attempted to minimize these TB pathology differences by rigorously selecting for the presence of specific pathological phenotypes, known to be relevant in human TB [50;51]. Finally, Rachman *et al*. analyzed *Mtb* gene expression in different sites of the lung from TB patients. Here, the necrosis associated Rv1607 (probable ionic transporter integral membrane protein, chaA) gene and granuloma associated Rv2465c (Ribose-5-phosphate isomerase, rpi) gene were upregulated in peri-cavity lesions [52], supporting the expression of these genes at the site of infection. Overall, limited data is available concerning *Mtb* gene-specific influences on TB disease phenotypes. The above data thus demonstrate that *Mtb* expresses genes specifically associated with pulmonary granulomatous structures.

HLA class I and class II restriction of new *Mtb* T-cell epitopes

Several recent studies have analyzed the presence of T-cell epitopes in *Mtb* antigens using *in silico* and *in vitro* peptide binding to HLA class I and II e.g. [53-56], which represent approaches that increasing the chance of detecting immunogenic *Mtb* antigens. An overview of immunogenic epitopes is available at the Immune Epitope DataBase (IEDB http://iedb.org) [57]. An *Mtb* genome-wide *in silico*, *ex vivo* and *in vitro* epitope analysis [54] described nine antigens that were also (specifically) expressed *in vivo* in our work as described in **chapter 4**. Two antigens (Rv0291 and Rv1243) were described as novel T-cell antigens. In addition, three other antigens were identified

from previous epitope screens [55;56] (Table 1). Thus these IVE-TB antigens contain epitopes with a high HLA binding affinity and are immunogenic, further underpinning their potential as vaccine candidates.

Table 1. Predicted HLA class I and class II epitopes overlapping with IVE-TB antigens

gene	protein	category selection chapter 4	HLA class	reference
Rv3615c	ESX-1 secretion-associated protein EspC	highly expressed	II	Lindestam Arlehamn *et al.*
Rv0288	EsxH	highly expressed	II	Lindestam Arlehamn *et al.*
Rv0287	EsxG	highly expressed	II	Lindestam Arlehamn *et al.*
Rv3875	ESAT-6	highly expressed	II	Lindestam Arlehamn *et al.*
Rv0291	Probable membrane-anchored mycosin MycP3	Inflammation	II	Lindestam Arlehamn *et al.*
Rv3015c	Conserved hypothetical protein	Relapse	II	Lindestam Arlehamn *et al.*
Rv3019c	EsxR	Resistance	II	Lindestam Arlehamn *et al.*
Rv3020c	EsxS (PE28)	Resistance	II	Lindestam Arlehamn *et al.*
Rv1243c	PE_PGRS23	resistance/low inflammation	II	Lindestam Arlehamn *et al.*
Rv2460c	ClpP2	highly expressed	II	Tang *et al.*
Rv0440	GroEL2	highly expressed	II	Tang *et al.*
Rv1482c	Conserved hypothetical protein	resistance/inflammation	I	Hammond *et al.*

Immune recognition of *Mtb* infection-phase related antigens by immune cells from mycobacteria exposed individuals

Both Rpf, DosR and IVE-TB antigens were recognized by $CD4^+$ and $CD8^+$ T cells from ltLTBI donors (**Chapter 2, 3 and 4**). $CD4^+$ T cells are known to be important in control of *Mtb* infection [58]. Less is known about the role of $CD8^+$ T cells in *Mtb* infection but they likely play an important role in TB protection, particularly during latency [59-61]. Our work showed that $CD8^+$ T cells are present in high frequencies in ltLTBI, suggesting that DosR, Rpf and IVE-TB antigens are presented either via (i) cross presentation; (ii) translocation to the cytoplasm [62]; or (iii) alternative MHC class I presentation pathways [63]. Furthermore, (iv) apoptosis of *Mtb* loaded cells can further enhance MHC-I antigen presentation and subsequent activation of $CD8^+$ T cells [64;65].

Using a short stimulation protocol (**chapter 2, 3 and 4**) Rpf, DosR-regulon and IVE-TB antigen-specific immune responses could be detected in ltLTBI donors. This approach readily detected effector and effector memory T cells (Teff and Tem, respectively) whereas longer stimulation periods are typically required to detect full activation of central memory T cells (Tcm). [66] Indeed IFN-γ^+/TNF-α^+ $CD8^+$ T cells were the most prominent DosR and Rpf specific T cells identified in the PBMC of ltLTBI population. Interestingly, this population showed mainly a Tem, ($CCR7^-$/$CD45RA^-$) and Teff ($CCR7^-$/$CD45RA^+$) $CD8^+$ T-cell phenotype, which are considered to be important to early-phase protection by inducing effector functions at the site of infection [67;68]. Two previous studies identified antigen-specific $CD4^+$ Tem in elderly with a

history of TB and also questioned why such effector cells remained present, even after effective treatment [69;70]. It was suggested that either endured longevity of the cells [69] or continued antigen exposure [70] due to insufficient clearance of the infection after treatment [69] accounted for their presence. Interestingly, a subset of untreated spontaneous 'healed' TB patients did not show IFN-γ specific T cells *ex vivo*, but had Tcm, suggesting clearance of the bacteria in these particular donors [70]. It has been proposed that Tem cells can convert to Tcm in the absence of antigen [71;72]. However, it is as yet not possible to detect viable *Mtb* bacilli in LTBI, such that true *Mtb* persistence cannot be verified in LTBI.

Although such effector and/or effector memory $CD4^+$ and $CD8^+$ T cells were present in healthy *Mtb* exposed individuals (our work and [54;69;70;73;74], they cannot be associated firmly to protection as multiple studies have shown Teff and Tem ($CD8^+$ [73]; $CD4^+$ [74]) in active TB patients as well. In addition, Tcm could be identified in LTBI (CD4 [2;10;54;74], CD8[2;10], TB patients (CD4[2;10], CD8[2;10]) and cured TB patients ($CD4^+$ [70;74], indicating that Tcm is not always associated with protection. The plasticity of particularly $CD8^+$ memory T-cell subset might account for this diverse spread of memory subsets among mycobacteria exposed individuals. $CD8^+$ memory T cells are suggested not to follow a linear differentiation model, in contrast to $CD4^+$ T cells [75;76]. Rather, activated $CD8^+$ T cells might directly develop into Tem or Tcm which subsequently develop into Teff. In addition, $CD8^+$ Tem seem to be able to differentiate into Tcm upon antigen clearance [75;77]. Interestingly, $CD8^+$ Teff, as defined by $CCR7^-/CD45^+$, were not as terminally differentiated as previously thought as they may become Tem upon TCR activation and finally return to Teff, or, as suggested by the authors, become transitional resting cells, based on CD45RA re-expression during continued lack of antigen [78]. The different $CD8^+$ memory subsets observed in LTBI as shown by the studies described in the latter may reflect these alternative explanations. Of note, the memory subsets of IFN-γ^+/TNF-α^+ $CD8^+$ T cells that were analyzed in this thesis were detected using a short stimulation protocol. It is possible that this might have biased towards the detection of effector cells (and this also accounts for other studies mentioned in the latter).

Taking the results of the previous studies into account ltLTBI individuals analyzed in **chapter 2 and 3** probably remained exposed to Rpf and DosR regulon encoded antigens either via *Mtb*, and thus *Mtb* infection was not fully cleared, or via cross-reactive immunity by, for example, exposure to (non-tuberculous) environmental mycobacteria (NTM).

Limited antigen-specific T-cell responses (by IFN-γ) were observed in TB patients when compared to TST^+ individuals (**chapter 4**), a phenomenon that has been described in other studies as well [10;79;80]. Sequestration (the preferential homing of T cells to the site of infection) [81] can result in a decrease in T cells in the periphery, thus leading to decreased antigen recognition by PBMC [82;83]. These peripheral blood responses normalize after successful treatment [81]. However, a continued lack of full responses after TB treatment can persist as well [80]. This may suggest that besides sequestration other factors might be involved in *Mtb* antigen

low responsiveness in TB patients [84;85]. Regulatory T cells (Tregs) are known to suppress T cell and APC activation, limiting the pro-inflammatory immune response [86;87]. In addition, anti-inflammatory macrophages might further enhance non-responsiveness by inducing Tregs which further suppress pro-inflammatory responses via *e.g.* TGF-β [88]. Indeed, PPD non-responding TB patients showed no or low IFN-γ production or T-cell proliferation, but expressed the anti-inflammatory cytokine IL-10 [89] which is known to dampen macrophage and T-cell activation [90]. Indeed, the absence of IL-10 increased protection against *Mtb* infection via enhanced Th1 immunity [91]. Other *Mtb* immune suppression mechanisms are described as well, including (i) specific inhibition and detoxification of ROS production [92;93], inhibition of phagosome maturation [93-95], avoiding of apoptosis and inhibition of autophagy [93]. The influence of additional, unknown host genetic factors [96-98] likely can also limit the development of a robust Th1 immune response and lastly, differential expression of *Mtb* antigens definitely influences the activation of antigen-specific T cells. Thus, many factors can be involved in the decreased antigen recognition observed in TB patients.

