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

Coppola, M.

CHAPTER 8

General discussion and future perspectives

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

Coppola M, Ottenhoff THM.

Dept. of Infectious Diseases, Leiden University Medical Center, The Netherlands

Semin Immunol. 2018 Oct; 39:88-101
doi: 10.1016/j.smim.2018.07.001

Every day Tuberculosis (TB) kills approximately three thousand people, a number that is on the rise due to the impact of the current COVID-19 pandemic on essential TB services (1). The causative agent of TB, *Mycobacterium tuberculosis* (*Mtb*), is an ancient pathogen that through its evolution developed complex mechanisms to evade immune surveillance and acquired the ability to establish persistent infection in its hosts (2). Currently, it is estimated that one-fourth of the human population is latently infected with *Mtb* and among those infected 3–10% are at risk of developing active TB disease during their lifetime (3). The available diagnostics cannot detect this risk group for prophylactic treatment to prevent disease progression. Anti-TB drugs can be used only as long (>six month) regimens with considerable side effects, which could both be reduced if adequate tests were able to monitor the response of TB to treatment (4). New vaccines are urgently needed to substitute or boost Bacillus Calmette-Guérin (BCG), the only approved TB vaccine, which however fails to impact the incidence of pulmonary TB in adults, and therefore has little effect on TB transmission (5).

To achieve TB eradication, the discovery of *Mtb* antigens that effectively correlate with the human response to infection, with the curative host response following TB treatment, and with natural as well as vaccine induced protection is critical. This thesis contributes to this ambitious aim through several findings. First, it uncovers multiple new *in vivo* expressed *Mtb* (IVE-TB) antigens by combining *Mtb*-transcriptomic data with advanced bioinformatics tools and medium throughput cytokine screening. Second, it deepens our understanding of the cellular and humoral immunity to *Mtb* antigens in latently *Mtb* infected donors (LTBIs) and TB patients as well as in animal models. Lastly, it demonstrates the feasibility of combining and integrating pre-clinical research of multiple mycobacterial diseases, which are endemic in the same areas and against which vaccines could induce cross-disease protection (i.e., TB and leprosy).

The present chapter integrates and discusses the main results of this thesis (summarized in Figure 1) and, by considering emerging evidence, drafts future perspectives on the search of *Mtb* antigens for target-based TB vaccines, diagnostics, and drug discovery.

IVE-TB genes: understanding the *Mtb* expressome to better control TB

The discovery of *Mtb* antigens that correlate with infection, protection, and vaccine immunogenicity is a complex process that has evolved over decades, and now is yielding important new results. In the early stages of antigen discovery, there were significant limitations in the resolution of biochemical technologies used to isolate

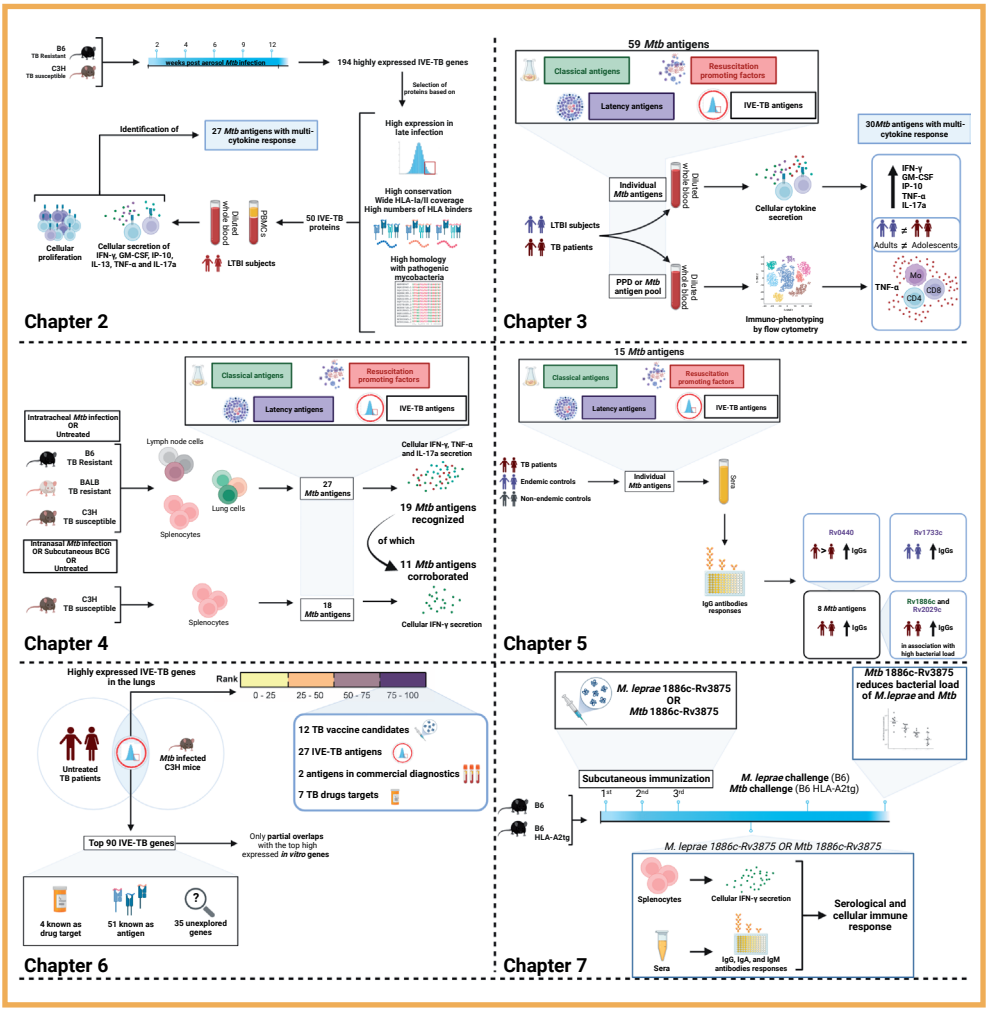

Figure 1. Schematic overview of the thesis results by chapter.

and characterize proteins from *Mtb in vitro* cultures. These limitations were overcome first by the availability of *Mtb* genome-wide expression libraries and subsequently by the availability of the whole *Mtb* genome sequence in 1998. This breakthrough allowed informational and experimental access to the entire *Mtb* antigenome, with its approximately 4,000 open reading frames (ORFs). These ORFs have now been probed extensively for their potential antigenicity, using several genome-wide strategies. However, little is known of whether and to which extent the genes encoding these antigens are expressed by *Mtb* in the primary TB target organ, the lung. Filling this knowledge gap is a crucial step for target-based vaccine and drug discovery. In 2013, Commandeur et al. determined a new class of *Mtb* antigens,

which were designated as IVE-TB (*in vivo* expressed) antigens (6). This approach identified *Mtb* genes highly expressed at six- and nine-weeks post-infection in the lung of four mouse strains associated with distinct TB susceptibility phenotypes. From this first pool of IVE-TB genes, Rv2034, which was highly expressed in the susceptible mouse strain B6.C3H-sst1 (super susceptibility to TB 1 locus), emerged as a potential vaccine candidate (7). To extend the set of putative IVE-TB antigens, **chapter 2** proposes a new genome-wide algorithm. Data included the relative gene copy numbers of 2,068 *Mtb* genes expressed in the lungs of the B6 (resistant to TB) vs. C3H (susceptible to TB) mouse strains, at multiple times post-infection (two, four, six, nine, and 12 weeks). A total of 194 genes was found to be consistently up-regulated in the lung, independently of the time of infection or the host genetic background. This new analysis, even though it differed from the first IVE-TB study for the characteristics described above (i.e., mouse strains and time points), confirmed 58 out of the 68 IVE-TB genes previously described. Interesting overlaps comprised genes important for virulence (e.g., Rv3615, Rv3616c, Rv3864, and Rv3865), iron acquisition (e.g., Rv2382, Rv0287, and Rv0288), glyoxylate shunt, and the methyl citrate pathway (e.g., Rv0467), which are all required for *in vivo* mycobacterial host adaptation (8).

