REASSESSING TWENTY YEARS OF VACCINE DEVELOPMENT AGAINST TUBERCULOSIS

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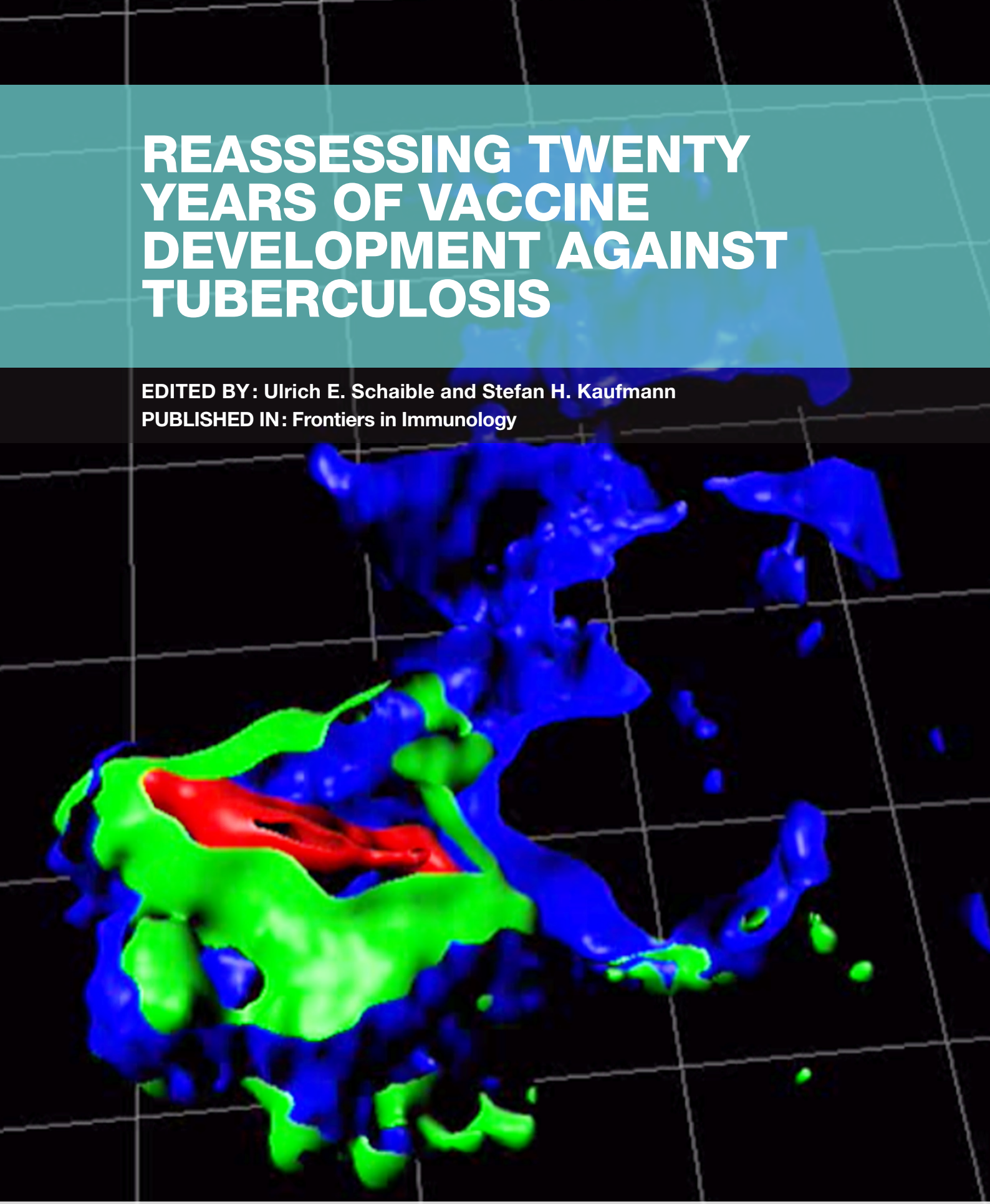

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REASSESSING TWENTY YEARS OF VACCINE DEVELOPMENT AGAINST TUBERCULOSIS

Topic Editors:
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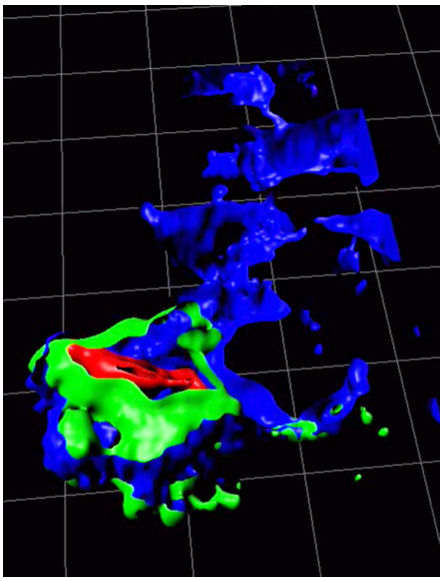
Tuberculosis (TB) remains the prime bacterial infection worldwide with 10.4 million infections and a death toll of 1.7 million people in 2016 according to WHO statistics. Tuberculosis is caused by members of the *Mycobacterium tuberculosis* complex, facultative intracellular bacteria able to thrive within otherwise potent innate defense cells, the macrophages. In a world of increasing numbers of infections with drug resistant *M. tuberculosis* strains, the daunting race between developing new therapeutics and emerging resistant strains will hardly produce a winner. This cycle can only be broken by enhancing population wide immune control through a better vaccine as the only one currently in use, *M. bovis* Bacillus Calmette Guerin (BCG). The protective efficacy of BCG against pulmonary tuberculosis in all age groups is dissatisfying and geographically highly diverse with the tropical areas showing the lowest efficacy rates. Despite worldwide vaccination coverage, the impact of BCG on the steep decrease of tuberculosis incidence rates in the developed world seems therefore questionable and can rather be attributed to improved social, housing and nutritional conditions, better health care, surveillance and treatment systems. The last 15 years saw tremendous efforts to improve vaccination strategies against tuberculosis. Different paths of vaccine approaches were followed including genetically improved BCG strains, attenuated *M. tuberculosis* variants, recombinant viral vectors and subunit vaccine candidates combined with novel more potent adjuvants. With the first novel vaccine candidates being evaluated in clinical phases II and III and initial results chastening the expectations, a critical reassessment of all candidates is inevitable. Here, we assembled experts to review and assess the current status of novel anti-tuberculosis vaccine candidates, their efficacy and prospects for implementation as well as the pitfalls and possible measures for improvement.



Human neutrophils infected with virulent *Mycobacterium tuberculosis* quickly succumb to necrotic cell death. However, when they get infected by attenuated *M. tuberculosis* strains such as those lacking the ESX1 secretion system they die of apoptosis. *M. tuberculosis* delivered into macrophages through necrotic neutrophils interfere with phagosome maturation and proliferate, whereas those enwrapped in apoptotic neutrophils end up in phagolysosomes and are controlled. Therefore, apoptotic host cell death is beneficial for protective immunity (See: Schaible et al. in this issue). The image depicts a LAMP1-positive (blue) phagolysosome containing an attenuated ESX1-deficient *M. tuberculosis* mutant (red) enwrapped in an apoptotic neutrophil (green).

Image: Tobias Dallenga, Forschungszentrum Borstel.

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EDITORIAL
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Editorial: Reassessing Twenty Years of Vaccine Development against Tuberculosis

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Editorial on the Research Topic

Reassessing Twenty Years of Vaccine Development against Tuberculosis

Tuberculosis remains the most threatening bacterial infection worldwide with 10.4 million infections and a death toll of 1.7 million people in 2016 according to World Health Organization (WHO) statistics (1). This makes tuberculosis the deadliest infectious disease of all. To those not familiar with this situation, this generally comes as a big surprise because drugs, diagnostics, and a vaccine are available as control measures. Currently, means of interventions, however, are insufficient, and better ones are urgently needed for early detection, successful treatment, and efficacious prevention.

20 years ago, in March 1997, the (WHO) had reported on the successful introduction of a novel therapeutic regiment, directly observed treatment, short course (DOTS). Compared with a 41% cure rate of other treatment programs, DOTS achieved a 77% rate. In a scenario, when tuberculosis was estimated to kill annually at least one-quarter of all patients worldwide, DOTS was therefore hailed as important milestone in tuberculosis control. It was hoped that the global tuberculosis epidemic could be controlled by the beginning of the twenty-first century, whereas otherwise an increase to nearly nine million annual cases by 2005 was projected. Indeed, numbers of deaths have been reduced by 2017; however, 10.8 million new tuberculosis cases in 2016, as well as the global increase in incidences of patients infected with multidrug resistant (MDR) strains spoiled the success story. Number of cases with MDR tuberculosis resistance against the two frontline drugs, such as rifampicin and isoniazid, tripled between 2009 and 2016 to 480,000 cases. Alarmingly, in several East European and Central Asian countries, the rate of MDR tuberculosis cases already exceeds 50% (1). Importantly, treatment of MDR patients comes with a significant economic burden to both the individual and the society (2). Despite the glimmer of hope provided by the recent introduction into the clinics of two new antimycobacterial drugs, such as Bedaquiline and Delamanid, patients with strains resistant to these new drugs have already been reported (3).

Consequently, the G20 Leader's Declaration from June 2017 explicitly singled out MDR tuberculosis to be tackled with highest priority over other infections (4). This has led to the WHO Global Ministerial Conference on "Ending TB in Sustainable Development Era: A Multisectoral Response" in Moscow, November 16–17,[1] which will be followed by The Stop TB Partnership Board Meeting in Delhi, March 2018.[2] These two meetings are paving the way for the planned high level meeting of heads of state on tuberculosis to be held at the UN in New York, September 2018. Thus, tuberculosis has finally gotten on the agenda of political decision makers at highest level.

[1] http://www.who.int/tb/features_archive/Online_Consultation_MinisterialConferenceDeclaration/en/.
[2] www.stoptb.org.

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Tuberculosis is caused by members of the *Mycobacterium tuberculosis* complex, facultative intracellular bacteria able to thrive within otherwise potent innate defense cells, such as macrophages. More than 90% of all individuals infected with *M. tuberculosis* remain in a lifelong latent stage by immunity-mediated sequestering of the mycobacteria within granulomas. Reactivation of a latent infection occurs in the remaining ~10%, usually due severe immune suppressive conditions. Thus, in principle, human immunity is able to control—but likely not sterilely eliminates—the infection.

Unfortunately, the daunting race between the development of new therapeutic compounds and the evolution of resistance mutations will hardly produce a winner. This cycle can only be broken by enhancing population wide immune protection through a better vaccine. Immune protection of the only antituberculosis vaccine currently in use, *Mycobacterium bovis* bacillus Calmette–Guerin (BCG) is limited to severe disseminated primary infections in early childhood. By contrast, its protective efficacy against pulmonary tuberculosis in all age groups is dissatisfying, and geographically highly diverse with the tropical areas showing the lowest efficacy rates. Despite high worldwide vaccination coverage, the impact of BCG on the steep decrease of tuberculosis incidence rates in the developed world is therefore questionable and can rather be attributed to improved social, housing, and nutritional conditions, better health care, surveillance, and treatment systems. Consequently, the last 20 years saw tremendous efforts to improve vaccination strategies against tuberculosis.

For this Research Topic, we assembled experts to review and assess the current status of novel antituberculosis vaccine candidates, their efficacy, and prospects for implementation as well as the pitfalls and possible measures for improvement. Different rational approaches of vaccine design were followed in recent years, and the first novel vaccine candidates are currently being evaluated in clinical phases II and III, and initial results chastening the expectations. Therefore, a critical reassessment of all candidates is inevitable. The different strategies followed include genetically improved BCG strains, attenuated *M. tuberculosis* variants, recombinant viral vectors, and subunit vaccine candidates combined with novel more potent adjuvants. In addition, immune targets of novel vaccine types and immune enhancing strategies to improve vaccination efficacy have been identified.

In our Research Topic, Cardona summarizes how our current knowledge of the immune responses to *M. tuberculosis* can instruct vaccine design. The lessons learned from the current BCG vaccine and its future potential is detailed by Dockrell and Smith. The TBVAC2020 Consortium, consisting of a large number of European researchers and funded by the European Union, summarizes the different tuberculosis vaccine candidates, which they are developing and where they stand. This review also includes a comprehensive table of all current tuberculosis vaccine types studied. Nieuwenhuizen et al. discuss the current state of the promising recombinant BCG VPM1002, which has advanced into clinical phase III. Gonzalo-Asensio et al. review the attenuated vaccine strain MTBVAC, which is based on the deletion of two virulence-associated genes in a clinical *M. tuberculosis* isolate. The role of polyfunctional CD4+ T cells in immune protection against *M. tuberculosis* is summarized by Lewinsohn et al. To enhance vaccine efficacy, different strategies, which can modulate innate immunity during vaccination, are discussed by Schaible et al., whereas Lang and Schick look into how helminth infections affect antituberculosis immunity at the macrophage level.

In summary, we believe that this Research Topic provides an excellent overview on the current state of antituberculosis vaccine and corresponding research and the prospect of vaccine success and failure.

AUTHOR CONTRIBUTIONS

SK and US together wrote the Editorial.

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Conflict of Interest Statement: SHEK is coinventor of a vaccine against tuberculosis currently undergoing clinical trial testing. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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What Have We Learnt about BCG Vaccination in the Last 20 Years?

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A number of new tuberculosis (TB) vaccines have been or are entering clinical trials, which include genetically modified mycobacteria, mycobacterial antigens delivered by viral vectors, or mycobacterial antigens in adjuvant. Some of these vaccines aim to replace the existing BCG vaccine but others will be given as a boosting vaccine following BCG vaccination given soon after birth. It is clear that the existing BCG vaccines provide incomplete and variable protection against pulmonary TB. This review will discuss what we have learnt over the last 20 years about how the BCG vaccine induces specific and non-specific immunity, what factors influence the immune responses induced by BCG, and progress toward identifying correlates of immunity against TB from BCG vaccination studies. There is still a lot to learn about the BCG vaccine and the insights gained can help the development of more protective vaccines.

Keywords: BCG, vaccination, tuberculosis, efficacy, biomarkers, correlates of protection, immune responses, infants

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INTRODUCTION

The BCG vaccine has been used since 1921 to prevent tuberculosis (TB) and is considered to be the world's most widely used vaccine (1). Yet, it is well established that the protective efficacy of BCG varies depending in which geographical location it is administered and we understand very little about why it protects when it does, or why it fails to protect when it does not. This review will survey what we have learnt about BCG over the last 20 years. Although a lot of progress has been made, the past two decades have shown that there is no simple correlate of BCG-induced protection against TB even though T-cells and IFNγ are clearly required.

We still need to understand why BCG gives such variable protection against pulmonary TB in different settings. The BCG vaccine remains critical for the development of new TB vaccines, many of which will be given as a booster vaccine after BCG vaccination, and any factors that affect how BCG works may also impact on these as well as on new genetically modified BCG vaccines or other live mycobacterial vaccines (2, 3).

Neonatal BCG provides good protection against disseminated and pulmonary TB disease in young children (4) but variable efficacy against pulmonary TB in adults when given later in life. However, when it does protect, this protection can be long-lived, lasting for up to 15 years in the United Kingdom (5), 30–40 years in Norway (6), and even as long as 50–60 years in Alaska (7). There is also evidence from outbreak settings to support the hypothesis that BCG vaccination can protect against infection, as well as disease; for example, an association has been observed between the presence of a BCG scar and not only less disease but also lower rates of interferon-gamma release assay (IGRA) positivity with relative risks for vaccinated compared with unvaccinated children of 0.61 for infection and 0.51 for disease (8).

Over the last 20 years most of the world's children have continued to receive BCG immediately after birth or when they are first in contact with health services. In 2005, the United Kingdom, which had previously vaccinated adolescents, switched to a targeted vaccination of infants at higher risk of infection (i.e., with parents or grandparents born in high incidence countries or who live in areas of the United Kingdom where the annual incidence of TB exceeds 40/100,000). Other countries have never given BCG, in order to retain the use of the tuberculin skin test (or the newer IGRA tests) as a means of assessing infection or because the risk of infection is low (9). BCG scars are often the best indication of prior vaccination that is available, but are an imperfect proxy for prior vaccination particularly if infants are vaccinated after birth (10).

Vaccination of young infants can, however, lead to disseminated BCGosis if the infant is HIV-infected (11). For this reason, the Global Committee on Vaccine Safety recommended that BCG vaccine should not be given to infants who were HIV infected. As HIV status is generally not known and as even in areas with higher HIV infection rates, most mothers will be on antiretroviral treatment, and their infants given post exposure prophylaxis, most infants are still being vaccinated without testing. As well as acquired immunodeficiencies, inherited genetic disorders of the immune system also confer susceptibility on infants to disseminated BCG infection following vaccination as well as to childhood TB [reviewed in detail here (12)]. A safer BCG for use in immunocompromised infants would therefore be beneficial.

WHAT HAVE WE LEARNT ABOUT THE CAUSES OF VARIABLE PROTECTION INDUCED BY BCG OVER THE LAST 20 YEARS?

Pre-Sensitization with Environmental Mycobacteria

BCG vaccination immediately or shortly after birth provides consistent protection against the disseminated forms of TB in young children (4, 13, 14), and is very cost effective (15). Neonatal BCG also protects against pulmonary TB in children (4) which contrasts with the variable protection seen against pulmonary TB in adolescents and young adults (16).

BCG vaccination is more protective against pulmonary disease in children and adults at higher latitudes and in those screened for prior sensitization by mycobacteria (4, 17). Environmental mycobacterial are often assumed to be the culprits, mycobacteria species that survive in different environmental niches, and colonize humans over time (18). The impact of prior sensitization, as detected by tuberculin skin testing, on the protective efficacy of BCG against pulmonary TB has been shown in a number of settings (4) but not whether this induces masking of the BCG vaccine effect (inducing protection that BCG cannot improve upon) or blocking (inducing pre-existing immune responses that prevent BCG vaccine-induced, protective responses) (19). One reason for limited progress has been the lack of antigens specific for environmental mycobacteria with which to dissect T-cell responses to the different mycobacteria other than tuberculosis or non-tuberculous mycobacteria (NTM; e.g., *Mycobacterium avium*) (20). Skin test positivity to *Mycobacterium tuberculosis* purified protein derivative (PPD or tuberculin) increases with age, although indurations >10 mm are less frequent in those who are BCG vaccinated (21). It is widely assumed that the effects of environmental mycobacteria or NTM would precede BCG vaccination, perhaps blocking BCG multiplication (22, 23); however, this is unlikely for infants vaccinated soon after birth, and too little work has focused on their effects post-BCG. In the mouse model, oral dosing with NTMs following BCG vaccination decreased protection (24).

BCG Strain Variations

Genetic variability between the different BCG vaccine strains might explain variable protection. The original attenuated *Mycobacterium bovis* bacille Calmette Guerin was kept in culture for many years before it was properly conserved and seed lots were preserved by lyophilization. It was distributed to different locations worldwide where further culture resulted in what are now known as BCG Russia, Japan, Copenhagen, etc. Improved sequencing methods have shown that further genetic events have occurred in these strains, including additional genetic deletions (25, 26). Although some countries have only used one strain of BCG, in others, two or more strains have been used making analysis difficult (27). The different vaccine strains induce varying degrees of T-cell response in *in vitro* peripheral blood mononuclear cell (PBMC) cultures from those vaccinated with different BCG vaccine strains (19, 28). However, to date there has been no evidence that such genetic variation between strains accounts for variability in efficacy or that the protection given by a particular BCG vaccine strain is related to the year in which the study was performed (4).

Route of Administration

BCG vaccine is delivered most often by the intradermal route. It can be given percutaneously by a multi-puncture device rather than intradermally but a large study in South Africa showed both delivery routes were equally protective (29, 30). Most data describing the impact of administration route on vaccine efficacy has come from animals. Originally BCG was given orally and studies in guinea pigs have shown that BCG can be as protective and with less pathology induced post-infection if given BCG is given orally (31). Giving both a BCG prime and an MVA85A boost to mice intranasally induced much more effective protection than seen using other routes of immunization (32), and more recently there is renewed interest in the mucosal route of vaccination (33). In rhesus macaques, improved BCG-induced protection was observed when the vaccine was administered endobronchially into the lungs when compared with parenteral vaccination (34).

There is also a body of data on the effect of administration route on the quantity and quality of the immune response induced. Percutaneous and intradermal routes induced similar immunogenicity in a study in South Korea (35). Giving BCG followed by the ID93 vaccine in GLA adjuvant intranasally induced tissue resident T-helper (Th)-17 T-cells rather than Th-1 T-cell immunity (36) and when BCG itself was given by the mucosal

route, this resulted in effector memory and resident memory T-cells in the mouse lung (37).

Vaccine Batch Variations

There have also been questions about viability or growth rate variations in different batches of vaccine, which are only required to have colony forming units between set limits (and thus may also contain varying numbers of dead bacilli). A study in West Africa found that batches of BCG showing slower growth were associated with larger BCG scars, and a higher prevalence of positive PPD skin test responses in the Mantoux skin test (38). Issues of batch or vaccine strain variability are very challenging to study at scale, as the EPI program will ship vaccine from one of several suppliers and thus, as mentioned above, many regions can have used three or more types of vaccine (9, 27), as well as different batches of the same vaccine strain.

Global Differences in Non-Specific Protection?

There has been interest in the benefits of administering BCG for reasons other than as a vaccine against TB for many years. For example, BCG vaccine can be instilled into the bladder where it is an effective immunotherapy against non-muscle invasive bladder cancer (39, 40). BCG vaccination is also associated with reduced rates of asthma but despite a number of observational studies, analyzed in two meta-analyses (41, 42), there have only been two randomized clinical trials using neonatal BCG vaccination and only one trial showed some beneficial effect (43, 44).

Studies in West Africa have indicated that BCG vaccination may have beneficial effects on all-cause mortality in infants, although the effects were most marked in low birth weight infants (45). Until recently, these effects had not been examined in infants in higher income or latitude settings, so there was a lot of interest in a study in Copenhagen that hypothesized that giving BCG at birth to Danish infants would reduce the number of somatic infections. In 2,129 BCG vaccinated and 2,133 unvaccinated controls, BCG vaccination had no effect on all-cause hospitalizations [hazard ratio 1.05 (95% confidence intervals 0.93–1.18)] or on parent-reported infections up to the age of 15 months. There was no difference between outcomes in normal birth weight infants or premature infants in this European setting (46). Some of the previously described non-specific effects of vaccination show sex differences; however, in this Danish study, there was no difference in outcomes by sex (46). Thus, any non-specific effects may be more marked in certain low and middle-income country settings.