Polyfunctional T cells and anti-mycobacterial immunity

The protective efficacy of IVE-TB antigen Rv2034 was described in **chapter 5**. Immunization with Rv2034/CpG resulted in a decrease of CFU after challenge with live *Mtb* when compared to non-immunized mice. Interestingly, immunization with Rv2034/CpG did not induce polyfunctional $CD4^+$ T cells producing IFN-γ, TNF-α and IL-2, but rather showed enhanced levels of $CD4^+$ T cells producing IFN-γ^+/TNF-α^+ and IFN-γ^+. Although triple positive $CD4^+$ T cells were not strongly induced, the vaccine nevertheless induced protection against *Mtb*. Therefore, triple positive (IFN-γ^+/TNF-α^+/IL-2^+) $CD4^+$ T cells do not seem to be strictly required for *Mtb* protection following vaccination. In addition, low frequencies of IFN-γ^+/TNF-α^+/IL-2^+ $CD4^+$ and triple positive $CD8^+$ T cells were identified within the ltLTBI donors who did not develop TB (**chapter 2, 3 and 4**). Together these studies imply a minor role of triple cytokine producing T cells in protection against *Mtb* during infection. Importantly, control SEB stimulation revealed substantial populations of $CD154^+$/$CD4^+$ T cells producing IFN-γ, TNF-α and IL-2, demonstrating that these ltLTBI donors are able to produce triple positive T cells.

Multiple studies have analyzed the presence of IFN-γ^+/TNF-α^+/IL-2^+ triple positive $CD4^+$ and/or $CD8^+$ T cells upon *Mtb* infection [99-102] and TB vaccination [103-109]. Contradictory results regarding the presence of these cells and their association to protection were reported. Technical variability in cytokine detection between the studies described may have been responsible in part for these contrasting data as both incubation period during antigen stimulation and cytokine accumulation using Golgi transport inhibitors can influence the distribution of *Mtb* specific polyfunctional T cells [110]. Furthermore, the timing of the assay may be relevant since T cells can change their cytokine expression pattern over time such that subsets based on cytokine secretion is not static but dynamic and plastic [111;112]. Also, as for memory subsets above, the

antigen/bacterial load could play a role in the variation of T-cell function and phenotype [100;113]. *Mtb* specific polyfunctional T cells were actually suggested to be correlates of antigen load rather than correlates of protection [114;115]. Hence, multiple factors may affect the development of polyfunctional T cells, complicating the interpretation of their exact role in TB protection.

TB vaccine candidates selection based on conservation between different mycobacterial strains

IVE-TB Rv2034 is conserved among multiple *Mtb* complex strains. It is important to vaccinate against conserved antigens in order to prevent *Mtb* escape from the immune system due to antigen variation (e.g. epitope mutation) as has been suggested for the PE and PPE families of *Mtb* proteins [116;117]. On the other hand, previously identified human T-cell epitopes of *Mtb* were found to be particularly strongly conserved as shown by Comas *et al.*[118]. This finding was interpreted to indicate that *Mtb* may benefit from host T-cell immunity as the bacterium seems to avoid escaping host immune pressure through antigenic variation. An underlying mechanism may well be that T-cell immunity eventually induces pathology [119], such as Th1 induced granulomatous necrosis, which can ultimately lead to further transmission and dissemination of infection [120]. This indicates that the balance between pathogen and host in *Mtb* infection is delicate and subtle.

Immunity directed against broadly conserved antigens might already be present in unvaccinated, *Mtb*-unexposed individuals, induced by non-mycobacterial or (non-tuberculous) environmental mycobacteria (NTM) species encoding these conserved antigens. DosR regulon specific responses were observed in NTM exposed individuals who had no history of TB exposure [121] and exposure to NTM can induce *Mtb* cross-protection [122-124] and enhance BCG protection [123]. However, NTM induced immunity might also limit the protective effect of subsequent TB vaccination [124]. Notably, NTM-induced immunity could block BCG multiplication, thus preventing the development of efficient BCG induced immunity [125]. Thus pre-existing memory induced by conserved antigens may act on live mycobacterial TB vaccines and as such influence the protective value of this vaccine.

Future perspective

Different *Mtb* infection**-**phase related antigens were analyzed as possible TB subunit vaccine antigens, taking into account four criteria as specified in section 1. Both Rpf and *Mtb* DosR regulon encoded antigens were recognized by mycobacteria exposed individuals and thus were immunogenic (first criterion). The antigens from both sets included immunogenic regions which can be used in future vaccines studies to determine their protective value against TB. A third set of antigens, identified and described in this thesis (IVE-TB antigens), expressed in the lungs of *Mtb* infected mice, was shown to be immunogenic as these were recognized by $CD4^+$ and $CD8^+$ T cells from *Mtb* exposed individuals, including long-term LTBI. IVE-TB protein Rv2034 fulfilled all four requirements for an efficient vaccine candidate. To further extend these results the Rv2034 protein

could be studied in the highly TB susceptible C3H mouse strain as a more relevant model for TB protection and disease than classically used C57BL/6, which is relatively TB resistant. Also future optimizing studies are required to define optimal adjuvant delivery strategy.

Based on our work and that of others, TB vaccine candidates should include multiple *Mtb* infection-phase related antigen sets, including active-infection- as well as latency-and *in vivo* expressed antigens. Although complete clearance of *Mtb* may be a challenging goal, it should be possible to prevent development of active TB by maintaining optimal immune pressure, which could be boosted by vaccines aiming to prevent reactivation of *Mtb* and thus help containing infection. To actually eradicate *Mtb* infection, other approaches may have to be used in combination with vaccine approaches. This might include the use of vaccination and host modulatory drugs followed by antibiotic treatment. Combining a therapeutic vaccine and antibiotic treatment already showed potential [126]. Hopefully, the future will show whether these multi-stage vaccines and cocktail treatments will lead to reduction in TB cases.

References

1. **Kassa,D., Ran,L., Geberemeskel,W., Tebeje,M., Alemu,A., Selase,A., Tegbaru,B., Franken,K.L., Friggen,A.H., van Meijgaarden,K.E., Ottenhoff,T.H., Wolday,D., Messele,T., and van,B.D.,** Analysis of immune responses against a wide range of Mycobacterium tuberculosis antigens in patients with active pulmonary tuberculosis. *Clin.Vaccine Immunol.* 2012. **19**: 1907-1915.
2. **Riano,F., Arroyo,L., Paris,S., Rojas,M., Friggen,A.H., van Meijgaarden,K.E., Franken,K.L., Ottenhoff,T.H., Garcia,L.F., and Barrera,L.F.,** T cell responses to DosR and Rpf proteins in actively and latently infected individuals from Colombia. *Tuberculosis.(Edinb.)* 2012. **92**: 148-159.
3. **Chegou,N.N., Black,G.F., Loxton,A.G., Stanley,K., Essone,P.N., Klein,M.R., Parida,S.K., Kaufmann,S.H., Doherty,T.M., Friggen,A.H., Franken,K.L., Ottenhoff,T.H., and Walzl,G.,** Potential of novel Mycobacterium tuberculosis infection phase-dependent antigens in the diagnosis of TB disease in a high burden setting. *BMC.Infect.Dis.* 2012. **12**: 10.
4. **Romano,M., Aryan,E., Korf,H., Bruffaerts,N., Franken,C.L., Ottenhoff,T.H., and Huygen,K.,** Potential of Mycobacterium tuberculosis resuscitation-promoting factors as antigens in novel tuberculosis sub-unit vaccines. *Microbes.Infect.* 2012. **14**: 86-95.
5. **Geluk,A., van den Eeden,S.J., van Meijgaarden,K.E., Dijkman,K., Franken,K.L., and Ottenhoff,T.H.,** A multistage-polyepitope vaccine protects against Mycobacterium tuberculosis infection in HLA-DR3 transgenic mice. *Vaccine* 2012. **30**: 7513-7521.
6. **Leyten,E.M., Lin,M.Y., Franken,K.L., Friggen,A.H., Prins,C., van Meijgaarden,K.E., Voskuil,M.I., Weldingh,K., Andersen,P., Schoolnik,G.K., Arend,S.M., Ottenhoff,T.H., and Klein,M.R.,** Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of Mycobacterium tuberculosis. *Microbes.Infect.* 2006. **8**: 2052-2060.
7. **Black,G.F., Thiel,B.A., Ota,M.O., Parida,S.K., Adegbola,R., Boom,W.H., Dockrell,H.M., Franken,K.L., Friggen,A.H., Hill,P.C., Klein,M.R., Lalor,M.K., Mayanja,H., Schoolnik,G., Stanley,K., Weldingh,K., Kaufmann,S.H., Walzl,G., and Ottenhoff,T.H.,** Immunogenicity of novel DosR regulon-encoded candidate antigens of Mycobacterium tuberculosis in three high-burden populations in Africa. *Clin.Vaccine Immunol.* 2009. **16**: 1203-1212.
8. **Demissie,A., Leyten,E.M., Abebe,M., Wassie,L., Aseffa,A., Abate,G., Fletcher,H., Owiafe,P., Hill,P.C., Brookes,R., Rook,G., Zumla,A., Arend,S.M., Klein,M., Ottenhoff,T.H., Andersen,P., and Doherty,T.M.,** Recognition of stage-specific mycobacterial antigens differentiates between acute and latent infections with Mycobacterium tuberculosis. *Clin.Vaccine Immunol.* 2006. **13**: 179-186.
9. **Geluk,A., Lin,M.Y., van Meijgaarden,K.E., Leyten,E.M., Franken,K.L., Ottenhoff,T.H., and Klein,M.R.,** T-cell recognition of the HspX protein of Mycobacterium tuberculosis correlates with latent M. tuberculosis infection but not with M. bovis BCG vaccination. *Infect.Immun.* 2007. **75**: 2914-2921.
10. **Rueda,C.M., Marin,N.D., Garcia,L.F., and Rojas,M.,** Characterization of CD4 and CD8 T cells producing IFN-gamma in human latent and active tuberculosis. *Tuberculosis.(Edinb.)* 2010. **90**: 346-353.
11. **Shi,C., Chen,L., Chen,Z., Zhang,Y., Zhou,Z., Lu,J., Fu,R., Wang,C., Fang,Z., and Fan,X.,** Enhanced protection against tuberculosis by vaccination with recombinant BCG over-expressing HspX protein. *Vaccine* 2010. **28**: 5237-5244.
12. **Jeon,B.Y., Kim,S.C., Eum,S.Y., and Cho,S.N.,** The immunity and protective effects of antigen 85A and heat-shock protein X against progressive tuberculosis. *Microbes. Infect.* 2011. **13**: 284-290.
13. **Taylor,J.L., Wieczorek,A., Keyser,A.R., Grover,A., Flinkstrom,R., Karls,R.K., Bielefeldt-Ohmann,H.,**

Dobos,K.M., and Izzo,A.A., HspX-mediated protection against tuberculosis depends on its chaperoning of a mycobacterial molecule. *Immunol.Cell Biol.* 2012. **90**: 945-954.