Both IVE-TB studies present obvious limitations. Mice were infected with the laboratory-adapted *Mtb* Erdman, which under host immune pressure might respond differently than clinical TB strains (9). Furthermore, IVE-TB genes were selected based on the *Mtb* transcriptomes conditioned by the interaction between the pathogen and the murine pulmonary (immune) environment. Thus, one could contend that the findings are not representative of the human *Mtb* expressome. Indirect proofs against this argument are that most IVE-TB candidate antigens identified were highly conserved among the genomes of >200 human isolated *Mtb* strains, and were recognized by immune cells from *Mtb* responsive human subjects (**chapters 2**, **3**, and **5**). Importantly, the evidence that these *Mtb* IVE-TB genes are expressed in the *Mtb* infected human lung is formally given in **chapter 6**. This chapter analyses and compares seven *in vivo* expressed *Mtb*-transcriptomic datasets derived from the airways from mice (n=23) and humans (n=35). A strong positive correlation was shown between the *Mtb* transcriptomes signatures from infected lungs of C3H mice (which corresponds to the C3H dataset used in **chapter 2**) and those identified from sputum and bronchoalveolar lavage fluid (BAL) of TB patients. This remarkable level of commonality in the *Mtb* expressome among species is in line with the recent finding that the host transcriptomic signatures induced upon infection translates across genetically diverse mice, macaques, and humans (10).

Furthermore, **chapter 6** defines a core set of 90 highly expressed *Mtb* genes during pulmonary infection that includes *Mtb* genes of potential interest as targets for vaccination, diagnostics, or antibiotic treatment. As expected, the top *Mtb* lung-expressed genes were enriched for biological processes consistent with active *Mtb* infection (i.e., growth, translation, pathogenicity, protein secretion, and response to hypoxia and oxidative stress). Of note, 67 out of 90 top expressed genes defined in **chapter 6** overlap with those identified in **chapter 2**. Among those are *Mtb* transcripts (Rv0005, Rv1305, Rv3854c, Rv3874, Rv3619c, Rv3620c, Rv2660c, Rv3875, Rv0288, Rv3614c, and Rv3615c) that encode for target of TB drugs (Moxifloxacin, Bedaquiline, Isoniazid, Prothionamide, and Ethionamide) (11), approved TB diagnostics (12) and TB subunit-vaccine candidates (ID93, RhCMV6, H4, H64, and H56) (www.tbvi.eu). This supports the hypothesis that highly expressed genes mostly translate in highly abundant transcripts and proteins, which are then valuable candidate targets for efficacious anti-TB measures. Corroborating this proposition is the observation that the poor or good performance of new diagnostics (TB7.7 vs. ESAT6 free immunodiagnostic tests) and candidate vaccines (MVA85A vs. H4 or M72) seems to reflect the low or high expression levels of the genes encoding their targets as described in **chapter 6**. In a large phase IIb trial, the vaccine MVA85A expressing the early secreted protein but lowly expressed Ag85A failed to induce additional protective efficacy against developing TB (in a PoD (prevention of disease) trial design) on top of initial standard BCG vaccination in children (13). In a more recent, prevention of infection (PoI) clinical study in the same area, H4 (a fusion protein of highly expressed secreted antigens Ag85B/TB10.4) was administered admixed with the Th1/Th17 inducing IC31 adjuvant to previously BCG vaccinated adolescents. Although H4:IC31 failed to prevent initial or sustained *Mtb* infection significantly, a clear trend towards reduced sustained infection (sustained infection here defined as three consecutive positive IGRA tests) was distinguishable in this PoI trial, providing a first "signal" for a TB subunit vaccine. Finally, the third and most recent clinical trial data with a TB subunit vaccine came from a PoD trial with the M72 (GSK) vaccine, a fusion protein consisting of rearranged antigen fragments from Rv0125 and the highly expressed Rv1196, adjuvanted in the Th1 promoting adjuvant AS01E. The results of this seminal study showed a highly encouraging 50% efficacy against active TB in the 3 years follow up time in a cohort of latently *Mtb* infected (LTBI) adults (14).

Although the definition of high or low gene expression is a relative concept, robust data on the *Mtb in vivo* expressome can help de-risking TB vaccine and drug development.

Several promising TB subunit-vaccine candidates contain at least one protein encoded by a highly expressed *Mtb* gene, as shown in **chapter 6**. Interestingly, all

the three antigens constituting H56, which is undergoing a phase IIb trial for efficacy, safety, and immunogenicity (NCT03512249), are highly expressed both in mice and humans. The results of this trial (expected by 2024) may provide additional indirect evidence of whether targeting antigens encoded by IVE-TB genes contribute to effectively reducing TB cases.

In vitro immune responses to IVE-TB proteins

Mtb proteins encoded by highly expressed genes are not necessarily immunogenic. To elicit humoral or cell-mediated immune responses, *Mtb* proteins should in fact be available during infection and containing epitopes that, by binding to HLA molecules, can be presented to immune cells. Out of the 50 IVE-TB proteins selected in this thesis for antigen screening, 21 were chosen because they were predicted to have wide HLA coverage and/or the highest numbers of predicted HLA class I and II binders (**chapter 2**). Although the presence of T-cell epitopes in these proteins was not tested *in vitro*, all of them were consistently recognized by T cells from LTBI subjects and TB patients (**chapters 2** and **3**), with the exception of Rv3583c. This highly expressed *in vivo* protein (**chapter 6**), also known as CarD, is essential in regulating the *Mtb* transcription machinery (15) and its inhibition has been recently proposed as a novel target for anti-TB therapy (16, 17). The scarce immunogenicity detected for this crucial *Mtb* protein raises the question of whether *Mtb* employs escape mechanisms to impede CarD's presentation by HLA or if CarD induces anti-/pro-inflammatory responses not explored in this thesis. Answering these questions could certainly increase our understanding of the *Mtb* survival machinery and reveal processes that could be redressed in favour of the host.

To expand the characterization of well-defined *Mtb* antigens and discover new IVE-TB antigens, multiple analytes (TNF-α, IL-17, IL-13, IP-10, GM-CSF, IL-9, IL-10, IL-22, and IL-32 alpha) were measured as immunological read-outs, next to classical standard IFN-γ read outs (**chapters 2**, **3**, and **4**). Although it would have been interesting to assess the cytokines and chemokines' kinetics in response to the *Mtb* antigens at early and late timepoints, the amount of blood that could be drawn from the participants (especially from the adolescent donors described in **chapter 3**) limited the possibility to investigate and evaluate those dynamics across different *Mtb* infected donors. Thus, the cytokines' responses were evaluated at day 6 after *Mtb* protein stimulation, a choice that may have biased the results towards cytokines optimally secreted at that time point.

Overall, blood cells from *Mtb* exposed individuals (n=72) recognized 37 IVE-TB proteins, which were able to induce multi-functional cytokine responses (Figure 1).

