Exploring the Mycobacterium tuberculosis antigenome: New insights for the development of vaccines, diagnostics and drugs

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CHAPTER 1

General introduction and thesis outline

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Genome wide approaches discover novel *Mycobacterium tuberculosis* antigens as correlates of infection, disease, immunity and targets for vaccination.

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WHY WE SHOULD HUNT FOR NEW *MYCOBACTERIUM TUBERCULOSIS* ANTIGENS

Mycobacterium tuberculosis (*Mtb*) is an ancient microbe that evolved in its ecological niche, namely the human host, for at least seventy thousand years (1). As a result of this prolonged co-evolution, *Mtb* is one of the most sophisticated human pathogens that, through elegant escape-mechanisms, can survive inside its host for many years even in the absence of clinical symptoms or any evidence of pathology (2). According to the latest estimations, 2.5 billion individuals latently harbour *Mtb*, a condition termed as latent TB infection (LTBI) (3). Of those LTBI, 3-10% will develop active tuberculosis (TB) disease, mostly for unknown reasons. Some well-known precipitating factors are the presence of comorbidities (e.g., diabetes), coinfections (e.g., HIV), aging or immunosuppressive agents (4). Considering that for each new TB case there are on average ten newly *Mtb* infected people, it is not surprising that the current *Mtb* infection cycle successfully sustains the global epidemic, which annually accounts for 10 million new TB patients and 1.4 million TB deaths (5, 6). Thus, despite the fact that global TB control efforts are averting millions of cases and thousands of deaths every year, TB remains the leading cause of mortality from a single bacterial agent worldwide (6).

Active TB is characterized by symptoms of weakness, weight loss, fever, and night sweats in addition to more specific symptoms based on the organ affected by *Mtb* which is, in adolescents and adults, primarily the lung (7). If not treated, TB leads to death in almost half of all HIV-uninfected people and in almost all HIV-positive individuals (6). Anti-TB drugs are available but treatments involve long regimens with considerable side effects (8). These could both be reduced by personalised medicinal approaches, if only adequate tests were available to monitor the response to, and predict the successful outcome of TB treatment. The protective efficacy of Bacillus Calmette-Guérin (BCG), the only licensed TB vaccine recommended at birth in over 180 countries, is variable and inconsistent, ranging from 0 to 80% (9, 10). Even though BCG is effective in reducing the incidence of disseminated TB in children, it is clearly insufficient in preventing the onset of pulmonary TB in adolescents and adults (10). Next to the difficulties in TB treatment, which are further complicated by the rapidly increasing numbers of drug resistant TB, and the lack of an effective vaccine, also current diagnostics for *Mtb* infection are of limited value. Active TB cases are defined as symptomatic patients with radiological, microbiological or molecular evidence of *Mtb* (6). Traditional tests include sputum smear microscopy and mycobacterial culture, which are accurate tests in detecting *Mtb* but hampered by poor sensitivity and long response time, respectively. New molecular diagnostics have been developed but not extensively implemented yet due to their high cost

and poor discriminating power in distinguishing viable from dead bacteria (11). The century old tuberculin skin test (TST) and the more recent interferon-gamma release assays (IGRAs) are indirect measures of infection as these tests determine previous host sensitization to *Mtb* antigens by detecting memory T cell responses. Moreover, their diagnostic performance in HIV/TB-co-infected patients is poor when CD4+ T cell counts are diminished. These tests also cannot distinguish between active TB and latent TB infection (LTBI), and have virtually no predictive value in estimating the risk of progression from latent to active state of disease (12). In the past decades, development of better diagnostics and vaccines has been a key goal in TB research, yet thwarted by the incomplete understanding of the complex and heterogeneous interplay between the human host and *Mtb* (13).

In order to provide better insights into this intricate host-pathogen interaction and to discover new *Mtb* antigens as vaccine candidates and TB biomarkers for improved diagnostic and predictive algorithms, intense searches for novel and relevant *Mtb*

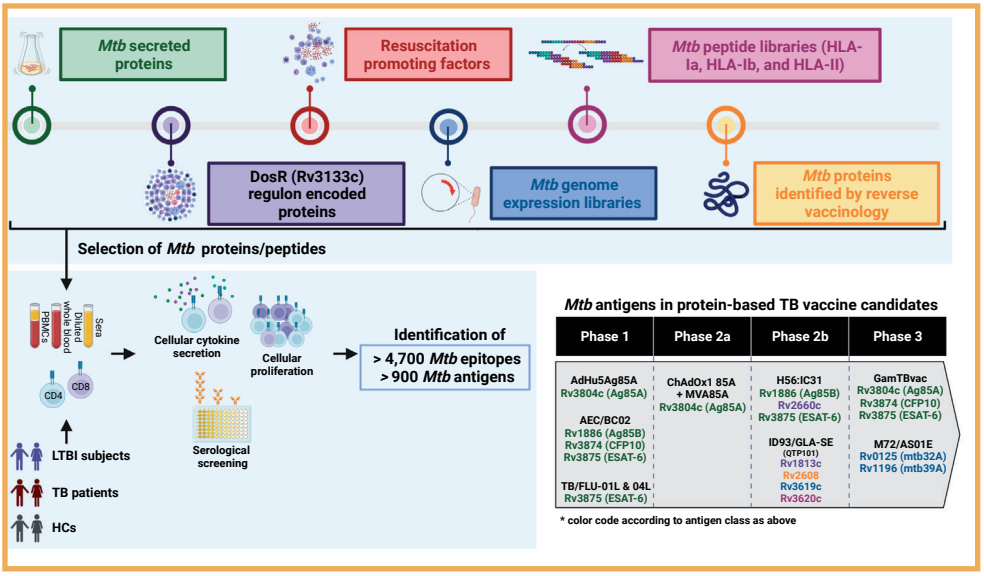

Figure 1. *Mtb* antigen discovery: approaches and outcome. *Mtb* proteins and peptides have been found/selected using several *Mtb* antigen discovery approaches, which are discussed thoroughly in this chapter. These strategies have led to the identification of more than 4,700 *Mtb* epitopes and 900 antigens. However, only a few of those antigens are currently part of TB vaccine candidates, as shown by the vaccine development pipeline in the lower right part of the figure. In this pipeline (based on the TAG 2021 report), vaccine names are colour coded in black while the composing antigens are colour coded according to antigen class as depicted in the upper part of the figure. *Mtb*: *Mycobacterium tuberculosis*; PBMCs: peripheral blood cells; LTBI: latently *Mtb* infected; TB: tuberculosis; HCs: healthy controls.

antigens have been undertaken, particularly since the *Mtb* genome sequence became available in 1998. Several *Mtb* antigen discovery strategies have been followed ranging from traditional to genome wide approaches (Figure 1). These strategies are discussed in the paragraphs below.

CLASSICAL ANTIGENS, LATENCY ANTIGENS, RESUSCITATION PROMOTING FACTORS, AND THEIR VACCINE POTENTIAL

Traditional *Mtb* antigen discovery approaches in the last century

Traditional *Mtb* antigen discovery approaches in the pre-genomics era relied on classical biochemical analyses of *in vitro* cultured *Mtb* from which fractions and secreted moieties were isolated and purified, based on much excellent work from many researchers (14). The antigenicity of the isolated components was typically tested using cells or sera from *Mtb* infected animals, including mice, rabbits, goats and cattle, as well as human beings exposed to mycobacteria such as LTBI, BCG vaccinees and active or treated TB patients. After the discovery of IFN-γ as an important cytokine in protective immunity to TB in animal models and humans (15, 16), and the development of suitable assays to measure it, the antigenicity of *Mtb* antigens started to be determined increasingly by the ability to induce IFN-γ from immune cells in mouse models and in blood cells of *Mtb* exposed individuals *in vitro*, assuming that IFN-γ could be a correlate of protection. These approaches in the 1980's and 1990's led to the identification of important *Mtb* antigens abundantly expressed *in vitro*, including Ag85C (fibronectin-binding protein C, Rv0129c), Ag85B (Rv1886), Ag85A (Rv3804c) (17-19), Mpb64 (Rv1980) (20), 38kDa (Rv0934) (21), ESAT6 (Rv3875) (22), *Mtb*8.4 (Rv1174) (23), CFP10 (Rv3874) (24), Mpt51 (Rv3803c) (25) and TB10.4 (Rv0288) (26). Virtually all of these proteins were secreted *Mtb* proteins, and therefore amenable to identification in the supernatants of *in vitro* grown *Mtb*. In addition, also heat shock proteins became a prominent focus of attention as will be discussed below.