DISSECTING IMMUNE RESPONSES TO BCG AT THE CELLULAR AND MOLECULAR LEVEL

Early Events following Vaccination

BCG is usually administered by intradermal injection. It has been possible, in animals, to investigate the vaccination site tissue for rapid innate immune responses induced by the array of pathogen-associated molecular patterns BCG is endowed with. These are largely components of the mycobacterial cell wall such as peptidoglycans, arabinogalactan, and mycolic acids that interact with Toll-like receptors (47) and other pattern recognition receptors such as complement receptor and mannose receptor as reviewed recently (48). The immediate response is characterized by inflammatory cytokines in BCG "lesions," including IL-1β, TNFα, MCP-1, and IL-8, as demonstrated in rabbits. A biphasic pattern of expression indicated that the source of the cytokines is local resident innate cells (days 1–3) followed later by infiltrating mononuclear cells (day 9) (49). Although most studies of the immune response to BCG in humans focus on peripheral blood, punch biopsies at the vaccination site have revealed that live BCG persists until at least 4 weeks post-vaccination in previously unvaccinated adults. The cellular infiltrate at the vaccination site, as revealed by suction blister analysis included lymphocytes (CD3+) and monocytes (CD14+) but predominantly comprised CD15+ neutrophils (50). Interestingly, human blood neutrophils that have recently captured BCG mycobacteria *in vitro* have been shown to cooperate with dendritic cells to enhance antigen-specific T-cell responses (51). Despite the chronic nature of intracellular mycobacterial infections, neutrophils are also present in the immune response in human TB. Gene expression signatures associated with neutrophils were found in TB disease (52) and in the lungs of patients with TB, many neutrophils containing *M. tuberculosis* are present (53).

BCG lacks the *esx-1* locus which, although providing sufficient attenuation compared with *M. bovis* or *M. tuberculosis* to allow its use as a vaccine, prevents phagosomal rupture, cytosolic contact, and host cell death following uptake by phagocytic antigen presenting cells (54). As such, there are a host of cytosolic immune detection systems that are underused by the vaccine but which may represent pathways for the induction of more potently protective immune responses (54). Clearly, in the case of *M. tuberculosis*, some of these responses are responsible for enhanced virulence *via* increased inflammation, granuloma formation, and damage to lung structure and function however there appears to be an *esx*-related balance to be struck between pathogenicity and immunity. Recombinant BCG expressing ESX-1 from *M. marinum* had much reduced virulence in immunocompromised mice when compared with *M. tuberculosis* itself or BCG expressing ESX-1 from *M. tuberculosis* however immune responses and protection against *M. tuberculosis* challenge were improved when compared with BCG (55). Interestingly for the design of new vaccines for TB, the ESAT-6 related immune responses that are important for the control of *M. tuberculosis* rely, at least in part, on antigen-non-specific, IFNγ-producing CD8+ T-cells, and natural killer cells that are induced *via* the NLRP3-inflammasome-IL-18 pathway (56).

Adaptive Immune Responses to BCG

The primary mechanism of action of BCG-induced protection against TB has long been thought to be Th-1 cell mediated and although the last 20 years have provided clues to the involvement of other effector mechanisms, the Th-1 paradigm remains in place. Resident dendritic cells travel to local lymph nodes bearing antigen/live BCG and activate antigen-specific CD4+ T-cells in the presence of type 1 polarizing cytokines such as IL-12 and IL-18. IFNγ-secreting T-cells therefore represent the

canonical immune response to BCG vaccination (57). Despite robust Th-1 responses in adults following BCG, our understanding of the newborn immune system dictated that an equivalent response to BCG would be unexpected in infants who demonstrate a marked bias toward regulatory and Th-2 response in their natural reaction to microbial challenge. Surprisingly, BCG also proved to be a potent inducer of Th-1 responses in infants (58). The hypothesis that delaying BCG vaccination of infants might circumvent some of the more profound regulatory effects of the newborn immune response and lead to improved type 1 immunity has come in for some scrutiny, however study results have been mixed (see below).

The antigen specificity of BCG-induced T-cells is broad, reflecting the plethora of mycobacterial antigens possessed by BCG which are presented to the immune system (59). Dendritic cells that have collected BCG antigens have the ability to cross-present antigen and BCG-specific CD8+ T-cells are detectable following BCG stimulation of PBMC from BCG-vaccinated donors *in vitro* (60, 61). Improving this component of the BCG-induced vaccine response is one of several approaches being taken to enhance the immunogenicity of BCG and develop a better TB vaccine. One strategy has been the insertion of the Listeriolysin gene designed to facilitate access of BCG antigen to the endogenous antigen processing pathway (62); the BCGΔureC:hly vaccine is more immunogenic in mice than parental BCG and induces Th-1 and IL-17-producing T-cells in man (63, 64). However, development of a recombinant BCG with a mutant Perfringolysin gene overexpressing Ag85A, Ag85B, and Rv3407 has been discontinued as varicella zoster virus was reactivated in 2/8 vaccinees, although the vaccine was immunogenic and induced anti-mycobacterial activity in man (65). Another approach is the use of adenovirus vectors rather than MVA to deliver *M. tuberculosis* antigens following BCG vaccination in a prime-boost regimen, as adenovirus vectors induce a more mixed CD4 and CD8 T-cell activation than MVA which is better at inducing CD4 T-cell responses (66).

One of the most prominent developments in the field of immunology in the last 20 years has been the rise of the Th-17 cell, initially identified in the context of autoimmune disease. Th-17 cell polarization is brought about by the activation of CD4+ T-cells in the presence of TGFβ and IL-6 and the Th-17 population is characterized by the expression of IL-17 and IL-22. Th-17 cells contribute to the granulomatous response to Mtb infection (67) and they are important for immune protection against more virulent, clinical isolates of *M. tuberculosis* (68). Th-17 cells were responsible for enhanced vaccine-induced protection against Mtb challenge in a mouse model (69, 70) and they are often taken as a sign of improved immunogenicity in pre-clinical models of novel TB vaccines. For example, the recombinant BCG vaccine BCGΔureC:hly, which induces increased protection compared with parental BCG in mice, induces increased numbers of IL-17 producing helper T-cells (63), with some increase in CD8 T-cells producing IL-17 in human vaccinees (64). BCG is not considered a potent inducer of Th-17 cells. Our group detected IL-17+ CD4+ T-cells following infant BCG vaccination in the United Kingdom but at much lower frequencies than polyfunctional Th-1 cells (71). One explanation for poor Th-17 responses to BCG may be the strain's lack of the RD1 region as discussed above. The RD1-encoded ESAT-6 protein, which is absent in BCG, is a potent inducer of Th-17 cells which are activated following interactions between ESAT-6 and TLR-2. Improved protective efficacy is seen when BCG is complemented with the ESAT-6 containing RD1 region; and enhanced Th-17 responses appear to be a component of this protection (72) as do antigen non-specific CD8+ and natural killer cells (see above).

B-cell and antibody responses in the context of TB have not been studied to the extent of their T-cell counterparts due to what may turn out to be an under-appreciation of their importance. Recent evidence suggests a role for functionally distinct antibody responses in latent TB infection (73, 74) and antibody and memory B-cell responses in BCG-vaccinated adults have been demonstrated (75, 76) and may correlate with a reduced risk of TB disease in infants (77, 78).

Despite evidence for longevity in the protective efficacy of BCG in some settings, this protection has yet to be attributed to a defined population of memory immune cells. It is difficult in humans to differentiate mycobacteria-specific, long-lived memory cells that are present because of an historic BCG vaccination from responses that are constantly re-stimulated by exposure to environmental mycobacteria. This is easier to study in animal models where exposure to mycobacteria is more easily controlled. In pre-clinical studies of the recombinant BCG vaccine candidate BCGΔureC:hly in mice, there was an association between vaccine-induced protection and CXCR5+ CCR7+ central memory CD4+ T-cells (79). Also, in mice, the quality of the T-cell response to vaccination was reliant upon the persistence of live BCG. Once drug-sensitive BCG bacteria were removed by drug treatment of mice, there was a reduction in antigen-specific T effector cells, and a reduction in protection (80). In man, BCG is still present in vaccination sites at 1 month after vaccination (50) but data on its longer-term survival are limited. Although there have been case reports of longer-term survival of BCG [i.e., where an HIV-infected individual developed disseminated BCG 30 years after vaccination (81)], disseminated BCG infections have not increased dramatically in countries where HIV infection is more prevalent, suggesting that in most cases BCG does not survive long-term in vaccinees.

BCG-Induced Trained Innate Immunity

Although interactions between the BCG vaccine and innate immune system receptors such as complement receptor-3 and TLRs 2 and 4 were previously known (82, 83), a more recent discovery is the ability of BCG to bestow a type of immunological memory on innate cells, so-called trained innate immunity (84, 85). Mediated by NOD2 receptor signaling and epigenetic modification of macrophages, the phenomenon is characterized by an enhanced ability of macrophages to produce cytokines such as TNFα and IL-6 in response to unrelated microorganisms and TLR ligands following BCG vaccination. Natural killer cells have also been demonstrated to contribute to the trained innate effect both in humans and in mice (86). Trained innate cells alter their metabolism, switching from oxidative phosphorylation to aerobic glycolysis (87). Trained immunity is reduced if monocytes from controls are stimulated with irradiated BCG bacilli rather than live BCG (88). Trained innate immunity is clearly a

prime candidate mechanism to explain the non-specific protective effects of BCG described above. To this end, our group and others have investigated whether BCG vaccination of infants has the same effect on the innate immune response. Jensen et al. in a cohort of West African infants and our group in United Kingdom infants both showed evidence of enhanced innate responses to non-specific stimuli following infant BCG vaccination (89, 90).

FACTORS THAT AFFECT BCG-INDUCED IMMUNE RESPONSES

Vaccination Setting

As noted above BCG vaccination is less effective against pulmonary TB in some settings. This phenomenon has been explored using immunological analysis, which confirmed that >80% of United Kingdom adolescents did not make an IFNγ response to PPD in diluted whole blood cultures before vaccination, whereas over 60% of Malawian adolescents and young adults did; 12 months post-vaccination there was still marked immunological memory present in most of the United Kingdom vaccinees whereas the Malawian vaccinees did not show a vaccine-induced enhancement of their responses (91). Less expected was the finding that Malawian infants, presumed to be immunologically naïve when vaccinated, also made lower IFNγ responses than United Kingdom infants (92) with reduced Th-1 and increased Th-2 cytokine responses (93). In the United Kingdom, BCG-vaccinated infants show increased BCG growth inhibition compared with unvaccinated infants (71); similar studies have not yet been reported for African infants. Again, there are other reports where growth inhibition is not associated with BCG vaccination, or with protection. Although BCG-induced immunity can be long-lived, there was no association between childhood BCG vaccination and mycobacterial growth inhibition in human bronchoaleolar lavage cells from TB contacts as assessed by colony counts (94). In a study of BCG-vaccinated South African infants, tested at 4–6 months of age, there was no significant association between growth inhibition of BCG and subsequent development of disease (77).

A number of other explanations have been suggested for varying immunogenicity and protective efficacy of the BCG vaccine. There are clearly factors related to location or setting. There are seasonal influences that determine how T-cell responses are induced or maintained, as shown for IFNγ responses in Malawi (92). Varying vaccination schedules may affect immunogenicity, as shown in some studies where, when BCG vaccine was given with oral polio vaccine (OPV) in Guinea Bissau, IFNγ, and IL-5 responses to PPD were reduced (95); however, any such immunosuppressive effects resulting from oral live polio vaccination should now reduce as OPV is replaced with inactivated polio vaccine.

Maternal Factors

In some of these settings, many mothers will be infected with latent TB, and this could lead to either *in utero* sensitization or tolerization to mycobacterial antigens. However, so far LTBI in mothers has only been shown to have a transient effect on responses to mycobacterial antigens in their infants (96, 97). Maternal BCG scar was associated with stronger proinflammatory cytokine responses to innate stimuli in infant cord blood, but not to stronger responses in the mothers themselves (98). Other maternal infections may also modulate BCG-induced immunity. Although infants will not acquire helminth infections until well after BCG vaccination, in LMIC many of their mothers will carry helminth infections. There was no clear association of maternal helminth infection with the infants immune responses to vaccination (99) and treating pregnant women in the second or third trimester with albendazole, praziquantel, or both did not affect infant responses to BCG, tetanus, or measles vaccination (100). Co-infections in the infants themselves could also modulate immunity. Cytomegalovirus infection is acquired early in life by most infants in Africa and has a profound effect on the immune system (101–103). Other factors could include nutrition, genetics, and season of birth (104, 105).

Delayed BCG Vaccination

Many infants do not receive BCG vaccination immediately after birth, and delays in vaccination might improve immunity, due to maturation of the immune system, which is skewed toward Th-2 T-cell immune responses at birth. However, delaying BCG vaccination has shown less consistent effects on BCG-induced immunogenicity than might have been expected (58, 106–110). Partly this lack of consensus results from variations in the approaches taken by different studies, including the age until which vaccination is delayed, the time points post-vaccination at which blood samples are obtained and analyzed and the range of assays used and immunological functions investigated. Delayed BCG may circumvent the risks associated with at birth vaccination of HIV-exposed infants. Tchakoute et al. reported that immune responses in HIV-exposed, uninfected infants vaccinated at 8 weeks of age were at least as robust as those in infants vaccinated at birth and in some respects (enhanced CD4 T-cell IFNγ responses and increase cytokine functionality in CD4 and CD8 T-cells) delayed vaccination was better (110). Kagina et al. demonstrated better T-cell cytokine responses (including polyfunctional CD4 T-cells) at 1 year in infants where BCG was delayed to 10 weeks of age. The differences detectable at an earlier time point of 10 weeks post-vaccination were more modest which agrees with the findings of Ritz et al. who conducted a similar study (107, 109).

In contrast to the studies mentioned above in which delaying BCG was either not different or in some cases better than vaccination at birth, two further studies reported that immune responses were enhanced in infants who received BCG at birth compared with those in which it was delayed. In the Gambia, Th-1 and Th-17 responses measured 4.5 months after vaccination were reduced when BCG was delayed to 4.5 months (approximately 19 weeks of age) however the differences became less apparent when samples were obtained later, at 9 months of age (106). In contrast Lutwama et al. found that at 9 months of age it was still possible to detect better CD4 and CD8 T-cell cytokine responses in infants vaccinated at birth compared with those vaccinated at 6 weeks of age (108). In addition to the variables mentioned above, the different settings of all these studies should be noted. With the exception of the study by Ritz

et al., performed in Australia (109), most of these studies were performed in Africa, although they were located in different settings; some of which (Uganda and the Gambia) were more equatorial than others (South Africa). The same factors that determine the geographical variation in BCG efficacy may also account for differences in the effect of delayed vaccination.

TOWARD CORRELATES OF PROTECTION

T-Cell and B-Cell Immune Responses as Correlates of Protection

Although most of these studies have investigated immunogenicity rather than protection, a large study based in South Africa that recruited 5,726 BCG-vaccinated infants and followed them for 2 years, has enabled correlates of risk of disease to be investigated more directly. In this cohort, the proportion of polyfunctional T-cells in BCG stimulated cultures was not associated with reduced risk of developing TB disease (111). Despite comprehensive analyses using gene expression, correlates of risk could not be identified. The protected and unprotected infants showed marked heterogeneity in gene expression patterns, with distinct subsets in both protected and unprotected groups with higher or lower monocyte:lymphocyte ratios and myeloid or lymphoid cell activation (112) (see below). In the only other BCG-vaccinated infant cohort followed for protection against the development of disease, infants from the MVA85A vaccine trial in which the MVA85A vaccination following BCG had not induced significant efficacy (113) were assessed for biosignatures associated with protection or progression. T-cell activation (CD4 T-cells expressing HLA DR) was associated with increased risk of progression to disease, but BCG-induced IFNγ producing T-cells were associated with reduced risk of TB (77).

Perhaps as a response to the lack of confirmed T-cell correlates of protection induced by BCG vaccination, there is renewed interest in B-cell immunity in TB, which as noted above has been under-studied. Although antibodies have not provided useful diagnostic tests for TB, expression of B-cell-associated genes are modulated during TB treatment (114) and the frequencies of plasmablasts are increased in those with active TB disease compared with healthy community controls, and community controls with evidence of latent TB infection have higher numbers of both plasmablasts and memory B cells (78). Memory B cells specific for PPD were higher in BCG-vaccinated adult donors than unvaccinated subjects, even from individuals vaccinated 13–45 years earlier (75). It is therefore interesting that in infants from the MVA vaccine trial higher concentrations of IgG antibodies to Ag85 at 5–7 months of age were associated with a reduced risk of subsequent TB (77).

Unbiased Functional Assays of BCG-Induced Protection: Mycobacterial Growth Inhibition

Functional assays that do not rely on prior knowledge of specific immune correlates of protection have been of interest for some time. Such assays can potentially indicate vaccine-induced protection in easily accessible samples (e.g., whole blood or PBMC) and are unbiased in the sense that they do not focus on any given immune component. Early interest in mycobacterial growth inhibition as a surrogate marker of protection involved the *in vitro* expansion of lymphocytes with relevant antigens prior to incubation with BCG-infected monocytes. PBMC previously stimulated with mycobacterial whole cell lysate or live BCG induced infected monocytes to control the growth of BCG better as did previous BCG vaccination of donors themselves (115). In another study using an assay that did not rely on *in vitro* stimulation of PBMC, *ex vivo* whole blood or fresh PBMC from adults who were recently BCG vaccinated (either primary vaccination or re-vaccination) were incubated with live BCG and growth inhibition determined by Bactec MGIT analysis. The study reported that PBMC from adults who had received primary BCG did control mycobacterial growth better *ex vivo* than unvaccinated controls (116). In a study of infants vaccinated in early life (at approximately 7 weeks of age), our group demonstrated better PBMC-mediated growth inhibition of BCG in vaccinated infants compared with unvaccinated controls 3 months after vaccination, although the effect had waned by 12 months (71). Despite these positive data supporting the use of mycobacterial growth inhibition as a surrogate marker of vaccine-induced protection, it should be noted that, as with vaccine-induced T-cell responses, growth inhibition was not associated with reduced risk of TB disease in BCG-vaccinated South African infants (77). The concept of mycobacterial growth inhibition has been taken a step further with human challenge models whereby protection is assessed following the administration of an *in vivo* mycobacterial challenge, usually an intradermal inoculation of live BCG (117, 118). Such an approach has allowed the association of *in vivo* control of live BCG with patterns of gene expression and cellular immune responses (119).

Variable Profiles of Response to BCG Vaccination: Hampering the Search for Correlates of Protection?

As discussed above, population differences exist in adult and infant immune response to BCG (91–93) however, even within populations, marked heterogeneity in the host response to BCG vaccination has been observed. South African infants who received BCG clustered into two groups of responders that displayed distinct myeloid or lymphoid activation patterns of gene expression (112). Furthermore, Boer and colleagues found that young adults receiving primary BCG vaccination responded in a surprisingly dichotomous manner. Either broadly proimflammatory responses with local reactogenicity and induction of polyfunctional CD4 T-cells or responses characterized by mild local inflammation, poor cytokine, and polyfunctional CD4 T-cell induction and a predominance of regulatory CD8 T-cells were detected (120).

Although there is opportunity in the fact that BCG vaccination demonstrates protective efficacy in infants and in adults in some geographical locations, there are clearly some host-related factors that modify immune responses to BCG and give rise to significant heterogeneity. Until we understand these factors better and take them into account, it will be difficult to identify broadly relevant correlates of protection in BCG-vaccinated cohorts.

CONCLUSION

Although there has been much progress over the last 20 years, we are still unable to identify BCG-induced correlates of protection in vaccinated infants. This remains a priority area for further research, as it is possible that the development of effective boosting TB vaccines could be abandoned simply because we have yet to discover the essential aspect of BCG-induced immunity to TB that should be boosted. Similarly, a better understanding of BCG-induced correlates of protection will help novel, live mycobacterial vaccines avoid the pitfalls that have caused BCG to fail in certain settings. To date, the search for BCG-induced correlates has been far too simplistic. However, the advent of more detailed immune and memory cell phenotyping obtained using mass cytometry (121) and the availability of more gene expression datasets in recent times is providing an increasingly nuanced picture. Coupled with sophisticated bioinformatic analyses, these new approaches may soon identify the complex biosignatures associated with BCG-induced protection against TB.

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HD and SS wrote, edited, and approved the final version of this article.

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REVIEW
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What We Have Learned and What We Have Missed in Tuberculosis Pathophysiology for a New Vaccine Design: Searching for the "Pink Swan"

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This is a call to encourage the search for a new vaccine to stop the progression of *Mycobacterium tuberculosis* infection to tuberculosis (TB) disease. TB is a highly discreet and stigmatized disease, with a massive impact on human health. It has killed 1.2 billion people in the last 200 years and still kills 1.5 million people per year. Over the last 20 years, the TB vaccine field has experienced spectacular developments, and we have learned about (1) the importance of the Th1 response in controlling infection, mainly against RD1 and Ag85 antigens; (2) the stability of the antigenic repertoire; (3) the dynamics of *M. tuberculosis* granulomas; or (4) the link between typical and atypical pulmonary TB and the immune status of the host. However, we still do not (1) know how to avoid *M. tuberculosis* infection and reinfection; (2) understand the major role of the increase in lesion size in progression from infection to disease; (3) the role of interlobular septa in encapsulating pulmonary lesions; or (4) the role of neutrophilic infiltration and an exaggerated inflammatory response in the development of TB disease. These are strong reasons to pursue new, imaginative proposals involving both the antibody response and a balanced, tolerant immune response that averts progression toward TB. So far, the scientific mindset has been quite monolithic and has mainly focused on the stimulation of conventional T cells. But this approach has failed. For that reason, we are seeking unconventional perspectives to find a "pink swan," a more efficacious and safer vaccine candidate.

Keywords: ***Mycobacterium tuberculosis*, bacillus Calmette–Guerin vaccine, pink swan, dynamic hypothesis, *Mycobacterium tuberculosis* tolerance, damage theory**

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TUBERCULOSIS CONTROL: A FAILED PROPHECY

> Discovery consists of seeing what everybody has seen, and thinking what nobody has thought—Albert Szent-György.

In 1993, the WHO declared tuberculosis (TB) a global emergency. The old, optimistic prophecy that had predicted its eradication by the year 2000 did not materialize. This enthusiasm was mainly

due to the short-term chemotherapy developed in the 1980s. Alongside relatively inefficient case detection, the problem was the relative nature of the "short-term" concept, meaning 6 months and the fact that treatment required the administration of at least three different chemotherapeutic drugs. This approach needed a certain level of organizational support, such as the Directly Observed Therapy (DOTS) strategy that was not always achieved, for various reasons, and which, together with a lack of patient compliance due to the lengthy, cumbersome treatments, led to the generation of multidrug resistant strains (1), with some *Mycobacterium tuberculosis* sublineages being more prone to developing resistance than others (2). The irruption of the HIV epidemic also had a substantial impact on the induction of new TB cases. Finally, the increase in human mobility around the planet has linked high-incidence areas with low-incidence ones, while growing urbanization, with the inherent rise in crowding, has done the rest (3).

One aggravating problem is TB's insidious nature, not only due to the clinical course of the disease but also because of the stigma linked to TB that leads patients and their families to hiding it (4). In consequence, social awareness of the magnitude of the problem of TB diminishes, leading to a reduction in the investments needed to defeat it.

Another reason for TB's elusiveness is that infection is totally asymptomatic and harmless and does usually not progress toward disease. Hence, in TB, it is very important to distinguish between infection and disease. Infection is caused by the inhalation of aerosols with *M. tuberculosis*, which reach the alveolar space and infect alveolar macrophages. Nevertheless, this infection only causes disease in a minority of cases, and the risk decreases exponentially over time, being the highest (80%) during the first 2 years after challenge (5). This is why there are no "explosive" TB epidemics over short-time periods, decreasing its visibility and reducing stakeholders' and politicians' interest in it. To tackle TB, short-term interventions and short-term solutions are far from being the answer.