Chapters 2 and 3	Chapter 3			Chapter 4		Chapter 5	Chapter 6
IVE-TB antigens recognized by a multi-cytokine signature in *Mtb* exposed subjects	IFN-γ LTBI>TB	IL-17a LTBI>TB	IP-10 LTBI>TB	Cytokine recognition profiles in TB resistant mice ≠ TB susceptible mice	Recognized by BCG vaccinated TB susceptible mice	↑ IgG in active TB	IVE-TB genes ranked in the upper quartile across mouse and human datasets
Rv0470c*						nt	
Rv3616c							
Rv1980c						nt	
Rv0287/88						nt	
Rv3614/15				nt	nt	nt	
Rv1131						nt	
Rv3615c						nt	
Rv3874/75							
Rv2873						nt	
Rv0440							
Rv2245				nt	nt	nt	
Rv3462c				nt	nt	nt	
Rv0991c						nt	
Rv1221						nt	
Rv1846c*						nt	
Rv0501*				nt	nt	nt	
Rv0824c				nt	nt	nt	
Rv0826						nt	
Rv1791						nt	
Rv2215*				nt	nt		
Rv0467				nt	nt	nt	
Rv0468*				nt	nt	nt	
Rv0640*				nt	nt	nt	
Rv0642c					nt	nt	
Rv1284				nt	nt	nt	
Rv1792				nt	nt	nt	
Rv1872c*					nt		
Rv2031c				nt	nt		
Rv2346c/47c				nt	nt	nt	
Rv2626c					nt		
Rv3865				nt	nt	nt	
Rv0423				nt	nt	nt	
Rv0645c				nt	nt		
Rv2007c				nt	nt	nt	
Rv2461c					nt	nt	
Rv2941				nt	nt	nt	
Rv3846					nt	nt	

Figure 2. Most promising IVE-TB antigens. Legend: *Selected in chapter 1 for high homology with *M. leprae*. *Mycobacterium tuberculosis* (*Mtb*) antigens previously identified by other antigen discovery strategies are colour coded in grey. IVE-TB: *in vivo* highly expressed *Mtb* genes; TB: tuberculosis; LTBI: latent TB; BCG: Bacillus Calmette–Guérin; nt: not tested.

To the best of our knowledge, among these 37 IVE-TB antigens, 21 were described for the first time as *Mtb* antigens (**chapters 2** and **3**) (Figure 1). Interestingly, almost half of those antigens were recognized by cells producing multiple cytokines and chemokines other than IFN-γ, including IP-10, GM-CSF, IL-17 and TNF-α. In agreement with this finding, non-IFN-γ responses were measured to a substantial number of antigens in splenocytes and cells from the mediastinal draining lymph nodes of *Mtb* infected C57BL/6 mice (**chapter 4**). This data suggests that IFN-γ based screening approaches may have significantly underestimated as well biased

Mtb antigen discovery studies. While useful, sensitive and robust, clearly many other molecules are secreted by immune cells, often in the absence of IFN-γ (18). This is also evident when examining alternative T cell responses such as those restricted by HLA-E, which often release Th2 rather than Th1 cytokines (19).

As for IFN-γ, there is no clear proof defining certain cytokines as indispensable in containing latent *Mtb* infection (20-23) or active TB disease in humans (24-27). However, IP-10 was recently showed to be mechanistically involved in controlling mycobacterial growth in MGIA assays in recently *Mtb* infected individuals (28). Likewise, GM-CSF and IL-17 have been associated with protective immunity both in *in vivo* and *in vitro* models (23, 29-35). In the absence of validated correlates of protection, multiple antigens included in TB vaccine candidates have been chosen because they were recognised by screening blood of LTBIs (36-38). Though the underlying immune mechanisms remain unknown, LTBIs contain *Mtb* infection better than TB patients (39) supporting the hypothesis that those *Mtb* antigens more strongly recognized by blood cells of LTBIs, can induce more effective host immune responses when used as vaccine targets. In **chapter 3**, the levels of IL-17A, IFN-γ, and IP-10 in response to several *Mtb* antigens were higher in the LTBI's blood cell supernatants compared to the concentrations found among TB patients (Figure 1). Additionally, when a set of these antigens was tested in mouse models, different cytokine recognition profiles were found between TB resistant and susceptible mice (**chapter 4**) (Figure 1). This is an interesting finding since, once verified that an antigen is recognised in a TB susceptible mouse strain, such as in the C3HeB/FeJ mice, appropriate adjuvant or vaccine formulation could redirect the immune system towards a protective response (e.g., the response found in TB resistant mouse strains, i.e., C57BL/6 mice). Therefore, if these assumptions were correct, multiple IVE-TB antigens identified in this thesis could serve as potential prophylactic or therapeutic TB vaccine candidates (Figure 1). Since, in most of the cases, subunit TB vaccines would be tested and used in areas where the BCG vaccination is given at birth, it would be important to assess also whether and how BCG vaccinated individuals recognize these antigens and compare those responses with what has been found in LTBIs from non-endemic TB countries. In animal models, it has been shown that BCG drives T cells towards a more differentiated phenotype that does not sustain long protection against *Mtb* (40). Thus, prior exposure to BCG might impact the way immune cell subsets respond to immunization and *Mtb* infection (41). In **chapter 4**, two out of 15 IVE-TB antigens (Rv0470 and Rv3616c) were recognized by BCG vaccinated C3HeB/FeJ mice. Rationally, BCG-shared antigens would be preferable for subunit TB vaccines aiming to boost BCG. However, if an antigen-based vaccine would contain BCG-shared epitopes and be co-administered with BCG, some antigens may inhibit BCG replication, as observed for the H4:IC31® vaccine (42).

Combining BCG with a subunit TB vaccine might be especially attractive if BCG re-vaccination will be introduced as additional measure to contain TB (ongoing trial: NCT04152161). Interest in this approach rose after the unexpected and encouraging results from a recent trial showing that BCG revaccination led to a significant reduction in sustained *Mtb* infection as determined by three consecutive QFG/IGRA tests (43). A recent study investigated the co-administration of BCG with the fusion protein H107 composed of eight *Mtb* proteins, which include five IVE-TB antigens described in this thesis (Rv1980c, Rv2873, Rv3615c, Rv3616c and Rv3875). Complementing BCG with H107 resulted in a less differentiated T cell repertoire, skewed towards a Th1/Th17 phenotype (as expected by the use of the CAF®01 adjuvant), and in a longer protective efficacy compared to BCG, H107 or H65 (42). The latter is composed by six *Mtb* proteins, which include Rv0287 and Rv0288, also described as IVE-TB antigens. None of the antigens included in H107 were recognised by BCG vaccinated CB6F1 mice, in contrast to what was found for the antigens comprised in H65. Therefore, the authors interestingly speculated that subunit vaccines with de novo antigens might act more synergistically with BCG than vaccine composed of BCG-shared antigens when co-administered (42). It would be informative to test this hypothesis also in TB susceptible mouse strains since the results of **chapter 4** suggest that immune responses against these proteins after BCG vaccination might differ in C3HeB/FeJ mice.

In **chapter 3**, TNF-α responses to *Mtb* antigens were found to be higher in TB patients than LTBI. Based on this observation, which was recently confirmed in an independent study analysing samples of TB cases and controls among HIV infected patients (44), measuring TNF-α could be useful to discriminate between active TB and LTBI. A recent study is in line with this proposition since it showed that a fluorospot-based IFN-γ/TNF-α dual release assay performed better than IFN-γ alone in diagnosing active TB (45).

TNF-α plays an intriguing role in TB: both in animal models and humans its dearth increases susceptibility to TB, while an excess correlates with TB pathogenesis (46-52). In **chapter 4**, lung and mediastinal lymph node cells from the TB susceptible C3HeB/FeJ mice did not secrete measurable TNF-α in response to *Mtb* antigens and several positive mitogen controls. As a result of this low TNF-α secretion, it is possible that those mice may develop necrotic lesions, as has been shown for other mouse strains (51, 52). However, that seems contrasting with the excess of TNF-α found in active TB patients also characterized by the same histopathology. The picture becomes even more complicated when considering that TNF-α responses seems to differ among age groups. In **chapter 3**, TNF-α+ cells in response to a selected group of IVE-TB antigens or PPD were more abundant in adult TB patients

than adult LTBIs, but an opposite trend was seen in adolescents. By zooming into these subsets, we found that these TNF-α+ cells displayed a different phenotype and functionality between the two age groups. A subset of TNF-α+ cells co-expressing CD14 was abundant in adolescent LTBIs but scarcely present in adult LTBIs. These cells might correspond to pro-inflammatory, non-classical monocytes, which have previously been associated with recent exposure to *Mtb* (28, 53). Differences in the amounts of myeloid-associated pro-inflammatory mediators have been also recently described between infants and young adults (54).