The completion of the sequencing and annotation of the *Mtb* (H37Rv) genome in 1998 (27), in the first decade of this century, was key to the initiation of the first *Mtb* post-genomic approaches, and allowed the discovery of new classes of *Mtb* antigens using unbiased approaches. One example of such approaches is the discovery of so called *Mtb* "latency antigens", based on genome-wide expression profiling of *Mtb* bacteria *in vitro* exposed to hypoxic culture conditions.

Mtb latency antigens: important tools for improving current TB vaccines

By using *in vitro* conditions that mimic the host environment encountered by *Mtb* during *in vivo* infection, such as hypoxia, nutrient starvation or IFN-γ-activated macrophages, a genetically regulated metabolic shift down was discovered in *Mtb* bacilli exposed to such conditions. *Mtb* downregulated most of its genes as an adaptive response to stress, but this appeared to be accompanied by a remarkable upregulation of the expression of 48 *Mtb* genes in response to various environmental stress factors including hypoxia. These genes were found to be regulated by a response regulator termed the DosR protein (encoded by Rv3133c) and hence this regulon was called the dormancy or DosR regulon (28, 29). One of the genes most strongly upregulated was Rv2031c/α-crystallin or hspX, which is involved in cell wall thickening and stabilisation under hypoxia (further discussed below). The DosR regulon encoded gene products were designated "latency antigens" and were tested as recombinant proteins for cellular and humoral recognition in LTBI individuals and TB patients from different geographical cohorts in Europe, Africa, South America and India. In all cohorts, the *Mtb* latency antigens were recognized preferentially by LTBI donors compared to (active or treated) TB patients, as measured by cellular responses (30-34). The data suggested that several of these antigens such as Rv1733c, Rv2029c, Rv2626 and R2628 could be useful antigenic targets to discriminate LTBI from TB patients, which is not well possible by IGRA or TST. Moreover, the fact that the BCG genome contains homologues of the DosR genes (35) suggested that latency antigens might be interesting TB booster vaccine candidates, particularly since they have been demonstrated in several studies to have protective efficacy in different animal models (36-40). However, a puzzling observation was that neither subcutaneous (s.c.) BCG vaccination in animals, nor intradermal (id) BCG vaccination in humans was found to induce T cell responses against latency antigens, suggesting that these routes of immunisation might not allow BCG to enter a state of latency (35, 41). The inability of BCG to induce immune responses to latency antigens may underlie its impaired ability to induce full protective immunity to *Mtb*. This shortcoming might be addressed either by recombinant overexpression or by subunit vaccine boosting, in order to induce improved protection. In support of this concept, it has been shown that an Rv1733c, Rv3407 and Rv2659 (representing latency and starvation antigens) expressing recombinant BCG strain (rBCGΔureC::hly) induced better protection against highly virulent *Mtb* (Beijing) in mice than did the regular recombinant BCG (rBCGΔureC::hly) lacking these antigens (42). In addition, recent data have demonstrated that Rv1733c vaccination following BCG significantly improved the protective efficacy of BCG in a mouse challenge model (43).

Another important latency antigen discovered around the same time was the 28kDa heparin-binding protein (HBHA, Rv0475), an antigen not regulated by DosR. The HBHA adhesin, identified using heparin-Sepharose chromatography from culture supernatant and extracts of *Mtb* and *M. bovis* (44), is expressed on the surface of *Mtb* and promotes its interaction with non-phagocytic cells, thereby facilitating *Mtb* extrapulmonary dissemination (45). It has been shown that when coated with anti-HBHA antibodies *Mtb* has a reduced ability to disseminate outside the lung in mice, suggesting that anti-HBHA antibodies in TB patients might help containing *Mtb* infection by blocking HBHA (45). Interestingly, stimulation of lymphocytes from LTBI but not TB patients with HBHA induced IFN-γ secretion (46, 47) and perforin-producing CD8+ T cells cytotoxic against mycobacterium infected macrophages (48). Based on these findings, HBHA induced T cell IFN-γ production has been proposed as a biomarker of LTBI (49). In addition, HBHA has been shown to enhance BCG protective efficacy both in adult and new-born mice when administrated in prime-boost regimens (50, 51). These data, supported by the evidence that BCG induces specific HBHA multi-cytokine responses (52), suggest that HBHA might be an interesting candidate antigen for a TB subunit vaccine candidate designed to boost BCG.

Mtb resuscitation-promoting factors (Rpf) are promising targets for diagnosis and vaccination

Next to latency antigens another class of *Mtb* antigens discovered were *Mtb*'s Rpfs (53, 54). Rpfs are secreted proteins that have hormone-like activity, and are able to promote the transition from a dormant into an active replicating state of bacteria, including mycobacteria (55). The five *Mtb* Rpfs, specifically RpfA (Rv0867c), RpfB (Rv1009), RpfC (Rv1884c), RpfD (Rv2389c), and RpfE (Rv2450c) were screened for immunological reactivity in *Mtb* exposed individuals and found to be recognized *in vitro* by IFN-γ producing cells preferentially from LTBI (34, 56, 57). Based on this property, these proteins could further help differentiating LTBI from TB, next to the above latency antigens.

Taken together, latency antigens (DosR regulon encoded antigens, HBHA) and Rpf are potent, novel *Mtb* antigens, which could, next to secreted antigens, find application in the design of new TB vaccines as well as in tests diagnosing LTBI.

New developments for TB vaccines with latency and Rpf antigens in non-human primate (NHP) and mouse models

With a TB vaccine perspective, several of the above discussed *Mtb* antigens (Rpfs, latency antigens and early secreted proteins) have been evaluated recently as combinatorial, multistage vaccines in mice and rhesus macaques. Vaccination with

a virally (i.e., modified RhCMV) vectored combination of six or nine *Mtb* antigens in rhesus macaques strongly reduced mycobacterial loads in the lung of the *Mtb* infected animals, even to the unprecedented extent of sterile eradication in half of the animals (58-60). The RhCMV vectored insert contained six or nine *Mtb* antigens, namely: Ag85A, Ag85B, ESAT6, Rv1733c, Rv2626c, Rv3407, RpfA, RpfC, and RpfD (60). Additionally, five of these antigens have been evaluated recently as part of another multi-antigenic vectored vaccine, MVATG18598, in this case in post-exposure BALB/c and C57BL/6 mouse models (61). MVATG18598 specifically expressed Rv2626c, Ag85B, CFP10, ESAT6, TB10.4, Rv0287, RpfB, RpfD, Rv3407, and Rv1813c. Mice were challenged with *Mtb*, treated with a standard antibiotic regimen (rifampin, isoniazid and pyrazinamide) and then "therapeutically" vaccinated. Two different vaccination regimens (i.e., during and after chemotherapy), two delivery routes (s.c. or intranasally) and different numbers of injections (1x, 3x, 5x, and 7x) were tested to evaluate the effect of MVATG18598 in this post-challenge model. The s.c. administration (3x) of MVATG18598 during treatment showed the strongest reduction in the occurrence of post treatment TB relapses and *Mtb* burden in BALB/c mice. This finding was confirmed also in C57BL/6 mice. In both mouse strains, the MVATG18598 vaccination triggered strong IFN-γ production and high levels of polyclonal antibodies against specific components of the multi-antigenic vaccine.

These preclinical TB vaccination data from NHP and mouse models confirm and extend the unexpectedly strong vaccine potential of the newly discovered latency, early secreted and Rpf antigens, and hold promise for application in humans in the nearby future, in both preventive and therapeutic fashion.