14. **Yuan,W., Dong,N., Zhang,L., Liu,J., Lin,S., Xiang,Z., Qiao,H., Tong,W., and Qin,C.,** Immunogenicity and protective efficacy of a tuberculosis DNA vaccine expressing a fusion protein of Ag85B-Esat6-HspX in mice. *Vaccine* 2012. **30**: 2490-2497.
15. **Niu,H., Hu,L., Li,Q., Da,Z., Wang,B., Tang,K., Xin,Q., Yu,H., Zhang,Y., Wang,Y., Ma,X., and Zhu,B.,** Construction and evaluation of a multistage Mycobacterium tuberculosis subunit vaccine candidate Mtb10.4-HspX. *Vaccine* 2011. **29**: 9451-9458.
16. **Lin,M.Y., Geluk,A., Smith,S.G., Stewart,A.L., Friggen,A.H., Franken,K.L., Verduyn,M.J., van Meijgaarden,K.E., Voskuil,M.I., Dockrell,H.M., Huygen,K., Ottenhoff,T.H., and Klein,M.R.,** Lack of immune responses to Mycobacterium tuberculosis DosR regulon proteins following Mycobacterium bovis BCG vaccination. *Infect.Immun.* 2007. **75**: 3523-3530.
17. **Jones,G.J., Pirson,C., Gideon,H.P., Wilkinson,K.A., Sherman,D.R., Wilkinson,R.J., Hewinson,R.G., and Vordermeier,H.M.,** Immune responses to the enduring hypoxic response antigen Rv0188 are preferentially detected in Mycobacterium bovis infected cattle with low pathology. *PLoS.One.* 2011. **6**: e21371.
18. **Tchilian,E.Z., Ronan,E.O., de,L.C., Lee,L.N., Franken,K.L., Vordermeier,M.H., Ottenhoff,T.H., and Beverley,P.C.,** Simultaneous immunization against tuberculosis. *PLoS.One.* 2011. **6**: e27477.
19. **Rustad,T.R., Harrell,M.I., Liao,R., and Sherman,D.R.,** The enduring hypoxic response of Mycobacterium tuberculosis. *PLoS.One.* 2008. **3**: e1502.
20. **Betts,J.C., Lukey,P.T., Robb,L.C., McAdam,R.A., and Duncan,K.,** Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol. Microbiol.* 2002. **43**: 717-731.
21. **Voskuil,M.I., Schnappinger,D., Visconti,K.C., Harrell,M.I., Dolganov,G.M., Sherman,D.R., and Schoolnik,G.K.,** Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. *J.Exp.Med.* 2003. **198**: 705-713.
22. **Chiappini,E., Della,B.C., Bonsignori,F., Sollai,S., Amedei,A., Galli,L., Niccolai,E., Del,P.G., Singh,M., D'Elios,M.M., and de,M.M.,** Potential role of M. tuberculosis specific IFN-gamma and IL-2 ELISPOT assays in discriminating children with active or latent tuberculosis. *PLoS.One.* 2012. **7**: e46041.
23. **Bold,T.D., Banaei,N., Wolf,A.J., and Ernst,J.D.,** Suboptimal activation of antigen-specific CD4+ effector cells enables persistence of M. tuberculosis in vivo. *PLoS. Pathog.* 2011. **7**: e1002063.
24. **Baena,A. and Porcelli,S.A.,** Evasion and subversion of antigen presentation by Mycobacterium tuberculosis. *Tissue Antigens* 2009. **74**: 189-204.
25. **Banerjee,S., Farhana,A., Ehtesham,N.Z., and Hasnain,S.E.,** Iron acquisition, assimilation and regulation in mycobacteria. *Infect.Genet.Evol.* 2011. **11**: 825-838.
26. **Johnson,E.E. and Wessling-Resnick,M.,** Iron metabolism and the innate immune response to infection. *Microbes.Infect.* 2012. **14**: 207-216.
27. **Moller,M., de,W.E., and Hoal,E.G.,** Past, present and future directions in human genetic susceptibility to tuberculosis. *FEMS Immunol.Med.Microbiol.* 2010. **58**: 3-26.
28. **Siegrist,M.S., Unnikrishnan,M., McConnell,M.J., Borowsky,M., Cheng,T.Y., Siddiqi,N., Fortune,S.M., Moody,D.B., and Rubin,E.J.,** Mycobacterial Esx-3 is required for mycobactin-mediated iron acquisition. *Proc.Natl.Acad. Sci.U.S.A* 2009. **106**: 18792-18797.
29. **Serafini,A., Boldrin,F., Palu,G., and Manganelli,R.,** Characterization of a Mycobacterium tuberculosis ESX-3 conditional mutant: essentiality and rescue by iron and zinc. *J.Bacteriol.* 2009. **191**: 6340-6344.
30. **Pere-Joan Cardona,** *Understanding Tuberculosis - Deciphering the Secret Life of the Bacilli*. 2013.
31. **Munoz-Elias,E.J. and McKinney,J.D.,** Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat.Med.* 2005. **11**: 638-644.

32. **McKinney,J.D., Honer zu,B.K., Munoz-Elias,E.J., Miczak,A., Chen,B., Chan,W.T., Swenson,D., Sacchettini,J.C., Jacobs,W.R., Jr., and Russell,D.G.,** Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* 2000. **406**: 735-738.
33. **Sirakova,T.D., Fitzmaurice,A.M., and Kolattukudy,P.,** Regulation of expression of mas and fadD28, two genes involved in production of dimycocerosyl phthiocerol, a virulence factor of Mycobacterium tuberculosis. *J.Bacteriol.* 2002. **184**: 6796-6802.
34. **Weigoldt,M., Meens,J., Bange,F.C., Pich,A., Gerlach,G.F., and Goethe,R.,** Metabolic adaptation of Mycobacterium avium subsp. paratuberculosis to the gut environment. *Microbiology* 2013. **159**: 380-391.
35. **Williams,K.J., Boshoff,H.I., Krishnan,N., Gonzales,J., Schnappinger,D., and Robertson,B.D.,** The Mycobacterium tuberculosis beta-oxidation genes echA5 and fadB3 are dispensable for growth in vitro and in vivo. *Tuberculosis.(Edinb.)* 2011. **91**: 549-555.
36. **Ward,S.K., Abomoelak,B., Marcus,S.A., and Talaat,A.M.,** Transcriptional profiling of mycobacterium tuberculosis during infection: lessons learned. *Front Microbiol.* 2010. **1**: 121.
37. **Waddell,S.J. and Butcher,P.D.,** Microarray analysis of whole genome expression of intracellular Mycobacterium tuberculosis. *Curr.Mol.Med.* 2007. **7**: 287-296.
38. **Gold,B., Rodriguez,G.M., Marras,S.A., Pentecost,M., and Smith,I.,** The Mycobacterium tuberculosis IdeR is a dual functional regulator that controls transcription of genes involved in iron acquisition, iron storage and survival in macrophages. *Mol.Microbiol.* 2001. **42**: 851-865.
39. **Rachman,H., Strong,M., Schaible,U., Schuchhardt,J., Hagens,K., Mollenkopf,H., Eisenberg,D., and Kaufmann,S.H.,** Mycobacterium tuberculosis gene expression profiling within the context of protein networks. *Microbes.Infect.* 2006. **8**: 747-757.
40. **Schnappinger,D., Ehrt,S., Voskuil,M.I., Liu,Y., Mangan,J.A., Monahan,I.M., Dolganov,G., Efron,B., Butcher,P.D., Nathan,C., and Schoolnik,G.K.,** Transcriptional Adaptation of Mycobacterium tuberculosis within Macrophages: Insights into the Phagosomal Environment. *J.Exp.Med.* 2003. **198**: 693-704.
41. **Tailleux,L., Waddell,S.J., Pelizzola,M., Mortellaro,A., Withers,M., Tanne,A., Castagnoli,P.R., Gicquel,B., Stoker,N.G., Butcher,P.D., Foti,M., and Neyrolles,O.,** Probing host pathogen cross-talk by transcriptional profiling of both Mycobacterium tuberculosis and infected human dendritic cells and macrophages. *PLoS.One.* 2008. **3**: e1403.
42. **Li,A.H., Waddell,S.J., Hinds,J., Malloff,C.A., Bains,M., Hancock,R.E., Lam,W.L., Butcher,P.D., and Stokes,R.W.,** Contrasting transcriptional responses of a virulent and an attenuated strain of Mycobacterium tuberculosis infecting macrophages. *PLoS.One.* 2010. **5**: e11066.
43. **Timm,J., Post,F.A., Bekker,L.G., Walther,G.B., Wainwright,H.C., Manganelli,R., Chan,W.T., Tsenova,L., Gold,B., Smith,I., Kaplan,G., and McKinney,J.D.,** Differential expression of iron-, carbon-, and oxygen-responsive mycobacterial genes in the lungs of chronically infected mice and tuberculosis patients. *Proc. Natl.Acad.Sci.U.S.A* 2003. **100**: 14321-14326.
44. **Mukhopadhyay,S., Nair,S., and Ghosh,S.,** Pathogenesis in tuberculosis: transcriptomic approaches to unraveling virulence mechanisms and finding new drug targets. *FEMS Microbiol.Rev.* 2012. **36**: 463-485.
45. **Stoop,E.J., Schipper,T., Huber,S.K., Nezhinsky,A.E., Verbeek,F.J., Gurcha,S.S., Besra,G.S., Vandenbroucke-Grauls,C.M., Bitter,W., and van der Sar,A.M.,** Zebrafish embryo screen for mycobacterial genes involved in the initiation of granuloma formation reveals a newly identified ESX-1 component. *Dis.Model.Mech.* 2011. **4**: 526-536.
46. **Volkman,H.E., Clay,H., Beery,D., Chang,J.C., Sherman,D.R., and Ramakrishnan,L.,** Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. *PLoS.Biol.* 2004. **2**: e367.
47. **Swaim,L.E., Connolly,L.E., Volkman,H.E., Humbert,O., Born,D.E., and Ramakrishnan,L.,** Mycobacterium marinum infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. *Infect.Immun.* 2006. **74**: 6108-6117.