Although both studies rely on relatively small size cohorts, these results show differences in age-associated immune responses that might play a role in the risk of developing active TB and reflect the spectrum of *Mtb* infection and *Mtb* exposure (55-57). Large longitudinal study following-up LTBIs and high TB risk household contacts across their lifespan could help understanding the optimal balance between pro- and anti-inflammatory responses to seek in anti-TB interventions.

The TNF-α+ cells from adult LTBIs were predominantly characterized by a terminal effector memory (TEMRA) like phenotype co-expressing CD8, IL-22, IL-32, and IFN-γ. TNF-α+ CD8+ TEMRA cells have been already proposed to play a role in antimicrobial activity against TB reactivation in adult LTBI (66, 67). While it is accepted that polyfunctional CD4+ cells are necessary but not sufficient in protective immunity against TB, the role of CD8+ T cell responses in TB remains less clear (41). Although CD8 depletion hampers the control of *Mtb* replication in BCG vaccinated non-human primates (NHPs), in murine models vaccine-induced CD8+ T cells failed to reduce *Mtb* proliferation (58, 59). In the context of vaccine studies, Ag85A-specific CD8+ cells were barely detectable in immunized MVA85A or H4:IC31 subjects (60-65), while M72/AS01E vaccination boosted detectable M72-specific polyfunctional CD8 T cells frequencies above pre-vaccination levels a week after the first immunization (66). This timepoint was not included in the M72/AS01E phase 2b trial, therefore it needs to be determined whether CD8+ cells contributed to the protective efficacy reported for this vaccine candidate (14). Similarly, VPM1002, another promising whole-cell (recombinant BCG) based candidate vaccine induces multifunctional CD8+ T cells in vaccinees (67). In line with these findings, prominent CD8+ T cell responses were also associated with the high protective efficacy elicited by the CMV-*Mtb* and intravenous BCG vaccination in NHPs (38, 68).

Besides conventional CD4+ and CD8+ cells, new high-dimensional cytometry analyses identified new cell subsets in differently exposed *Mtb* adolescents. Those new populations included for instance CD4 CD8 double positive T cells (**chapter 3**), NK cells, CD27–CD8+ αβ T cells, ILC3, and B cells (69, 70).

The contribution of B cells and humoral immunity in TB host defence is becoming progressively more recognized (71-74). The association of Ag85A-specific IgG boosted by MVA85A vaccination with a significantly reduced risk of developing TB, underlined the potential protective role of antigen-specific antibodies in TB (75). Furthermore, recent studies in NHPs showed that that after mucosal or intravenous BCG vaccination, a robust expansion of antigen-specific IgA and IgM in the BAL was associated with protection against *Mtb* infection and TB progression (35, 68, 76) (ref). In **chapter 5**, the IgG titres against ESAT/CFP10, Rv3616c and Rv0867c were increased in active TB patients compared to controls at high-risk for TB. Only antibodies directed to Rv1733c were higher in the controls compared to TB patients, indicating a possible role of these specific antibodies in controlling TB progression. Thus, these antigens are targeted by the humoral response during natural *Mtb* infection, TB disease and potentially TB control. It would be important to characterize these antibodies in term of isotypes, subclasses, avidity and glycosylation patterns, as well as to extend this analysis to other promising IVE-TB antigens. Accumulating evidence shows in fact striking differences in the glycosylation profiles, the Fc functions and the subclasses of mycobacteria-specific IgG between LTBIs, active and treated TB patients (77, 78).

Knowing which cellular and humoral responses mediate TB disease or protection is fundamental to rationally guide future vaccine design. The identification of the key immune components against TB could be accelerated by a broad and high dimensional evaluation of the immunogenicity elicited by protective TB vaccines. Hopefully, new insights will result from on-going analysis of successful clinical vaccine studies (41). The discovery of mediators of TB protection will help selecting the best antigens and vaccine formulations to contain TB. Yet, a deep knowledge gap still exists, hampering this quest. While impressive advances have been made in determining the cells, molecules and pathways involved in TB, there is still not a full explanation to account for TB control vs. progression and subsequent pathogenesis (2). Only when it will be clear why certain people are susceptible to TB, it will be possible to develop tools to protect them.

IVE-TB antigens: one toolbox against several mycobacterial diseases?

To select the most promising genes for functional and immunological evaluation, several criteria were applied. Among others, genes encoding proteins predicted to share high homology with other pathogenic mycobacteria were selected. Of note, high priority was given to homologs of *M. leprae* proteins. Despite *M. leprae*, which causes leprosy, being the second most pathogenic mycobacterium after *Mtb* (79), there is an extreme gap between the number of vaccines developed against TB and leprosy. Notwithstanding the new WHO Global Leprosy Strategy, which

aims to interrupt leprosy transmission and achieve zero leprosy cases by 2030 (80), the disparity described in **chapter 7** increased in the last years (www.tbvi.eu). Repurposing TB vaccines against leprosy could narrow this inequality. The idea of preventing TB and leprosy with one vaccine is per se quite old if considering that BCG has been largely used in this fashion (5). However, for none of the current 16 TB candidate vaccines in clinical evaluation studies (www.tbvi.eu), the impact on leprosy is currently being taken into account. This thesis demonstrates that novel TB vaccine candidates can cross-protect against leprosy. Specifically, **chapter 7** shows cross-protection induced by two *Mtb* antigens, ESAT6 (Rv3875) and Ag85B (Rv1886c), against *M. leprae* in mice. This finding supports integrating pre-clinical research against several mycobacterial diseases (i.e., TB, leprosy, and Buruli ulcer) at an early stage of antigen discovery, as done in **chapter 2** and calls for harmonizing efforts in future clinical trials.

Mtb antigens: concluding remarks and future perspectives

Up to date, 4,727 *Mtb* (ID1773) epitopes and more than 900 *Mtb* antigens (IEDB—immune epitope database—, www.iedb.org) have been identified. However, the majority of the *Mtb* epitopes reported disproportionally (56%) belongs to a relatively small proportion of proteins (n = 45) (IEDB). Furthermore, the antigenicity of these recombinant peptides and proteins has been typically defined by *in vitro* measuring of IFN-γ and proliferative T cell responses using PBMC, T cell lines, or whole blood from LTBI donors. This thesis contends that additional parameters need to be included as well, based in part on the observations that many new *Mtb* antigens were recognized by cells producing cytokines other than IFN-γ, and often no IFN-γ at all. This suggests that IFN-γ-based screening approaches may not have captured the *Mtb* antigenome adequately.

Current diagnostic tests, including TST and IGRA, have poor prognostic capacity in predicting which *Mtb* infected individuals will progress towards TB, which would allow rapid preventive treatment of these subjects to decrease the risk of *Mtb* progression and subsequent transmission. Differences in *Mtb* antigen specific IFN-γ production and in polyfunctionality of T cell responses, such as to Rpf or DosR regulon antigens, have been found repeatedly between LTBI and TB patients (81, 82). However, not many of such antigens have been assessed in longitudinal follow-up studies of TB household contacts to examine whether they could predict TB progression. The novel epitopes or antigens identified by wide genome screenings have been evaluated sporadically in multiple TB cohorts. When analysed, very few differences in the magnitude and frequency of responses, which were mostly IFN-γ centred, were found between TB patients and LTBI (6, 83-85). However, the number of subjects included in those studies was generally quite low and future studies would need to screen

larger cohorts including follow-up analyses to capture their disease -or protection- association. Such studies could also be interesting as they might elucidate how the immune response repertoire against antigens/epitopes is shaped during the natural course of infection. That is important considering that antigens highly expressed at an early stage of infection can lead to T cell exhaustion and dysfunction (40, 86) while others not evoking exhaustion could induce long term memory. Interestingly, new published data indicate that antigens presenting a delayed *in vivo* expression induce less differentiated CD4 T cells but also low protection when used for vaccination in murine models (87). On the contrary, highly *in vivo* expressed antigens drive CD4+ cells toward terminal differentiation during natural infection. However, immunization with such antigens in appropriate formulations can sustain T cells in a less differentiated state and induce better protection than the one elicited by delayed *in vivo* expressed antigens (87).