Tuberculosis is the most underestimated major killer of humankind. TB has caused 1.2 billion deaths over the last 200 years. Even nowadays, in the twenty-first century, TB caused 1.5 million deaths and 9 million new cases, 480,000 of which were MDR-TB, in 2016 (6). Most importantly, a third of these are not even detected, a proportion that reaches 50% in some areas, especially in Asia and Africa (6).

THE QUEST FOR A NEW VACCINE AGAINST TB

Immediately after the declaration of the global emergency, the idea of having a more efficacious TB vaccine became a major goal. The Bacillus Calmette–Guerin (BCG) vaccine has been available since 1927. It is the world's most widely used vaccine and has been administered massively to neonates, with over 3 billion vaccinations performed so far (7). BCG can avoid the development of quickly progressing, deadly forms of the disease such as meningitis and disseminated (miliary) TB, but it neither avoids infection nor lung disease. In addition, BCG revaccination of teenagers, at a time when the effect of the first administration has dissipated, does not offer any additional protection to the population (8).

The enormous effort made over the last 20 years, seeking for a new vaccine based on a conventional T cell response, appears to have been of limited value. For that reason, the idea of searching for a "pink swan" is spreading in the TB field. It is inspired by the concept of "black swan," used in the field of economics by N.N. Taleb referring to an unpredictable event with massive, ground-breaking consequences. The "pink swan" illustrates a fresh, unconventional approach, driven by a currently unknown mechanism that will bring us a new, more efficacious and safer vaccine (9).

What We Have Learned So Far

The Th1 Cellular Immune Response: The Mouse Model

The reappraisal of TB vaccine development in the 1990s coincided with the availability of new, powerful tools for cellular immune analysis, like the flow cytometer, and the technology needed to generate knockout (KO) mice. Thanks to this, the paramount role of IFN-γ in the control of *M. tuberculosis* infection was soon demonstrated. IFN-γ-KO mice were the ones with the lowest survival ratio (10).

The experimental mouse model gained a lot of relevance and became the first gateway for every new candidate for vaccine development, while BCG became the positive gold standard. Mice vaccinated with BCG showed a reduction of around 0.7 logs in colony-forming units in the lung 3 weeks after challenge, when the highest bacillary load was reached. This was determined in inbred, immunocompetent mice, usually C57BL/6 (11).

It led to a search for vaccines able to induce a Th1 response against *M. tuberculosis* antigens. In fact, all vaccines currently in clinical trials were selected on the basis of their capacity to induce Th1 biased CD4 T cells and, to a lesser extent, on CD8 T cell responses (12). Nevertheless, it soon became evident that *M. tuberculosis* infection could, in itself, induce a protective Th1 immune response, at least as effective as the one induced by BCG vaccination. This prompted the need to look for candidates able to induce "something else" to ensure protection against *M. tuberculosis* infection (13).

This "something else" could be multifunctional T cells. These cells have a prolonged memory and effector potential and can produce not only IFN-γ but also IL-2 and TNF-α, reinforcing the surveillance against *M. tuberculosis*. The protective effect of these cells was described in the *Leishmania* model, where their presence was linked to a significant reduction in the number of lesions and amastigotes (14).

Unfortunately, in the majority of cases, the presence of these cells did not appear to make a difference in TB disease development between protected and non-protected subjects (15). They can be considered a marker determining the presence of long-lasting memory cells, but this does not mean that they have to be protective. There are still several authors delving into this issue. Recently, it has been found that multifunctional cells specific for DosR and Rfp antigens can be linked to protection after using a long-term culture assay (16).

Antigenic Discovery: The RD1-Encoded Antigen and the Ag85 Complex Proteins

At the beginning of the 1990s, two groups of *M. tuberculosis* antigens, the ones triggering the greatest Th1 response, gained great relevance. As dead bacilli were not able to induce a protective response in the mouse model, attention was turned to the proteins secreted by active growing bacilli (17). It was soon determined that there were two groups of proteins capable of triggering the greatest Th1 response. These were, on the one hand, the RD1-encoded antigens, mostly linked to the production of ESAT-6 and its secretion *via* its carrier: CFP-10. ESAT-6 is responsible for inducing the lysis of host cell phagosomes, thus being a paramount virulence factor (18). On the other hand, the Ag85 complex, which has a major role in the construction of the cell wall *via* its mycolyltransferase activity (19).

The Genomic Sequence

Mycobacterium tuberculosis sequencing is one of the milestones in the field of TB research. It has allowed a better comprehension of the physiology of *M. tuberculosis* and its regulatory mechanisms against stress (20). It has also been a key to a better understanding of the origin and evolution of the species as well as the characterization of the different geographical families, linked in turn to the evolution of humankind (21). Interestingly, sequencing has also shown the extraordinary stability of the antigenic repertoire and the immune response against this repertoire, which does not vary according to the different families defined (22).

Granuloma Dynamics

Twenty years ago, there was the tendency to consider granulomas as a sort of "bunker" where bacilli were kept enclosed. Currently, intravital imaging offers the possibility of obtaining real-time videos of the organization of these structures, showing their dynamics, with a constant movement of cells entering and leaving BCG-induced granulomas (23). In addition, the discovery of the formation of foamy macrophages in the granuloma and their drainage, infected with dormant bacilli, toward the bronchioles, explained the continuous reinfection and the constant growth of pulmonary infiltration in the murine model, while the bacillary concentration remained stable (24–26). This observation led to the "dynamic hypothesis" concept, based on the constant drainage from the granulomas (27). This process is driven by old macrophages that, after phagocyting the cellular debris of the intragranulomatous necrosis together with the mixed dormant bacilli, become foamy macrophages and are drained with the alveolar fluid *via* the mucociliary escalator. Thus, the majority of dormant bacilli are drained toward the gastrointestinal tract, but there is a small proportion that, after the destruction of foamy macrophages in the bronchi, infect the internal aerosols, enabling pulmonary reinfection (**Figure 1**). Recent data using positron emission tomography in the experimental non-human primate model (28) and in the evaluation of TB treatment in humans (29) have demonstrated this endogenous reinfection process.

This phenomenon is the basis for at least two well-known practices: TB diagnosis in children, by looking for bacilli in stomach aspirates, even when open lesions are lacking (30), and the current latent tuberculosis infection (LTBI) treatment. The latter is based on the administration of isoniazid for 9 months (31). This drug is only active against growing bacilli. So how can it display efficacy killing dormant bacilli, which are responsible for LTBI? The answer is that dormant bacilli are eliminated *via* the physiological drain, and what isoniazid does is avoid reinfection caused by infected internal aerosols. Overall, the complete drainage of LTBI lesions takes around 9 months, demonstrating the dynamic nature of granulomas (27).

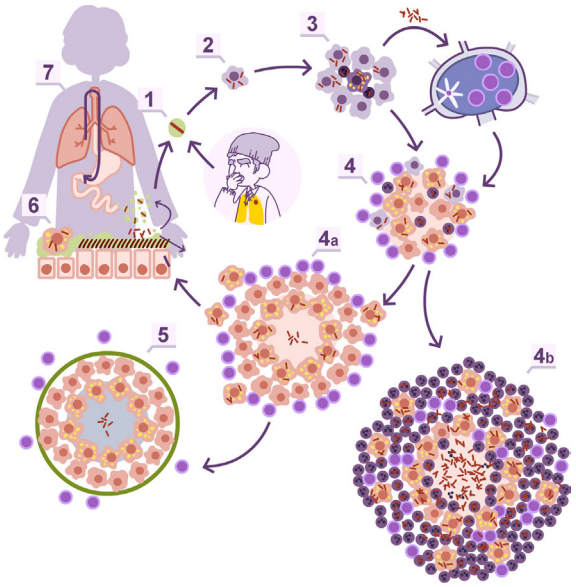

FIGURE 1 | Dynamic hypothesis of *Mycobacterium tuberculosis* infection. Bacilli contained in an aerosol droplet of endogenous or exogenous origin (1) enter the alveolar space, infect it, and grow inside the alveolar macrophage (2). After necrotizing it, the bacilli infect neighboring alveolar macrophages and grow again, unleashing an inflammatory response that allows for the entry of monocytes and neutrophils in the lesions, and the drainage of bacilli *via* the lymphatic vessels (3). Once in the lymph node, the bacilli antigens are presented and a Th1 response is generated, resulting in the proliferation of specific effector lymphocytes that enter the systemic circulation toward the right heart. They reach the lung and are attracted to the infected lesions thanks to local chemokine production (4). The lymphocytes activate the infected macrophages, which kill most intracellular bacilli. A percentage of the bacilli become dormant and survive inside the necrotic tissue or the phagosomes of the macrophages (4a). Macrophages age and fill with lipid inclusions, turning into foamy macrophages, and are drained with the alveolar fluid toward the bronchial tree (6) entering the esophagus and reaching the gastrointestinal system where they are finally expelled from the body (7). Some of these foamy macrophages are destroyed on the way toward the pharynx, liberating the bacilli that become part of the aerosols and can reenter the lung again (1). This cycle is interrupted with the encapsulation and calcification of the granuloma, due to the action of the interlobular septa (5), or when neutrophils are attracted to the lesion, initially around the infected foamy macrophages, progressing toward a large degree of infiltration and cavitation (4b).

The Relevance of Primary and Postprimary TB

Along with the massive chest X-ray surveillance programmes after the Second World War (32) came the concept of primary TB: the disease developed in childhood, spontaneously healed, and calcified and thus easily seen on an X-ray. Generally, primary

TB showed a pattern with a nodule in the lower lobes and lymph node calcification. Postprimary (adult) disease was characterized by the presence of infiltration with or without cavitation in the upper lobe, without affecting the lymph nodes. It was then speculated that postprimary TB was the consequence of the reactivation of a secondary seeding of *Mycobacteria* that escaped *via* the blood circulation from the primary lesions in the lower lobe during childhood. This theory (the "Unitary Concept") also considered that, once infected, subjects were always infected and protected against external reinfection (33).

The analysis of the relationship between recently acquired and remotely acquired pulmonary TB, clinical and demographic variables, and radiographic features by using molecular fingerprinting and conventional epidemiology has proven that adults can develop upper lobe infiltration—cavitation as a consequence of a recent infection and that the radiological pattern is related to the immune status of the patient and not to the time lapse after infection (34). Radiologically speaking, today, we define two patterns: the atypical one, when the lower lobes plus the lymph nodes are affected, which is seen in immunosuppressed persons, and the typical pattern, observed in immunocompetent persons who display upper lobe infiltration without the lymph nodes being affected.

What We Have Missed

M. tuberculosis Can Always Infect and Reinfect Us, Regardless of Our Immune Status

So far, up to 13 vaccine candidates are already in the clinical pipeline (35). None of them have ever proven the capacity to avoid *M. tuberculosis* infection (36). Such a demonstration would represent the greatest scientific achievement in the twenty-first century. But it will not happen.

Mycobacterium tuberculosis infection takes place in the alveolar macrophage. This cell specializes in keeping the alveoli clean and is constantly infected. Fortunately, this cell does not recruit help from the immune system every time it is infected, otherwise our lungs would be constantly inflamed and the gas exchange function seriously hampered. The special anatomy of the alveolar space also has to be considered. Alveoli are sealed from the bloodstream to preserve the correct surface tension and avoid their collapse. Therefore, the presence of antibodies in this space is impossible, and an antibody-based immune response cannot avoid infection (37).

Moreover, the growth of *M. tuberculosis* is quite peculiar. It is slow, with a doubling time of around 24 h, and it blocks the macrophage apoptosis mechanism (38). This means that the bacilli can benefit from the internal milieu of the macrophage for growing until they cause its necrosis. As the maximum capacity of a human alveolar macrophage is around 60 bacilli, necrosis after infection by only 1 bacillus takes place around 6 days after its phagocytosis (39). Once the first macrophage has been destroyed, the bacilli infect the neighbor alveoli, aided by the constant movement of the lung, and the process starts all over again. However, after the destruction of several macrophages, there is enough chemokine production to induce an inflammatory response, drain the bacilli, and trigger the immune response in the lymph node.

This immune response is based on the proliferation of specific Th1 lymphocytes in the lymph node. It can happen quicker if there are already specific memory lymphocytes but, in any case, those cells have to locate the lesion, and they will be attracted to it only if a big enough inflammatory response is generated (40, 41).

All in all, this means that alveolar macrophages can be continually infected, even when specific effector lymphocytes are available, because they require a local inflammatory response strong enough to attract them (42).

In this regard, it is well known that the higher the TB incidence in a region, the higher the reinfection rate its inhabitants suffer and the greater the chance of TB induction (43). This is a very important point. Traditionally, disease induction caused by reactivation has been overemphasized to the point that vaccine efficacy is only tested against one challenge. High-incidence areas need a vaccine able to offer protection against multiple reinfections. This is obvious. In fact, it is probably the most important reason why BCG does not work in several geographic areas (42), and there is a simple explanation. As mentioned earlier, *M. tuberculosis* infection itself induces a protective immune response equal to the one elicited by BCG (13). Therefore, BCG vaccination only provides an advantage against the initial *M. tuberculosis* infection, by eliciting a faster immune response. However, in the context of several reinfections, there is no such advantage, as reinfection itself induces the immune boost, so there is no advantage of being BCG vaccinated. Of course other factors might also play a role, like coinfection with parasites (44) or environmental mycobacteria (45), together with individual genetic susceptibility (46). On the contrary, what seems to be unrelated is the varying virulence exhibited by specific *M. tuberculosis* strains (47).

Size Matters: The Role of Interlobular Septa

Standard medical practice for TB diagnosis entails the tuberculin skin test or the interferon gamma release assay to determine if a person is infected with *M. tuberculosis*. In case of positivity, the next step is a chest X-ray to look for lesions in the lungs. TB lesions can be distinguished in the lung with this technique once they reach a diameter of 10 mm (48).

Traditionally, when attempting to illustrate the progression from infection to disease, the size of TB lesions in both cases was shown to be the same (49); only the bacillary bulk harbored in each of them varied. This could be anecdotal, but it has certainly influenced many researchers.

Looking at the progression of lesions in large mammals, infection lesions are not bigger than the ones we see in mice, around 0.5–1 mm at the time the immune response appears, 3 weeks after challenge (25). The major difference is that large mammal's lungs are structured in cubes of around 1 cm^3 surrounded by a collagen bag, the interlobular septum. These septa make up a net that conveys the forces induced by the movement of the diaphragm to inflate the lung, without tearing the delicate structure of the parenchyma designed for gas exchange (50). Fibroblasts in the septa can detect tiny lesions in the parenchyma, even those with a diameter of less than 1 mm. Once detected, they encapsulate

them within 1–2 weeks, as has been demonstrated in the TB experimental model in minipigs (51) (**Figure 2**).

The size of lesions in disease should be depicted at least 10 times larger than the infected ones, to aid understanding of the process (**Figure 3**). In fact, one of the most important questions when addressing the issue of the progression from infection to disease should be how can a lesion of less than 1 mm become a 10 mm one. Especially taking into account the special circumstances found in the human lung, where the encapsulation process is so efficient.

Precisely why the encapsulation process has merited so little interest in the TB field should be questioned, seeing how important it is for interrupting bacillary drainage, ensuring the infection remains latent, and avoiding progression from infection to disease.

Neutrophils Are Key to Disease Development

Neutrophils are cells associated with acute infectious disease, mainly by extracellular pathogens (52), and linked to explosive inflammatory reactions that develop over the course of hours and usually end with the destruction of the infiltrated tissue, which fills with apoptotic or necrotic neutrophils, producing pus.

Mycobacterium tuberculosis is a slowly growing intracellular pathogen. This is the reason why, for a long time, and especially over the last 20 years, the role of neutrophils in TB has not been considered. This is clearly illustrated by the virtual models that have been built: neutrophils are not even mentioned (53).

Looking at the TB murine model, the authors have observed the presence of neutrophils during the acute phase, inducing tiny infiltrates, and have even related their presence to the presentation of antigens to the lymph nodes, but mainly as a limited phenomenon. This limited role is observed in all inbred mice but one: the C3HeB/FeJ. Ten years ago, it was observed that C3HeB/FeJ mice infected with *M. tuberculosis* surprisingly developed massive intragranulomatous necrosis (54). Further research was conducted to ascertain if this necrosis was linked to liquefaction, in which case, the model could be considered a "human-like" TB disease model. Consequently, the evolution of the lesions was carefully followed. The results showed how the size of the lesions increased rapidly, in a matter of days, thanks to peripheral growth linked to infected foamy macrophages heavily surrounded by neutrophils. From these original lesions, new ones appeared, and they all coalesced to induce massive lesions (55). This process illustrated how a lesion of less than 1 mm could become a 10 mm one, avoiding the encapsulation processes seen in large mammals. The process takes place in around 10 days (**Figure 4**). A parallelism can be drawn with the way soap bubbles are formed, and this has aided its modeling, becoming known as the "bubble model" (56).

At the beginning of the twentieth century, authors demonstrated that TB patients present two kinds of lesions: proliferative

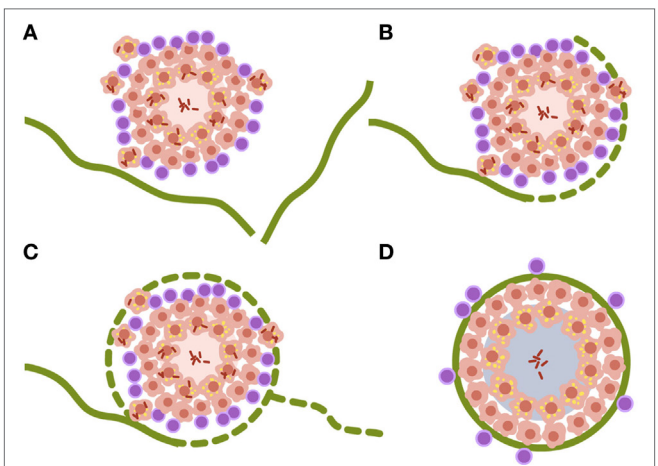

FIGURE 2 | Encapsulation process of *Mycobacterium tuberculosis* lesions. **(A–D)** Interlobular septa are represented in green. Once the lesion is contacted, the encapsulation process starts surrounding it. The capsule completely envelops the lesion and aids the organization of miofibroblasts that stabilize the lesion. Non-drained necrotic tissue finally becomes calcified. Data are obtained from Gil et al. (51).

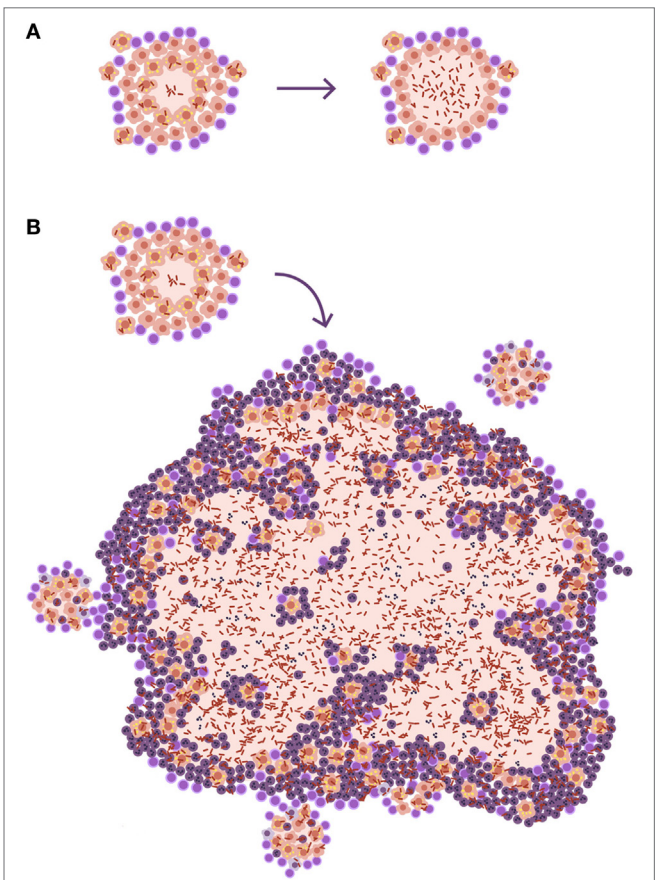

FIGURE 3 | Size matters in progression toward tuberculosis (TB). **(A)** Disease development as it is usually represented in publications. The only difference shown is that, in the lesion corresponding to disease, there is a higher bacillary concentration. **(B)** A more realistic approach. The TB disease lesion is exudative, with a predominance of neutrophilic infiltration together with massive necrosis, and it is much larger than the infected lesion. It must be taken into account that lesions need to be at least 10 mm in diameter to be visible on a chest X-ray and that infection lesions measure between 0.5 and 2 mm.

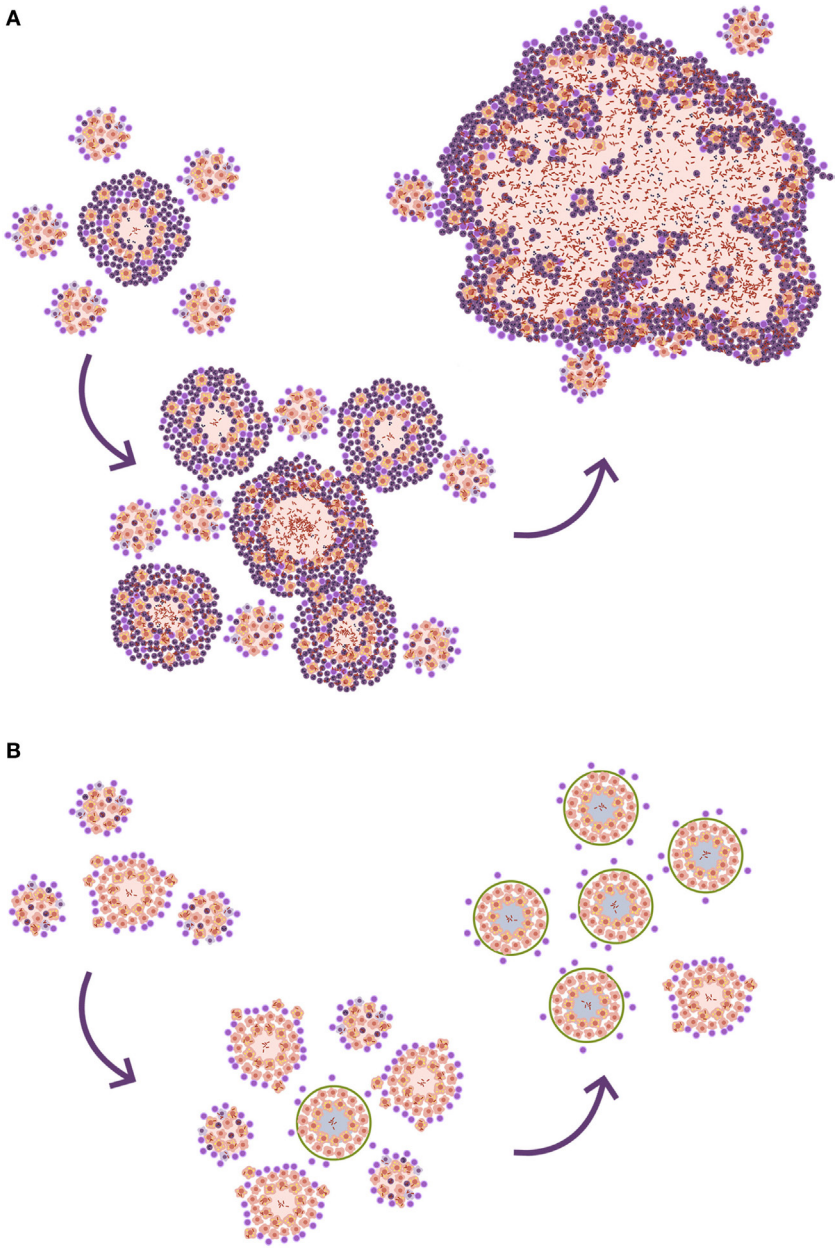

FIGURE 4 | **Evolution toward active tuberculosis (TB)**. **(A)** The process toward a neutrophilic infiltrated lesion favors the induction of new lesions and their rapid growth; this leads to lesion coalescence and the induction of an active TB lesion. On the contrary, **(B)** shows a well-controlled lesion, without neutrophilic infiltration, which hardly induces new lesions and soon becomes encapsulated, blocking the induction of active TB.

and exudative. The former are well structured and controlled, based on the presence of epithelioid cells and lymphocytes, like the ones developed by infected mice. The latter are mainly infiltrated with neutrophils and related to the expansion of the lesions and the induction of cavitation and liquefaction (57). A recent review on human pathology run in parallel by Hunter, searching through a wider selection of authors, has led to the same conclusion (58). In addition, the role of neutrophils has recently been reinforced by the data obtained from a search for a biosignature for TB progression (59, 60).