BROADENING THE DISCOVERY OF THE *MTB* ANTIGENOME USING GENOME WIDE APPROACHES

Advances in DNA technologies, genome sequencing and bioinformatics tools now offer unprecedented opportunities to rapidly and exhaustively mine the potential *Mtb* antigenome, at least at the proteome level. Here, the results of the most representative studies are summarized and discussed.

The first whole genome based *Mtb* antigenome search was innovative in that it generated the first *Mtb* genome expression libraries using newly developed recombinant DNA technologies. This *Mtb* genome expression library was built by shearing *Mtb* Erdman strain's DNA, that was subsequently linked to EcoRI linkers and inserted into λgt11 vectors, allowing access to *Escherichia coli*'s transcriptional

and translational machinery to produce *Mtb* protein fragments (62). The potential to detect "all" (note: the library almost certainly was incomplete) *Mtb* proteins, including those not always expressed by *Mtb* depending on its phase, constituted an important novelty. By screening this library with murine monoclonal antibodies (63-65) and polyclonal sera (66, 67) several new *Mtb* proteins such as HspX/ α-crystallin (Rv2031c) were identified and further validated using lymphocytes or T cell clones from TB patients (68). In addition, also other heat shock proteins such as hsp60 (Rv0440) and hsp70 (Rv0350) (64, 69, 70) were extensively studied, in part due to their high immunogenicity and in part due to their sequence similarities with mammalian hsp, raising the interesting possibility of being involved in inducing cross-reactivity and the subsequent precipitation of auto-immunity (71-73).

Expression cloning technologies used in subsequent studies, including those employing DNA from virulent *Mtb* strains (74), resulted in the discovery of additional immunogenic proteins comprising Rv1510 (74), *Mtb*39a (Rv1196) (75), *Mtb*32a (Rv0125) (76), *Mtb*9.9a (Rv1793c) (77), *Mtb*9.8 (Rv0287) (78) and *Mtb*41 (Rv0915c) (79-84). Obviously, these methods are biased towards protein antigens and do not offer the possibility to evaluate non-protein antigens like polysaccharides, lipopolysaccharides, and glycolipids. In addition, the gene products synthesized by recombinant expression in heterologous hosts does often not replicate post-translational modifications such as lipidation and glycosylation, which are naturally occurring in mycobacteria. Whether and how those changes impact protein immunogenicity has been studied to some extent, and it was recently shown that glycoconjugate derivatives of Ag85B, obtained after glycosylation of its lysine residues, were less recognised by BCG vaccinees and TB patients compared to the unmodified variant (85). This finding suggests that recombinant products lacking post-translational modifications could lead to misinterpret the immunological activity of antigens compared to the variants present during natural *Mtb* infection. The use of other vectors phylogenetically closer to *Mtb*, such as *M. smegmatis* has been proposed as an alternative host to overcome such post-translational issues (86).

Although allowing the identification of the *Mtb* antigens Rv1196 and Rv0125 which are now included in the advanced TB vaccine candidate, M72/AS01E (87, 88), the above discussed *Mtb* genome expression libraries have nevertheless unlocked only a small portion of the *Mtb* antigenome. This is probably due to issues of low expression in heterologous systems and the use of small DNA fragments, precluding the expression of conformationally more complex structures such as antibody epitopes.

This scenario was revolutionized by the completion of the *Mtb* genome sequence that now permitted access to the entire *Mtb* protein antigenome (27). The *Mtb*

genome sequence made it possible to identify novel candidate antigens without having to cultivate *Mtb* (14). A breakthrough was also that all genome information was made available via open-source internet genomic and proteomic databases such as TubercuList (http://genolist.pasteur.fr/TubercuList/), Mycobrowser (https://mycobrowser.epfl.ch/) and later TB Database (http:// http://tbdb.bu.edu/) (89). In addition, the increasing accessibility of highly powerful bioinformatics tools also allowed *in silico* genomic analyses and comparative evaluation (e.g.: BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi), and to predict new *Mtb* B cell epitopes as well as T cell epitopes, based on predictive HLA binding motifs (e.g.: ProPred, http://crdd.osdd.net/raghava/propred/; HLA_BIND, https://www-bimas.cit.nih.gov/molbio/hla_bind/; EpiMatrix, http://i-cubed.org/tools/ivax/ivax-tool-kit/epimatrix/; DTU prediction servers, http://www.cbs.dtu.dk/services/). These developments and technologies have revolutionized the field of antigen discovery in general, and that of *Mtb* in particular (*Mtb* is a complex pathogen to work with due to its slow growth, its phase variation and its restriction to BSL3 safety level laboratories).

MINING THE COMPLETE *MTB* ANTIGENOME FOR EPITOPE AND ANTIGEN DISCOVERY

In search of new *Mtb* epitopes using peptide libraries

Experimental methods to identify new *Mtb* epitopes have been classically based on the screening of overlapping peptides spanning the full sequence of the relevant target proteins, followed by assaying antibody and/or T cell immune responses in *in vitro* assays and/or *in vivo* models (90-96). This approach has also been applied to interrogate novel interesting candidate antigens selected from the whole *Mtb* genome sequence. Some of the most studied proteins have been those encoded by the so-called regions of differences (RDs). RDs were identified by comparative genome analysis and consist of 16 genomic segments that are present in *Mtb* (H37Rv strain) but absent from most BCG vaccines and non-tuberculous mycobacterial strains (97-99). Although those genomic regions together encode 129 predicted open reading frames (ORFs), only a few of these have been studied in detail–at least according to accessible published information. Moreover, those that were translated into approved TB diagnostic tests had already been discovered through conventional approaches described above (100). Very recently only, a new overlapping peptide cocktail covering the RD7 encoded esx member antigen Rv2348 in combination with CFP10 and two other secreted proteins (EspC (Rv3615s) and EspF (Rv3865)) was identified using this approach, and subsequently developed into an ESAT6 free diagnostic IGRA test (101). The ESAT6 family comprises 23 small proteins (from EsxA to EsxW) that are mostly secreted in pairs (with the only exception of EsxQ).

Not only ESAT6 or CFP10 but also other members of the ESAT6 family are highly antigenic as demonstrated across several independent post-genomic studies. Furthermore, several ESAT6 family members were found to have strong vaccine efficacy in mouse models: immunization with the fusion protein H65 (containing the ESX dimer substrates EsxD-EsxC, ExsG-EsxH, and ExsW-EsxV) had comparable protective efficacy with BCG (102).

Conventional HLA class I restricted CD8+ T cell targeting peptide libraries and predictive algorithms: complementary approaches successfully identify Mtb epitopes for Mtb specific CD8+ T cells

Other new candidate *Mtb* antigens were studied for antigenicity using conventional peptide arrays. These candidates were selected based on specific criteria such as MHC binding algorithms that were used to mine public genomic databases (mainly TubercuList) or published proteomic/transcriptomic data obtained from *in vitro Mtb* intracellular cultures. One of the first libraries built on such an approach consisted of 15-mer peptides covering the sequences of 389 proteins (103), and aimed to identify CD8+ T cell stimulating epitopes by using a small panel of human CD8+ T cell indicator clones restricted to the classical HLA class Ia alleles B*5701, B*3905 and B*3514. The proteins included were selected based on three parameters: (I) gene products described in TubercuList as "PPE/PE", "cell wall and cell processes", "virulence, detoxification, adaptation" and "secreted"; (II) genes highly expressed during *in vitro Mtb* intracellular infection; and (III) genes not expressed by BCG strains. This resulted in the synthesis of 39,499 peptides, which were then pooled and tested for T cell recognition by IFN-ɣ ELISPOT. The peptide pools were screened in the presence of up to nine different CD8+ T cell clones from two different donors (one TB patient and one LTBI), autologous dendritic cells and IL-2. In this way, new CD8+ epitopes encoded by the *Mtb* antigens EsxJ (Rv1038c), PE9 (Rv1088) and PE_PGRS42 (Rv2487c) could be identified. When this same 15-mers library was screened with a panel of more than 30 non-classically HLA class I (that is: non-HLA class Ia A, B or C) restricted *Mtb*-reactive CD8+ T cell clones, no epitopes presented by the HLA class Ib molecules MR-1 or HLA-E were found.