48. **Volkman,H.E., Pozos,T.C., Zheng,J., Davis,J.M., Rawls,J.F., and Ramakrishnan,L.,** Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. *Science* 2010. **327**: 466-469.
49. **Chan,K., Knaak,T., Satkamp,L., Humbert,O., Falkow,S., and Ramakrishnan,L.,** Complex pattern of Mycobacterium marinum gene expression during long-term granulomatous infection. *Proc.Natl.Acad.Sci.U.S.A* 2002. **99**: 3920-3925.
50. **Apt,A. and Kramnik,I.,** Man and mouse TB: contradictions and solutions. *Tuberculosis.(Edinb.)* 2009. **89**: 195-198.
51. **Pichugin,A.V., Yan,B.S., Sloutsky,A., Kobzik,L., and Kramnik,I.,** Dominant role of the sst1 locus in pathogenesis of necrotizing lung granulomas during chronic tuberculosis infection and reactivation in genetically resistant hosts. *Am.J.Pathol.* 2009. **174**: 2190-2201.
52. **Rachman,H., Strong,M., Ulrichs,T., Grode,L., Schuchhardt,J., Mollenkopf,H., Kosmiadi,G.A., Eisenberg,D., and Kaufmann,S.H.,** Unique transcriptome signature of Mycobacterium tuberculosis in pulmonary tuberculosis. *Infect.Immun.* 2006. **74**: 1233-1242.
53. **Axelsson-Robertson,R., Magalhaes,I., Parida,S.K., Zumla,A., and Maeurer,M.,** The immunological footprint of Mycobacterium tuberculosis T-cell epitope recognition. *J.Infect.Dis.* 2012. **205 Suppl 2**: S301-S315.
54. **Lindestam Arlehamn,C.S., Gerasimova,A., Mele,F., Henderson,R., Swann,J., Greenbaum,J.A., Kim,Y., Sidney,J., James,E.A., Taplitz,R., McKinney,D.M., Kwok,W.W., Grey,H., Sallusto,F., Peters,B., and Sette,A.,** Memory T Cells in Latent Mycobacterium tuberculosis Infection Are Directed against Three Antigenic Islands and Largely Contained in a CXCR3(+) CCR6(+) Th1 Subset. *PLoS.Pathog.* 2013. **9**: e1003130.
55. **Tang,S.T., van Meijgaarden,K.E., Caccamo,N., Guggino,G., Klein,M.R., van,W.P., Kazi,F., Stryhn,A., Zaigler,A., Sahin,U., Buus,S., Dieli,F., Lund,O., and Ottenhoff,T.H.,** Genome-based in silico identification of new Mycobacterium tuberculosis antigens activating polyfunctional CD8+ T cells in human tuberculosis. *J.Immunol.* 2011. **186**: 1068-1080.
56. **Hammond,A.S., Klein,M.R., Corrah,T., Fox,A., Jaye,A., McAdam,K.P., and Brookes,R.H.,** Mycobacterium tuberculosis genome-wide screen exposes multiple CD8 T cell epitopes. *Clin.Exp.Immunol.* 2005. **140**: 109-116.
57. **Salimi,N., Fleri,W., Peters,B., and Sette,A.,** The immune epitope database: a historical retrospective of the first decade. *Immunology* 2012. **137**: 117-123.
58. **Ottenhoff,T.H. and Kaufmann,S.H.,** Vaccines against tuberculosis: where are we and where do we need to go? *PLoS.Pathog.* 2012. **8**: e1002607.
59. **Chen,C.Y., Huang,D., Wang,R.C., Shen,L., Zeng,G., Yao,S., Shen,Y., Halliday,L., Fortman,J., McAllister,M., Estep,J., Hunt,R., Vasconcelos,D., Du,G., Porcelli,S.A., Larsen,M.H., Jacobs,W.R., Jr., Haynes,B.F., Letvin,N.L., and Chen,Z.W.,** A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. *PLoS.Pathog.* 2009. **5**: e1000392.
60. **Bruns,H., Meinken,C., Schauenberg,P., Harter,G., Kern,P., Modlin,R.L., Antoni,C., and Stenger,S.,** Anti-TNF immunotherapy reduces CD8+ T cell-mediated antimicrobial activity against Mycobacterium tuberculosis in humans. *J.Clin.Invest* 2009. **119**: 1167-1177.
61. **van Pinxteren,L.A., Cassidy,J.P., Smedegaard,B.H., Agger,E.M., and Andersen,P.,** Control of latent Mycobacterium tuberculosis infection is dependent on CD8 T cells. *Eur.J.Immunol.* 2000. **30**: 3689-3698.
62. **Welin,A. and Lerm,M.,** Inside or outside the phagosome? The controversy of the intracellular localization of Mycobacterium tuberculosis. *Tuberculosis.(Edinb.)* 2012. **92**: 113-120.
63. **Harriff,M.J., Purdy,G.E., and Lewinsohn,D.M.,** Escape from the Phagosome: The Explanation for MHC-I Processing of Mycobacterial Antigens? *Front Immunol.* 2012. **3**: 40.
64. **Winau,F., Weber,S., Sad,S., de,D.J., Hoops,S.L., Breiden,B., Sandhoff,K., Brinkmann,V., Kaufmann,S.H., and Schaible,U.E.,** Apoptotic vesicles

crossprime CD8 T cells and protect against tuberculosis. *Immunity.* 2006. **24**: 105-117.