Most of the novel *Mtb* antigens/epitopes identified have high homologies to antigens from NTM or other bacteria (85, 88, 89), including those present in the human microbiota. How this impacts their reactogenicity in TB needs to be clarified. In fact, it should be recognized that prior sensitization to mycobacterial antigens can influence the nature and course of all *Mtb* subsequent infections (2). Additionally, the currently used rather narrowly focused immunological read-outs (mostly IFN-γ or polyfunctional CD4 + T cell centred) are unlikely to detect immunological changes in other domains of immunity, which widely occur in *Mtb* carrying hosts as they transit from a stage of controlling *Mtb* infection to a process culminating in active TB disease (90-92). Identifying such changes and defining the corresponding biomarkers of TB risk would greatly facilitate early TB diagnosis and prediction of TB onset at an early stage. Innovative animal models, like cynomolgus macaques that can display the entire human TB clinical spectrum (93, 94), and which can also recognize CD4+ epitope pools defined in humans (95) would be of great value to help identifying such markers in translational studies.

Most *Mtb* genome wide antigen discoveries have relied on samples from LTBI donors. Those individuals are interesting from a vaccine development point of view, since LTBI results in an almost 80% lower risk of developing active TB than non LTBI subjects upon re-infection (96). However, the underlying biological mechanisms and immune correlates remain unknown (97). As discussed, most studies today still follow IFN-γ oriented approaches although we know that the presence of activated and polyfunctional (IFN-γ+/IL-2+/TNF-α+) T cells are not correlates or sufficient mediators of protection (75, 98). A recent study in mice demonstrated that CD4 + T cells activated by systemic peptide administration was able to reach the lung parenchyma but, critically, failed to act directly with *Mtb* infected cells (99). *Mtb*

infected cells have the ability to decoy immune cells through different mechanisms such as suboptimal antigen presentation, antigen camouflage, exporting antigens to bystander uninfected cells, thereby reducing the recognition of those cells containing the mycobacteria, the release of inhibitory cytokines or the induction of inhibitory mechanisms such as regulatory T cells (100-104). It would be interesting to study whether the decoy activity is restricted to certain antigens, such as secreted *Mtb* antigens, which are more exposed to the immune system. If so, vaccine strategies might need to be focused on non-secreted antigens, which would be contrary to most current thinking.

To advance novel antigens into the TB vaccine pipeline, it will be necessary to prove their immunogenicity and especially their protective efficacy in preclinical animal models of increasing complexity and relevance to human TB. To our knowledge, from all antigens identified by recent genome wide strategies, only few antigens (http://www.tbvi.eu/what-we-do/pipeline-of-vaccines/) have been tested *in vivo*. Mice are generally used as first line *in vivo* model and usually the protective effects of adjuvanted/vectored proteins are tested alone and compared to BCG. This might not be the best strategy since most protein-based subunit vaccine candidates aim to boost BCG vaccination, and there could be antigens able to improve the protective efficacy of BCG but not as much reduce the bacterial load as stand-alone vaccines. Moreover, the diversity in mouse strains, regimens, adjuvants, infection challenges and doses used differ widely and impede a comparison between different vaccine candidates. In that regard, a head-to-head comparison of vaccine candidates in the same models and experiments should be strongly promoted to provide more solid and consistent data in the pre-clinical stage of vaccine development. TBVI is one of the first organisations that has been promoting such a TB vaccine selection process during the past decade (105). Also interesting is the so called "Collaborative Cross" (CC) panel composed of mouse recombinant inbred lines displaying genetic diversities ad thereby susceptibility to TB and TB vaccination (106). Even more attractive for evaluating new TB vaccines are lung-oriented mycobacterial controlled human infection models using live BCG and PPD, which have been recently shown to be practicable and safe (107).

TB vaccines have been generally formulated as viral-vectored vaccines, subunit vaccines, and whole-cell live or non-live mycobacterial vaccines. The recent approval of several mRNA-based COVID-19 vaccine (108), prompted efforts to introduce such a new platform in the TB vaccine pipeline (109). This novel vaccine approach will in all likelihood open new exciting possibility for TB vaccination.

In conclusion, genome wide strategies, including the one proposed in this thesis, have discovered a wealth of new *Mtb* antigens and epitopes that have escaped detection by previous classical methodologies, with interesting overlaps among those identified by independent approaches (Supplementary Table 1 available online at: https://doi.org/10.1016/j.smim.2018.07.001).

Unlocking their potential as vaccine and drug targets as well as TB biomarker antigens, e.g., for diagnostic or prognostic purposes, will be the next fascinating challenge.

REFERENCES

1. WHO. WHO Global TB Report 2021.
2. Hunter RL. The Pathogenesis of Tuberculosis-The Koch Phenomenon Reinstated. Pathogens. 2020;9(10).
3. Houben RM, Dodd PJ. The Global Burden of Latent Tuberculosis Infection: A Re-estimation Using Mathematical Modelling. PLoS medicine. 2016;13(10): e1002152.
4. Drain PK, Bajema KL, Dowdy D, Dheda K, Naidoo K, Schumacher SG, et al. Incipient and Subclinical Tuberculosis: a Clinical Review of Early Stages and Progression of Infection. Clinical microbiology reviews. 2018;31(4).
5. Fritschi N, Curtis N, Ritz N. Bacille Calmette Guérin (BCG) and new TB vaccines: Specific, cross-mycobacterial and off-target effects. Paediatr Respir Rev. 2020;36:57-64.
6. Commandeur S, van Meijgaarden KE, Prins C, Pichugin AV, Dijkman K, van den Eeden SJ, et al. An unbiased genome-wide *Mycobacterium tuberculosis* gene expression approach to discover antigens targeted by human T cells expressed during pulmonary infection. Journal of immunology (Baltimore, Md : 1950). 2013;190(4):1659-71.
7. Commandeur S, van den Eeden SJ, Dijkman K, Clark SO, van Meijgaarden KE, Wilson L, et al. The *in vivo* expressed *Mycobacterium tuberculosis* (IVE-TB) antigen Rv2034 induces $CD4^+$ T-cells that protect against pulmonary infection in HLA-DR transgenic mice and guinea pigs. Vaccine. 2014;32(29):3580-8.
8. Serafini A, Tan L, Horswell S, Howell S, Greenwood DJ, Hunt DM, et al. *Mycobacterium tuberculosis* requires glyoxylate shunt and reverse methylcitrate cycle for lactate and pyruvate metabolism. Mol Microbiol. 2019;112(4):1284-307.
9. De Groote MA, Gruppo V, Woolhiser LK, Orme IM, Gilliland JC, Lenaerts AJ. Importance of confirming data on the *in vivo* efficacy of novel antibacterial drug regimens against various strains of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2012;56(2):731-8.
10. Ahmed M, Thirunavukkarasu S, Rosa BA, Thomas KA, Das S, Rangel-Moreno J, et al. Immune correlates of tuberculosis disease and risk translate across species. Science translational medicine. 2020;12(528).
11. Cohen KA, Manson AL, Desjardins CA, Abeel T, Earl AM. Deciphering drug resistance in *Mycobacterium tuberculosis* using whole-genome sequencing: progress, promise, and challenges. Genome Med. 2019;11(1):45.
12. WHO. Global tuberculosis report 2020. Geneva: World Health Organization; 2020. 2020.
13. Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. Lancet (London, England). 2013;381(9871):1021-8.
14. Tait DR, Hatherill M, Van Der Meeren O, Ginsberg AM, Van Brakel E, Salaun B, et al. Final Analysis of a Trial of M72/AS01E Vaccine to Prevent Tuberculosis. The New England journal of medicine. 2019;381(25):2429-39.
15. Zhu DX, Garner AL, Galburt EA, Stallings CL. CarD contributes to diverse gene expression outcomes throughout the genome of *Mycobacterium tuberculosis*. Proceedings of the National Academy of Sciences of the United States of America. 2019;116(27):13573-81.
16. Stefan MA, Velazquez GM, Garcia GA. High-throughput screening to discover inhibitors of the CarD·RNA polymerase protein-protein interaction in *Mycobacterium tuberculosis*. Scientific reports. 2020;10(1):21309.
17. Kaur G, Kapoor S, Kaundal S, Dutta D, Thakur KG. Structure-Guided Designing and Evaluation of Peptides Targeting Bacterial Transcription. Front Bioeng Biotechnol. 2020; 8:797.