More recently, the same peptide library has been tested again in IFN-ɣ ELISPOT assays using *ex vivo* peripheral blood CD8+ T cells from 20 ethnically diverse individuals including five TB patients and 15 LTBI donors (104). That study did not show differences in the magnitude of response between TB and LTBI, but validated 17 know CD8+ T cell epitopes and identified several new ones scattered across 58 antigens. Peptide pools were considered immunodominant when able to induce positive IFN-ɣ ELISPOT responses in three or more *Mtb* exposed individuals. Four antigens, specifically PPE15 (Rv1039c), PPE51 (Rv3136), PE12 (Rv1172c) and PE3

(Rv0159c), discovered in this CD8+ T cell library and three of them independently by other two post-genomic approaches (discussed below) (105, 106) have been recently evaluated for protective efficacy in C57BL/6 and BALB/c mice models using a ChAdOx vector platform (107). Two of them, PPE15 and PPE51 were able to reduce the *Mtb* pulmonary load as stand-alone vaccines, but only PPE15 boosted BCG protective efficacy in the C57BL/6 (but not in BALB/c) mice. The reason why antigens selected using similar criteria (based e.g., on protein category, antigenic or immunogenic properties) have different protective effects *in vivo* is a common finding but still not fully understood.

HLA restricted CD8+ and CD4+ T cell targeting peptide libraries based on in silico predictive algorithms successfully identified epitopes for Mtb specific CD4+ and CD8+ T cells

In silico epitope prediction programs, even when unable to consider the effects of 3D peptide structures, post-translation modifications or alternative peptides sequences generated by proteasome-catalysed splicing (108, 109), can be used to select putative antigenic peptides without the need to screen the entire amino acid (aa) sequence of target proteins. Using these bioinformatics tools, novel CD8+ or CD4+ T cell epitopes of known *Mtb* proteins were found, which were validated by MHC binding assays and reactogenicity of lymphocytes from *Mtb* exposed individuals, and/or used to generate *Mtb* epitope specific induced T cell clones (110-114). These pioneering studies, although limited to previously studied *Mtb* proteins and to the most common HLA alleles, provided the proof of concept for later application of *in silico* epitope screenings to the full *Mtb* antigenome. In search of new potential CD4+ T cell epitopes, 73 secreted proteins were directly selected from the *Mtb* genome and screened for HLA-II binding motifs (115). The positive hits were then ranked and the top 17 novel peptides synthesized. One of these epitopes (belonging to the Rv2223c protein) was promiscuously and broadly recognized by IFN-γ producing peripheral blood cells (PBMC) from LTBI donors (n=11) and proved to be immunogenic in HLA-DR B*0101 transgenic mice when administered in a DNA plasmid vector co-expressing other 23 *Mtb* epitopes previously identified.

Using a similar approach, which instead was CD8+ T cell epitope oriented, 235 *Mtb* proteins were screened using genome-based bioinformatics for potential binding peptide epitopes to HLA-A2, -A3, and -B7 as HLA class Ia superfamily members (116). The *Mtb* proteins included were: (I) proteins already used in TB vaccine trials; (II) proteins with already known CD8+ T cell epitopes; (III) proteins with the highest HLA class I binding prediction values; (IV) conserved proteins; (V) proteins encoded by the *Mtb* DosR regulon; (VI) proteins with known B cell epitopes; (VII) secreted proteins; and (VIII) proteins predicted to be secreted (116). Out of 432 *Mtb* 9-mer

peptides tested, 70 (of which 58 were unknown epitopes) were found to induce proliferative responses in CD8+ T cells from two or more individuals out of the 41 purified protein derivative responder (PPD+) healthy donors screened. Based on the frequency and the magnitude of CD8+ T cell responses, 18 out of these 70 epitopes, mainly including peptides from secreted antigens, were then selected for further testing in ten HLA-A*0201 positive TB patients and an equal number of HLA-A*0201 positive controls, using *Mtb* specific peptide/HLA-A*0201 tetramers and functional peptide stimulation assays. All 18 selected epitopes were confirmed to be antigenic in TB patients (but not negative controls) as visualised by specific tetramer staining, and to induce specific polyfunctional (IFN-γ+/IL-2+/TNF-α+) CD8+ T cells. Of note, half of the 18 proteins encoding these epitopes had also been screened in the 15-mer CD8+ peptide library mentioned above, but only three antigens (Rv1966, Rv1997 and Rv2780) were found in both approaches to contain *Mtb* antigenic peptides stimulating CD8+ T cells. This significant yet limited overlap could be explained by differences in the design of the two studies: the criteria applied to determine potential *Mtb* antigens (this resulted in only 56 putative antigens shared between the ones selected in both studies); the length of the peptides generated from these proteins (9-mers vs. 15-mers); the way in which the peptides were evaluated (single peptides vs. peptide pools); and the read-outs chosen to evaluate the immunodominance of the peptides (proliferation / CD8+ polyfunctionality / HLA class Ia tetramer staining in the second vs. the use of selected T cell clones / IFN-γ ELISPOT in the first study).

The first *in silico* genome-wide screening that did not apply an arbitrary criteria-driven selection of *Mtb* proteins was performed in search of putative HLA-B*3501 T cell stimulating *Mtb* epitopes (117). This allowed the identification of both known as well as novel antigens (Rv0670, Rv1280c, Rv1464, Rv1641, Rv2182c, Rv2476, Rv2823c, Rv3378c and Rv3689) encoding new epitopes recognised by CD8+ T cells from BCG vaccinated healthy donors in the context of one single HLA allele. Another study, applying a similar methodology but restricted rather to HLA-A*0201, reported two other new antigens (Rv1490 and Rv1614) that contained CD8+ T cell stimulating epitopes, and that were recognized by CD8+ T cells from LTBI and TB patients (118).

Additional across-genome epitope predictions identify many novel Mtb epitopes for CD4+ T cells

With the better definition of HLA-I supertypes and corresponding alleles (119), the increased knowledge of the global distribution of the most common HLA-II molecules (120), and the availability of additional *Mtb* genome sequences, the comprehensiveness and sizes of HLA allele based *in silico* epitope predictions for *Mtb* sharply increased. One of the largest *in silico* genome-wide screening efforts used an approach parsing all predicted protein sequences of 21 *Mtb* strains into all their

possible 15-mers and subsequently selecting the most conserved and best-predicted promiscuous binders for 22 different HLA-DR, -DP and -DQ class II alleles (106). A total of 20,610 peptides was then selected for synthesis and tested in pools of 20 peptides to assess the *in vitro* IFN-ɣ response elicited in PBMC of 28 LTBI donors by ELISPOT. Based on peptide pools recognised by three or more LTBI donors, 369 individual *Mtb* epitopes, covering 82 different *Mtb* antigens, were identified. Of note, it was reported that 47% of those peptides accounted for 90% of the total responses. By depleting PBMC of either CD4+ or CD8+ T cells, it could be confirmed that 97% of the epitopes were recognized exclusively by CD4+ T cells. It is not fully clear why so few CD8+ T cell epitopes were found, but suboptimal peptide length and assay criteria might have contributed to favouring CD4+ T cell epitope identification. In any case, out of the 82 *Mtb* antigens, 34 were recognized as CD4+ T cell targets for the first time. The majority of the *Mtb* antigens identified were associated with most categories indicated in TubercuList, but with a relative overrepresentation of antigens from the PE/PPE family, a large and unique family of genes representing over 10% of the entire *Mtb* genome (27). Interestingly, the authors introduced the concept of antigenic clusters which they defined as groups of at least four proteins recognized by at least two LTBI donors and encoded within a five gene-interval. Based on this approach, they then identified three antigenic islands that mainly included known components of the type VII secretion systems (T7SS) Esx-1 and Esx-3, and ten novel antigens were reported for the first time. The researchers then further investigated the antigenicity of those new peptide pools by cytokine induction (IFN-ɣ/TNF-α/IL-2) and by assessing T cell memory phenotypes. No differences in cytokine production were observed between the proteins identified (106). Of particular interest, the authors found a new memory CD4+ T cell subset to be involved in the recognition of *Mtb* antigens in LTBI donors. This Th1* cell subset expressed a unique, CD4+CXCR3+CCR6+CCR4- phenotype, and had a lineage-specific transcriptional signature shared with both Th1 (such as T-bet (TBX21), granzymes A and K, perforin (PRF1), and the transcription factor EOMES) and Th17 cells (RORC, DAM12, PTPN13, and IL17RE, the receptor for IL-17C) (121). Furthermore, the CD4+CXCR3+CCR6+CCR4−T cells selectively expressed genes involved in cytokine/receptor interactions (CCR2, IL12RB2, IL23R, KIT [CD117, c-Kit], BAFF [CD257, TNFSF13B]), cell persistency and proliferation (i.e., BAFF, MDR1 (ABCB1), and KIT) as well as genes previously reported associated with TB susceptibility (CCR2 and IL12RB2). In addition to IFNG, TNF and IL2, the activated CD4+CXCR3+CCR6+CCR4−T cells expressed cytokine transcripts (CSF1/2, CCL3/4, GZMB, IL6/17A/22, CXCL9, and VEGFA, CSF1 (M-CSF) and GM-CSF) many of which have been shown to play a role in TB containment previously. This makes these CD4+ T cell subsets also interesting as potential *Mtb* specific correlates of protective immunity.