Tuberculosis tropism in the upper lobes can be also explained by neutrophilic infiltration. Upper lobes tend to mobilize less than the lower ones, simply because that is the region where the lungs "hang," while the lower region is more directly influenced by the diaphragmatic contraction. The lack of mobilization in the upper lobes causes lower blood circulation and lower gas exchange, leading to an increase in oxygen pressure that had traditionally and erroneously been linked to higher bacillary growth (61). What is relevant about the lack of mobilization in the upper lobes is that bacilli tend to accumulate locally after the

destruction of alveolar macrophages. This accumulation means that new, incoming macrophages have to phagocyte higher bacillary concentrations than in the lower lobes. This in turn causes more necrosis and the attraction of neutrophils that lead to the rapid increase in the size of the lesion (**Figure 5**). This fact also induces a Th17 immune response that stabilizes the neutrophilic infiltration at that site (62).

In this regard, it must be assumed that some genetic polymorphism must determine a more intensive reaction to this challenge, thus explaining why certain families were more prone to suffer TB than others. Unfortunately, precisely which polymorphism promotes this exaggerated inflammatory response has not been yet clarified, but it would certainly signify a valuable improvement in the field.

The Role of Antibodies in Progression toward Disease

The presence of antibodies does not offer protection against *M. tuberculosis* infection, because they cannot enter the alveolar space. However, the study of progression of infection in C3HeB/FeJ mice has demonstrated that neutrophilic infiltration builds neutrophilic extracellular traps that favor the extracellular growth of bacilli (63). In this phase, the presence of antibodies might certainly help to stop the progression of the lesion (**Figure 5**), enabling the encapsulation process and avoiding progression toward disease.

Several authors have proven the protective effect of antibodies. The article that reflects this process best investigated the protective effect of passive transference of *M. tuberculosis*-specific antibodies in a model of TB reactivation in SCID mice. The results clearly showed a protective effect, with a threefold reduction in lung infiltration and a 2-log reduction in pulmonary bacillary load (64).

Unfortunately, there is little information on the protective role of antibodies in humans. Only two projects have linked their level to disease prognosis: the case of antibodies against lipoarabinomannan and against the 38-kDa antigen, while the presence of IgG against Ag85A has been related to a reduced risk of developing TB disease in a case–control study in infants (65). Recently, an antibody-related signature has been identified for latent-infected or active TB patients, where glycosylation plays a key role (66).

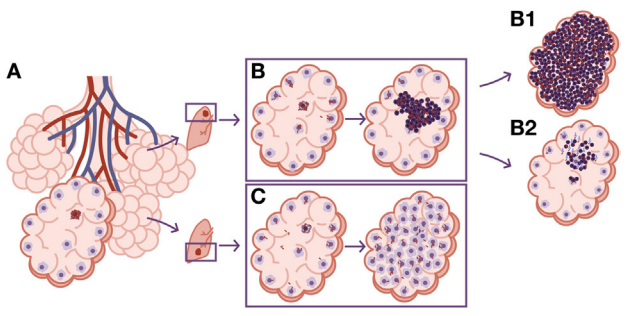

FIGURE 5 | Differences in local drainage determine the nature of the lesion. **(A)** The necrosis of an alveolar macrophage releases intracellular bacilli. The rate of bacilli drainage follows the breathing amplitude of the lobe, which is lower in the upper lobes, resulting in lower bacillary drainage **(B)**. As a result, new incoming macrophages must face higher bacillary concentrations that, once the inflammatory response is triggered, cause a predominance of neutrophilic infiltration and the induction of neutrophilic extracellular traps that favor extracellular bacillary growth. This is the perfect scenario for the protective role of circulating antibodies, which can neutralize the bacilli and reduce the inflammatory response, controlling progression toward TB disease. **(B1)** The progression without antibodies. **(B2)** The control achieved by the presence of neutralizing antibodies. On the contrary, the larger breathing amplitude in the lower lobes causes significant bacillary dissemination, so macrophages face lower bacillary loads, favoring a less inflammatory lesion dominated by macrophages (**C**).

Should We Tolerate *M. tuberculosis*?

Why do a minority of patients infected by *M. tuberculosis* develop TB disease? As **Figure 6** shows, mice will never develop lesions of the same magnitude as humans. The difference resides in the fact that although both bacilli and host cells have the same size; humans have a higher number of cells so, potentially, a certain amount of tissue can be destroyed without hampering our health status, at least more than in the case of mice.

Traditionally, mice have been considered "resistant" to *M. tuberculosis* infection because they show prolonged survival, usually quite similar to that of non-infected mice. In guinea pigs, considered susceptible, the contrary happens, because they generally die within weeks of infection. However, if the bacillary load is examined, it is found to be very high in mice compared to guinea pigs. This means that mice are not resistant, but tolerant to the infection, because they do not control the bacillary load, but it does not hamper their health status (67).

This led to the theory of the "damage-response" framework of microbial pathogenesis, which classifies all infectious diseases according the virulence potential of the pathogens and the response of the host to the pathogen. In the case of TB, the response against *M. tuberculosis* is a double-edged sword, advocating the need for a balanced immune response. Both a too weak or too strong immune response can cause active TB (68). It appears that in TB this concept has been poorly addressed. Invariably,

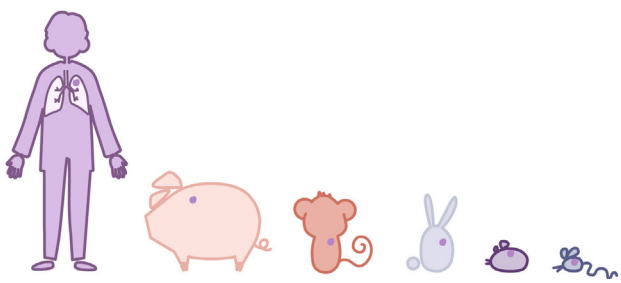

FIGURE 6 | Relationship between the volume of a cavity and the whole-body mass of a host. Several laboratory animals plus humans are represented, comparing their mass with that of a cavitation. This is to illustrate the fact that in mice they would hardly develop, because they would disappear. Unable to eliminate the bacillary load, the majority of mouse strains (except C3Heb/FeJ) tolerate the presence of a constant, relatively high bacillary concentration in their lobes, controlling the induction of an excessive inflammatory response.

induction of TB disease is linked to some sort of immunosuppression, but the consequences of an exaggerated immune response are poorly understood (**Figure 7**).

The relationship between a greater immune response and progression toward TB disease has recently been determined by an increased frequency of activated HLA-DR+ CD4+ T cells being linked to a heightened risk of TB disease (69). This higher immunological activation is probably originated by intercurrent infections like those caused by cytomegalovirus (CMV). Similarly, one of the major risks fueling TB is being overweight, which is related to type II diabetes (70), and in turn to a systemic pro-inflammatory response (71).

The work by Green et al. in the cynomolgus macaque model demonstrated that the animals that did not progress toward TB disease had significantly higher frequencies of Tregs in peripheral blood prior to infection, compared to macaques that developed active disease, supporting the idea that more Tregs prior to infection correlates with a better infection outcome (72). This made others consider protection against TB the consequence of a balanced immune response, able to stimulate a Th1 immune response but without causing too much damage, especially without causing excessive neutrophil infiltration (67, 73). In this regard, the proof of concept that a decreased inflammatory response causes a reduction of the bacillary load was experimentally demonstrated in a TB murine model after treatment with ibuprofen (74), a clear indication of the new approach towards the use of host-directed therapies in TB (75). Following this logic, a promising strategy to stop progression toward TB disease appears to be the induction of *M. tuberculosis* protein purified derivative-specific memory Tregs to counterbalance the Th17 (76–78). The role of Tregs has been controversial for a long time, as it was related to counterbalance Th1 response (79). This concept was progressively refined, placing Tregs in a more neutral role, without increasing the bacillary load in lesions at all (80), or even a protective one (81, 82). The fact is that the models that were used did not develop human-like lesions, and therefore, the role of pathology and exudative progression was not explored. When this parameter is included, as in C3HeB/FeJ mice (55) or NHP (72), Tregs response is clearly beneficial and is related to the interruption of progression toward active TB.

In conclusion, after 30 years of investing heavily in "bacilli-oriented therapies" to destroy *M. tuberculosis*, there now seems to be a new opportunity to address the control of TB epidemics using "host-oriented therapies," which would enable a physiological approach to living with the presence of this bacillus: simply tolerating it!

One World, Ecology, Coinfection

Studies in wild-type animals have highlighted the role of coinfection in TB. In this case, it is important to note the impact of parasitosis as a source of Tregs response, balancing the immune response and thus protecting against progression from infection to disease. This concept must be approached cautiously as, so far, parasitosis has been related to a neutral (83, 84) effect in humans with regard to progression toward TB disease. On the contrary, concurrent infections such as CMV are potent immunity activators favoring progression toward TB (85, 86).

TB IS A COMPLEX INFECTION: THE QUEST FOR THE "PINK SWAN"

Tuberculosis is the consequence of a major, complex process and, even though we have accumulated a vast amount of information

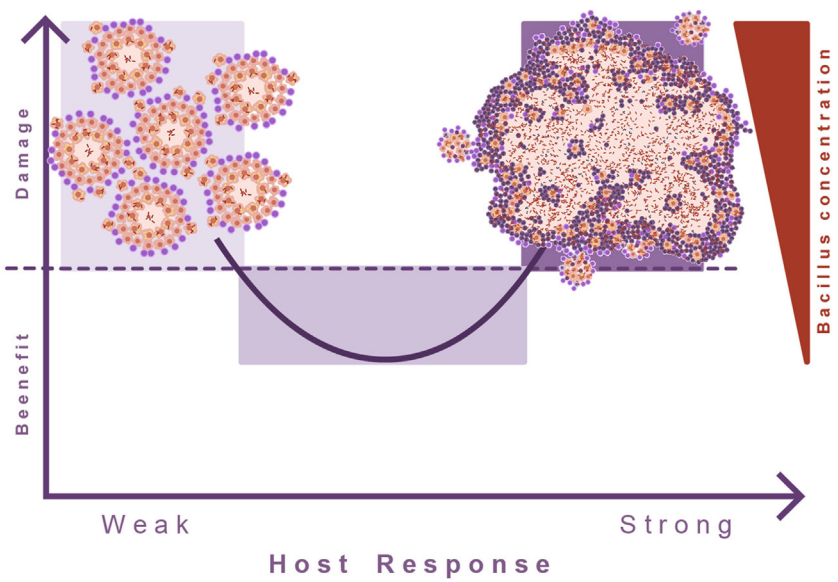

FIGURE 7 | Damage theory of infectious diseases. This picture illustrates Arturo Casadevall's "damage theory" (68). In the case of TB, a weak immune response leads to the proliferation of lesions and dissemination of the bacilli. However, the induction of an excessive inflammatory response also leads to exudative lesions characterized by massive tissue destruction. In this regard, we can consider that TB develops two kinds of diseases, and in between there is a wide range of situations where the immune response is balanced and allows the host to live with the bacilli without hampering its health status.

and knowledge about it, we desperately need new tools and novel, groundbreaking concepts to aid us in the search for more efficacious strategies to curtail TB progression.

As a consequence of a lack of resources, TB researchers tend to organize in synergistic, monolithic consortiums to act coordinately and obtain funding. This is a positive and intelligent organizational set up, but it can be potentially deleterious from the intellectual point of view, as it can lead to homogeneous thinking.

It has been claimed that "out of the box thinking" is needed and that we are seeking for a "pink swan" (9): a unique and revolutionary concept that will have a massive impact in the field. The recruitment of all research groups under one single strategy may be incompatible with reaching this target. Large organizations need to harmonize to seek consensus. This is very important to complete file-intensive projects, requiring many samples, many subjects, etc. such as clinical trials. However, it probably is not that convenient when searching for new concepts, blue-sky thinking, because unifying, harmonizing exercises tend to have too great influence on the group, and marginalize divergent approaches.

In this sense, the fresh approaches to old methodological systems for testing new vaccines are very interesting. Mycobacterial Growth Inhibition Assay (MGIA), to ascertain mycobactericidal capacity, in spite of not yet understanding the mechanism of action (87) and the use of the monocyte:lymphocyte ratio in peripheral blood to predict the induction of TB disease are two clear examples of novel, imaginative ways of looking for a protective surrogate marker (88).

In a nutshell, it is necessary to encourage the creation of instruments to fund "free-thinking" concepts to advance TB research. In particular, it would be interesting to recruit knowledge from other disciplines, with fresh concepts and new ideas, to drive innovative projects capable of leading us to our "pink swan."

AUTHOR CONTRIBUTIONS

P-JC is responsible for the whole review. This is an inaugural article.

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MINI REVIEW
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MTBVAC: Attenuating the Human Pathogen of Tuberculosis (TB) Toward a Promising Vaccine against the TB Epidemic

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Bacille Calmette-Guérin (BCG) is a live-attenuated strain of *Mycobacterium bovis* developed a century ago by repeated subculture. It remains the only vaccine against tuberculosis (TB) in use today, and it offers variable protection against the respiratory forms of TB responsible for transmission. The principal genetic basis for BCG attenuation is the loss of the region of difference 1 (RD1) that includes the genes codifying for production and export of the major virulence factor ESAT6. Today more than 13 TB vaccine candidates are in clinical evaluation. One of these candidates is MTBVAC, which is based on a rationally attenuated *Mycobacterium tuberculosis* clinical isolate belonging to modern lineage 4, one of the most widespread lineages among humans. MTBVAC conserves most of the T cell epitopes described for TB including the major immunodominant antigens ESAT6 and CFP10 of the RD1, deleted in BCG. After almost 20 years of discovery and preclinical development, MTBVAC is the only live attenuated vaccine based on a human pathogen that has successfully entered clinical trials as a preventive vaccine in newborns, aiming to replace BCG, and as a preventive vaccine in adolescents and adults (BCG-vaccinated at birth). Our recent preclinical studies have demonstrated that MTBVAC-induced immunity to ESAT6 and CFP10 correlate with improved efficacy relative to BCG encouraging exploration of these responses in human clinical trials as potential biomarkers and identification of these antigens as possible correlates of vaccine-induced protection. Such data would be extremely valuable as they would greatly accelerate clinical development to efficacy trials.

Keywords: tuberculosis, Bacille Calmette-Guérin, live vaccines, ESAT6, CFP10, MTBVAC

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INTRODUCTION

The current tuberculosis (TB) vaccine, Bacille Calmette-Guérin (BCG), is an attenuated strain of *Mycobacterium bovis* based on the etiologic agent of TB in cattle (1). BCG was introduced for the first time into clinical use almost a hundred years ago, when in 1921 it was given orally to an infant whose mother had died of TB a day after delivery. The infant showed no adverse events to vaccination with BCG and importantly, did not develop disseminated TB, the common form of disease at that time acquired mainly form unpasteurized milk of infected cows (2).

Today, BCG remains the only licensed vaccine against TB, given by the intradermal route of administration at birth. In infants, BCG is acknowledged to afford substantial protection against disseminated (miliary and meningeal) forms of TB (3, 4), whereas in adolescents and adults, lack of BCG efficacy against respiratory (pulmonary) TB, the most common form of the disease responsible for transmission, is considered to be a result of waning BCG immunity with time that is gradually lost 10 years after vaccination (5).

Tuberculosis has reached alarming proportions of 10.4 million incidence cases and 1.7 million deaths attributed to the disease as reported by the latest World Health Organization (6) global TB report 2017. Globally, some 50 million individuals are already latently infected with MDR *Mycobacterium tuberculosis* strains creating a remarkable resource for future cases of active TB with insufficient treatment options. Nevertheless, the WHO End TB Strategy has vowed to reduce TB morbidity by 90% and TB mortality by 95% by 2035 and recognizes the urgent need for more accessible diagnostic tools that are rapid and reliable, new less toxic and more efficacious antibiotics to shorten therapy and ultimately new vaccines to prevent pulmonary TB in order to achieve this ambitious goal (7).

There are a total of 12 candidates in the current clinical TB vaccine pipeline and they can be classified into three groups (2), including preventive pre- and postexposure subunit vaccines: viral or subunit vaccines that aim to boost immunity to BCG, killed or fractioned whole cell vaccines, and live-attenuated mycobacterial vaccines, which target either BCG-replacement at birth or prevention of TB in adolescents and adults.

Currently, the most advanced live-attenuated vaccine in clinical development is the recombinant BCG VPM1002, which is now entering efficacy trials aimed to prevent TB in infants with and without HIV exposure (8). The other candidate is the live-attenuated *M. tuberculosis* MTBVAC, which is the first vaccine of its kind to successfully enter human clinical evaluation in the history of human vaccinology. These two candidates could eventually replace BCG, if able to demonstrate improved safety and or greater efficacy than BCG when administered in newborns in high-burden TB-endemic settings.

The main advantage of using rationally attenuated live *M. tuberculosis* as vaccine is that many genetic regions encoding important immunodominant antigens absent in BCG are still present in attenuated *M. tuberculosis*, while chromosomal deletions in virulence genes provide assurance for safety and genetic stability (**Figure 1**). These vaccines are expected to safely induce more specific and longer lasting immune responses in humans that can provide protection against all forms of the disease (9). This is the rationale that has been followed in the development of the live-attenuated MTBVAC.

MTBVAC

MTBVAC was constructed by rational attenuation of the *M. tuberculosis* clinical isolate Mt103 (10), belonging to the modern *M. tuberculosis* Lineage 4, which together with Lineage 2 (Beijing strains) represent the most geographically widespread lineages of MTBC transmitted by the aerosol route between humans (11). Following the Geneva consensus safety requirements for progressing new live attenuated mycobacterial vaccines to clinical trials (12, 13), MTBVAC was constructed by generating two independent stable genetic deletions, without antibiotic resistance markers, in the genes *phoP* and *fadD26* encoding two major virulence factors (10). The gene *phoP* encodes the transcription factor of the two-component virulence system PhoPR and the gene *fadD26* participates in biosynthesis and export of phthiocerol dimycocerosates (PDIM), the main virulence-associated cell-wall lipids of *M. tuberculosis* (14–16). PhoP has been shown to regulate more than 2% of *M. tuberculosis* genome, most of which implicated in virulence (17, 18), and this regulation could be mediated through other transcription factors, e.g.,

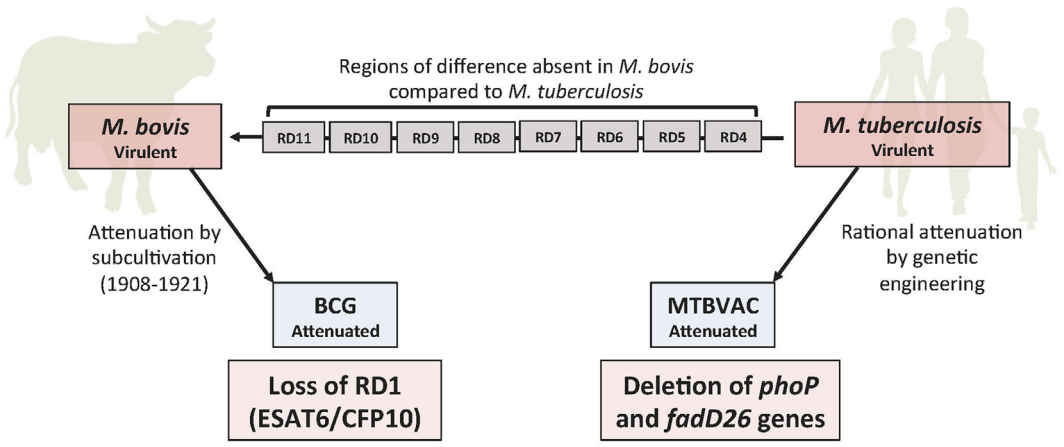

FIGURE 1 | Genomic deletions between human and cattle tuberculosis pathogens. Eight regions of difference (RD) deleted in the bovine pathogen *Mycobacterium bovis* with respect to the human pathogen *Mycobacterium tuberculosis*. Repeated subcultivation of an *M. bovis* strain for 13 years (1908–1921), following classical Pasteur's postulates, led to attenuation due to loss of RD1, giving rise to Bacille Calmette-Guérin (BCG). MTBVAC is the result of the rational attenuation of an *M. tuberculosis* clinical isolate by genetic deletions of the two independent virulence genes *phoP* and *fadD26*, following molecular Pasteur's postulates for attenuated vaccines.

WhiB6 of the ESX-1 system (19) or *via* non-coding RNAs, such as the recently described *mcr7* and its role in activating the TAT secretion system in MTBVAC leading to over secretion of major antigens such as those of the Ag85 complex (20). Other relevant virulence genes regulated by PhoP include lipid metabolism genes (e.g., *pks2, pks3*) involved in biosynthesis of the polyketide-derived acyltrehaloses (DAT, PAT) and sulfolipids, which are front-line lipid constituents of the cell wall thought to have a role in host immune modulation (21, 22). PhoP also regulates genes within ESX-1 (e.g., the *espACD* locus) implicated in the secretion of the major antigen and virulence factor ESAT6, so that *phoP*-mutants can produce but are unable to export ESAT6 (23).

Since 2001, rigorous preclinical safety, efficacy and immunogenicity studies of prototype SO2 and final vaccine construct MTBVAC were conducted in recognized animal models by independent (national and international) laboratories. Both MTBVAC and its prototype SO2 vaccine demonstrated to be at least as safe as BCG in SCID mice, and to confer greater or equivalent efficacy to BCG in different animal models (10, 24–27). SO2 conferred greater efficacy than BCG in a high-dose challenge, long-term survival experiment in guinea pigs (100% survival in SO2 group vs. 33% in the BCG group) (24). In guinea pigs, MTBVAC given as boost to BCG demonstrated significantly greater protection compared to BCG alone (27). In adult and newborn mouse models, MTBVAC conferred improved protection compared to BCG (25, 26) (**Table 1**).

The preclinical data with MTBVAC and SO2 provided a proof-of-concept to progress MTBVAC to first-in-human Phase 1 clinical evaluation. For this purpose, MTBVAC was developed and characterized as a freeze-dried vaccine product by the Spanish Biopharmaceutical company, Biofabri, in compliance with current Good Manufacturing Practices, fulfilling regulatory guidelines for assuring the quality, safety and stability, and efficacy of BCG freeze-dried vaccine (10).