18. Lu LL, Smith MT, Yu KKQ, Luedemann C, Suscovich TJ, Grace PS, et al. IFN-gamma-independent immune markers of *Mycobacterium tuberculosis* exposure. Nature medicine. 2019;25(6):977-87.
19. van Meijgaarden KE, Haks MC, Caccamo N, Dieli F, Ottenhoff TH, Joosten SA. Human CD8+ T-cells recognizing peptides from *Mycobacterium tuberculosis* (*Mtb*) presented by HLA-E have an unorthodox Th2-like, multifunctional, *Mtb* inhibitory phenotype and represent a novel human T-cell subset. PLoS pathogens. 2015;11(3): e1004671.
20. Coulter F, Parrish A, Manning D, Kampmann B, Mendy J, Garand M, et al. IL-17 Production from T Helper 17, Mucosal-Associated Invariant T, and gammadelta Cells in Tuberculosis Infection and Disease. Frontiers in immunology. 2017; 8:1252.
21. Shen H, Chen ZW. The crucial roles of Th17-related cytokines/signal pathways in *M. tuberculosis* infection. Cellular & molecular immunology. 2018;15(3):216-25.
22. Villar-Hernandez R, Latorre I, De Souza-Galvao ML, Jimenez MA, Ruiz-Manzano J, Pilarte J, et al. Use of IP-10 detection in dried plasma spots for latent tuberculosis infection diagnosis in contacts via mail. Scientific reports. 2019;9(1):3943.
23. Robinson RT. T Cell Production of GM-CSF Protects the Host during Experimental Tuberculosis. mBio. 2017;8(6).
24. Tebruegge M, Ritz N, Donath S, Dutta B, Forbes B, Clifford V, et al. Mycobacteria-Specific Mono- and Polyfunctional CD4+ T Cell Profiles in Children With Latent and Active Tuberculosis: A Prospective Proof-of-Concept Study. Frontiers in immunology. 2019; 10:431.
25. Wang S, Li Y, Shen Y, Wu J, Gao Y, Zhang S, et al. Screening and identification of a six-cytokine biosignature for detecting TB infection and discriminating active from latent TB. Journal of translational medicine. 2018;16(1):206.
26. Balcells ME, Ruiz-Tagle C, Tiznado C, Garcia P, Naves R. Diagnostic performance of GM-CSF and IL-2 in response to long-term specific-antigen cell stimulation in patients with active and latent tuberculosis infection. Tuberculosis (Edinburgh, Scotland). 2018; 112:110-9.
27. Latorre I, Diaz J, Mialdea I, Serra-Vidal M, Altet N, Prat C, et al. IP-10 is an accurate biomarker for the diagnosis of tuberculosis in children. The Journal of infection. 2014;69(6):590-9.
28. Joosten SA, van Meijgaarden KE, Arend SM, Prins C, Oftung F, Korsvold GE, et al. Mycobacterial growth inhibition is associated with trained innate immunity. The Journal of clinical investigation. 2018;128(5):1837-51.
29. Gonzalez-Juarrero M, Hattle JM, Izzo A, Junqueira-Kipnis AP, Shim TS, Trapnell BC, et al. Disruption of granulocyte macrophage-colony stimulating factor production in the lungs severely affects the ability of mice to control *Mycobacterium tuberculosis* infection. J Leukoc Biol. 2005;77(6):914-22.
30. Dou J, Tang Q, Yu F, Yang H, Zhao F, Xu W, et al. Investigation of immunogenic effect of the BCG priming and Ag85A- GM-CSF boosting in Balb/c mice model. Immunobiology. 2010;215(2):133-42.
31. Bryson BD, Rosebrock TR, Tafesse FG, Itoh CY, Nibasumba A, Babunovic GH, et al. Heterogeneous GM-CSF signaling in macrophages is associated with control of *Mycobacterium tuberculosis*. Nature communications. 2019;10(1):2329.
32. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. Nature immunology. 2007;8(4):369-77.
33. Ahmed M, Smith DM, Hamouda T, Rangel-Moreno J, Fattom A, Khader SA. A novel nanoemulsion vaccine induces mucosal Interleukin-17 responses and confers protection upon *Mycobacterium tuberculosis* challenge in mice. Vaccine. 2017;35(37):4983-9.
34. Woodworth JS, Cohen SB, Moguche AO, Plumlee CR, Agger EM, Urdahl KB, et al. Subunit vaccine H56/CAF01 induces a population of circulating CD4 T cells that traffic into the *Mycobacterium tuberculosis*-infected lung. Mucosal Immunol. 2017;10(2):555-64.

35. Dijkman K, Sombroek CC, Vervenne RAW, Hofman SO, Boot C, Remarque EJ, et al. Prevention of tuberculosis infection and disease by local BCG in repeatedly exposed rhesus macaques. Nature medicine. 2019;25(2):255-62.
36. Aagaard C, Hoang T, Dietrich J, Cardona PJ, Izzo A, Dolganov G, et al. A multistage tuberculosis vaccine that confers efficient protection before and after exposure. Nature medicine. 2011;17(2):189-94.
37. Geluk A, van den Eeden SJ, van Meijgaarden KE, Dijkman K, Franken KL, Ottenhoff TH. A multistage-polyepitope vaccine protects against *Mycobacterium tuberculosis* infection in HLA-DR3 transgenic mice. Vaccine. 2012;30(52):7513-21.
38. Hansen SG, Zak DE, Xu G, Ford JC, Marshall EE, Malouli D, et al. Prevention of tuberculosis in rhesus macaques by a cytomegalovirus-based vaccine. Nature medicine. 2018;24(2):130-43.
39. Weiner J, 3rd, Kaufmann SH. Recent advances towards tuberculosis control: vaccines and biomarkers. Journal of internal medicine. 2014;275(5):467-80.
40. Lindenstrøm T, Moguche A, Damborg M, Agger EM, Urdahl K, Andersen P. T Cells Primed by Live Mycobacteria Versus a Tuberculosis Subunit Vaccine Exhibit Distinct Functional Properties. EBioMedicine. 2018; 27:27-39.
41. Andersen P, Scriba TJ. Moving tuberculosis vaccines from theory to practice. Nature reviews Immunology. 2019;19(9):550-62.
42. Woodworth JS, Clemmensen HS, Battey H, Dijkman K, Lindenstrøm T, Laureano RS, et al. A novel *Mycobacterium tuberculosis*-specific subunit vaccine provides synergistic immunity upon co-administration with Bacillus Calmette-Guérin. bioRxiv. 2021:2021.03.18.435784.
43. Nemes E, Geldenhuys H, Rozot V, Rutkowski KT, Ratangee F, Bilek N, et al. Prevention of *M. tuberculosis* Infection with H4:IC31 Vaccine or BCG Revaccination. The New England journal of medicine. 2018;379(2):138-49.
44. Meier NR, Battegay M, Ottenhoff THM, Furrer H, Nemeth J, Ritz N. HIV-Infected Patients Developing Tuberculosis Disease Show Early Changes in the Immune Response to Novel *Mycobacterium tuberculosis* Antigens. Frontiers in immunology. 2021; 12:620622.
45. Kim JY, Kang YA, Park JH, Cha HH, Jeon NY, Lee SW, et al. An IFN-γ and TNF-α dual release fluorospot assay for diagnosing active tuberculosis. Clin Microbiol Infect. 2020;26(7):928-34.
46. Clifford V, Tebruegge M, Zufferey C, Germano S, Forbes B, Cosentino L, et al. Cytokine biomarkers for the diagnosis of tuberculosis infection and disease in adults in a low prevalence setting. Tuberculosis (Edinburgh, Scotland). 2019; 114:91-102.
47. Harari A, Rozot V, Bellutti Enders F, Perreau M, Stalder JM, Nicod LP, et al. Dominant TNF-alpha+ *Mycobacterium tuberculosis*-specific CD4+ T cell responses discriminate between latent infection and active disease. Nature medicine. 2011;17(3):372-6.
48. Mootoo A, Stylianou E, Arias MA, Reljic R. TNF-alpha in tuberculosis: a cytokine with a split personality. Inflamm Allergy Drug Targets. 2009;8(1):53-62.
49. Roca FJ, Ramakrishnan L. TNF dually mediates resistance and susceptibility to mycobacteria via mitochondrial reactive oxygen species. Cell. 2013;153(3):521-34.
50. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MP. The immune response in tuberculosis. Annual review of immunology. 2013; 31:475-527.
51. Kindler V, Sappino AP, Grau GE, Piguet PF, Vassalli P. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell. 1989;56(5):731-40.
52. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, et al. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. Immunity. 1995;2(6):561-72.