65. **Schaible,U.E., Winau,F., Sieling,P.A., Fischer,K., Collins,H.L., Hagens,K., Modlin,R.L., Brinkmann,V., and Kaufmann,S.H.,** Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. *Nat.Med.* 2003. **9**: 1039-1046.
66. **Sallusto,F., Lenig,D., Forster,R., Lipp,M., and Lanzavecchia,A.,** Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999. **401**: 708-712.
67. **Sallusto,F., Geginat,J., and Lanzavecchia,A.,** Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu.Rev.Immunol.* 2004. **22**: 745-763.
68. **Sallusto,F., Lanzavecchia,A., Araki,K., and Ahmed,R.,** From vaccines to memory and back. *Immunity.* 2010. **33**: 451-463.
69. **Tapaninen,P., Korhonen,A., Pusa,L., Seppala,I., and Tuuminen,T.,** Effector memory T-cells dominate immune responses in tuberculosis treatment: antigen or bacteria persistence? *Int.J.Tuberc.Lung Dis.* 2010. **14**: 347-355.
70. **Millington,K.A., Gooding,S., Hinks,T.S., Reynolds,D.J., and Lalvani,A.,** Mycobacterium tuberculosis-specific cellular immune profiles suggest bacillary persistence decades after spontaneous cure in untreated tuberculosis. *J.Infect.Dis.* 2010. **202**: 1685-1689.
71. **Woodland,D.L. and Kohlmeier,J.E.,** Migration, maintenance and recall of memory T cells in peripheral tissues. *Nat.Rev.Immunol.* 2009. **9**: 153-161.
72. **Sarkar,S., Teichgraber,V., Kalia,V., Polley,A., Masopust,D., Harrington,L.E., Ahmed,R., and Wherry,E.J.,** Strength of stimulus and clonal competition impact the rate of memory CD8 T cell differentiation. *J.Immunol.* 2007. **179**: 6704-6714.
73. **Rozot,V., Vigano,S., Mazza-Stalder,J., Idrizi,E., Day,C.L., Perreau,M., Lazor-Blanchet,C., Petruccioli,E., Hanekom,W., Goletti,D., Bart,P.A., Nicod,L., Pantaleo,G., and Harari,A.,** Mycobacterium tuberculosis-specific CD8 T cells are functionally and phenotypically different between latent infection and active disease. *Eur.J.Immunol.* 2013.
74. **Petruccioli,E., Petrone,L., Vanini,V., Sampaolesi,A., Gualano,A., Girardi,E., Palmieri,F., and Goletti,D.,** IFNgamma/TNFalpha specific-cells and effector memory phenotype associate with active tuberculosis. *J.Infect.* 2013.
75. **Seder,R.A., Darrah,P.A., and Roederer,M.,** T-cell quality in memory and protection: implications for vaccine design. *Nat.Rev.Immunol.* 2008. **8**: 247-258.
76. **Lanzavecchia,A. and Sallusto,F.,** Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science* 2000. **290**: 92-97.
77. **Wherry,E.J., Teichgraber,V., Becker,T.C., Masopust,D., Kaech,S.M., Antia,R., von Andrian,U.H., and Ahmed,R.,** Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat.Immunol.* 2003. **4**: 225-234.
78. **Carrasco,J., Godelaine,D., Van,P.A., Boon,T., and van der Bruggen,P.,** CD45RA on human CD8 T cells is sensitive to the time elapsed since the last antigenic stimulation. *Blood* 2006. **108**: 2897-2905.
79. **Shams,H., Wizel,B., Weis,S.E., Samten,B., and Barnes,P.F.,** Contribution of CD8(+) T cells to gamma interferon production in human tuberculosis. *Infect.Immun.* 2001. **69**: 3497-3501.
80. **Delgado,J.C., Tsai,E.Y., Thim,S., Baena,A., Boussiotis,V.A., Reynes,J.M., Sath,S., Grosjean,P., Yunis,E.J., and Goldfeld,A.E.,** Antigen-specific and persistent tuberculin anergy in a cohort of pulmonary tuberculosis patients from rural Cambodia. *Proc.Natl.Acad.Sci.U.S.A* 2002. **99**: 7576-7581.
81. **Dieli,F., Friscia,G., Di,S.C., Ivanyi,J., Singh,M., Spallek,R., Sireci,G., Titone,L., and Salerno,A.,** Sequestration of T lymphocytes to body fluids in tuberculosis: reversal of anergy following chemotherapy. *J.Infect.Dis.* 1999. **180**: 225-228.
82. **Sable,S.B., Goyal,D., Verma,I., Behera,D., and Khuller,G.K.,** Lung and blood mononuclear cell responses of tuberculosis patients to mycobacterial proteins. *Eur.Respir.J.* 2007. **29**: 337-346.
83. **Brighenti,S. and Andersson,J.,** Local immune responses in human tuberculosis: learning from the site of infection. *J.Infect.Dis.* 2012. **205 Suppl 2**: S316-S324.

84. **Almeida,A.S., Lago,P.M., Boechat,N., Huard,R.C., Lazzarini,L.C., Santos,A.R., Nociari,M., Zhu,H., Perez-Sweeney,B.M., Bang,H., Ni,Q., Huang,J., Gibson,A.L., Flores,V.C., Pecanha,L.R., Kritski,A.L., Lapa e Silva JR, and Ho,J.L.,** Tuberculosis is associated with a down-modulatory lung immune response that impairs Th1-type immunity. *J.Immunol.* 2009. **183**: 718-731.
85. **Roberts,T., Beyers,N., Aguirre,A., and Walzl,G.,** Immunosuppression during active tuberculosis is characterized by decreased interferon- gamma production and CD25 expression with elevated forkhead box P3, transforming growth factor- beta , and interleukin-4 mRNA levels. *J.Infect.Dis.* 2007. **195**: 870-878.
86. **Joosten,S.A. and Ottenhoff,T.H.,** Human CD4 and CD8 regulatory T cells in infectious diseases and vaccination. *Hum. Immunol.* 2008. **69**: 760-770.
87. **Joosten,S.A., van Meijgaarden,K.E., van Weeren,P.C., Kazi,F., Geluk,A., Savage,N.D., Drijfhout,J.W., Flower,D.R., Hanekom,W.A., Klein,M.R., and Ottenhoff,T.H.,** Mycobacterium tuberculosis peptides presented by HLA-E molecules are targets for human CD8 T-cells with cytotoxic as well as regulatory activity. *PLoS.Pathog.* 2010. **6**: e1000782.
88. **Savage,N.D., de,B.T., Walburg,K.V., Joosten,S.A., van,M.K., Geluk,A., and Ottenhoff,T.H.,** Human anti-inflammatory macrophages induce Foxp3+ GITR+ CD25+ regulatory T cells, which suppress via membrane-bound TGFbeta-1. *J.Immunol.* 2008. **181**: 2220-2226.
89. **Boussiotis,V.A., Tsai,E.Y., Yunis,E.J., Thim,S., Delgado,J.C., Dascher,C.C., Berezovskaya,A., Rousset,D., Reynes,J.M., and Goldfeld,A.E.,** IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. *J.Clin.Invest* 2000. **105**: 1317-1325.
90. **Redford,P.S., Murray,P.J., and O'Garra,A.,** The role of IL-10 in immune regulation during M. tuberculosis infection. *Mucosal.Immunol.* 2011. **4**: 261-270.
91. **Redford,P.S., Boonstra,A., Read,S., Pitt,J., Graham,C., Stavropoulos,E., Bancroft,G.J., and O'Garra,A.,** Enhanced protection to Mycobacterium tuberculosis infection in IL-10-deficient mice is accompanied by early and enhanced Th1 responses in the lung. *Eur.J.Immunol.* 2010. **40**: 2200-2210.
92. **Ehrt,S. and Schnappinger,D.,** Mycobacterial survival strategies in the phagosome: defence against host stresses. *Cell Microbiol.* 2009. **11**: 1170-1178.
93. **Kim,K.H., An,D.R., Song,J., Yoon,J.Y., Kim,H.S., Yoon,H.J., Im,H.N., Kim,J., Kim,d.J., Lee,S.J., Kim,K.H., Lee,H.M., Kim,H.J., Jo,E.K., Lee,J.Y., and Suh,S.W.,** Mycobacterium tuberculosis Eis protein initiates suppression of host immune responses by acetylation of DUSP16/MKP-7. *Proc.Natl.Acad.Sci.U.S.A* 2012. **109**: 7729-7734.
94. **Mishra,A.K., Driessen,N.N., Appelmelk,B.J., and Besra,G.S.,** Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in Mycobacterium tuberculosis physiology and host-pathogen interaction. *FEMS Microbiol. Rev.* 2011. **35**: 1126-1157.
95. **Vergne,I., Chua,J., Singh,S.B., and Deretic,V.,** Cell biology of mycobacterium tuberculosis phagosome. *Annu.Rev.Cell Dev. Biol.* 2004. **20**: 367-394.
96. **Sousa,A.O., Salem,J.I., Lee,F.K., Verrcosa,M.C., Cruaud,P., Bloom,B.R., Lagrange,P.H., and David,H.L.,** An epidemic of tuberculosis with a high rate of tuberculin anergy among a population previously unexposed to tuberculosis, the Yanomami Indians of the Brazilian Amazon. *Proc.Natl.Acad.Sci.U.S.A* 1997. **94**: 13227-13232.
97. **Magira,E.E., Papasteriades,C., Kanterakis,S., Toubis,M., Roussos,C., and Monos,D.S.,** HLA-A and HLA-DRB1 amino acid polymorphisms are associated with susceptibility and protection to pulmonary tuberculosis in a Greek population. *Hum.Immunol.* 2012. **73**: 641-646.
98. **Vannberg,F.O., Chapman,S.J., and Hill,A.V.,** Human genetic susceptibility to intracellular pathogens. *Immunol.Rev.* 2011. **240**: 105-116.
99. **Sutherland,J.S., Adetifa,I.M., Hill,P.C., Adegbola,R.A., and Ota,M.O.,** Pattern and diversity of cytokine production differentiates between Mycobacterium tuberculosis infection and disease. *Eur.J.Immunol.* 2009. **39**: 723-729.

100. **Caccamo,N., Guggino,G., Joosten,S.A., Gelsomino,G., Di,C.P., Titone,L., Galati,D., Bocchino,M., Matarese,A., Salerno,A., Sanduzzi,A., Franken,W.P., Ottenhoff,T.H., and Dieli,F.,** Multifunctional CD4(+) T cells correlate with active Mycobacterium tuberculosis infection. *Eur.J.Immunol.* 2010. **40**: 2211-2220.

101. **Qiu,Z., Zhang,M., Zhu,Y., Zheng,F., Lu,P., Liu,H., Graner,M.W., Zhou,B., and Chen,X.,** Multifunctional CD4 T cell responses in patients with active tuberculosis. *Sci.Rep.* 2012. **2**: 216.

102. **Marin,N.D., Paris,S.C., Rojas,M., and Garcia,L.F.,** Functional profile of CD4+ and CD8+ T cells in latently infected individuals and patients with active TB. *Tuberculosis.(Edinb.)* 2013.

103. **Derrick,S.C., Yabe,I.M., Yang,A., and Morris,S.L.,** Vaccine-induced anti-tuberculosis protective immunity in mice correlates with the magnitude and quality of multifunctional CD4 T cells. *Vaccine* 2011. **29**: 2902-2909.

104. **Aagaard,C., Hoang,T., Dietrich,J., Cardona,P.J., Izzo,A., Dolganov,G., Schoolnik,G.K., Cassidy,J.P., Billeskov,R., and Andersen,P.,** A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nat.Med.* 2011. **17**: 189-194.