The first-in-human Phase 1 trial designed to evaluate the safety, local tolerance and immunogenicity of three escalating dose of MTBVAC relative to BCG, was conducted in healthy, BCG-naive, HIV-negative adults. MTBVAC showed excellent safety and tolerability profile comparable to BCG. MTBVAC was at least as immunogenic as BCG, and at the same dose level as BCG, MTBVAC group showed greater frequency of polyfunctional $CD4^+$ central memory T cells. This first Phase 1 trial marks a historic milestone in human vaccinology, as for the first time a live-attenuated *M. tuberculosis* vaccine has entered clinical trials showing excellent safety profile and differential immunogenicity profile compared to BCG supporting clinical development in high-burden TB endemic countries. Today MTBVAC follows two clinical development pathways, the primary supporting disease prevention at birth, as BCG-replacement strategy, and the secondary pathway supports prevention of pulmonary disease in adolescents and adults.

TABLE 1 | Comparison of Bacille Calmette-Guérin (BCG), MTBVAC, and H37Rv properties.

	BCG	MTBVAC	H37RV
Source	**Virulent *M. bovis***	**Virulent *M. tuberculosis***	**Reference lab strain**
Attenuation process	Subcultivation	Deletions in *phoP* and *fadD26* virulence genes	None
Number of epitopes	••	•••	•••
Immunogenicity	•	•••	•••
Protection	••	•••	
Attenuation/safety	•••	•••	

ESAT6: A DOUBLE-EDGED SWORD

During the *in vitro* subcultivation process of BCG attenuation between 1908 and 1921, more than 100 genes were lost from BCG, relative to *M. tuberculosis* (28). Deletion of the region of difference 1 (RD1) region, which encodes the protein secretion system ESX-1, is considered the deletion responsible for BCG attenuation (28) (**Figure 1**). In a study by Copin et al. (29), where 1,530 experimentally verified human T cell epitopes were compared, it was demonstrated that 23% of the known MTBC T cell epitopes are absent in BCG. Despite their low molecular weight, the two major virulence factors ESAT6 and CFP10 encoded in the RD1 and absent in BCG, have been shown to contain the greatest number of human T cell epitopes recognized by the immune system (29). These data suggest the intensive interaction maintained throughout evolution between human hosts and these major RD1-encoded antigens of *M. tuberculosis*, which is likely a consequence of the crucial role of these proteins in virulence.

The role of ESAT6 in virulence has been demonstrated by experiments where complementation of BCG with RD1 restores full virulence (30), whereas deletion of RD1 in *M. tuberculosis* strains leads to a profoundly attenuated profile (31). ESAT6 has been described to interfere in different host–pathogen interaction processes: ESAT6 inhibits autophagy (32), a crucial cellular mechanism to eliminate intracellular pathogens, including mycobacteria (33). There exist strong evidences about the ability of ESAT6 to trigger cell death on host cells. A recent study demonstrated the contribution of the ESX-1 system to induce necrosis on infected neutrophils, in mechanism meditated by reactive-oxygen species (34); in addition, ESAT6 results also crucial for the bacteria to induce apoptosis on infected macrophages (35, 36), an event that provides the pathogen the capacity to spread from cell-to-cell maintaining an intracellular status (36), which might be important to establish early infection in the absence of initial recognition by the immune system (37, 38). During the last years, a role of ESAT6 in the escape of *M. tuberculosis* from the phagosome to the cytosol has been demonstrated to occur both *in vitro* and *in vivo* in mouse models of infection. This event has been considered relevant for infection outcome as strains unable to escape to cytosol have an attenuated phenotype (39–41). ESAT6 also stimulates the induction of type I interferon responses (42), which has been reported by some authors as detrimental for TB infection control (43).

Despite its great antigenic capacity, experiments in mice have shown that *esat6* and *cfp10*, as well as genes involved in their secretion, are highly expressed during different stages of lung

infection (26, 44). In contrast, antigenic proteins such as those from the Ag85 complex, without an apparent role in virulence, have been shown to be downregulated upon infection (26, 45, 46). Antigen expression following infection might have an impact on the peptide repertoire presented by the MHC molecules from infected cells, which should be coated with peptides derived from the most represented proteins by the bacteria, including ESAT6 and CFP10, and not from those under expressed as Ag85. Indeed, human and mouse data indicate a higher degree of differentiation of ESAT6-specific T cells compare to Ag85B, due to the different antigen availability (47).

A classical approach for rational vaccine development has been the construction of vaccines that target the stimulation of a specific immune response against the most abundant proteins of the pathogen at each stage of infection. Thus, presence of a preexisting immunity against ESAT6 and CFP10 upon pathogen encounter with host might allowed rapid recognition of infected cells and would increase the probabilities to control infection before dissemination. In the particular case of BCG, which lacks of ESAT6 and CFP10, complementation of this strain with RD1 increases substantially its protective efficacy in different animal models (48, 49), suggesting that the deficient protection of BCG could rest on the absence of these major antigens. In addition, some subunit vaccines expressing ESAT6 have demonstrated good protection in mice and are currently under clinical evaluation (44). In contrast, the MVA85A vaccine demonstrated no improved protection compared to BCG in a phase IIb clinical trial, despite the good immunogenic profile shown (50). There are multiple reasons that could explain this failure, but it is plausible that the T cells reactive to Ag85A generated following boosting vaccination deficiently recognized infected cells due to the poor representation of Ag85A-derived peptides on cellular surface.

In the case of MTBVAC, this vaccine represents a unique candidate in the pipeline of vaccines currently under clinical evaluation, as it is the only one containing the whole antigen repertoire of *M. tuberculosis*, including the major antigens ESAT6 and CFP10 (28). Recently, we have demonstrated that an MTBVAC substrain mutant for these two proteins confers similar protection to BCG and lower than MTBVAC parental strain, suggesting a link between vaccine-induced protection and ESAT6/CFP10-mediated response, which could result in the definition of a biomarker for correlates-of-protection of MTBVAC (26).

Data available from MTBVAC-clinical trials indicated a significative CFP10-specific response in humans at six months post MTBVAC vaccination (26, 51). In this regard, it is crucial to elucidate the potential interference of MTBVAC in the quantiFERON (QFT) test used nowadays to discern infected individuals, as this assay is based on specific reactogenicity against ESAT6 and CFP10. Current MTBVAC clinical trials in TB endemic countries will be key to define this important question. In case of interference, we should be cautious with respect of the interpretation of the results. A direct translation of our preclinical data to humans could indicate that positive individuals to QFT induced by vaccination could be protected. The current practices where QFT-positive individuals are directly treated with isoniazid could be detrimental to identify these potential MTBVAC-mediated protection, as MTBVAC is a live vaccine, and therefore, sensitive to antibiotics. Interestingly, a recent study describes that mainly individuals with a high QFT value (higher than 4 IU/ml) have significantly a higher risk of develop active TB, whereas individuals with a value between 0.35 and 4 have no more probabilities than negative individuals (52). These data probably reveal the need to reconsider the cutoff of this assay in order to treat with isoniazid only individuals with a real risk to develop TB.

MTBVAC CARRIES THE EPITOPE REPERTOIRE OF *M. TUBERCULOSIS*

Pioneering studies exploring the genomic basis behind BCG attenuation have identified several genetic regions present in *M. bovis* but deleted in BCG (1) (**Figure 1**). These deletions arose during subcultivation in the laboratory probably because the parental *M. bovis* strain was not subjected to the immune pressure of the host and hence it gradually lost virulence factors. BCG continued being passaged in the laboratory until lyophilization process was established, resulting in the emergence of different BCG "daughter" strains (53). These substrains differ in genome polymorphisms and show variable efficacy (54). Today, we know that BCG polymorphisms affect the synthesis of important virulence factors, such as the *phoPR* virulence regulator or PDIM (55–57) which might explain the different efficacy of BCG strains.

It is important to remark that regions lost in BCG strains also contain potent antigenic proteins. After an updated analysis based on previous studies (29, 58), we found that of the 1,603 experimentally validated human T cell epitopes, 433 (27%) of them are located in RD regions and absent in BCG Pasteur (**Figure 2**). These epitopes are distributed as follows: RD1 307 epitopes (19.15%), RD2 59 epitopes (3.68%), RD11 42 epitopes (2.62%), RD 7 5 epitopes (0.31%), RD10 and RD14 4 epitopes each (0.5%), RD5 3 epitopes (0.19%), RD3, RD8, and RD13 2 epitopes each (0.36%), and RD4, RD6, and RD9 1 epitope each (0.18%). The vast number of epitopes absent in BCG is located in only five antigenic proteins: ESAT6 (112 epitopes), CFP10 (95 epitopes), and PPE68 (79 epitopes) within the RD1 region and MPT64 (24 epitopes) and Rv1985c (23 epitopes) within RD2 (**Table 2**).

It has been described that Ag85B carries an F140L substitution predicted to affect protein stability (29). Moreover, our recent results demonstrated that Ag85B is absent in the secreted fraction of BCG but not of MTBVAC (26). Ag85B contains 86 epitopes which account for up to 5.36% of the total epitope content of *M. tuberculosis*. Remarkably, vaccination of C57BL/6 mice with BCG resulted in lack of Ag85B-specific response (26).

Of the whole epitope repertoire of the *M. tuberculosis* chromosome, BCG has lost 519 epitopes during its evolution distributed in 433 epitopes in RD regions and 86 epitopes in Ag85B. In other words, attenuated vaccines based on the human pathogen *M. tuberculosis* as MTBVAC, contains up to 48% more epitopes than BCG and consequently they are expected to produce a more robust stimulation of the immune system than BCG. It was previously demonstrated that MTBVAC-like vaccines exhibit a long-term maintenance of $CD4^+$ T cell memory compared to

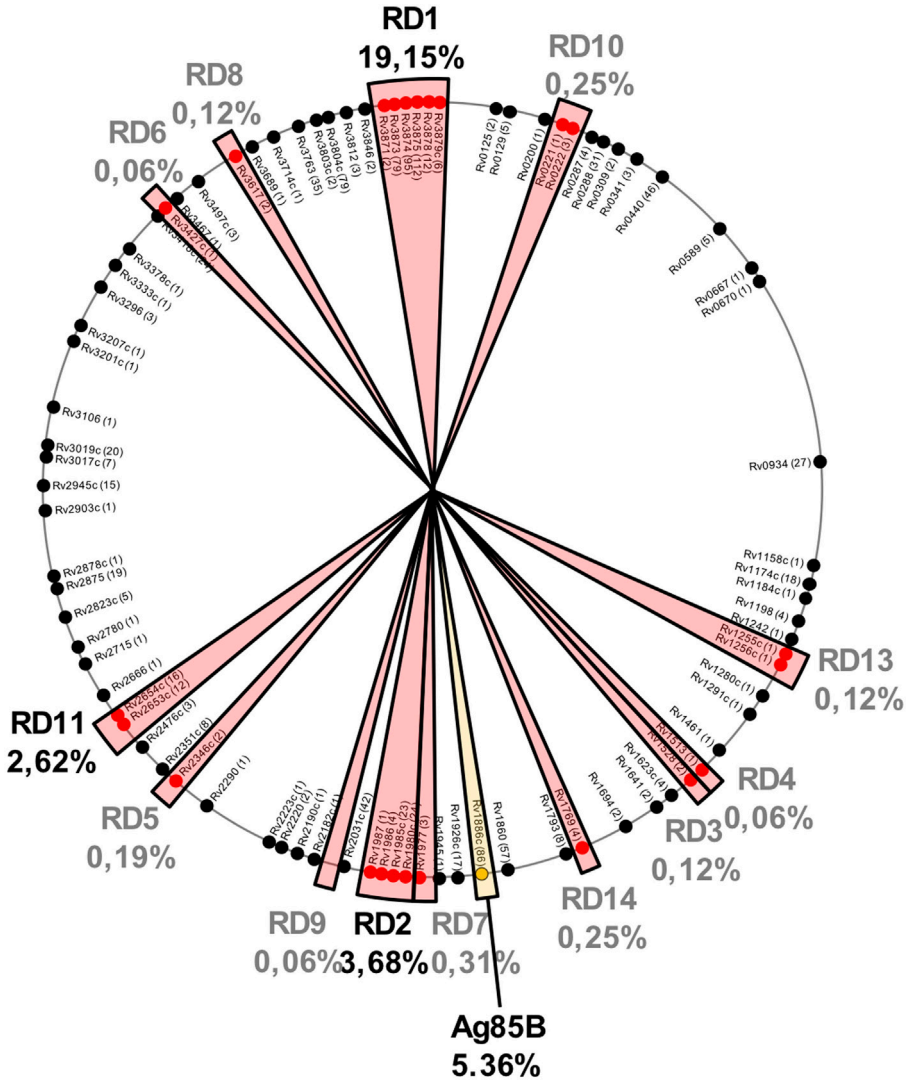

FIGURE 2 | Localization of 1,603 experimentally demonstrated T cell epitopes (according to http://IEDB.org) in the *Mycobacterium tuberculosis* H37Rv chromosome. Those regions absent in Bacille Calmette-Guérin (BCG) but present in *M. tuberculosis* are shown by red sectors and the percentage of epitopes contained in each region is indicated. Three of these regions (RD1, RD2, and RD11) contain >25% of the total *M. tuberculosis* epitopes. It is also important to note that Ag85B (shown by a yellow sector) produced but not secreted by BCG also contains a high percentage of epitopes. Taken together, these results indicate that live attenuated vaccines based on the human pathogen, as MTBVAC, would present almost 40% more T cell epitopes than BCG.

BCG. Notably, this phenotype is specific of Ag85B since these experiments were performed in transgenic mice that exclusively respond to the p25 peptide of this protein (59).

MTBVAC not only produces all the antigens present in *M. tuberculosis* but it is also able to secrete some of these antigens more efficiently than other vaccines. We recently demonstrated that among the PhoP-regulated genes, the more tightly regulated region corresponds to a small RNA named *mcr7*. Consequently, *mcr7* is absent in *phoP* mutants such as the MTBVAC vaccine. Since *mcr7* exert a negative effect over translation of *tatC*, this protein involved in the TAT (Twin Arginine Translocation) secretion system exhibit higher levels in *M. tuberculosis phoP* mutants and results in more efficient secretion of TAT substrates (20). Among these, we found Ag85A (79 epitopes) and Ag85C (5 epitopes). Future experiments will demonstrate whether this enhanced antigenic secretion translate into more efficient immunity against these antigens.

Exploration of animal-adapted species of the MTBC such as *M. bovis* have revealed some epitope polymorphisms compared to *M. tuberculosis* (60) and this list is expected to increase as more animal isolates become sequenced. Two recent studies have demonstrated that these epitope polymorphisms result in differential immune responses (61) or MHC recognition (60). Consequently, even if the antigen repertoire from the human and the cow pathogen is highly similar, we can hypothesize that a

TABLE 2 | Summary table of described tuberculosis T cell-epitopes present in RD regions absent in Bacille Calmette-Guérin (BCG).

RD region	Rv number/gene name		# Epitopes	Sum
RD10	Rv0221		1	4
	Rv0222	*echA1*	3	
RD13	Rv1255c		1	2
	Rv1256c	*cyp130*	1	
RD4	Rv1513		1	1
RD3	Rv1582c		2	2
RD14	Rv1769		4	4
RD7	Rv1965	*yrbE3B*	1	5
	Rv1973		1	
	Rv1977		3	
RD2	Rv1979c		6	59
	Rv1980c	*mpt64*	24	
	Rv1984c		1	
	Rv1985c		23	
	Rv1986		4	
	Rv1987		1	
RD9	Rv2074		1	1
RD5	Rv2346c		2	3
	Rv2350c	*plcB*	1	
RD11	Rv2645		13	42
	Rv2653c		12	
	Rv2654c		16	
	Rv2658c		1	
RD6	Rv3427c		1	1
RD8	Rv3617	*ephA*	2	2
RD1	Rv3871	*eccCb1*	2	307
	Rv3873	*ppe68*	79	
	Rv3874	*cfp10*	95	
	Rv3875	*esat6*	112	
	Rv3876	*espI*	1	
	Rv3878	*espJ*	12	
	Rv3879c	*espK*	6	
			Total	433

vaccine for human use will benefit from stimulating the immune system with the pathogen from human origin.

CONCLUDING REMARKS

In the current pipeline of TB vaccine candidates in clinical evaluation, MTBVAC is the only vaccine able to induce CFP10- and ESAT6-specific immune responses. What is more, our most recent results (26) suggest that these responses could be effective in protecting from pulmonary TB, which would have an evident impact on TB transmission. In support of this hypothesis, prospective cohort studies with individuals exposed to patients with active TB indicate that persons with latent TB infection (i.e., those reactive to CFP10 and ESAT6 stimulation) could be more protected against secondary *M. tuberculosis* infection than non-infected people (62). Thus, analysis of CFP10- and ESAT6-positive cells after vaccination in future clinical efficacy trials with MTBVAC would be highly valuable in the search of a possible biomarker of protection.

AUTHOR CONTRIBUTIONS

NA, DM, JG-A, and CM contributed equally in the elaboration of the manuscript.

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REVIEW
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The Recombinant Bacille Calmette–Guérin Vaccine VPM1002: Ready for Clinical Efficacy Testing

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The only licensed vaccine against tuberculosis (TB), bacille Calmette–Guérin (BCG), protects against severe extrapulmonary forms of TB but is virtually ineffective against the most prevalent form of the disease, pulmonary TB. BCG was genetically modified at the Max Planck Institute for Infection Biology to improve its immunogenicity by replacing the urease C encoding gene with the listeriolysin encoding gene from *Listeria monocytogenes*. Listeriolysin perturbates the phagosomal membrane at acidic pH. Urease C is involved in neutralization of the phagosome harboring BCG. Its depletion allows for rapid phagosome acidification and promotes phagolysosome fusion. As a result, BCGΔ*ureC*::*hly* (VPM1002) promotes apoptosis and autophagy and facilitates release of mycobacterial antigens into the cytosol. In preclinical studies, VPM1002 has been far more efficacious and safer than BCG. The vaccine was licensed to Vakzine Projekt Management and later sublicensed to the Serum Institute of India Pvt. Ltd., the largest vaccine producer in the world. The vaccine has passed phase I clinical trials in Germany and South Africa, demonstrating its safety and immunogenicity in young adults. It was also successfully tested in a phase IIa randomized clinical trial in healthy South African newborns and is currently undergoing a phase IIb study in HIV exposed and unexposed newborns. A phase II/III clinical trial will commence in India in 2017 to assess efficacy against recurrence of TB. The target indications for VPM1002 are newborn immunization to prevent TB as well as post-exposure immunization in adults to prevent TB recurrence. In addition, a Phase I trial in non-muscle invasive bladder cancer patients has been completed, and phase II trials are ongoing. This review describes the development of VPM1002 from the drawing board to its clinical assessment.

Keywords: tuberculosis, bacille Calmette–Guérin, VPM1002, vaccine, listeriolysin, immune response

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Abbreviations: AIM2, absent in melanoma 2; BCG, bacille Calmette–Guérin; CFUs, colony forming units; CTL, human cytotoxic lymphocyte; DC, dendritic cell; EPI, expanded program of immunization; GBP, guanylate binding protein; HEU, HIV-exposed uninfected; IgG, immunoglobulin G; LC3, microtubule-associated protein light chain 3; LLO, listeriolysin O; MHC, major histocompatibility complex; *Mtb*, *Mycobacterium tuberculosis*; NLRP3, NLR family pyrin domain-containing 3; NMIBC, non-muscle-invasive bladder cancer; rBCG, recombinant BCG; RD1, Region of Difference 1; SCID, severe combined immunodeficiency; STING, stimulator of interferon genes; TB, tuberculosis; T_{CM}, central memory T cells; T_{EM}, effector memory T cells; T_{FH}, follicular T cells; Th, T helper cell.

INTRODUCTION

Infection with *Mycobacterium tuberculosis* (*Mtb*) led to 10.4 million recorded cases of tuberculosis (TB) in 2015, with 1.8 million recorded deaths [World Health Organization (WHO) report 2016]. The current therapy involves 6–9 months of antibiotics, with the emergence of multiple drug resistant strains being a continuing obstacle. An attenuated form of the bovine *Mycobacterium* species, *Mycobacterium bovis* bacille Calmette–Guerin (BCG) has been in clinical use since 1921 and remains the only licensed vaccine against TB. BCG partially protects against TB meningitis and disseminated TB in infants and has non-specific immunostimulatory effects (1), which reduce general infant mortality by enhancing responses to other infectious diseases (2, 3). However, in all age groups, BCG does not adequately protect against pulmonary TB, the most prevalent form of disease and the route of disease transmission. In addition, BCG can cause severe adverse effects in immunocompromised individuals (4) and hence is contraindicated in HIV-infected individuals, the group that is most vulnerable to TB. However, in the absence of an alternative, BCG continues to be used in the immunization programs of several countries.

To overcome these issues, several TB vaccine candidates are under development (5). One of the most advanced among them is BCG *ΔureC*::*hly* (VPM1002) (6).

VPM1002 is a recombinant BCG (rBCG) in which the urease C gene has been replaced by the listeriolysin O (LLO) encoding gene (*hly*) from *Listeria monocytogenes* (7). Urease C drives neutralization of phagosomes containing mycobacteria by generation of ammonia, thereby inhibiting phagolysosomal maturation and contributing to the survival of mycobacteria inside the macrophage (8, 9). Its depletion allows for rapid phagosome acidification, which promotes phagolysosome fusion and provides the optimal pH for LLO stability (10). LLO is a cholesterol-dependant cytolysin that forms transmembrane β-barrel pores in the phagolysosome membrane, allowing escape of *L. monocytogenes* into the cytosol (10, 11). Its expression in VPM1002 results in the release of antigens and bacterial DNA into the cytosol, triggering autophagy, inflammasome activation, and apoptosis. VPM1002 has demonstrated substantially increased immunogenicity, efficacy, and safety in preclinical studies, successfully passed Phase I and II clinical trials, and will now enter a Phase II/III clinical trial in India in 2017. This review summarizes the development, preclinical, and clinical testing of VPM1002 (**Figure 1**).