Screening large cohorts of *Mtb* exposed individuals in different geographic settings obviously is essential to corroborate the antigenicity of *Mtb* peptides whose immunodominance has been based on positive IFN-γ responses limited to few donors in a single site. To accomplish this, 15-mer peptide pools (20 per protein per pool) of 25 novel antigens identified in the last study described were further examined by ELISPOT in 128 LTBI from nine different geographical locations. Importantly the results validated the antigenicity of the selected epitopes (122). It is interesting to note that peptide pools from four novel antigens (Rv1172c, Rv1788, Rv1791, and Rv3135) that were shown to be recognised by (presumably CD4+ T cells from) most of the LTBI cohorts, were among those subsequently described to contain also CD8+T cell epitopes in another study (104). Recently, IFN-γ based recognition of 40 *Mtb* antigens identified in the previously described CD4+ library together with 20 *Mtb* antigens selected from other studies was evaluated in a diluted whole blood assay in Atlanta and Kenya (123). The peptide pools of proteins previously described as antigenic (such as Rv1172c and Rv1872c among others) did not always induce high IFN-γ responses in the cohorts of LTBI, TB household contacts and TB patients included in this study. This difference could be explained by the different composition of peptide pools used (18-mer based), the read-out implemented (1:4 diluted whole blood stimulated seven days with 1ug/ml), environmental or genetic (HLA) differences between the populations, amongst others.

The *in vitro* reactogenicity of human T cells to *Mtb* peptides restricted to diverse HLA molecules can indeed be highly heterogeneous due to the extensive HLA polymorphisms (124). To solve those limitations, a recent study tested a peptide pool of 300 *Mtb* epitopes and showed that this comprehensive megapool, consisting of peptides from 52 *Mtb* antigens (including 45 antigens discovered in the above described CD4+ peptide library, of which seven were also evaluated as peptide pools in the nine different cohorts of LTBI discussed above), accounted for 80% of *Mtb*-specific T cell responses in LTBI, including adults and adolescents (125). The study cohort included LTBI with and without a self-reported history of active TB. Interestingly, the *Mtb* epitope reactivity, based on IFN-γ ELISPOT and intracellular cytokine staining, was around ten-fold lower among subjects that had experienced past TB disease in the last six years, for a specific set of epitopes, which was designated "type 2" or "post TB sensitive" *Mtb* epitopes (126). No such difference was found for "type 1" or "persistent" epitopes, which were recognized equally well by LTBI with or without previous TB. Confounding factors such as an active TB disease process (which was evaluated by comparing responses between type 1 and type 2 epitopes in 16 TB patients), differences in T cell memory phenotypes, or differences in gene expression in responding T cells could be excluded. Also, numbers of *in silico* predicted HLA-II binding motifs did not differ among the different

classes of epitopes. The only difference found was that *Mtb* epitopes that were less recognised by LTBI with past-TB were those with the highest sequence homologies to proteins from the human microbiota or from nontuberculous mycobacteria (NTM). Based on their data, the authors concluded that the diversity in type 2 epitope reactogenicity among LTBI with or without past TB could be related to changes induced in the microbiota by TB treatment, although some mycobacterial specific proteins, such as ESAT6 or CFP10, still contained several type 2 peptides. Data on the microbiota compositions of TB patients followed pre-, during and after treatment will, however, be needed to validate this hypothesis. It would also be important to determine the general homology between other proposed *Mtb* antigens and the human microbiota. The fact that NTM homologous *Mtb* epitopes were found to be less reactogenic in the post-TB donor group is also quite intriguing especially considering previous reports showing a strong reactogenicity to such epitopes in LTBI and in healthy individuals from non-endemic areas (127). Moreover, the low recognition of NTM homologue epitopes in the post-TB LTBI donors is in line with the finding that other NTM homologues, such as the DosR regulon encoded proteins (128), are less well recognized in treated TB patients than in LTBI (30). A role for NTM exposure in inhibiting BCG vaccine induced protective immunity has been repeatedly proposed (10) and experimentally proven in mouse models (129). The relation to the phenomenon of type 2 epitope remains to be clarified.

Unconventional HLA class Ib restricted CD8+ T cells: identification of Mtb epitopes for CD8+ T cells genetically restricted by HLA-E, with an unusual phenotype and function

In addition to conventional HLA-I and HLA-II binding epitopes, peptides presented by non-classical HLA molecules have been proposed as interesting *Mtb* antigenic targets. For HLA-E only two coding variant molecules are known, which differ in a single amino acid localized outside the peptide binding groove. Thus, HLA-E molecules can essentially be seen as virtually monomorphic antigen presentation molecules, suggesting the interesting possibility that a relatively small number of *Mtb* peptides can be used for presentation via HLA-E for e.g., vaccination purposes. Based on initial evidence that HLA-E molecules could present *Mtb* derived peptides to two human T cell clones (130), the *Mtb* (H37Rv) genome was screened *in silico* for candidate epitopes predicted to bind to HLA-E molecules (131). This effort resulted in the selection of 69 potential HLA-E binding peptides, which were tested for HLA-E binding and CD8+ T cell recognition (by T cell proliferation and cytokine induction) in PPD+ donors, PPD- healthy controls, BCG vaccinated infants and cord blood samples. Unexpectedly, almost all predicted epitopes could be validated and some were recognized by as many as 40% of the PPD+ responsive donors. Several epitopes were also recognized by the BCG vaccinated infants from South Africa, but not

by cells from cord blood, suggesting vaccine induced T cell memory. Subsequent investigations focussed on the most frequently recognized immunodominant HLA-E presented *Mtb* peptides using T cell clones. This analysis revealed that these HLA-E restricted CD8+ *Mtb* specific T cells had a Th2 like phenotype and function. This was confirmed in the circulation of TB patients and LTBI (132-134). Although numbers were small, there was a tendency towards higher frequencies of HLA-E restricted *Mtb* specific T cells in TB/HIV coinfected individuals. Using a new expansion protocol these results could be corroborated using HLA-E tetramers combined with specific functional phenotyping in the blood of TB patients and LTBI (134). The low polymorphism of the HLA-E locus and the stable expression of the HLA-E molecule even upon infection with HIV may render HLA-E peptides to be attractive antigenic targets (135).