53. Belge KU, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, et al. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. Journal of immunology (Baltimore, Md: 1950). 2002;168(7):3536-42.
54. Baguma R, Mbandi SK, Rodo MJ, Erasmus M, Day J, Makhethe L, et al. Inflammatory Determinants of Differential Tuberculosis Risk in Pre-Adolescent Children and Young Adults. Frontiers in immunology. 2021; 12:639965.
55. Lin PL, Flynn JL. Understanding latent tuberculosis: a moving target. Journal of immunology (Baltimore, Md: 1950). 2010;185(1):15-22.
56. Cadena AM, Fortune SM, Flynn JL. Heterogeneity in tuberculosis. Nature reviews Immunology. 2017;17(11):691-702.
57. Lin PL, Ford CB, Coleman MT, Myers AJ, Gawande R, Ioerger T, et al. Sterilization of granulomas is common in active and latent tuberculosis despite within-host variability in bacterial killing. Nature medicine. 2014;20(1):75-9.
58. Chen CY, Huang D, Wang RC, Shen L, Zeng G, Yao S, et al. A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. PLoS pathogens. 2009;5(4):e1000392.
59. Day CL, Tameris M, Mansoor N, van Rooyen M, de Kock M, Geldenhuys H, et al. Induction and regulation of T-cell immunity by the novel tuberculosis vaccine M72/AS01 in South African adults. American journal of respiratory and critical care medicine. 2013;188(4):492-502.
60. Nemes E, Hesseling AC, Tameris M, Mauff K, Downing K, Mulenga H, et al. Safety and Immunogenicity of Newborn MVA85A Vaccination and Selective, Delayed Bacille Calmette-Guerin for Infants of Human Immunodeficiency Virus-Infected Mothers: A Phase 2 Randomized, Controlled Trial. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. 2018;66(4):554-63.
61. McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, Huygen K, et al. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. Nature medicine. 2004;10(11):1240-4.
62. Tameris M, Geldenhuys H, Luabeya AK, Smit E, Hughes JE, Vermaak S, et al. The candidate TB vaccine, MVA85A, induces highly durable Th1 responses. PloS one. 2014;9(2): e87340.
63. Lindenstrøm T, Aagaard C, Christensen D, Agger EM, Andersen P. High-frequency vaccine-induced $CD8^+$ T cells specific for an epitope naturally processed during infection with *Mycobacterium tuberculosis* do not confer protection. European journal of immunology. 2014;44(6):1699-709.
64. Yang JD, Mott D, Sutiwisesak R, Lu YJ, Raso F, Stowell B, et al. *Mycobacterium tuberculosis*-specific CD4+ and CD8+ T cells differ in their capacity to recognize infected macrophages. PLoS pathogens. 2018;14(5):e1007060.
65. Bekker LG, Dintwe O, Fiore-Gartland A, Middelkoop K, Hutter J, Williams A, et al. A phase 1b randomized study of the safety and immunological responses to vaccination with H4:IC31, H56:IC31, and BCG revaccination in *Mycobacterium tuberculosis*-uninfected adolescents in Cape Town, South Africa. EClinicalMedicine. 2020; 21:100313.
66. Penn-Nicholson A, Geldenhuys H, Burny W, van der Most R, Day CL, Jongert E, et al. Safety and immunogenicity of candidate vaccine M72/AS01E in adolescents in a TB endemic setting. Vaccine. 2015;33(32):4025-34.
67. Nieuwenhuizen NE, Kulkarni PS, Shaligram U, Cotton MF, Rentsch CA, Eisele B, et al. The Recombinant Bacille Calmette-Guérin Vaccine VPM1002: Ready for Clinical Efficacy Testing. Frontiers in immunology. 2017; 8:1147.
68. Darrah PA, Zeppa JJ, Maiello P, Hackney JA, Wadsworth MH, 2nd, Hughes TK, et al. Prevention of tuberculosis in macaques after intravenous BCG immunization. Nature. 2020;577(7788):95-102.