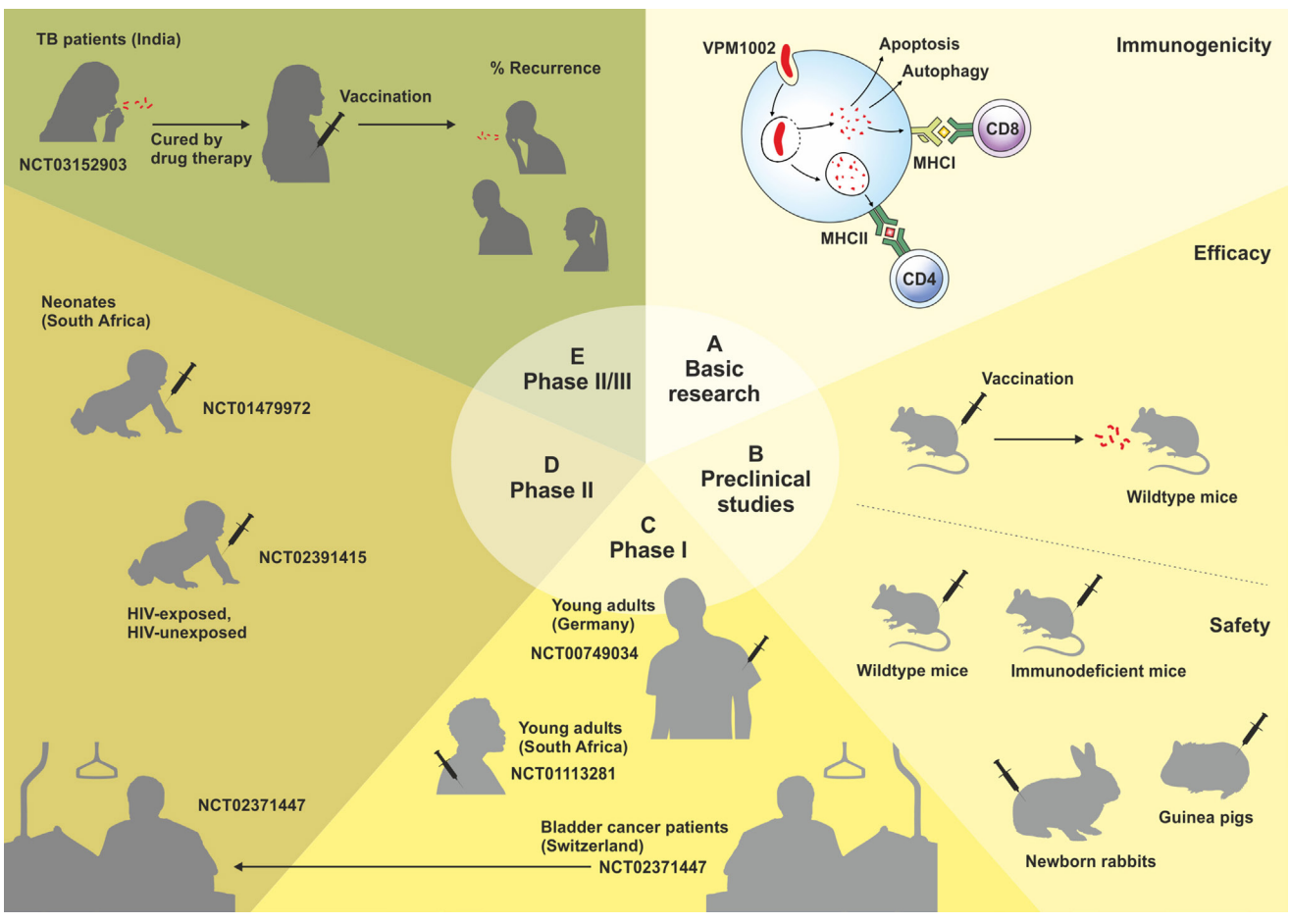

FIGURE 1 | Schematic overview of the development of the VPM1002 vaccine candidate. Clinical trials are labeled by their ClinicalTrials.gov Identifier number.

DESIGN AND GENERATION OF VPM1002

The attenuation of BCG was achieved by passaging virulent *M. bovis* in bile-containing medium for 13 years in the laboratory (12), during which time several genome segments were lost, including a segment known as Region of Difference 1 (RD1) which encodes the unique mycobacterial ESX-1 type VII secretion system (13, 14). ESX-1-dependent perturbation of host cell membranes requires direct contact with pathogenic mycobacteria such as *Mtb*, allowing the bacilli or their antigens to egress the phagosome into the cytosol (15). *Mtb* antigens are thus accessible to both the endocytic major histocompatibility complex (MHC) class II antigen presentation pathway and the MHC I antigen presentation pathway in the cytosol, and consequently can stimulate $CD4^+$ and $CD8^+$ T-cell subsets, respectively, both of which are required for optimal protection against TB (16–21). In addition, ESX-1 dependent release of *Mtb* DNA into the cytosol can be detected by host sensors, leading to activation of NLR family pyrin domain-containing 3 (NLRP3) and absent in melanoma 2 inflammasomes, release of interferons, increased autophagy and apoptosis (22–25). Induction of apoptosis in infected host cells generates vesicles carrying mycobacterial antigens that can be phagocytosed by bystander antigen presenting cells, mainly dendritic cells (DCs) and trafficked through MHC I antigen processing pathways to stimulate $CD8^+$ T cells in a process known as cross-priming (26, 27). Mice with deficient cross-presentation due to the absence of annexin 1 show impaired *Mtb*-specific $CD8^+$ T cells and are highly susceptible to TB (28). Lacking the ESX-1 secretion system, BCG is restricted to the phagosome of host cells, therefore its antigens and bacterial DNA do not enter the cytosol and the antigens are primarily processed by MHC class II pathways, stimulating $CD4^+$ T cell responses (13, 14, 29, 30). BCG induces only weak apoptosis and $CD8^+$ T cell responses (26). Furthermore, both BCG and *Mtb* inhibit surface MHC II expression, as urease-dependent alkalinization of the phagosome causes intracellular sequestration of MHC II dimers, resulting in suboptimal $CD4^+$ T cell responses (31–33). Phagosomal biology is therefore a clear target for interventions aimed at enhancing T cell responses against mycobacteria.

Originally, VPM1002 was designed to improve accessibility of mycobacterial antigens to the MHC I pathway via cytosolic egression of antigens mediated by LLO perturbation of phagosomal membranes in order to improve induction of $CD8^+$ T cells by the parental BCG strain (34, 35). In addition, leakage of phagolysosomal proteases such as cathepsins into the cytosol could activate caspases, leading to apoptosis and subsequent cross-presentation of mycobacterial antigens, which promotes both MHC I and MHC II restricted T cell stimulation (36). Studies with *L. monocytogenes* have shown that pore formation by LLO also triggers many downstream effects such as activation of the NLRP3 inflammasome, induction of cytokine expression, activation of kinases, triggering of endocytosis, histone modification and release of calcium from intracellular stores (37). An Hly recombinant strain, *hly*$^+$ $rBCG^+$, was generated by integrating the *hly* gene into BCG using the mycobacteria-*Escherichia coli* shuttle vector pMV306 (34). LLO was detected in the membrane structures, phagosomal space, and cytoplasmic vacuoles of macrophages infected with BCG pMV306::*hly*, and intracellular persistence of this strain was reduced compared with the parental BCG strain. MHC I presentation of co-phagocytosed soluble protein was improved in macrophages infected with this strain compared to BCG (34) and an *in vitro* human cytotoxic T lymphocyte (CTL) assay using cultured DCs and T cells from healthy human donors demonstrated that *hly*$^+$ BCG infection was better at inducing CTL responses than BCG infection (38). In the next generation strain, deletion of *ureC* was performed to ensure an optimal (acidic) pH for LLO stability; however, absence of *ureC* also promotes MHCII trafficking to the macrophage surface (31), which would also stimulate $CD4^+$ T cell responses. To generate Δ*ureC hly*$^+$ BCG, the chromosomal integrative shuttle vector pMV306*hyg-hly* (8) was used to transform *M. bovis* BCG Δ*ureC*::*aph*, and hygromycin-resistant clones were selected (35). The vaccine was licensed to Vakzine Projekt Management, and named "VPM1002." The resistance cassette was subsequently successfully removed, although VPM1002 is equally sensitive to the antimycobacterial agents isoniazid, rifampicin, and ethambutanol in the presence or absence of the hygromycin resistance gene (39).

HOST CELL RESPONSES TO VPM1002 *IN VITRO*

Increased quantities of mycobacterial antigen were detected in VMP1002 infected macrophages compared to BCG infected macrophages (35), and mycobacterial DNA was detected only in the cytosol of VPM1002 infected but not BCG infected macrophages (29), indicating that expression of LLO in BCG Δ*ureC*::*hly* allows the escape of bacterial products to the cytosol, presumably by perturbation of the phagosomal membrane. The bacteria themselves do not escape to the cytosol, unlike *Mtb* bacilli (29, 35). Infection of primary human and mouse macrophages demonstrated increased apoptosis after infection with VPM1002 compared to both BCG and BCG::*hly*, demonstrating the additional benefit of urease C deletion (35). Membrane disruption can facilitate the release of phagolysosomal proteases such as cathepsins into the cytosol, which are known to induce apoptosis (36, 40). Both the presence of mycobacterial proteins in the cytosol and the induction of apoptosis by perforation of the phagosomal membrane could cause increased trafficking of antigens to MHC I pathways (35). Apoptosis results in an increase in both $CD8^+$ and $CD4^+$ T cell responses in mycobacterial infection, suggesting that DCs may transfer efferocytosed antigens to the endocytic system (27, 36). The priming potential of apoptotic vesicles isolated from BCG and VPM1002 infected mouse macrophages was investigated in a co-culture system with splenic DCs and T cells, and VPM1002-infected apoptotic vesicles induced more profound $CD4^+$ and $CD8^+$ T cell responses compared to those infected with BCG (41). Vesicles from VPM1002 infected macrophages also induced higher production of the T helper type (Th)17-polarizing cytokines interleukin (IL)-6 and IL-23, and the immunoregulatory cytokine IL-10 by bone marrow-derived DCs.

Experiments in THP1 macrophages demonstrated that VPM1002 infection leads to activation of multiple caspases (29). The apoptotic effector caspases 3 and 7 were highly activated by VPM1002 in comparison to BCG, as well as caspase 1, which mediates pyroptosis, an inflammatory form of cell death and is an important regulator of the inflammatory response (42). Inflammasomes are multi-protein complexes composed of intracellular sensors and caspase 1. They control activation of caspase 1, which in turn cleaves the precursors of the cytokines IL-1β and IL-18 into their active forms (43). VPM1002 infection increased production of IL-1β and IL-18, which was dependent on AIM2 inflammasome activation but not on NLRP 1 and 3 inflammasome activation. Furthermore, VPM1002 induced increased levels of the autophagy marker microtubule-associated protein light chain 3 in an AIM2- and stimulator of interferon genes (STING)-dependent manner. The AIM2 inflammasome senses cytosolic DNA and is involved in the induction of caspase 1-dependent pyroptosis (44, 45), while STING acts as an essential adaptor protein in the induction of autophagy by cytosolic DNA (25). Autophagy, a protein degradation process induced by stress conditions such as infection, promotes the delivery of cytosolic antigens to MHC trafficking pathways (46, 47). It has also been shown to contribute to innate immunity against mycobacteria and other intracellular pathogens (48, 49). While autophagy was originally thought to be non-specific, it is now known that it can selectively target intracellular pathogens in a process known as xenophagy that involves ubiquitination of pathogen proteins or pathogen-containing endosomes (50). Intriguingly, gene expression of guanylate-binding proteins (GBPs) was also elevated in VPM1002 infected THP-1 macrophages compared to BCG infected macrophages. Interferon-inducible GBPs have multiple roles in inflammasome activation, autophagy, and lysis of pathogen-containing vacuoles and can even directly target the pathogens themselves (51–54). Whether they play a role in the translocation of mycobacterial components from the phagosome into the cytosol during VMP1002 infection remains to be determined.

Disruption of the VPM1002-containing phagosome membrane by LLO and release of mycobacterial DNA into the cytosol appears to have effects in inducing immune responses that are similar to the effects of ESX-1 activity in *Mtb* or *M. marinum*. ESX-1 of *M. marinum* stimulates autophagosome formation and recruitment to the vacuole; however, unlike LLO it also inhibits autophagic flux, thereby preventing bacterial degradation (49). Testing of vaccine candidates expressing ESX-1 such as *Mtb* Δ*ppe25-pe19* (55) and BCG expressing ESX-1 of *M. marinum* (BCG:ESX-1^{Mmar}) (56) demonstrated that ESX-1 was critical for enhancing innate immune responses via phagosome rupture. BCG:ESX-1^{Mmar} induced the cGas/STING/TBK1/IRF-3/type I interferon axis and promoted AIM2 and NLRP3 inflammasome activation, resulting in increased frequencies of antigen-specific CD8$^+$ and CD4$^+$ T cells and increased protection against *Mtb* compared to BCG (56), while *Mtb* Δ*ppe25-pe19* also led to enhanced protection. ESX-1 may induce protective immunity by an additional mechanism, as ESAT6 is required for rapid, non-cognate IFN-γ production by CD8$^+$ T cells, mediated by the NLRP3/caspase-1/IL-18 axis (57).

PRECLINICAL EFFICACY AND SAFETY

Aerosol challenge of vaccinated BALB/c or C57BL6 mice with 100–200 colony-forming units (CFUs) of *Mtb* H37Rv or a clinical isolate of the Beijing/W genotype family demonstrated that VPM1002 immunization has significantly greater protective efficacy than the parental BCG strain, with bacterial loads in the lungs typically reduced by one to two logs in late stages of infection (35, 58–61). In a low dose infection (30 CFU), VPM1002 led to an almost 1000-fold reduction of *Mtb* in the lungs compared to naïve mice at day 200 after infection (35). Homologous boosting with VPM1002 did not improve protection compared to a single immunization (60). However, a post-exposure vaccination model using antibiotics for an extended period and then allowing bacterial regrowth demonstrated that mice with subclinical TB had lower bacterial burdens when vaccinated with VPM1002 compared to BCG, suggesting that VPM1002 could also be considered for use as a post-exposure vaccine (60).

The safety profile of VPM1002 has been evaluated in animal models including mice, guinea pigs, rabbits, and non-human primates (6). In *RAG1-/-* immunodeficient mice lacking mature T and B cells, bacterial loads were not significantly different in lungs and spleen after vaccination with VPM1002 compared to BCG (35). However, VPM1002 demonstrated substantially lower virulence in severe combined immunodeficiency mice, most likely due to the reduced intracellular persistence of this strain (35, 61). After immunization of wildtype BALB/c or C57BL6 mice, VPM1002 was more rapidly cleared from the draining lymph nodes than BCG and disseminated less to the spleens, where it was also quickly cleared (59, 61). Dissemination to the lungs was observed in BCG vaccinated but not VPM1002 vaccinated mice. Enhanced adaptive immune responses after VPM1002 vaccination are therefore likely to play a role in the reduced dissemination of VPM1002 in immunocompetent mice. Overall, the data demonstrate increased safety and protective efficacy of VPM1002 compared to parental BCG in mice.

In guinea pigs and non-human primates, the safety of VPM1002 was comparable to that of BCG (6, 39). As the primary target population for vaccination against TB is newborns, the safety profiles of VPM1002 and BCG were also compared in newborn rabbits (39). No dissemination to tissues was observed after VPM1002 administration, and the body weight gain was not affected during the 90 days observation period, whereas the body weight was reduced in the BCG vaccinated group compared to the saline control group. No premature mortality was observed in either group. The preclinical safety of VPM1002 is thus supported by a large body of evidence.

ANALYSIS OF IMMUNE RESPONSES TO VPM1002 IN MICE

Analysis of gene expression in mice early after immunization with VPM1002 demonstrated that, as in THP-1 cells, expression of IL-18 and IL-1β was increased, as well as expression of IFN-inducible genes such as *Tmem173* (STING), *Gbp*'s,

and other GTPases (29, 61). Apoptosis was increased in the lymph nodes of VPM1002 immunized mice compared with BCG immunized mice by day 14 (61). Immunization with VMP1002 induced both type 1 and type 17 cytokine responses in mice, whereas BCG induced type 1 responses only (58). After restimulation with PPD, levels of IFN-γ, IL-17, IL-2, IL-6, and GM-CSF were increased in lung cells isolated from VPM1002 immunized mice compared to those from BCG immunized mice, and splenocytes from VPM1002-vaccinated mice also produced more IL-17. Furthermore, percentages of γδ T cells producing IFN-γ and IL-17 were increased after vaccination with VPM1002 (58). Seven days after *Mtb* challenge, IL-2$^+$TNF$^+$ double cytokine producing cells were increased in the lungs of VPM1002-immunized mice compared with BCG-vaccinated mice, suggesting recall responses, because newly generated T cells take 12–14 days to reach the lungs during *Mtb* infection (62). IL-2$^+$TNF$^+$ CD4$^+$ T cells typically show a central memory phenotype (T_{CM}) (21), and further studies demonstrated that VPM1002 immunization indeed induces higher frequencies of T_{CM} than immunization with BCG (59, 61). Ag85B-specific CD4$^+$ T_{CM} were significantly increased in the draining lymph nodes of VPM1002-vaccinated compared to BCG-vaccinated mice at day 14 (59).

Bacille Calmette–Guérin induces effector memory CD4$^+$ T (T_{EM}) cells that can control acute infection but appears to induce insufficient numbers of T_{CM} cells for long-term protection (21). Transfer studies demonstrated that T_{CM} cells from VPM1002 infected mice conferred protection against TB infection whereas T_{EM}, T follicular helper (T_{FH}), and naïve T cells did not, at least at the numbers of cells tested (59). These findings concur with other studies in which T_{CM} cells were associated with protection (21). While T_{EM} cells appear early after infection and provide protection by the secretion of effector cytokines such as IFN-γ and TNF-α, T_{CM} cells proliferate in the LN and generate new pools of T_{EM} cells after re-exposure to antigen (59, 63, 64). The T_{CM} cells generated by subcutaneous vaccination with VPM1002 or BCG were found to reside over the long term in the secondary lymphoid organs, rather than in the lung, and to be recruited to the lungs after *Mtb* challenge (58, 59). Waning of BCG-induced immunity correlates with a decline in T cell functions such as cytokine production and CTL activity and an increase in terminally differentiated, dysfunctional T cells (65). Thus, systemic maintenance of T_{CM} populations over the long term and the rapid recruitment of T_{CM} cells to the lung following *Mtb* infection remains a key goal in the development of more effective vaccine candidates (59). VPM1002 also induced an increase in mycobacteria-specific immunoglobulin G levels after vaccination compared to BCG, and a concomitant increase in CXCR5-expressing T_{FH} cells (59, 61), which have been associated with decreased lung pathology (66) and stimulate germinal center B cell responses (63). Passive transfer of serum from VPM1002- or BCG-immunized mice on the day of *Mtb* infection and thrice weekly did not reduce bacterial load at day 14 (59), but growing evidence suggests that antibodies may play a role in protection against *Mtb* (67–71). Overall, increased protection conferred by VPM1002 immunization in the mouse model was associated with increased numbers of T_{CM} and T_{FH} cells, increased Th17 responses, earlier recruitment of T cells to the lungs following *Mtb* challenge and increased levels of anti-mycobacterial antibodies (58, 59, 61).

CLINICAL TRIALS WITH VPM1002: A STEP TOWARD A SAFER, MORE EFFICACIOUS TB VACCINE

Human data on VPM1002 are available from three clinical trials, all performed with the original hygromycin-resistant strain of VPM1002. Two Phase I studies were performed in healthy adult volunteers, and one Phase IIa study was conducted in healthy newborn infants, one of the intended target populations. In the first Phase I clinical trial (ClinicalTrials.gov Identifier: NCT00749034) conducted in Germany, healthy Caucasian adult males with (W) or without (WO) a history of BCG vaccination received VPM1002 randomized to three escalating doses ($N = 30W + 30WO$) or BCG at the standard vaccine dose ($N = 10W + 10WO$) and were followed for 6 months. Single vaccination with VPM1002 up to 5 × 10e5 CFU was safe and well tolerated. The immunogenicity of VPM1002 as measured by IFN-γ release by stimulated T cells was dose dependent. Both VPM1002 and BCG induced multifunctional CD4$^+$ and CD8$^+$ T cell subsets, which are thought to play a role in protection against TB (72–74), with VPM1002 showing an earlier increase in double and triple cytokine producing T cells which remained at heightened levels throughout the study (7). Furthermore, only VPM1002 induced serum antibodies against mycobacterial antigens (7), echoing preclinical studies in which VPM1002 induced higher levels of mycobacteria-specific antibodies than BCG in mice (59, 61). In the second Phase I clinical trial (ClinicalTrials.gov Identifier: NCT01113281), performed in South Africa, 24 healthy male and female adults with a history of BCG immunization, predominantly from the indigenous African population, were vaccinated with VPM1002. The study showed that a single vaccination with VPM1002 is safe, well tolerated and elicits a profound immune response in an African adult population (6).

The Phase IIa clinical trial (ClinicalTrials.gov Identifier: NCT01479972) was the first investigation of VPM1002 in newborns (75). It was conducted in Cape Town, South Africa, a region with a high TB burden. Forty-eight HIV-unexposed, newborn infants were vaccinated with either VPM1002 ($n = 36$) or BCG ($n = 12$) through an open label, randomized, controlled design. Polyfunctional CD4$^+$ and CD8$^+$ T cell responses were similar between the groups, and both groups had increased IFN-γ responses after 7 h PPD stimulation at all measured time points post vaccination compared to baseline. Both vaccines induced IL-17 responses; though, unlike BCG, VPM1002 induced increased proportions of CD8$^+$ IL-17$^+$ T cells at day 14 and month 6 time points compared to the baseline. The incidence of abscess formation was lower for VPM1002 compared to BCG. Thus, VPM1002 was safe, well tolerated, and immunogenic in newborn infants.

In addition, a Phase IIb clinical trial is currently ongoing in South Africa (ClinicalTrials.gov Identifier: NCT02391415). This trial is a double-blind, randomized, controlled study to evaluate

the safety and immunogenicity of VPM1002 in comparison with BCG in HIV-exposed uninfected (HEU) and HIV-unexposed, BCG-naive newborn infants. The inclusion of HEU infants in the trial is important, as this group comprises 30% of the newborns requiring BCG vaccination in South Africa, and they may be at higher risk of *Mtb* infection than HIV-unexposed infants. The proportion of HEU may vary in different countries. Previous work from Brazil suggests that HEU infants have poorer T-cell proliferation and lower levels of IFN-γ production compared to HIV-unexposed infants (76). Enrollment of 416 infants has been completed and follow-up is in progress. Follow-up will continue for 12 months, as opposed to 6 months in NCT01479972, enabling collection of preliminary efficacy data.

In addition to its development as a vaccine for newborns, VPM1002 is also being assessed as a post-exposure vaccine for adults, since preclinical studies in mice demonstrated that it reduced bacterial loads in a post exposure model (60). A phase II/III trial has received regulatory approval by the Indian authorities. Once ethics committee approvals are received for all sites, the trial will commence across India (ClinicalTrials.gov Identifier: NCT03152903). The study will be conducted in 2000 adults who were TB patients, but received drug treatment and were cured of disease. In such populations, there is a high risk of recurrence (including re-infection and relapse), especially within 12 months after completing treatment. The multi-centric, placebo-controlled, randomized, controlled study will assess whether VPM1002 can prevent such TB recurrence over a 1-year follow-up period. Currently, no intervention is licensed for this indication, including BCG, which means there is clearly an unmet medical need. The study will also expand the safety database on VPM1002.

EVALUATION OF VPM1002 AS A BLADDER CANCER THERAPY

Bladder cancer is the ninth most common cancer in the world, and is four times more common in men than in women (77). The main risk factors for developing bladder cancer include smoking, *Schistosoma* infection (bilharzia), and exposure to industrial chemicals (77, 78). Tumors can be non-muscle invasive, i.e., confined to the mucosa of the bladder wall, or muscle-invasive. More than seventy percent of bladder cancers are detected while they are still non-muscle invasive (79). Due to its immunostimulatory properties, repeated intravesical BCG instillation is the standard adjuvant treatment for intermediate to high-risk non-muscle-invasive bladder cancer (NMIBC) after transurethral resection of the tumors (80–82). BCG therapy reduces the risk of recurrence and the progression to muscle invasive bladder cancer. The repeated instillations require much higher doses and volumes of BCG than vaccination against TB does, and some patients have adverse events that lead to discontinuation of the therapy (83, 84). Adverse events include fever, bladder irritation, decreased bladder capacity, incontinence, hematuria, flu-like symptoms and in approximately 5% of cases, BCG infection (85, 86). Patients undergoing traumatic catheterization are at risk for intraluminal BCG dissemination, resulting in a potentially lethal systemic infection (87).