105. **Forbes,E.K., Sander,C., Ronan,E.O., McShane,H., Hill,A.V., Beverley,P.C., and Tchilian,E.Z.,** Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against Mycobacterium tuberculosis aerosol challenge in mice. *J.Immunol.* 2008. **181**: 4955-4964.

106. **Lindenstrom,T., Agger,E.M., Korsholm,K.S., Darrah,P.A., Aagaard,C., Seder,R.A., Rosenkrands,I., and Andersen,P.,** Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. *J.Immunol.* 2009. **182**: 8047-8055.

107. **Abel,B., Tameris,M., Mansoor,N., Gelderbloem,S., Hughes,J., Abrahams,D., Makhethe,L., Erasmus,M., de,K.M., van der Merwe,L., Hawkridge,A., Veldsman,A., Hatherill,M., Schirru,G., Pau,M.G., Hendriks,J., Weverling,G.J., Goudsmit,J., Sizemore,D., McClain,J.B., Goetz,M., Gearhart,J., Mahomed,H., Hussey,G.D., Sadoff,J.C., and Hanekom,W.A.,** The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults. *Am.J.Respir.Crit Care Med.* 2010. **181**: 1407-1417.

108. **Scriba,T.J., Tameris,M., Mansoor,N., Smit,E., van der Merwe,L., Isaacs,F., Keyser,A., Moyo,S., Brittain,N., Lawrie,A., Gelderbloem,S., Veldsman,A., Hatherill,M., Hawkridge,A., Hill,A.V., Hussey,G.D., Mahomed,H., McShane,H., and Hanekom,W.A.,** Modified vaccinia Ankara-expressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional CD4+ T cells. *Eur.J.Immunol.* 2010. **40**: 279-290.

109. **Kagina,B.M., Abel,B., Scriba,T.J., Hughes,E.J., Keyser,A., Soares,A., Gamieldien,H., Sidibana,M., Hatherill,M., Gelderbloem,S., Mahomed,H., Hawkridge,A., Hussey,G., Kaplan,G., and Hanekom,W.A.,** Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guerin vaccination of newborns. *Am.J.Respir.Crit Care Med.* 2010. **182**: 1073-1079.

110. **Kaveh,D.A., Whelan,A.O., and Hogarth,P.J.,** The duration of antigen-stimulation significantly alters the diversity of multifunctional CD4 T cells measured by intracellular cytokine staining. *PLoS.One.* 2012. **7**: e38926.

111. **Han,Q., Bagheri,N., Bradshaw,E.M., Hafler,D.A., Lauffenburger,D.A., and Love,J.C.,** Polyfunctional responses by human T cells result from sequential release of cytokines. *Proc.Natl.Acad.Sci.U.S.A* 2012. **109**: 1607-1612.

112. **Haining,W.N.,** Travels in time: assessing the functional complexity of T cells. *Proc.Natl.Acad.Sci.U.S.A* 2012. **109**: 1359-1360.

113. **Day,C.L., Abrahams,D.A., Lerumo,L., Janse van,R.E., Stone,L., O'rie,T., Pienaar,B., de,K.M., Kaplan,G., Mahomed,H., Dheda,K., and Hanekom,W.A.,** Functional capacity of Mycobacterium tuberculosis-specific T

cell responses in humans is associated with mycobacterial load. *J.Immunol.* 2011. **187**: 2222-2232.

114. **Mattila,J.T., Diedrich,C.R., Lin,P.L., Phuah,J., and Flynn,J.L.,** Simian immunodeficiency virus-induced changes in T cell cytokine responses in cynomolgus macaques with latent Mycobacterium tuberculosis infection are associated with timing of reactivation. *J.Immunol.* 2011. **186**: 3527-3537.
115. **Pere-Joan Cardona,** *Understanding Tuberculosis - Analyzing the Origin of Mycobacterium Tuberculosis Pathogenicity*. 2013.
116. **Talarico,S., Cave,M.D., Foxman,B., Marrs,C.F., Zhang,L., Bates,J.H., and Yang,Z.,** Association of Mycobacterium tuberculosis PE PGRS33 polymorphism with clinical and epidemiological characteristics. *Tuberculosis.(Edinb.)* 2007. **87**: 338-346.
117. **McEvoy,C.R., Cloete,R., Muller,B., Schurch,A.C., van Helden,P.D., Gagneux,S., Warren,R.M., and Gey van Pittius,N.C.,** Comparative analysis of Mycobacterium tuberculosis pe and ppe genes reveals high sequence variation and an apparent absence of selective constraints. *PLoS.One.* 2012. **7**: e30593.
118. **Comas,I., Chakravartti,J., Small,P.M., Galagan,J., Niemann,S., Kremer,K., Ernst,J.D., and Gagneux-,S.,** Human T cell epitopes of Mycobacterium tuberculosis are evolutionarily hyperconserved. *Nat.Genet.* 2010. **42**: 498-503.
119. **Torrado,E., Robinson,R.T., and Cooper,A.M.,** Cellular response to mycobacteria: balancing protection and pathology. *Trends Immunol.* 2011. **32**: 66-72.
120. **Ehlers,S., Benini,J., Held,H.D., Roeck,C., Alber,G., and Uhlig,S.,** Alpha/beta T cell receptor-positive cells and interferon-gamma, but not inducible nitric oxide synthase, are critical for granuloma necrosis in a mouse model of mycobacteria-induced pulmonary immunopathology. *J.Exp.Med.* 2001. **194**: 1847-1859.
121. **Lin,M.Y., Reddy,T.B., Arend,S.M., Friggen,A.H., Franken,K.L., van Meijgaarden,K.E., Verduyn,M.J., Schoolnik,G.K., Klein,M.R., and Ottenhoff,T.H.,** Cross-reactive immunity to Mycobacterium tuberculosis DosR regulon-encoded antigens in individuals infected with environmental, nontuberculous mycobacteria. *Infect.Immun.* 2009. **77**: 5071-5079.
122. **Fine,P.E., Floyd,S., Stanford,J.L., Nkhosa,P., Kasunga,A., Chagaluka,S., Warndorff,D.K., Jenkins,P.A., Yates,M., and Ponnighaus,J.M.,** Environmental mycobacteria in northern Malawi: implications for the epidemiology of tuberculosis and leprosy. *Epidemiol.Infect.* 2001. **126**: 379-387.
123. **Edwards,M.L., Goodrich,J.M., Muller,D., Pollack,A., Ziegler,J.E., and Smith,D.W.,** Infection with Mycobacterium avium-intracellulare and the protective effects of Bacille Calmette-Guerin. *J.Infect.Dis.* 1982. **145**: 733-741.
124. **Beverley,P., Ronan,E., Lee,L., Arnold,I., Bolinger,B., Powrie,F., and Tchilian,E.,** Environmental effects on protection against Mycobacterium tuberculosis after immunization with Ad85A. *Vaccine* 2013. **31**: 1086-1093.
125. **Brandt,L., Feino,C.J., Weinreich,O.A., Chilima,B., Hirsch,P., Appelberg,R., and Andersen,P.,** Failure of the Mycobacterium bovis BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infect.Immun.* 2002. **70**: 672-678.
126. **Coler,R.N., Bertholet,S., Pine,S.O., Orr,M.T., Reese,V., Windish,H.P., Davis,C., Kahn,M., Baldwin,S.L., and Reed,S.G.,** Therapeutic Immunization against Mycobacterium tuberculosis Is an Effective Adjunct to Antibiotic Treatment. *J.Infect.Dis.* 2013. **207**: 1242-1252.

ADDENDUM

Nederlandse samenvatting
Curriculum Vitae
List of publications

Nederlandse samenvatting

Algemene inleiding

Tuberculose (TB) wordt veroorzaakt door de bacterie *Mycobacterium tuberculosis* (*Mtb*). Men schat dat er in 2011 8.7 miljoen nieuwe TB gevallen bij zijn gekomen en dat het de levens van 1.4 miljoen mensen heeft gekost. Hiermee blijft TB de op één na grootste veroorzaker van sterfgevallen door infectieziekten, waarbij *human immunodeficiency virus* (HIV) de lijst aanvoert.

Na infectie nestelt de tuberkel bacterie zich in de long en kan zich hier jarenlang schuilhouden. Dit leidt tot een latente infectie, zonder zichtbare klinische kenmerken. Echter 3-10% van de *Mtb* geïnfecteerde mensen zal in de loop der tijd TB ontwikkelen, met nieuwe *Mtb* besmettingen als gevolg.

De bestrijding van TB wordt bemoeilijkt door; (i) de ontwikkeling van antibiotica resistentie, (ii) co-infectie van TB en HIV, (iii) beperkte methodes om latente en vroeg klinische *Mtb* infectie op te sporen en (iii) de afwezigheid van een effectief TB vaccin. Op dit moment is er slechts één TB vaccin beschikbaar; *Mycobacterium bovis* Bacille Calmette-Guérin (BCG). Dit vaccin induceert echter zeer variabele, en incomplete bescherming tegen de meest voorkomende en besmettelijke vorm, open long TB. Daarbij is het vaccin een verzwakt, maar levende bacterie. Dit kan leiden tot ernstige BCG infectie in HIV geïnfecteerde kinderen en mensen met genetische afwijkingen in genen die betrokken zijn bij anti-TB immuun reacties. Daarom is de vraag naar een effectief TB vaccin groter dan ooit.