69. Roy Chowdhury R, Vallania F, Yang Q, Lopez Angel CJ, Darboe F, Penn-Nicholson A, et al. A multi-cohort study of the immune factors associated with *M. tuberculosis* infection outcomes. Nature. 2018;560(7720):644-8.
70. Ardain A, Domingo-Gonzalez R, Das S, Kazer SW, Howard NC, Singh A, et al. Group 3 innate lymphoid cells mediate early protective immunity against tuberculosis. Nature. 2019;570(7762):528-32.
71. Joosten SA, van Meijgaarden KE, Del Nonno F, Baiocchini A, Petrone L, Vanini V, et al. Patients with Tuberculosis Have a Dysfunctional Circulating B-Cell Compartment, Which Normalizes following Successful Treatment. PLoS pathogens. 2016;12(6): e1005687.
72. Phuah J, Wong EA, Gideon HP, Maiello P, Coleman MT, Hendricks MR, et al. Effects of B Cell Depletion on Early *Mycobacterium tuberculosis* Infection in Cynomolgus Macaques. Infection and immunity. 2016;84(5):1301-11.
73. Rijnink WF, Ottenhoff THM, Joosten SA. B-Cells and Antibodies as Contributors to Effector Immune Responses in Tuberculosis. Frontiers in immunology. 2021; 12:640168.
74. Hermann C, King CG. TB or not to be: what specificities and impact do antibodies have during tuberculosis? Oxford Open Immunology. 2021;2(1).
75. Fletcher HA, Snowden MA, Landry B, Rida W, Satti I, Harris SA, et al. T-cell activation is an immune correlate of risk in BCG vaccinated infants. Nature communications. 2016; 7:11290.
76. Irvine EB, O'Neil A, Darrah PA, Shin S, Choudhary A, Li W, et al. Robust IgM responses following vaccination are associated with prevention of *Mycobacterium tuberculosis* infection in macaques. bioRxiv. 2021:2021.05.06.442979.
77. Lu LL, Chung AW, Rosebrock TR, Ghebremichael M, Yu WH, Grace PS, et al. A Functional Role for Antibodies in Tuberculosis. Cell. 2016;167(2):433-43. e14.
78. Grace PS, Dolatshahi S, Lu LL, Cain A, Palmieri F, Petrone L, et al. Antibody Subclass and Glycosylation Shift Following Effective TB Treatment. Frontiers in immunology. 2021; 12:679973.
79. Strong EJ, Lee S. Targeting Autophagy as a Strategy for Developing New Vaccines and Host-Directed Therapeutics Against Mycobacteria. Frontiers in microbiology. 2020; 11:614313.
80. Towards Zero Leprosy. Global Leprosy (Hansen's disease) Strategy 2021–2030. New Delhi: World Health Organization, Regional Office for South-East Asia; 2017. Licence: CC BY-NCSA 3.0 IGO.
81. Serra-Vidal MM, Latorre I, Franken KL, Diaz J, de Souza-Galvao ML, Casas I, et al. Immunogenicity of 60 novel latency-related antigens of *Mycobacterium tuberculosis*. Frontiers in microbiology. 2014; 5:517.
82. Arroyo L, Marín D, Franken K, Ottenhoff THM, Barrera LF. Potential of DosR and Rpf antigens from *Mycobacterium tuberculosis* to discriminate between latent and active tuberculosis in a tuberculosis endemic population of Medellin Colombia. BMC Infect Dis. 2018;18(1):26.
83. Lewinsohn DA, Swarbrick GM, Park B, Cansler ME, Null MD, Toren KG, et al. Comprehensive definition of human immunodominant CD8 antigens in tuberculosis. NPJ Vaccines. 2017;2.
84. Tang ST, van Meijgaarden KE, Caccamo N, Guggino G, Klein MR, van Weeren P, et al. Genome-based *in silico* identification of new *Mycobacterium tuberculosis* antigens activating polyfunctional CD8+ T cells in human tuberculosis. Journal of immunology (Baltimore, Md: 1950). 2011;186(2):1068-80.
85. Scriba TJ, Carpenter C, Pro SC, Sidney J, Musvosvi M, Rozot V, et al. Differential Recognition of *Mycobacterium tuberculosis*-Specific Epitopes as a Function of Tuberculosis Disease History. American journal of respiratory and critical care medicine. 2017;196(6):772-81.
86. Moguche AO, Musvosvi M, Penn-Nicholson A, Plumlee CR, Mearns H, Geldenhuys H, et al. Antigen Availability Shapes T Cell Differentiation and Function during Tuberculosis. Cell host & microbe. 2017;21(6):695-706.e5.

87. Clemmensen HS, Dube JY, McIntosh F, Rosenkrands I, Jungersen G, Aagaard C, et al. *In vivo* Antigen Expression Regulates CD4 T Cell Differentiation and Vaccine Efficacy against *Mycobacterium tuberculosis* Infection. mBio. 2021;12(2).
88. Shah JA, Lindestam Arlehamn CS, Horne DJ, Sette A, Hawn TR. Nontuberculous Mycobacteria and Heterologous Immunity to Tuberculosis. The Journal of infectious diseases. 2019;220(7):1091-8.
89. Lindestam Arlehamn CS, Paul S, Mele F, Huang C, Greenbaum JA, Vita R, et al. Immunological consequences of intragenus conservation of *Mycobacterium tuberculosis* T-cell epitopes. Proceedings of the National Academy of Sciences of the United States of America. 2015;112(2): E147-55.
90. Pai M, Behr MA, Dowdy D, Dheda K, Divangahi M, Boehme CC, et al. Tuberculosis. Nature Reviews Disease Primers. 2016;2(1):16076.
91. Zak DE, Penn-Nicholson A, Scriba TJ, Thompson E, Suliman S, Amon LM, et al. A blood RNA signature for tuberculosis disease risk: a prospective cohort study. Lancet (London, England). 2016;387(10035):2312-22.
92. Suliman S, Thompson EG, Sutherland J, Weiner J, 3rd, Ota MOC, Shankar S, et al. Four-Gene Pan-African Blood Signature Predicts Progression to Tuberculosis. American journal of respiratory and critical care medicine. 2018;197(9):1198-208.
93. Flynn JL, Gideon HP, Mattila JT, Lin PL. Immunology studies in non-human primate models of tuberculosis. Immunol Rev. 2015;264(1):60-73.
94. Thompson EG, Shankar S, Gideon HP, Braun J, Valvo J, Skinner JA, et al. Prospective Discrimination of Controllers From Progressors Early After Low-Dose *Mycobacterium tuberculosis* Infection of Cynomolgus Macaques using Blood RNA Signatures. The Journal of infectious diseases. 2018;217(8):1318-22.
95. Mothé BR, Lindestam Arlehamn CS, Dow C, Dillon MBC, Wiseman RW, Bohn P, et al. The TB-specific CD4(+) T cell immune repertoire in both cynomolgus and rhesus macaques largely overlap with humans. Tuberculosis (Edinburgh, Scotland). 2015;95(6):722-35.
96. Andrews JR, Noubary F, Walensky RP, Cerda R, Losina E, Horsburgh CR. Risk of progression to active tuberculosis following reinfection with *Mycobacterium tuberculosis*. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. 2012;54(6):784-91.
97. Boom WH, Schaible UE, Achkar JM. The knowns and unknowns of latent *Mycobacterium tuberculosis* infection. The Journal of clinical investigation. 2021;131(3).
98. Urdahl KB. Understanding and overcoming the barriers to T cell-mediated immunity against tuberculosis. Seminars in immunology. 2014;26(6):578-87.
99. Ernst JD, Cornelius A, Desvignes L, Tavs J, Norris BA. Limited Antimycobacterial Efficacy of Epitope Peptide Administration Despite Enhanced Antigen-Specific CD4 T-Cell Activation. The Journal of infectious diseases. 2018;218(10):1653-62.
100. Moise L, Gutierrez AH, Bailey-Kellogg C, Terry F, Leng Q, Abdel Hady KM, et al. The two-faced T cell epitope: examining the host-microbe interface with JanusMatrix. Hum Vaccin Immunother. 2013;9(7):1577-86.
101. Ottenhoff TH, Verreck FA, Lichtenauer-Kaligis EG, Hoeve MA, Sanal O, van Dissel JT. Genetics, cytokines and human infectious disease: lessons from weakly pathogenic mycobacteria and salmonellae. Nature genetics. 2002;32(1):97-105.
102. Ottenhoff TH. New pathways of protective and pathological host defense to mycobacteria. Trends Microbiol. 2012;20(9):419-28.
103. Srivastava S, Grace PS, Ernst JD. Antigen Export Reduces Antigen Presentation and Limits T Cell Control of *M. tuberculosis*. Cell host & microbe. 2016;19(1):44-54.
104. Grace PS, Ernst JD. Suboptimal Antigen Presentation Contributes to Virulence of *Mycobacterium tuberculosis In vivo*. Journal of immunology (Baltimore, Md: 1950). 2016;196(1):357-64.

105. Kaufmann SHE, Dockrell HM, Drager N, Ho MM, McShane H, Neyrolles O, et al. TBVAC2020: Advancing Tuberculosis Vaccines from Discovery to Clinical Development. Frontiers in immunology. 2017; 8:1203.
106. Smith CM, Proulx MK, Olive AJ, Laddy D, Mishra BB, Moss C, et al. Tuberculosis Susceptibility and Vaccine Protection Are Independently Controlled by Host Genotype. mBio. 2016;7(5).
107. Davids M, Pooran A, Hermann C, Mottay L, Thompson F, Cardenas J, et al. A Human Lung Challenge Model to Evaluate the Safety and Immunogenicity of PPD and Live Bacillus Calmette-Guérin. American journal of respiratory and critical care medicine. 2020;201(10):1277-91.
108. Pardi N, Hogan MJ, Porter FW, Weissman D. mRNA vaccines–a new era in vaccinology. Nat Rev Drug Discov. 2018;17(4):261-79.
109. Frick M. The TB vaccine pipeline. New York: Treatment Action Group; 2020.