The precise immune mechanisms by which BCG promotes anti-tumor activity in bladder cancer are not completely resolved, but it is well-established that the ability of BCG to promote Th1 responses is important, as well as the recruitment of neutrophils and innate lymphocytes including natural killer cells (82, 88, 89). Activation of immune cells may lead to elimination of the urothelial cancerous cells that have internalized BCG (82, 90). Increased $CD4^+$ T cell responses have been measured during BCG therapy, and BCG was shown to promote secretion of both Th1- and Th2-type cytokines (88, 91–93). A positive response to BCG therapy (no recurrence or evidence of disease during follow-up examinations) has been associated with an intratumoral Th2 predisposition (increased GATA3) and decreased concentrations of IL-10, combined with a Th1 functional phenotype indicated by increased levels of Th1-related inflammatory metabolites (88). In another study, increased regulatory T cells and tumor-associated macrophages in the tumor microenvironment were also associated with non-responsiveness, while increased $GATA3^+$ and $CD4^+$ T cells were associated with responders (88, 94). BCG Connaught conferred greater 5-year recurrence-free survival than BCG Tice and induced stronger Th1 type responses, BCG-specific $CD8^+$ T cells and T cell recruitment to the bladder (93). Genetic analysis demonstrated several differences between the two strains, including the absence of RD15 in BCG Connaught (93).

Because approximately 30–40% of patients do not respond to BCG therapy and others suffer from adverse events, rBCG technology has been tested for improving the efficacy and tolerability of BCG in bladder cancer therapy (82). rBCGs that have been modified to express immunostimulatory molecules, cytokines, or antigens have been tested in mice for their capacity to induce stronger and more specific immune responses. VPM1002 is currently being evaluated in SAKK 06/14, a Phase I/II trial for immunotherapy in patients with NMIBC (ClinicalTrials.gov Identifier: NCT02371447). The phase I part of the trial has been completed in Switzerland. Intravesical application of VPM1002BC demonstrated that the product is safe and well tolerated in NMIBC patients. The recommended phase II dose has been established as 1–19.2 × 10e8 CFUs of VPM1002BC. The phase II part has been approved by the Swiss and German regulatory authorities and is currently ongoing in both countries.

OUTLOOK

The available preclinical and clinical data reveal that VPM1002 is immunogenic and may be better than BCG in terms of safety. VPM1002 could be a safe, well-tolerated and efficacious alternative to the BCG vaccine in the future. With an annual capacity of 100 million doses, Serum Institute of India Pvt. Ltd. can meet the global demand for a BCG vaccine and is well poised to supply the new vaccine if efficacy trials are successful. While this vaccine progresses through efficacy trials, next-generation derivatives are being designed and tested in preclinical models aimed at optimizing efficacy and/or safety (61, 95). Furthermore, VPM1002 is currently being tested in goats by the Friedrich Loeffler Institute in Germany for the prevention of *M. caprae* infection (Menge et al. unpublished data). Infections with *M. caprae* and *M. bovis*, closely related species of the same clade that cause TB in goats and cattle, respectively, are of agricultural importance, and can potentially be transmitted to humans (96, 97).

Almost 100 years after the first immunization with BCG, a rBCG vaccine candidate is ready for clinical efficacy testing. This marks a major step forward in the long journey that began when the recombinant vaccine was constructed in the late 1990s and tested in different animal models to determine its safety and protective effect.

AUTHOR CONTRIBUTIONS

NN, LG, and SK wrote and reviewed the manuscript. All other authors (BE, PK, US, CR, and MC) reviewed the manuscript.

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REVIEW
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TBVAC2020: Advancing Tuberculosis Vaccines from Discovery to Clinical Development

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TBVAC2020 is a research project supported by the Horizon 2020 program of the European Commission (EC). It aims at the discovery and development of novel tuberculosis (TB) vaccines from preclinical research projects to early clinical assessment. The project builds on previous collaborations from 1998 onwards funded through the EC framework programs FP5, FP6, and FP7. It has succeeded in attracting new partners from outstanding laboratories from all over the world, now totaling 40 institutions. Next to the development of novel vaccines, TB biomarker development is also considered an important asset to facilitate rational vaccine selection and development. In addition, TBVAC2020 offers portfolio management that provides selection criteria for entry, gating, and priority settings of novel vaccines at an early developmental stage. The TBVAC2020 consortium coordinated by TBVI facilitates collaboration and early data sharing between partners with the common aim of working toward the development of an effective TB vaccine. Close links with funders and other consortia with shared interests further contribute to this goal.

Keywords: tuberculosis, bacille Calmette–Guérin, vaccination, biomarker, clinical trial, portfolio management, discovery

INTRODUCTION

One hundred years ago, Albert Calmette (1863–1933) and Camille Guérin (1872–1961) had almost reached their goal (1). They had passaged *Mycobacterium bovis* 200 times at 14 d intervals on potato slices soaked with ox gall in their attempt to generate an attenuated vaccine against tuberculosis (TB). They had started their program in 1906 and had already obtained preliminary evidence for safety and protection of their candidate in 1913. Yet, they continued until they had completed the 230th passage before verifying its efficacy and safety in experimental animals including guinea pigs, rabbits and non-human primates (NHPs) as well as in the natural host, cattle. The data obtained were highly promising so Calmette and Guérin started the first vaccination of a human neonate born

into a household with a TB patient. Risk of TB in a household-contact infant at those times was extremely high. Yet, the baby did not develop TB and after additional encouraging results, vaccinations of more than 20,000 neonates in households with at least one TB patient were performed between 1921 and 1924. The results of this trial revealed that of the vaccinees 5% had died and 1% of these of TB, whereas knowledge of the time held that a quarter of non-vaccinated newborns would die in the first years of life, many of them of TB (1). This is the start of Bacille Bilié Calmette-Guérin, later shortened to Bacille Calmette–Guérin (BCG), which remains the only licensed vaccine against TB until today. Although not perfect, this vaccine partially fulfills the goal set by Calmette and Guérin, since it protects against extra-pulmonary forms of disease in infants, including life threatening TB meningitis. Unfortunately, in most settings, BCG does not reliably prevent pulmonary TB, the most prevalent and the most contagious form in all age groups from the neonatal to the elderly population. The lack of an efficient vaccine against pulmonary TB is a major obstacle to control TB satisfactorily until today.

In 1993, the World Health Organization (WHO) declared TB a global emergency at a time when some 4–6 million new TB cases per year were notified. With the introduction of a new treatment regimen, called directly observed treatment, short-course (DOTS) program, WHO claimed to bring TB under control and predicted a decline from 6–8 million new cases in 1997 to 2–3 million new cases by 2017 by implementing highly intensive DOTS (2). Unfortunately, this hope failed miserably. The most recent notifications reveal a stunning 10.4 million new cases and 1.8 million deaths in 2015. Building on better drug regimens alone not only proved to be insufficient in controlling TB, it also led to increasing incidences of multi-drug-resistant forms of TB.

At this time, researchers interested in the mechanisms underlying immunopathology and immune protection of TB were concerned that the efficacy of DOTs might be overestimated and considered a better TB vaccine a necessity for efficient TB control. Thus, a proposal on TB vaccines was submitted to the European Commission (EC) funded framework program (FP) 5 and this was funded from 1998 to 2002. The subsequent FP 6 and FP 7 programs continued to support TB vaccine-related research until 2013. During this period, enormous progress was achieved, not only resulting in the development of a number of promising vaccine candidates but also providing deeper understanding of immune mechanisms underlying protection and pathology in TB as well as identifying new TB biomarkers (BMs). These FP5 to FP7 activities were succeeded by the Horizon 2020 program which further supported TB vaccine research and development. It is this continuous support over the past 20 years which has welded together researchers from all over Europe and other parts of the globe to harness cutting-edge knowledge in the immunology, microbiology, and high end technology platforms for the development of novel TB vaccine candidates. This sustained EC funding made it possible to establish a unique European consortium responsible for most of the global innovation in TB research.

This EC-funded activity proved extremely successful. Over 50% of the global pipeline in TB vaccine candidates currently evaluated in clinical trials originate from EC funded projects (3). The EC has played a key leadership role in accelerating TB vaccine research and development and ensured a well-coordinated and efficient TB vaccine research consortium.

At the same time, the pipeline needs to be fed constantly with new innovative candidates since it is unlikely that one single vaccine could protect against all different forms of TB disease in all age groups and for all indications. Thus, the concept of diversification gained increasing importance. Different vaccine types (subunit vaccines vs. whole cell vaccines), different administration schedules (pre-exposure vs. post-exposure), different age groups (neonates vs. adults) and different purposes (prevention of infection vs. disease vs. recurrence of disease), and perhaps even therapeutic TB vaccines have all to be considered.

Already before the beginning of TBVAC2020, it was realized that an isolated vaccine development approach was inadequate and needed to be complemented by deeper knowledge of pathomechanisms, immunology, and microbiology; by established as well as novel animal models; by identifying biosignatures of TB disease, risk of disease, vaccine efficacy, and safety (3–6). Finally, with increasing numbers of vaccines being ready for clinical testing, early clinical trials needed to be integrated. Although TBVAC2020 and its predecessors respect individual researchers' autonomy, it was deemed of increasing importance to also provide a portfolio management process, including consensus gating criteria, to objectively and transparently guide further product and clinical development of TB vaccine candidates (7). This article describes the different activities of the Horizon 2020 funded TBVAC2020 project which is coordinated by TBVI summarizing recent achievements and future goals as part of the European effort to successfully combat TB. **Figure 1** provides an overview of the close interactions between different WorkPackage (WP) activities and progression of promising vaccine candidates from discovery *via* preclinical testing to clinical trial. Although TBVI has coordinated much of the European research efforts on TB vaccine development to date, there are other ongoing activities, for example, through the Aeras Foundation[1] and the EMI-TB Consortium.[2]

WP1 DISCOVERY OF NOVEL TB VACCINE STRATEGIES

A major objective of TBVAC2020 is the identification of novel vaccine candidates for subsequent preclinical and early clinical development. To this end, the objective of WP1 is to develop innovative approaches and platforms for TB vaccination in three areas: (i) the discovery of novel antigens and live vaccines; (ii) the exploration and implementation of novel immunization strategies and vaccination platforms; and (iii) the optimization of formulation and delivery of available subunit and live vaccine candidates.

Antigen Discovery

The identification of novel antigens is the initial and critical step in developing new vaccination strategies. It is well established

[1] www.aeras.org.
[2] www.emi-tb.org.

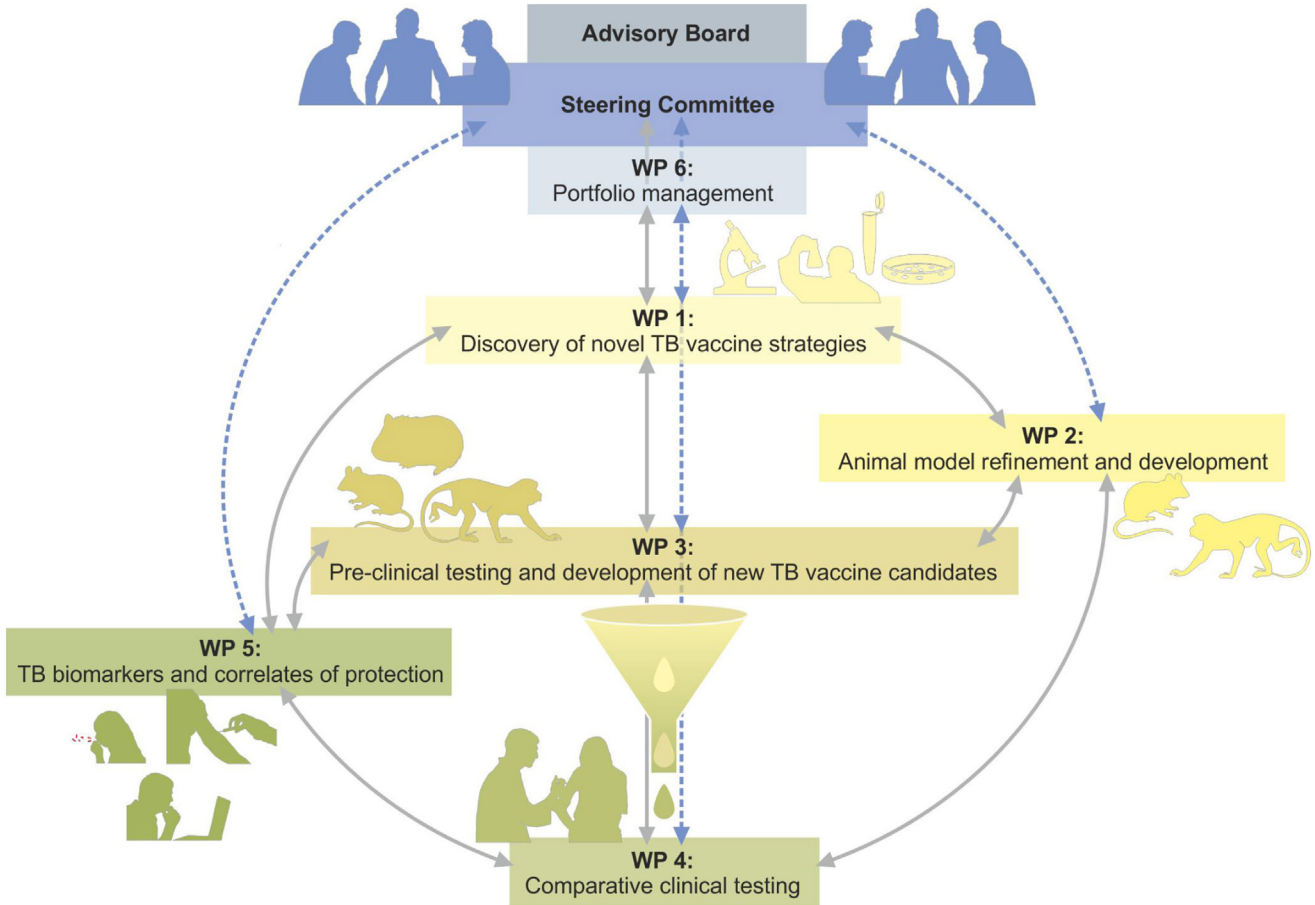

FIGURE 1 | Overview of the TBVAC2020 project structure indication interactions between WorkPackages (WPs) and flow from discovery *via* preclinical testing to clinical trial of most promising vaccine candidates.

that polyfunctional effector T cell subsets act in concert to activate macrophages to limit mycobacterial replication and prevent progression of latent infection to active disease. Our key objective is, therefore, to identify antigens that trigger protective lymphocyte subsets and to improve their delivery to antigen-presenting cells. Specifically, this sub-work package aims to identify: (i) new immunogenic epitopes derived from *Mycobacterium tuberculosis* (*Mtb)*-infected macrophages; (ii) new immunogenic proteins and lipoproteins of *Mtb*; (iii) new immunogenic lipids of *Mtb*; and (iv) antigens that induce antibody-mediated protection.

A high level of innovation is being achieved by applying sophisticated techniques, e.g., for the elution of peptides from human leukocyte antigen (HLA)-molecules from *Mtb*-infected antigen-presenting cells using a data-independent acquisition (DIA) method which aims to complement traditional mass spectrometry-based proteomics techniques and SRM methods (SWATH-MS) (8). This unbiased approach allows the identification of immunogenic antigens that are presented during natural infection with *Mtb*. The use of transcriptomic analysis is another unbiased approach to identify antigens expressed at the site of infection. Unbiased transcriptomic approaches were also used to identify antigens expressed at the site of infection. A number of new *in vivo* expressed (IVE) *Mtb* antigens were identified that were highly expressed in the lungs of TB susceptible (C3HeB/FeJ) mice. Using various predictive algorithms, including potential HLA-I and -II presented epitopes many new antigens were identified that activated both conventional and unconventional T-cells from latently infected individuals (9). Of note, many IVE-TB antigen-directed T cell responses were characterized by the production of cytokines other than IFNγ. Studies are ongoing to dissect antibody recognition of this novel class of antigens.

In parallel to these unbiased approaches, WP1 is following hypothesis-driven approaches for the discovery of vaccine candidates. For example, stage-specific, subdominant mycobacterial antigens encoded by genes co-regulated with those encoding latency antigens, such as the heparin-binding hemagglutinin (HBHA) (10) are being evaluated. Recently, the importance of *Mtb*-specific chemokine receptor expression ($CXCR3^+$ and $CXCR5^+$) lung-homing T lymphocytes in protection against TB has been demonstrated (11). This knowledge has been exploited to show that the liposome-adjuvanted fusion protein H56/CAF01 confers durable protection against TB by eliciting protective T-cells expressing CXCR3 in the lung, while limiting

the induction of non-protective intravascular T-cells (12). The identification and evaluation of lipid antigens is another key objective of WP1. Using protease cleavable CD1b-molecules, formulation of lipid-containing liposomes (13) or inclusion of α-galactosyl ceramide (α-GalCer) (14) to activate iNKT cells the potential of lipid antigens as vaccines is being evaluated. Liposomes are a particularly attractive delivery system, because they offer a simple and flexible platform to combine antigens and adjuvants, including immune modulators triggering the toll-like receptor (TLR) pathways (15).

Another innovative concept addressed in WP1 is the hitherto almost completely neglected search for *Mtb* antigens that trigger protection-associated antibody responses (16). To address this issue, *Mtb*-specific antibodies are being isolated from patients and immunized mice, and their mycobacterial targets are being identified.

Delivery Systems and Immunization Strategies

Mucosal Delivery

The induction of protective immune responses in the lung, which is the site of *Mtb*-entry, is elementary for vaccine development. Previous data showed that BCG vaccination *via* the pulmonary route confers superior protection when compared to the subcutaneous route, which relies at least in part on Th17 cells (17–19). Mimicking the natural route of infection, the protective efficacy of whole-cell live-attenuated *Mtb* Δ*phoP*Δ*fadD26* (MTBVAC) delivered intranasally is being explored. A specific lung-targeted approach is to develop an inert pulmonary delivery platform, which can "pull" antigen-specific Th17 cells from the circulation into the lungs to form a depot of tissue-resident memory cells.

Designing Novel Adjuvants

Local pulmonary immunity could also be induced by parenteral delivery of adjuvants designed to promote site-specific homing of protective T cells. In this regard $CD4^+CXCR5^+$ T-cells promote early and efficient macrophage activation, and their recruitment requires a pre-existing Th17 response (20). This knowledge is translated into a new strategy by implementation of the Th17-inducing adjuvant, CAF01, combined with stimulators of CXCR5, CXCR3, and CCR4 known to favor pulmonary homing.

Synthetic glycolipids also have potent adjuvant activity and homologs of various mycobacterial glycolipids induce the inflammasome and have adjuvant activity *in vivo* (21). In particular, vaccination of mice with recombinant PPE44 formulated in dimethyldioctadecylammonium and synthetic glucose monomycolate (GMM) induced protective immunity against *Mtb*, with comparable efficacy to BCG. Induction of CD1b-restricted GMM-specific responses by this formulation is currently being tested in guinea pigs.

Developing Novel Inert and Viral Vector-Based Delivery Systems

Pre-existing anti-*Mtb* antibodies in the lung mucosa have also been suggested as a strategy to prevent infection, but so far it has been difficult to induce high-titer and long-lived antibody responses against structural antigens. A novel nanoparticle-based technology is being exploited for the delivery of synthetic cell wall antigens, a technology that has shown excellent results in the field of influenza (22). Improved viral vectors are currently being evaluated in WP1, including non-integrative lentiviral vectors (LV), chimpanzee adenoviral (ChAd) vectors, lymphocytic choriomeningitis virus and influenza virus. In particular, a vaccine regime using two viral vectors has been optimized: a ChAd vector (ChAdOx1) and a modified vaccinia virus Ankara (MVA), both expressing Ag85A. A boost of BCG with intranasal (i.n.) ChAdOx1.85A followed by a second boost with i.n. or intradermal MVA85A, significantly improved BCG vaccination in mice (23).

Novel Live Vaccines

Past research programs (TBVAC and NEWTBVAC) have succeeded in developing novel live vaccines, including BCGΔ*ureC:hly* (VPM1002) (24), MTBVAC (25, 26), which already progress through clinical trials, and BCGΔ*zmp1* which is considered for clinical testing in the near future. One of the aims of WP1 is to build upon these first-generation live vaccines as well as to provide novel live vaccine candidates based on strong scientific rationale.

Mtb Δ*phoP*Δ*fadD26* will be used as a basis to search for safer and more effective mycobacteria-based vaccines to be used in immune-compromised individuals. Specifically, an additional virulence gene, *erp*, has been deleted from MTBVAC resulting in increased attenuation (27). To improve protection by MTBVAC, a genome-wide screening approach is being performed, in which random mutants are screened in mice for safety and protective efficacy. Deletion of the anti-apoptotic factor *nuoG* in VPM1002 resulted in improved safety and protection as compared to BCG and VPM1002 in the mouse model (28). A safer VPM1002 derivative was created by deletion of *pdx1* rendering the vaccine auxotrophic for vitamin B6 (29). Finally, an attenuated *Mtb* ESX-5 mutant, *MtbΔppe25-pe19*, is safe and more protective than BCG in mice (30). The mechanism of attenuation is yet unknown, but likely results from phagosomal-rupture-induced innate immune signaling.

To summarize, WP1 has identified promising novel adjuvants, delivery systems, and live vaccine candidates that are currently evaluated for safety, immunogenicity, and protective efficacy in appropriate animal models, in collaboration with partners in other WPs.

WP2 ANIMAL MODEL REFINEMENT AND DEVELOPMENT

In the absence of unambiguous markers that correlate with and, thus, predict vaccine-induced protective immunity against *Mtb* infection and TB disease (31, 32), experimental infection models remain an indispensable tool in the process of TB vaccine research and development. And while at present no animal-free systems exist that fully recapitulate the complexity of host defense cascades and pathogenesis (33), it is imperative to exploit animals at various (staggered) stages of this vaccine Research and

Development (R&D) process and in particular to demonstrate vaccine efficacy (34).

Typically, several lower and also higher vertebrate animal species are utilized as models from as early as discovery up to building qualification dossiers and down selecting the best candidate vaccines toward resource-consuming, advanced stage clinical testing (Phase II and later) (35). These models are not only used to investigate the protective efficacy against an infectious challenge but also to assess vaccine tolerability (or, in specific cases, attenuation of live vaccines as a parameter of vaccine safety) and immunogenicity of new approaches, which build upon or extend beyond the current BCG vaccine.

Strictly speaking, no single animal model has been validated for the ultimate predictive assessment of TB vaccines toward the various target populations that can be identified globally. Models by definition imply a simplification of real-life complexity. Especially in the light of developing new preventive/prophylactic vaccine strategies, we, therefore, explore candidate vaccine performance in several different models to strengthen its proof-of-concept and to de-risk the development process for possible failures (35). In recognition of the undoubted limitations of any specific model, centralized and independent preclinical evaluation modalities are established in mouse, guinea pig, and NHP hosts, as described in WP3.1.