Subunit vaccins zijn niet-levende vaccins die bestaan uit immunogene componenten zoals eiwitten en/of peptiden en kunnen worden toegediend in combinatie met een adjuvant of via een viraal vector systeem. Er zijn verschillende TB subunit vaccins in ontwikkeling die bestaan uit *Mtb* eiwitten die door actief groeiende *Mtb* bacteriën tot expressie worden gebracht. Echter, *Mtb* kan ook in een minder actieve, sluimerende/ "dormant" staat verkeren. Men neemt aan dat deze sluimerende staat van *Mtb* aanwezig is in latent geïnfecteerde individuen. Deze sluimerende staat is een aanpassingsmechanisme dat *Mtb* gebruikt om te kunnen overleven in moeilijke, stressvolle situaties. *Mtb* bacteriën worden opgenomen door antigeen presenterende cellen zoals macrofagen en verblijven in een zogenoemd fagosoom, dat zorgt voor een voor de bacteriën onaangenaam milieu. Hier wordt *Mtb* onder andere bloot gesteld aan zuurstoftekort en een gebrek aan voedingsstoffen. Om deze omstandigheden toch te kunnen overleven schakelt *Mtb* over op een sluimerende, rustende fase die wordt gekenmerkt door een veranderd gen expressie patroon. Een gen cluster dat specifiek verhoogd tot expressie komt gedurende zuurstof depletie is het 'dormancy regulon encoded' (DosR) gen cluster. Verder blijkt dat er ook genen selectief tot expressie komen bij een tekort aan voedingsstoffen. Daarnaast is een bepaalde eiwit groep, 'resuscitation promoting factors' (Rpfs), zeer waarschijnlijk betrokken bij het ontwaken of reactiveren van sluimerende *Mtb*.

Dergelijke bovengenoemde eiwitten zijn dus betrokken bij specifieke fasen waar *Mtb* in kan verkeren. Het is nog niet volledig duidelijk welke andere genen en eiwitten de *Mtb* bacterie verder reguleert tijdens een *in vivo* infectie. Deze eiwitten zouden goede subunit vaccin kandidaten zijn, die zowel profylactisch als therapeutisch kunnen worden toegediend.

Nieuwe en relevante antigenen die mogelijk als TB subunit vaccin kandidaat kunnen dienen, moeten voldoen aan een aantal voorwaarden:

i) Het antigen moet immunogeen zijn, dat wil zeggen: het eiwit zal efficiënt moeten worden herkend door specifieke T cellen in mycobacterie blootgestelde individuen.

ii) Het eiwit moet *in vivo* tot expressie worden gebracht door *Mtb* in geïnfecteerde organen, zodat immuun cellen geïnduceerd tegen het antigen *Mtb* geïnfecteerde cellen ook daadwerkelijk kunnen herkennen.

iii) Het antigen moet protectief zijn, dat wil zeggen: het eiwit moet in staat zijn een immuun respons op te wekken die de groei van *Mtb* kan remmen of *Mtb* kan doden.

iv) De eiwit sequentie van het antigen moet onveranderd aanwezig zijn in alle stammen van *Mtb*, zodat een vaccin gebaseerd op dit antigeen effectief kan zijn tegen alle verschillende *Mtb* stammen.

Het onderzoek in dit proefschrift beschrijft de analyse van *Mtb* fase gerelateerde antigenen als mogelijke TB subunit vaccin kandidaten met specifieke focus op bovengenoemde factoren.

Dit proefschrift

'Resuscitation promoting factors (Rpfs)', zijn kleine eiwitten die zeer waarschijnlijk betrokken zijn bij het ontwaken van *Mtb* uit zijn sluimerende staat. Zij kunnen dus een belangrijke rol spelen in de ontwikkeling van TB. Het toepassen van deze antigenen in een TB vaccin zou dan kunnen leiden tot afweer responsen die reactiverende *Mtb* meteen herkennen en bedwingen, en op die manier actieve TB ziekte helpen voorkomen. Deze antigenen zijn daarom mogelijk interessante TB vaccin kandidaten. In **hoofdstuk 2** is de immunogeniciteit van twee Rpf antigenen (Rv0867c en Rv2389c) bepaald en zijn tevens immunogene regio's in deze eiwitten geïdentificeerd. Beide antigenen, vooral Rv2389c, werden herkend door witte bloedcellen van donoren die aan verschillende mycobacterium stammen waren blootgesteld (BCG gevaccineerde personen, latent geïnfecteerde personen (LTBI) en TB patiënten), zoals bepaald door de antigeen-specifieke productie van T helper 1 cytokines. De antigenen, maar nu vooral Rv0867c, bleken ook te worden herkend door de witte bloedcellen van een specifieke groep individuen die al tientallen jaren latent geïnfecteerd waren met *Mtb*, maar nooit TB ontwikkeld hadden (lt (long-term) LTBI). $CD4^+$ en $CD8^+$ T cellen van deze ltLTBIs reageerden op de antigenen en produceerden één of twee verschillende cytokines, wat betekent dat deze immuun cellen lang na *Mtb* blootstelling aanwezig

blijven. Ook bleken beide antigenen immunogene regio's te bevatten, zoals bepaald door middel van het gebruik van T cel lijnen. Al met al voldoen deze antigenen dus al aan één belangrijke vaccin voorwaarde.

'Dormancy regulon encoded genes' (DosR), zijn *Mtb* genen die sterk verhoogd worden afgeschreven wanneer de *Mtb* bacil wordt blootgesteld aan een tekort aan zuurstof. Sommige van de DosR eiwitten, afgeschreven van deze genen, worden vaker herkend door LTBI dan door TB patiënten. Het richten van een vaccin tegen deze antigenen zou dan ook mogelijk kunnen helpen bij het onder controle houden van de *Mtb* infectie. In **hoofdstuk 3** zijn de immunogeniciteit en immunogene regio's van drie verschillende DosR antigenen (Rv1733c, Rv2029c en Rv2031c) bepaald. Alle drie de antigenen werden herkend door dezelfde ltLTBI groep, en zowel $CD4^+$ en $CD8^+$ T cellen reageerden op de antigenen. De grootste populatie van T cellen produceerde één of twee verschillende cytokines. Verder bleken deze T cellen "uitvoerende geheugen cellen" (effector memory cells) of "uitvoerende cellen" (effector cells) te zijn. Dit betekent wederom dat ook immuun cellen specifiek voor deze DosR antigenen lange tijd na blootstelling aan *Mtb* aanwezig blijven. Alle drie antigenen bevatten ook immunogene regio's, specifiek herkend door $CD4^+$ en/of $CD8^+$ T cellen. Ook deze drie DosR antigenen blijken immunogeen en dus te voldoen aan enkele belangrijke, bovengenoemde subunit vaccin voorwaarden.

De boven genoemde *Mtb* fase gerelateerde antigenen werden alle ontdekt in *in vitro* studies welke de *in vivo* stress omstandigheden nabootsen. Een directe analyse van *Mtb* genen expressie tijdens een echte *in vivo* infectie is echter een krachtiger methode om relevante, hoog geexpresseerde *Mtb* genen te identificeren omdat hierbij meerdere factoren tegelijk van invloed zijn zoals het effect van het immuun systeem. Daarbij is het wellicht ook mogelijk om belangrijke respons systemen te identificeren die *Mtb* gebruikt om *in vivo* te overleven. Wij veronderstelden dat *in vivo* geexpresseerde genen tot de selectie van belangrijke nieuwe vaccin kandidaten zou kunnen leiden. In **hoofdstuk 4** is daarom een set van *in vivo Mtb* geexpresseerde (IVE-TB) genen beschreven die zijn geïdentificeerd in de longen van *Mtb* geïnfecteerde muizen. Vier verschillende, genetische verwante muizen stammen zijn geanalyseerd. Deze stammen lieten een divers *Mtb* infectie patroon of spectrum van TB gevoeligheid zien. Een associatie kon worden gelegd tussen gen expressie en TB ziekte fenotypes. Ook werd de immunogeniciteit van een selectie van deze IVE-TB antigenen bepaald in mycobacterie blootgestelde individuen. Deze selectie bevat antigenen waarvan de eiwit sequenties geconserveerd zijn tussen de verschillende *Mtb* stammen. De IVE-TB antigenen werden goed herkend door *Mtb* blootgestelde individuen met een positieve IGRA en TST, terwijl weinig herkenning zichtbaar was in IGRA negatieve, TST positieve donoren of in negatieve controles. In TB patiënten werd ook herkenning gezien, zij het enigszins lager dan in de $IGRA^+TST^+$ donoren. Een aantal IVE-TB antigenen werden ook herkend door $CD4^+$ en $CD8^+$ T cellen van ltLTBI, dus immuun responsen tegen deze eiwitten waren wederom zeer langdurig aanwezig na infectie. Concluderend, de geteste IVE-TB antigenen zijn immunogeen, zijn geconserveerd en worden tot expressie gebracht *in vivo*, en voldoen hiermee aan drie van de gestelde vaccin voorwaarden.