In this context it is relevant to mention that several of the *Mtb* peptide specific HLA-E restricted T cell clones inhibited *Mtb* growth in human macrophages, in line with a possible protective function. Furthermore, several studies in animals now suggest that HLA-E like Qa-1 restricted CD8+ T cells contribute to protective immunity *in vivo*. The murine homologue of HLA-E, Qa-1, was found capable of presenting the same HLA-E binding *Mtb* peptides to CD8+ T cells, and Qa-1 knockout mouse were more susceptible to *Mtb* and died earlier with higher bacterial burdens (136). This was not due to engagement of NK receptors, as demonstrated in genetic ablation experiments. In addition, as mentioned above, NHP vaccination studies with genetically modified RhCMV vectors expressing *Mtb* antigens revealed that MHC-E restricted T cells can be induced and mediate part of the strongly protective response against TB I the NHP model (60). Clearly HLA-E restricted T cell immunity needs to be investigated in more detail in the context of TB vaccination.

Besides HLA-E restricted T cells there are also other groups of so-called donor unrestricted T cells or DURT T cells (137). These cells are mostly CD8+ T cells that are genetically restricted by highly conserved, non-polymorphic presentation molecules: MR1 (presenting metabolites to mucosal associated invariant T cells or MAITs); CD1a, b and c molecules presenting mostly lipid antigens; NKT cells restricted by CD1d; TCRγδ cells, some which recognize phospho-antigens in the context of butyrophilin 3A1 (138). However, none of these cells recognize peptide antigens and therefore are not discussed here.

Hyperconserved or variable Mtb epitopes

Most of the studies discussed so far have aimed at identifying immunodominant *Mtb* peptides and protein antigens for T cells. However, the focusing of T cell responses exclusively on conserved immunodominant epitopes has been under discussion lately

after human T cell *Mtb* epitopes were found to be evolutionary more conserved than essential *Mtb* genes as a whole, or than the non-epitope encoding sequences of the same *Mtb* antigens (139) (in this work 491 experimentally verified peptides were studied covering 78 *Mtb* antigens). This discussion also impacts TB vaccine development strategies since it was proposed that the observed T cell epitope hyperconservation suggested that natural T cell immunity might be beneficial for *Mtb* transmission in some critical steps of the infection cycle in the human host (140). Although highly speculative, this phenomenon might play a role in TB cavitation, which is considered to be due to excessive inflammation and plays a key role in transmitting *Mtb* bacteria to new susceptible hosts.

Another possibility may be that *Mtb* has evolved to drive T cell responses preferentially to highly expressed *Mtb* epitopes, which would likely skew reactive T cells from a central memory towards a terminally exhausted phenotype, as suggested by observations in mice (141), thereby limiting the formation of a pool of long-term memory cells. Under such a scenario an alternative approach towards vaccine design would then be to mobilize subdominant epitopes that are less recognized during natural infection, thus not only circumventing the induction of exhaustion but also to broaden the *Mtb* antigenic repertoire that can be recognized beyond the natural infection induced repertoire. A first study to assess the protective efficacy of subdominant *Mtb* peptides was performed in B6CBAF1 mice using ESAT6 peptides (142). It was demonstrated that *Mtb* peptides not highly recognized after natural *Mtb* infection could induce better protection when used as vaccine antigens than the naturally immunodominant epitopes. These results have been subsequently extended to other mouse models (CB6F1; C57BL/6 and BALB/c) (143, 144) and other antigens (145). Although the protective efficacy differed among mouse strains, it was shown that indeed these ESAT6 subdominant epitopes led to less terminally differentiated T cell profiles, with a greater ability to sustain polyfunctional cytokine responses and proliferative capacity over time (144).

Alternative interpretations to the role of hyperconservation of T cell epitopes, however, exist: hyperconservation could be caused by a strong evolutionary selection pressure on *Mtb* protein domains due to indispensable functions rather than T cell immune pressure. It has been argued that if T cell recognition was driving epitope conservation, this should have been restricted mainly to HLA molecules associated with genetic TB disease susceptibility. However, in clinical *Mtb* strains isolated from TB endemic areas, *Mtb* T cell epitopes rarely recognised by TB patients or restricted to infrequent HLA molecules are not less conserved than those binding to the most common HLA molecules and more strongly recognised by TB patients (146).

Recent results have confirmed the low rate of *Mtb* epitope sequence variation and found only a small number of variable regions in the *Mtb* genome (147). From the encoded sequences of seven variable genes (Rv0001c, RimJ (Rv0995), Rv0012, LldD2 (Rv1872c), Rv0990c, Rv2719c and TB7.3 (Rv3221c)), HLA-I and HLA-II binding motifs were predicted *in silico*. Comparative analyses showed amino acid changes in predicted CD8+ but not CD4+ T cell epitopes. Interestingly, those substitutions affected the antigenicity of epitopes *in vitro*, as demonstrated by measuring the INF-γ production induced in dilute whole blood of 82 TB patients. Hence, variant *Mtb* epitopes, generated by aa substitutions, could be either less or more efficiently recognized than the ancestral peptide. Whether in a vaccine setting the hypervariable *Mtb* epitopes would be more efficacious than conserved *Mtb* epitopes still needs to be determined. Furthermore, additional studies, including other regions such as Asia will be needed to lend further support for hyperconservation and variation of *Mtb* epitopes. This need is strongly supported by a recent report showing the first evidence of region-specific T-cell epitope variation (148). In this study, 57 new mutated CD4 T-cell epitopes were identified from 79 *Mtb* strains isolated in India, while only seven additional ones had been previously described in clinical isolates from other geographic regions, mostly African (148). Interestingly, on the basis of an *in silico* epitope binding analysis, the authors suggested the HLA-DR alleles highly prevalent in India are the main drivers of region-specific T-cell epitope variation (148). If the T-cell epitope landscape will be confirmed to be regional host genetic background, this will have a major impact on the design of protein–and epitope–based TB diagnostics and vaccines.

Searching for novel *Mtb* Antigens

The prior section introduced different approaches used to search for novel *Mtb* peptide epitopes. However, other studies have used complementary data driven approaches to interrogate the *Mtb* genome seeking new *Mtb* antigens. In several of these approaches arbitrary criteria were used to mine genomic and proteomic databases, which in one study resulted in the selection of 94 *Mtb* genes predicted to encode secreted proteins, in particular members of the Esx or PE/PPE families, expressed in *Mtb* infected macrophages and up- or down-regulated in response to hypoxia or carbon starvation (149). IFN-γ production in response to these recombinant proteins was tested in PBMC from PPD− and PPD+ individuals and 48 proteins were found to be highly reactogenic. It is interesting to note that ten of those antigens were confirmed to contain CD4+ or CD8+ T cell epitopes in subsequent studies (Rv1789, Rv1813c, Rv1886c, Rv1984c, Rv2220, Rv2608, Rv2875, Rv3020c, Rv3478, Rv3619c, and Rv3620c) (93, 104, 106, 116, 131). The 48 reactogenic antigens were then adjuvanted with CpG and each individually evaluated for protective efficacy in *Mtb* infected C57BL/6 mice. None of the antigens, although eliciting both

antibodies (IgG1 and IgG2) and cytokines (IFN-γ and TNF-α), however, resulted in better reduction in lung CFU than BCG. Nevertheless, four were selected (Rv1813c, Rv2608, Rv3619c and Rv3620c) and fused to form the ID93 vaccine, which is now under clinical evaluation in various trials and trial designs (PoD as well as Prevention of Recurrence (PoR)) in combination with the glucopyranosyl lipid adjuvant (GLA) formulated in SE (150). The ID93/GLA-SE fusion protein vaccine induced pro-inflammatory cytokines (MCP-1, IL-8, IL-6, IL-5, TNF-α, and GM-CSF) in cynomolgus macaques and decreased mortality and lung pathology in guinea pigs, when used in a BCG prime-boost strategy (151).