Een belangrijke parameter voor een efficiënt TB vaccin kandidaat is de inductie van beschermende immuniteit. IVE-TB antigen Rv2034 uit hoofdstuk 4 werd verder bestudeerd als mogelijke vaccin kandidaat *in vivo* in HLA transgene muizen in **hoofdstuk 5**. Zoals beschreven in hoofdstuk 4 werd het antigen Rv2034 herkend door aan mycobacterie blootgestelde donoren maar niet door negatieve controles, wat betekent dat Rv2034 tot expressie wordt gebracht tijdens infectie én dat het immuun systeem in staat is om dit antigen te herkennen. Om Rv2034 als vaccin te gebruiken zal ook eerst de immunogeniciteit van het vaccin verder moeten worden bepaald. Hiertoe werden HLA transgene muizen gevaccineerd met Rv2034. Het bleek dat het antigen sterk herkend werd en een aantal HLA-DR3 specifieke epitopen bevatte. De geïnduceerde $CD4^+$ T cellen produceerden voornamelijk een of twee verschillende soorten cytokines. Vervolgens werd aangetoond dat in Rv2034 gevaccineerde muizen het aantal *Mtb* bacteriën in de longen sterk gereduceerd was na intranasale infectie met de pathogene H37Rv *Mtb* stam. Dit is in overeenstemming met de expressie van Rv2034 *in vivo* in de long, na infectie. Verder bleek de Rv2034 eiwit sequentie geconserveerd. Rv2034 voldoet zo aan alle gestelde vaccin voorwaarden.

Om de functie van de Rv2034 T cellen nader te kunnen bestuderen hebben we een CD154 klonerings methode gebruikt om Rv2034 specifieke T cel klonen te genereren (**hoofdstuk 6**). $CD4^+$, $CD8^+$ en $CD3^+CD4^-CD8^-$ T cel klonen konden worden gegenereerd. De specificiteit van een $CD4^+$ Rv2034 T cel kloon was verder onderzocht en bleek specifiek voor peptide p81-100 welke gepresenteerd wordt via HLA-DR. Verder herkende de T cel kloon ook Rv2034 in *Mtb* lysaat, wat aantoont dat de T cel kloon niet alleen recombinant eiwit en synthetische peptiden, maar ook het natuurlijke eiwit geproduceerd door *Mtb* herkent. Daarbij bleek de T cel kloon T helper 1 en cytotoxische markers tot expressie te brengen. Om de mechanismen achter TB bescherming en infectie te ontrafelen is het van essentieel belang om de rol van (fase gerelateerde) antigeen specifieke $CD4^+$ en $CD8^+$ T cellen in dit proces te begrijpen. Het werk beschreven in hoofdstuk 6 kan daar een bijdrage aan leveren wat uiteindelijk zal leiden tot meer efficiënte TB vaccins en verbeterde behandelingsmethodes met als gevolg een afname van of zelfs het elimineren van TB.

Concluderend/toekomst perspectief

In dit proefschrift zijn verschillende *Mtb* fase gerelateerde antigenen geanalyseerd als mogelijke TB subunit vaccin kandidaten, met een specifieke focus op vier vooraf gestelde voorwaarden. Rpf en DosR eiwitten werden herkend door mycobacterie blootgestelde individuen en bleken immunogeen. De antigenen bevatten immunogene regio's welke mogelijk kunnen worden gebruikt in vaccin studies om verder het beschermende effect van deze antigenen te bestuderen. Een derde groep van antigenen, gevonden in dit proefschrift (IVE-TB antigenen) werden door *Mtb* tot expressie gebracht in de longen van muizen en bleken tevens immunogeen omdat ze werden herkend door *Mtb* blootgestelde individuen. Het IVE-TB antigen Rv2034 voldeed aan alle gestelde voorwaarden voor een effectieve vaccin kandidaat, dit zal verder moeten worden geoptimaliseerd en bestudeerd in andere *Mtb* modellen om de resultaten beschreven in dit proefschrift te valideren.

Om het effect van een vaccin te vergroten, zal het moeten bestaan uit meerdere antigenen welke in verschillende fasen van *Mtb* tot expressie komen. Op deze manier heeft het vaccin ook effect op de verschillende stadia, door het gebruik van deze fase gerelateerde antigenen en zal daarom ook toepasbaar zijn als profylactisch als ook therapeutisch vaccin. Tevens zullen immunogene epitopen aanwezig moeten zijn die via een wijd scala aan HLA moleculen kunnen worden gepresenteerd. De toekomst zal uitwijzen of deze multi-fase vaccins inderdaad zullen kunnen leiden tot het verminderen van TB.

Curriculum Vitae

Susanna Commandeur, de auteur van dit proefschrift, werd geboren op 27 december 1984 te Kreileroord. In 2002 behaalde zij haar HAVO diploma aan de RSG Wiringherlant te Wieringerwerf. Het jaar daarop ontving zij haar propedeuse van de opleiding Sociaal Pedagogische Hulpverlening aan de Hogeschool van Amsterdam. Hetzelfde jaar startte zij, na succesvol afronden van het toelatingsexamen, aan de opleiding Biomedische Wetenschappen aan de Vrije Universiteit van Amsterdam. Tijdens de 3-jarige bachelor liep zij stage op de afdeling Medische Microbiologie en Infectiepreventie aan het VU medisch centrum onder begeleiding van prof. dr. Wilbert Bitter. Tijdens deze periode onderzocht zij welke genen van het ESX-5 cluster betrokken waren bij het secreteren van het eiwit PPE41. Zij vervolgde haar 2-jarige master aan dezelfde universiteit in de richting Celbiologie en Immunologie. Haar stage in het eerste masterjaar werd uitgevoerd op de afdeling Immunohematologie en Bloedtransfusie aan het Leids Universitair Medisch Centrum, onder supervisie van dr. Nigel Savage. Dit werk betrof een structuur-activiteitsrelatie studie naar gastheer-georiënteerde chemische stoffen welke mogelijk een bactericide effect induceren. Haar laatste masterstage liep zij op het departement Infectieziekten en Immunologie (afdeling virologie) aan de Diergeneeskunde faculteit van de Universiteit van Utrecht, onder begeleiding van dr. Berend Jan Bosch. Hier werkte zij aan de ontwikkeling van 'virus-like particles', een vaccin platform, welke Rift Valley fever virus eiwitten tot expressie brengen. Na het schrijven van haar scriptie behaalde zij haar master diploma in 2008 en halverwege dat jaar begon zij, onder begeleiding van prof. dr. T.H.M. Ottenhoff en dr. A. Geluk, haar promotie onderzoek dat is beschreven in dit proefschrift.

Sinds mei 2013 is Susanna werkzaam als post-doctoraal onderzoeker op de afdeling Medische Microbiologie en Infectiepreventie aan het VU medisch centrum waar ze haar werk en interesse in de bestrijding van *Mycobacterium tuberculosis* continueert.

List of publications

Susanna Commandeur, Karin Dijkman, Annemieke H. Friggen, Krista E. van Meijgaarden, Susan J.F. van den Eeden, Louis Wilson, Jolien J. van der Ploeg-van Schip, Kees L.M.C Franken, Annemieke Geluk and Tom H.M. Ottenhoff. *Clonal analysis of the T-cell response to in vivo expressed Mycobacterium tuberculosis protein Rv2034, using a CD154 expression based T-cell cloning method.* **Submitted.**

Susanna Commandeur, Susan J.F. van den Eeden, Karin Dijkman, Kees L.M.C. Franken, Krista E. van Meijgaarden, Louis Wilson, Tom H.M. Ottenhoff and Annemieke Geluk. *The newly identified Mycobacterium tuberculosis antigen Rv2034 induces CD4+ T cells that protect against pulmonary infection in HLA-DR transgenic mice.* **Submitted.**

Susanna Commandeur, Krista E. van Meijgaarden, Corine Prins, Alexander V. Pichugin, Karin Dijkman, Annemieke H. Friggen, Kees L.M.C. Franken, Gregory Dolganov, Igor Kramnik, Gary K. Schoolnik, Fredrik Oftung, Gro Ellen Korsvold, Annemieke Geluk and Tom H.M. Ottenhoff. *An unbiased genome-wide Mycobacterium tuberculosis gene expression approach to discover antigens targeted by human T cells expressed during pulmonary infection.* **J Immunol. 2013. Feb 15;190(4):1659-71.**

Susanna Commandeur*, May Young Lin*, Krista E. van Meijgaarden*, Annemieke H. Friggen, Kees L.M.C. Franken, Jan W. Drijfhout, Gro Ellen Korsvold, Fredrik Oftung, Annemieke Geluk and Tom H.M. Ottenhoff. *Double- and monofunctional CD4+ and CD8+ T-cell responses to Mycobacterium tuberculosis DosR antigens and peptides in long-term latently infected individuals.* **Eur J Immunol. 2011. Oct;41(10):2925-36.**

Susanna Commandeur*, Krista E. van Meijgaarden*, May Young Lin, Kees L.M.C. Franken, Annemieke H. Friggen, Jan Wouter Drijfhout, Fredrik Oftung, Gro Ellen Korsvold, Annemieke Geluk and Tom H.M. Ottenhoff. *Identification of human T-cell responses to Mycobacterium tuberculosis resuscitation-promoting factors in long-term latently infected individuals.* **Clin Vaccine Immunol. 2011. Apr;18(4):676-83.**

Annemieke Geluk, Jolien J. van der Ploeg-van Schip, Krista E. van Meijgaarden, **Susanna Commandeur**, Jan Wouter Drijfhout, Willemien E. Benckhuijsen, Kees L.M.C. Franken, Bernard Naafs and Tom H.M. Ottenhoff. *Enhancing sensitivity of detection of immune responses to Mycobacterium leprae peptides in whole-blood assays.* **Clin Vaccine Immunol. 2010. Jun;17(6):993-1004.**

* These authors contributed equally to the study

Notes