Additional broad screening of the *Mtb* antigenome was performed by using high-throughput proteome microarray technology. In one study, the screening of 3,480 *Mtb* proteins with pooled TB patients' sera reported significant immunoreactivity to 249 proteins; these included proteins belonging to the PE/PPE, DosR and RD families, as well as known "conventional" antigens (105). From these screens, three novel proteins (Rv1987, Rv3807c, and Rv3887c) were found to be particularly highly reactogenic and to provide better diagnostic sensitivity and accuracy than commercial serological tests. Through a similar approach, the recognition of 4,099 *Mtb* proteins was tested with sera from 561 TB suspects. This study identified 484 *Mtb* proteins that were recognized by the sera of at least one TB patient, and recognition of 13 proteins was significantly associated with active TB (Rv3881c, Rv3804c, Rv3874, Rv1860, Rv1411c, Rv2031c, Rv0934, Rv3616c, Rv3864, Rv1980c, Rv0632c, Rv1984c, and Rv2773) (152). There was no overlap with the proteins identified in the just mentioned previous study. Although both screenings identified novel antigens reactive with TB patients' sera, current global recommendations from WHO and others do not encourage the use of serological tests for TB diagnosis, since the high intertest variability leads to high rates of false positive and false negative results (153).

In another study, which was based on the assumption that CD4+ T cells are required for the maturation of long-lived plasma cells, 164 *Mtb* proteins that had been identified through serological screening (152), were interrogated in a search for novel *Mtb* CD4+ T cell antigens (154). Polyclonal *Mtb*-reactive CD4+ T cell lines, generated from PBMC of 12 LTBI (three from USA and nine from India) and two healthy controls (from USA) exposed to *Mtb* lysate, were used to test this proteome set. The immunological responses were measured by IFN-γ release after three days of culturing the T cell lines with irradiated antigen presenting cells and the respective unpurified antigen. Forty-three proteins were recognized by CD4+ T cells from at least one LTBI or healthy control, resulting in a total of 27 newly characterized CD4+ T cell *Mtb* antigens. Although performed in a small group of individuals, this study proposed a scalable system that could be used as a workflow to screen *Mtb* antigens

directly in endemic areas, and proved that antigens recognized by immunoglobulins often also react with CD4+ T cells.

Integrating Mtb antigen discovery with Mtb infection biology and in vivo expression in the infected lung

The various approaches described so far have expanded our knowledge of the *Mtb* antigenome for T cells and (to a lesser extent) antibodies from LTBI or TB patients, but did not examine whether and how those antigens were expressed in the primary TB target organ, the lung. This characteristic might be not essential for TB biomarkers detectable in the peripheral circulation, but it could be crucial for antigens proposed as potential vaccine candidates. A minimal prerequisite for a vaccine antigen is that it is expressed by infected cells in the infected target organ, in this case the lung. Immune responses directed against *Mtb* antigens expressed in the lung could restrain the *Mtb* immunological life cycle at an early stage and prevent the onset of TB (2). Based on this hypothesis, a new class of *Mtb* antigens, which were designated IVE-TB (*in vivo* expressed) antigens, was introduced (155). The first IVE-TB antigen set was based on the analysis of RNA expression patterns of 2,170 *Mtb* genes in the lung of four mouse strains at six- and nine-weeks post-infection. Based on the distinct TB susceptibility phenotypes of the mouse strains examined (relatively resistant C57BL6 vs. super susceptible C3H/FeJ as polar extremes) *Mtb* genes were then selected to represent genes expressed: (I) independently of host genetic background; (II) in association with necrosis; (III) in association with severe necrotic infection or susceptibility (expressed in the C3H but not B6, C3H.B6-sst1, or B6.C3H-sst1); (IV) in association with dense granuloma development; (V) in association with diffuse granuloma development; (VI) in association with resistance; (VII) in association with low inflammation; (VIII) inflammation; and (IX) relapse. This resulted in the first selection of 16 IVE-TB genes, which were tested as recombinant proteins *in vitro* and *in vivo*. IFN-γ responses to seven IVE-TB antigens were observed *in vitro* by screening 133 TST+ donors. A further in-depth study using PBMC from six LTBI revealed that the most pronounced T cell subsets recognizing IVE-TB antigens were IFN-γ+/TNF-α+ CD8+ T cells and TNF-α+/IL-2+ CD154+CD4+ T (central memory) cells. Further experiments with one of those IVE-TB antigens, Rv2034, confirmed its *in vivo* immunogenicity in HLA-DR3 transgenic mice by strong induction of T cells and antibodies (156). Moreover, immunization with Rv2034 or the hybrid-protein Ag85B-ESAT6-Rv2034 adjuvanted with CpG or CAF09 induced over one log reduction in lung CFU compared to unvaccinated controls both in *Mtb* challenged HLA-DR3 transgenic mice and guinea pigs (in the latter model the hybrid antigen was tested in CAF09) (156).

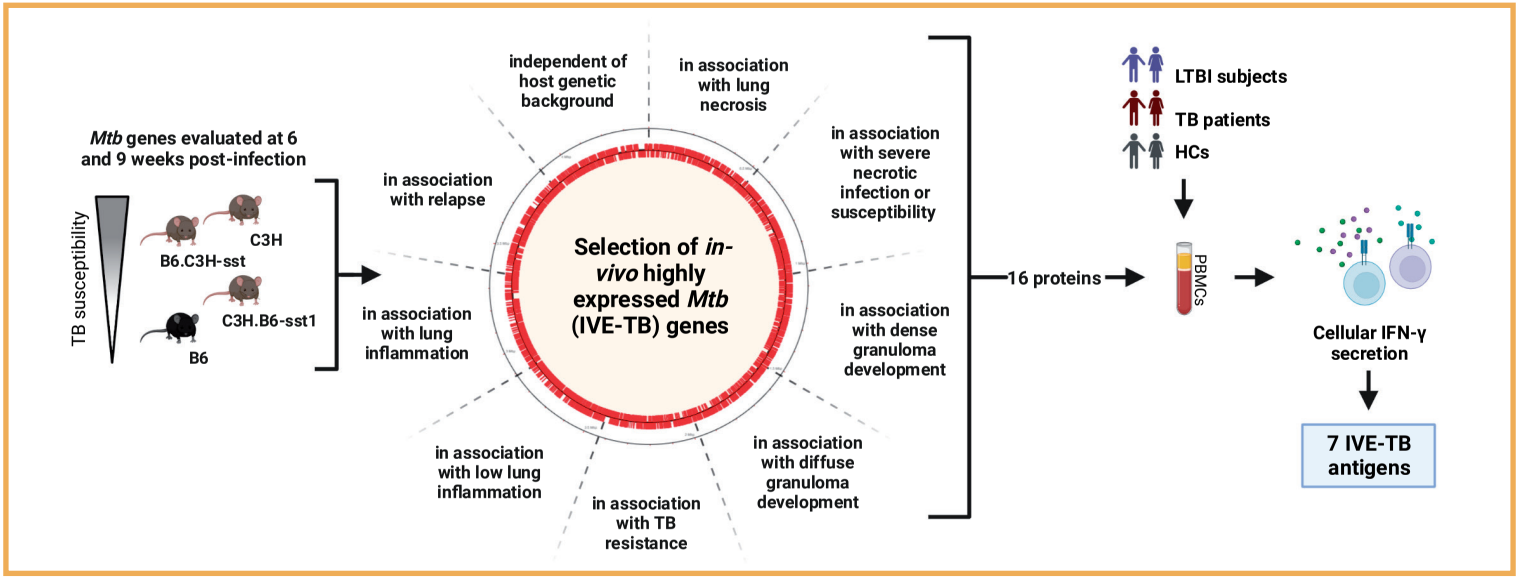

Figure 2. First selection of IVE-TB antigens. 2,170 *Mtb* genes were analysed from the lung of four mouse strains at six- and nine-weeks post-infection. Sixteen *in vivo* highly expressed *Mtb* (IVE-TB) genes were selected according to the nine criteria listed in the figure. This selection included IVE-TB genes associated to distinct TB susceptibility phenotypes of the mouse strains examined (resistant C57BL6 vs. susceptible C3H/FeJ as polar extremes) or shared across mice. Seven IVE-TB antigens induced IFN-γ responses in blood cells of latently *Mtb* infected (LTBI) donors. *Mtb*: *Mycobacterium tuberculosis*; PBMCs: peripheral blood cells; LTBI: TB: tuberculosis; HCs: healthy controls.

Together, these data suggest the potential of IVE-TB antigens for future TB vaccination.

MVA85A VACCINE TRIAL: THE FALL OF PARADIGMS AND THE RISE OF CHALLENGES

To date, three clinical studies have reported promising human efficacy data, namely: following BCG revaccination (157); following vaccination with *Mtb* antigen-based subunit vaccines H4:IC31 (157) and M72 vaccine (65). These two candidates and BCG revaccination will be further discussed in the last chapter of this thesis.

When this PhD project started, a pivotal moment in TB vaccine development was the just published result from the MVA85A vaccine trial. Although in the early 2000's the TB vaccine pipeline was quite narrow, in 2013 there were 14 new TB vaccine candidates under clinical evaluation (the most recent TB vaccine pipeline can be found at this link: www.tbvi.eu). These included: live recombinant- (VPM1002; *MTB*VAC), whole-cell or fragmented whole mycobacteria (RUTI; Dar-901; *Mycobacterium vaccae*; *Mycobacterium indicus parnii*), virally vectored- (MVA85A; Ad35; Ad5Ag85A) and adjuvanted subunit- (M72+AS01; H1, H56 and H4 + IC31; ID93+GLA-SE) vaccine candidates (158). Among those, MVA85A at that time had reached the most advanced stage in clinical evaluation for any new TB candidate vaccine at that time. MVA85A's vaccine potential had been supported by several pre-clinical findings: it could reduce TB pathology and increase survival of animals following *Mtb* challenge (159-161) and induced specific immune response against the *Mtb* specific antigenic component, Ag85A (Rv3804c), in both children and adolescents (162, 163). In these studies, immunogenicity was mainly assessed by measuring CD4+ T helper-associated immune responses by IFN-γ, TNF-α, IL-2 and IL-17 induction. Despite these encouraging preclinical findings and its immunogenicity profile in humans, MVA85A administrated as a booster vaccine in infants (4-6 months) who had received neonatal BCG vaccination did not significantly reduce TB incidence compared to an equal dose of Candida skin test antigen (164). This disappointing result from this large phase 2b efficacy study called for revisiting the paradigms behind TB vaccine development.

Which antigens to target?

MVA85A contained one single protein, Ag85A (Rv3804c) that, as described above, was identified through a traditional antigen discovery approach. Although abundantly secreted *in vitro*, the transcript levels of Ag85A were drastically decreased in the lung of mice at three weeks post *Mtb* challenge (165), suggesting that its expression

may be downregulated during later phases of *Mtb* infection, which might negatively impact the efficacy of vaccines designed to induce immunity to Ag85A. The availability of Ag85A had also not been evaluated in the sputum of TB patients, including TB cases among the MVA85A vaccinees. If the transcript dynamics found in mice reflected those of *Mtb* human pulmonary infection, the resulting poor antigen availability at the site of *Mtb* infection, the human lung, could partially explain the failure of the MVA85A vaccine. Those observations supported the rationale for this thesis to consider alternative, stage specific and IVE-TB based antigen discovery approaches for the design of novel TB vaccines.

What type of immune response defines an efficacious antigen?

Traditionally, TB vaccines have been designed to elicit T-cell-based immunity, with a major focus on IFN-γ centred responses (166). However, the failure of the MVA85A efficacy trial spurred a rethinking of traditional approaches, including which immune responses should be induced to control *Mtb* infection and TB transmission (167). The lack of immunological correlates of protection has been, and still is a crucial challenge in TB vaccine development (168). Although the pivotal role of IFN-γ and CD4+ T cells in mycobacterial control has been extensively established, these immune responses are clearly not sufficient in preventing TB disease (164). This is supported by the fact that although people with IL-12/IL-23/IFN-γ axis immunodeficiencies are more susceptible to mycobacterial infections, most TB patients have no clear T cell defects or impairments in *Mtb* specific IFN-γ responses (166). Additionally, the role of BCG induced IFN-γ producing T cells in infant vaccinees is still debated due to contrasting results supporting or not the association of IFN-γ responses with the risk of developing TB (169, 170).

Therefore, there is a clear rationale for including at an early stage of antigen discovery a number of alternative immune readouts, besides conventional IFN-γ responses, including additional cytokines, antibodies and high dimensional single cell analysis.

Which animal models to use?

Without clear correlates of TB protection, animal models are still crucial in selecting and prioritizing TB vaccine candidates. In those models, TB protection is generally defined by the reduction of *Mtb* bacterial load, tissue damage and death in vaccinated animals compared with control groups (171). Although those endpoints are commonly used in TB research, it is unclear how they translate into a confident assessment of vaccine protection in humans. The results of the MVA85A vaccine, that as mentioned met the conventional TB protection criteria in animals but failed in enhancing protection in humans, suggested that the current animal models are not

sufficiently predictive and thus often inadequate, which prompted a re-evaluation of those animal models (167).

For several research questions addressed in this thesis, more representative and likely more adequate mouse models were used. Most of the experiments included wild type mice, similarly to those used in other labs such as C57BL/6j (BL/6) and BALB mice; but importantly, also mouse strains displaying characteristics of human TB, notably the C3HeB/FeJ ("Kramnik-model") mouse model that develops centrally necrotizing lesions approximating the pathology of human lung TB. This approach allowed to study host-pathogen interactions occurring in mice that are more closely related to those in humans.

THESIS SCOPE AND OUTLINE

The first aim of this thesis was to innovate *Mtb* antigen discovery and identify a novel repertoire of IVE-TB proteins, encoded by *Mtb* genes highly and consistently expressed in the lung of susceptible (C3HeB/FeJ) as well as resistant (C57BL/6J) mice following aerosol *Mtb* (Erdman) challenge. Besides their high expression in the *Mtb* infected lung, the IVE-TB proteins constitute a rationally selected group of candidate antigens for various reasons: (I) they are conserved among 219 *Mtb* clinical isolates and thus cover a wide array of *Mtb* clades; (II) they share high homology with BCG and other pathogenic mycobacteria, including *M. leprae*; (III) they contain a large number of epitopes predicted to bind to HLA-Ia and HLA-II alleles (coverage of 85% of the human population); (IV) they are well recognized by immune blood cells from *Mtb* exposed subjects (as shown in 36 *Mtb* exposed individuals); and: (V) they elicit immune cells that are producing multiple cytokines besides IFN-γ, which is known to be necessary but not sufficient in conferring protection against TB (**chapter 2**).

Next (**chapter 3**), the recognition of several *Mtb* antigens, including IVE-TB, latency, and Rpf stage-specific antigens, was extended and validated in an independent South European cohort, which included both adults and adolescents with either LTBI or TB. As shown initially in **chapter 2**, many *Mtb* antigens induced responses marked by multiple cytokines besides IFN-γ. Interestingly, through unbiased analyses of high dimensional single-cell data, several clusters of antigen-specific TNF-α+ cells were distinctively abundant in frequencies between LTBI and TB patients of different age groups.

After proving that most of the novel IVE-TB antigens were recognized by immune cells from *Mtb* exposed individuals, findings were translated back to animal models.

More specifically, the recognition of IVE-TB, latency, and Rpf stage-specific antigens was characterized using cells from the lung, draining lymph nodes and spleens of three different *Mtb* infected or BCG vaccinated strains of mice (C57BL6, BALB/c, and C3H) (**chapter 4**).

To further characterize IFN-γ-independent immune responses towards *Mtb* antigens, in a TB-endemic setting IgG levels were investigated against 15 *Mtb* antigens, representing various *Mtb* IVE-TB, latency, and Rpf stage-specific antigens (**chapter 5**).

In addition, **chapter 6** provides the first comprehensive lung-oriented *Mtb* gene expression comparative analysis. This analysis offers new and important insights into the pulmonary *Mtb* transcriptome signature and the biological pathways involved. Understanding these processes is a crucial step toward TB vaccine and drug development.

Finally, considering that a future subunit TB vaccine will likely impact other mycobacterial infections, the last research chapter reflects on the importance of early preclinical integration and harmonization of related diseases, focusing on leprosy and TB and showing cross-protective efficacy of TB subunit vaccines against leprosy (**chapter 7**).

The major findings of this work are summarized and discussed in the context of current literature and remaining future challenges are addressed (**chapter 8**).

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