Mostly, preclinical studies assess the prophylactic profile of vaccine candidates in healthy animal hosts, which are experimentally challenged with *Mtb* and that are immunologically naive to mycobacterial antigens and typically free from any other disease or infectious pressure. In the clinic, pre-exposure, co-infection, and/or co-morbidity conditions can be expected to have an impact on susceptibility and also vaccine efficacy (36). The TBVAC2020 consortium, which at this point just passed mid-term of its 4-year work program, has set out in a specific WP to develop and refine models toward various clinically relevant conditions. Grossly, we have defined three areas as follows: (A) modeling vaccination conditions post-*Mtb* exposure, (B) modeling disease risk factors and clinically relevant conditions, and (C) refinement of existing models. With an eventual goal of implementing alternative models to provide proof-of-concept in support of preclinical vaccine development and candidate portfolio management, animal studies are explicitly useful also for investigating fundamental aspects of TB infection and immunity and may contribute to the identification and validation of BM candidates as one of the other key objectives of this consortium (covered in WP5).

Modeling Vaccination Conditions Post-*Mtb* Exposure

From the first year of the program onward activities have been initiated (i) to test alternative approaches to provoke reactivation of low-dose disease in mice and in the face of vaccination, (ii) to set up a so-called Cornell-type of post-exposure vaccination in guinea pigs, and (iii) to evaluate *Mtb* infection in cattle as a novel model of latent *Mtb* infection for post-exposure vaccine evaluation.

While both spontaneous reactivation and immunosuppressive regimens pose shortcomings in the context of modeling and testing efficacy of post-exposure vaccination strategies, we have explored "energy deprivation" by protein malnutrition (PEM) as an alternative to reactivate TB in mice. It was demonstrated that PEM, both in the infection and the vaccination phase, abrogates vaccine-induced protection, which is reversible by protein supplementation (37). Abrogation of vaccine efficacy correlated with the loss of cytokine positive memory populations from the periphery and in particular with the loss of IL2 producing $CD4^+$ T cell subsets. Although prototype vaccination did overcome and protect from malnutrition-induced reactivation, the results also suggested that more stringent malnutrition conditions would be required to lend more robustness to this model. In the light of animal welfare, however, this line will not be pursued. In the guinea pig model, the test conditions for *in vivo* bacterial expansion (regrowth) after antibiotic treatment have been established. The dynamic window of this model has been established using RUTI™ as a therapeutic vaccine candidate (38), with promising results that merit a follow-up study testing other candidates. This model may also be a valuable tool for studying and developing TB treatment regimes toward drug-resistant TB. Using *Mtb* (rather than *M.bovis*) to infect cattle provides great potential for obtaining a large (outbred) animal model that is hall-marked by latent, low burden *Mtb* infection and that can be manipulated to induce disease reactivation (39). Although in its early days, if successful, this effort may provide a platform for analyzing vaccine efficacy in a state of latent TB infection and a perspective on identification of associated BMs.

Toward Modeling of Risk Factors and Clinically Relevant Conditions

The workplan comprises the following conditions: (i) exploring differential monocyte/myeloid phenotypes in mice as a parameter of disease susceptibility, (ii) co-morbidity of obesity either or not in inflammation-prone mice and with multiple consecutive challenge conditions, (iii) stringent mouse modeling against clinical (Beijing-type) isolates of *Mtb*, and (iv) simian immunodeficiency virus co-infection in NHPs.

While monocyte frequency and phenotype have been associated with TB risk in the clinic (40–42), different strains of mice, which express differential profiles of myeloid cell compartment markers, have been used in BCG vaccination and protection experiments. In the face of variable efficacy in a selected range of host strains (43), flow cytometric analyses allow correlation of monocyte numbers and (functional) phenotypes with BCG-induced efficacy. Also in mice, we have exploited commercial high-fat diet to induce obesity (not formally characterized as a diabetic condition, yet showing impaired glucose tolerance). In a matrix-type study design, varying parameters relating to obesity, low-dose aerosol infection and multiple consecutive infections, disease susceptibility, and standard BCG efficacy are investigated. Further to experimental TB modeling in mice, multiple clinical isolates with different clinical and genotypic appearance were cultured and selected from Beijing, Beijing subtype and non-Beijing strains. Supported by molecular typing and *in vitro* virulence analyses using bone-marrow-derived macrophage cultures, seed lots were prepared for future vaccine evaluation testing from a limited number of strains, as well as a standard laboratory strain for comparison. Mouse infection experiments

further corroborated the notion that HN878 and M2 strains provide highly pathogenic phenotypes both *in vitro* and *in vivo* (44, 45). A study toward the definition of a TB/HIV co-infection model for vaccine evaluation using TB/SIV co-infection in NHP is in progress. All cynomolgus macaques in the study controlled a stable low grade chronic *Mtb* infection for 16 weeks prior to SIV challenge. Co-infection with SIV leads to a reduction in CD4 T cells and trends for higher disease burden with mycobacteria-specific and SIV-specific immune responses detected in all co-infected macaques.

Refinement of Existing Models

To strengthen the use of the well-established guinea pig model in TB vaccine R&D, methods have been developed to quantify and characterize antigen-specific T cell responses. Specific T lymphocytes are expanded, isolated, and re-stimulated to investigate functional markers by quantification of cytokine and other effector molecule expression levels. Further vaccine experiments will assess anti-mycobacterial immunity by these new tools in this stringent model. This refinement is particularly relevant for the analysis of unconventional vaccine candidates based on mycobacterial lipid antigens. Guinea pigs, in contrast to mice, do express a functional CD1 type 1 antigen presentation system, thus, representing the only small animal model to study these unconventional vaccine research approaches.

As another means of refinement, new NHP modeling tools and conditions are being established for refined infectious challenge, advanced flow cytometric analyses (including that of innate subsets), and advanced imaging of disease and host response dynamics by PET/CT imaging (46). To the latter in particular, the consortium also collaborates with external partners in a global network [governed by the Bill & Melinda Gates Foundation (BMGF)[3]]. The work in NHP so far has revealed pro- vs. anti-inflammatory/regulatory signatures that correlate with disease susceptibility and that hint toward a key role for differential innate immune orchestration. While, typically, NHP studies and protective efficacy assessment rely on measuring peripheral immunity and parameters of disease reduction, we currently apply standard and non-conventional vaccination to investigate early and local, innate and adaptive immune response parameters, and toward possible proof-of-concept for protection against infection (rather than protection against disease) in this highly susceptible species.

Overall, new modalities for modeling reactivation of disease (rather than primary infection) have been evaluated and are ongoing in different species (mouse, guinea pig, and cattle). More stringent and clinically relevant conditions for preclinical *in vivo* analysis of vaccines have been established. Benchmarking by standard/prototype vaccination is ongoing in mice, guinea pigs, and NHP toward diversification and refinement of preclinical vaccine R&D strategies. All these activities, as a two-pronged attack, add to our continuous and concerted effort to strengthen preclinical evaluations of candidates as well as our basic understanding of pathogen–host interaction in TB.

[3]https://www.ctvd.co.

WP3 PRECLINICAL TESTING AND DEVELOPMENT OF NEW TB VACCINE CANDIDATES

The overall aim of WP3 is to support and facilitate the progression of vaccine candidates from discovery through to preclinical development. This is achieved in two sub-WPs. WP3.1 enables the selection of successful candidates through comparative head-to-head testing in standardized animal models. These studies provide crucial information for vaccine developers and also support activities in WP3.2 which aim to take the prioritized, most promising preclinical TB vaccine candidates and accelerate their advancement through the TB vaccine pipeline. WP3.2 provides expertise in all aspects of product development which enables relevant advice *via* tailor-made Product and Clinical Development Teams (P&CDTs) to be given to developers in order to advance their candidates in an efficient manner. Specific issues are identified and guidance/recommendations are given to developers. Close interaction between the sub-WPs is essential to ensure effective use of resources and this is facilitated by the Portfolio Management Committee (PMC) (WP6).

WP3.1 HEAD-TO-HEAD TESTING IN STANDARDIZED PRECLINICAL MODELS

Demonstration of the safety, immunogenicity, and efficacy of vaccine candidates in animal models is required for preclinical development. This WP aims to provide evidence that the candidate is safe and elicits:

- an immune response that can be justified as being anti-mycobacterial and is of appropriate magnitude in terms of strength and duration, compared to a benchmark such as BCG;
- a protective effect against experimental challenge with *Mtb* which is demonstrated by a quantifiable reduction in disease burden compared to no treatment and/or BCG or another relevant benchmark vaccine.

Systematic, independent, and objective comparison of TB vaccines in well-characterized animal models enables the identification of the vaccines that have the greatest potential to be immunogenic and to show efficacy in the clinic. WP3.1 provides capacity for animal testing, building upon the concept that was developed in previous FP5, FP6, and FP7 projects for head-to-head comparisons of candidates in standardized animal models. In addition, there is an aim to offer models that better represent the target product profiles of the vaccines being developed.

The objective of WP3.1 is to compare and prioritize vaccines from the candidate discovery work package of this project (WP1) which supports decision-making at the individual partner and WP level and provides objective evidence for decision-making in portfolio management using the stage-gating criteria (WP6). Wherever feasible, samples are retained for selected evaluation of potential correlates of protection (CoP; WP5). The partners providing the models all have experience and a track record in TB R&D and importantly are recognized providers of unbiased,

high quality evaluation of vaccines in their respective models. A summary of each of the models provided is given below.

SCID Mouse Model for Safety of Live Vaccines

Assessing the residual virulence of live vaccine candidates in the context of an immunodeficient host is an essential part of modern pre-clinical vaccine evaluation. Vaccine candidates are administered into severe combined immune deficient (SCID) mice using methodologies and study designs described (47). This has become an accepted standard for safety evaluation of live vaccines (48) and has been successfully used to benchmark the safety of live vaccine candidates against BCG.

Standard Immune Competent Mouse Model for Protective Efficacy

The first screen of vaccine candidates for immunogenicity and efficacy is usually performed by individual laboratories during the discovery phase. Capacity has been provided in WP3.1 for testing in a standard mouse model to assist laboratories, which lack facilities for *Mtb* challenge studies, and/or to facilitate decision-making within the project. For example, within WP1 where different adjuvants and delivery systems are being developed, a head-to-head comparison could allow prioritization based on protective efficacy. The model entails vaccination of CB6F1 (H-$2^{b/d}$) hybrid mice, subsequently challenged with *Mtb* H37Rv *via* the respiratory route with protection determined by bacterial load in lungs and spleen at 6 weeks post infection.

Mouse Model for Protective Efficacy against Clinical (Beijing) Strains of *Mtb*

It is important to demonstrate that new TB vaccine candidates can protect against challenge with clinically relevant and highly virulent strains of *Mtb*, including Beijing strains, which are prevalent in East Asia. Highly virulent *Mtb* strains (one Beijing strain and one Euro-American lineage, see also activities in WP2), showing a lower efficacy of the BCG vaccine (49), are used to challenge by inhalation, vaccinated mice (either pre- or post-exposure). Protection is determined at 4 weeks and 10 weeks after challenge.

Mouse Model for Efficacy of Vaccine Candidates Given Post-Exposure

Robust animal models are needed to be able to test that vaccines can be safe and efficacious in individuals who are already infected with *Mtb*. A well-established mouse model for therapeutic vaccination is provided. This model has been validated previously (50, 51). The model involves aerosol infection followed by chemotherapy administered for 8 to 10 weeks post-infection with vaccination within weeks 12–17 and efficacy determined at week 23.

Guinea Pig Model for Protective Efficacy

The aerosol infection guinea pig model of TB has been widely used to determine vaccine efficacy, being regarded as a more stringent test than the standard mouse model. Data generated in previous EU-funded consortia demonstrated the utility of a standardized model, where the main read-out of efficacy is reduction in bacterial load, compared to control groups at 4 weeks post-challenge (47).

Mouse Immunogenicity for Formulation Studies

In combination with a vaccine formulation service where TB vaccine candidates are optimized with various type of adjuvants, there is provision of immunogenicity data in a harmonized mouse model which enables rational selection through comparative assessment of promising adjuvanted TB vaccine candidates.

To provide further flexibility in the preclinical evaluation of candidates, an aim of the project is to develop new animal models and improve read-outs of existing models such that they recapitulate a broader range of relevant conditions as they appear in the human populations being targeted. This model development is being conducted in WP2 with a view to incorporate a newly developed animal model or readouts into WP3.1 in order to offer a wider range of head-to-head testing.

PROGRESS TO DATE

Up to June 2017, 51 tests (comprised of 21 different vaccine candidates/approaches) of safety, immunogenicity and/or efficacy have been completed (33 tests) or are on-going (18 tests) in the various models. Outcomes of completed studies are summarized below:

- In the standard mouse or guinea pig challenge models, three candidates have shown protective efficacy and four were not efficacious.
- In post-exposure or Beijing challenge models, eight candidates afforded significant protection, four gave partial protection and three did not protect.
- Safety data on four candidates using the SCID mouse model, showed that three were attenuated.
- Data on the stability and immunogenicity of seven adjuvant formulations have been provided for one antigen.

These data (both positive and negative) have informed the portfolio management (WP6) and supported the vaccine discovery activities (WP1).

WP3.2 PRECLINICAL DEVELOPMENT

The main objective of this sub work package is to manage and facilitate the advance of the preclinical development pathway of new TB vaccine candidates. It supports the progression of the pipeline by advancing candidates prioritized through preclinical toward an early clinical stage of development using a portfolio management approach (as described in WP6). Tailor-made P&CDTs, including experts in regulatory, manufacturing, and clinical studies of vaccines, are established together with the selected vaccine developers. Currently, there are four vaccine candidates in preclinical development. For HBHA and BCGΔ*zmp1* candidates, joint P&CDTs with Aeras (as part of the joint global

effort in TB vaccine development) have also been established to support these candidates in the context of the activities of this project. More information about the P&CDT can be found in the chapter describing WP6.

Heparin-Binding Hemagglutinin

Heparin-binding hemagglutinin (52, 53) in combination with adjuvant giving the best level of immunogenicity and protection in animal models and in *ex vivo* models are compared and evaluated, in order to select the best combination. For vaccine optimization, a thermo-stabilized form of HBHA, which will make it less dependent on the cold chain and, therefore, more logistically accessible for most of the TB endemic countries, will also be developed and evaluated.

Recombinant BCGΔzmp1

Confirmation of identity, drug susceptibility and genetic stability of BCGΔ*zmp1* mutant (54, 55) strain(s) over serial passages (>12) has been achieved. An unmarked Δ*zmp1* mutant in a background strain with full background history and freedom to operate in terms of product development to licensure has been generated and characterized with respect to safety, immunogenicity and efficacy in various animal models within the consortium.

Combination of M72/AS01$_E$ and ChAd3M72

The M72 vaccine, containing both human $CD4^+$ and $CD8^+$ T cell epitopes, is an adjuvanted (AS01E) fusion protein vaccine candidate and has been evaluated in clinical trials (56, 57). The use of ChAd vector has been shown to elicit strong $CD8^+$ T cell responses. The ChAd3M72, using a viral vector for delivery, is developed as a boost for M72/AS01E prime. This heterologous prime boost approach has been assessed in a mouse model to induce a higher number of antigen-specific $CD4^+$ and $CD8^+$ T cells in the lung and peripheral blood with a different profile of $CD4^+$ T cell polyfunctionality as compared to two doses of M72/AS01E (unpublished data).

MVA–TB Vaccine Candidates

Preclinical development of the MVA vectors expressing multiple *Mtb* antigens specifically targeted as a therapeutic vaccine is to improve treatment of active TB (in particular linked to drug-resistant strains) and to prevent reactivation and/or re-infection in the adult population, in particular from endemic countries. Genetically stable MVA–TB vaccine candidates (58) are currently being evaluated using different murine post-exposure models (with collaborators in the USA and Spain). In addition, a manufacturing process for MVA–TB from a cell line and not primary cell cultures (NIH grant with Emergent BioSolutions) is also under development.

Additional support service is provided for specific vaccine formulation optimization and characterization of TB vaccine candidates selected within the consortium (59). A panel of adjuvants (including emulsions, liposomes, aluminum salts, TLR agonists, and others) and different production methods are available for formulation. This will allow vaccine developers to investigate and improve the compatibility and stability of their antigen with different adjuvants, and to define the optimal production methods to generate physico-chemically stable and immunogenic TB vaccine candidates.

WorkPackage 3 provides a valuable resource for vaccine developers especially those from an academic environment to progress their vaccine candidates from discovery to the preclinical development stage. The developers with selected vaccine candidates have access to various preclinical animal models and the support of the P&CDT. The advice from the tailor-made P&CDT is not restricted to regulatory, manufacturing, and early clinical studies, but also includes project management. These activities will lead to a more diverse and more promising pipeline of novel vaccine candidates at preclinical stages, and subsequently also for early clinical stages.

WP4 COMPARATIVE CLINICAL TESTING IN TBVAC2020

Early phase I and first-in-man clinical testing is an essential bridge between the demonstration of safety and efficacy in preclinical animal models and field testing in clinical trials in TB endemic countries. Such early clinical testing focuses on evaluating safety, usually in healthy adults, and immunogenicity, using a variety of standardized immunological assays. The overall concept underpinning WP4 is that standardized early clinical testing is essential, given the increasing numbers of diverse candidate TB vaccines being evaluated in preclinical animal models. There is currently a significant portfolio of diverse candidate vaccines in clinical and late preclinical development, which illustrates the progress made over the last 15 years (60). With the increasing number of diverse candidate vaccines currently in preclinical development, it is important to develop standardized methods and facilities for early clinical testing, in order that the most promising candidates can be selected for progression. There will never be sufficient resources to progress all the current candidates through to field testing in TB endemic countries. The use of standardized clinical trial protocols, standardized safety reporting, and standardized immunological evaluation according to published recommendations will allow comparison between different candidate vaccines (61, 62). This comparison will facilitate the rational selection of which candidates should be progressed to further clinical testing and efficacy testing in field trials. The aim of WP4 is to focus on early clinical TB vaccine development and to build on the successful standardized preclinical vaccine testing developed in EC supported FP6 TBVAC and FP7 NEWTBVAC projects. Over the last 10 years, the TBVAC/NEWTBVAC consortia have conducted many standardized head-to-head preclinical animal studies in mice, guinea pigs and NHPs. The data arising from these studies have facilitated vaccine selection (63, 64). This WP takes this successful approach a step further and applies it to early clinical testing.

Furthermore, there is an opportunity provided by this WP to embrace human experimental medicine studies, where early clinical trials can be used to demonstrate proof-of-concept. An example of this would be the evaluation of the safety and immunogenicity of a recombinant viral vector delivered by aerosol, compared with systemic administration (65).

PROCESS FOR VACCINE SELECTION

Vaccine candidates are selected for inclusion in this WP by the PMC, in conjunction with the TBVI P&CDT. This advice is independent of the vaccine developer. TBVI and Aeras have agreed on a set of portfolio management criteria which include stage-gating criteria (7, 34), and these criteria are used to guide vaccine selection in this WP4. The data arising from the trials conducted as part of this WP4 will be reviewed by the PMC for subsequent decisions about progression to safety and immunogenicity trials in TB high burden countries. Ongoing interactions with The European & Developing Countries Clinical Trials Partnership (EDCTP) and clinical sites in Africa, including those that are part of the EDCTP Networks of Excellence, will ensure that, subject to full and independent peer review, promising candidates are progressed as efficiently and effectively as possible in an endemic setting. Support and advice by tailor-made P&CDT will be provided to each vaccine developer to accelerate the development of the respective vaccine candidates through phase I/first-in-man clinical trial stage.

IMMUNOLOGICAL EVALUATION

Although we do not have an immunological correlate of protection with which to guide vaccine design and evaluation, there are several well-defined parameters of the host immune response which are known to be essential for protective immunity against *Mtb*. The immunological evaluation will be conducted using standardized operating procedures (SOP). Assays will include *ex vivo* IFNγ ELISpot assays, whole blood polychromatic flow cytometry with intracellular cytokine staining to measure other T cell cytokines, including IL-2, TNF-α, and IL17 from antigen-specific $CD4^+$ and $CD8^+$ T cells, and serological testing for antibody responses. The focus of this immunological evaluation is to use standardized assays to generate information about immune functions considered to be important in protective immunity. Work conducted within FP6 TBVAC, FP7 NEWTBVAC, and FP7 TRANSVAC has resulted in a harmonized SOP for an *ex vivo* ELISpot, which will be used here (66). In addition, a harmonized ICS flow protocol developed in these consortia will be used here as well (67). We will not use more exploratory assays in this WP, as they are less standardized, but PBMC and serum will be stored from all subjects at all time-points using harmonized SOP, so that further immunological evaluation can be performed as the field of BMs develops. In this regard, this WP will work closely with WP5 to monitor progress in the field of BM development (4, 5, 40, 41). Furthermore, ongoing work, for example, within the infrastructural FP7 EURIPRED project, to standardize the PBMC mycobacterial growth inhibition assay (MGIA), will be monitored and this assay will be conducted on cryopreserved material if the assay is deemed sufficiently fit for this purpose (68).

PROGRESS TO DATE

There are two broad categories of vaccines currently in development: "live" whole organism BCG replacement vaccines and subunit vaccines designed to boost BCG. Some live vaccines are also being evaluated as booster candidates (69, 70). It is anticipated that both types of candidate vaccines will be included and tested within this WP. Centre Hospitalier Universitaire Vaudois (CHUV), Leiden University Medical Center and University of Oxford clinical trial units will be collaborating in the planning and execution of the future candidate vaccine testing. Clinical and immunological SOPs have been agreed between the three partners. Following an open call to all partners, a first vaccine candidate has been selected and is planned to enter into phase I testing in CHUV in Q4 2017. It is expected that a second candidate will be tested in Q1 2018.

WP5 RESEARCH INTO TB BMs AND CoP

Next to TB vaccine development and evaluation, an important second TBVAC2020 objective is to identify TB BMs, in particular CoP. CoP are measurable BMs indicating that the host is immune, or protected against developing TB disease. Correlates of Risk (CoR) are BM indicating the host has an increased risk of developing active TB disease. Such TB BMs can be transcriptomic, cellular, or soluble analytes. The identification of CoP in particular will help to develop vaccines that target and strengthen protective immunity. Importantly, such correlates will facilitate the selection and prioritization of candidate TB vaccines for human efficacy testing, and will reduce the protracted time scale, size, and expenses of human efficacy trials by allowing the demonstration of vaccine immunogenicity and potential efficacy at an early stage. In addition, CoP will permit selection of antigens that induce protective immune responses, optimization of dose, vehicle, adjuvants, and immunization schedules of new candidate vaccines at an early stage and, thus, minimize the need for preclinical animal studies. WP5 has a strategically designed workflow, which combines TB BM discovery with parallel-specific assay development, followed by testing and validation in carefully characterized, complementary and unique human cohorts from genetically diverse populations. WP5 involves key partners from European, African, and East Asian laboratories.

This WP first aims to develop core assays for measuring CoP and CoR (#1 in **Figure 2**) and to evaluate these in carefully characterized, unique human cohorts from genetically diverse populations (#2 in **Figure 2**). Complementary to these efforts is the discovery of new TB BMs, using innovative approaches (#3 in **Figure 2**). A long-term goal is to move the best performing correlates forward toward validated immune correlate assays, ideally in the format of point-of-care tests for use in areas with high TB burden. Future activities will include active engagement of assay developers. Finally, the WP5 is building a database to capture the most important (transcriptomic) TB BMs (#4 in **Figure 2**), and there are close interactions between this WP and other efforts in this area.

SPECIFIC OBJECTIVES OF WP5

The first key objective is the development, evaluation, and improvement of core assays measuring CoP in TB. To identify TB

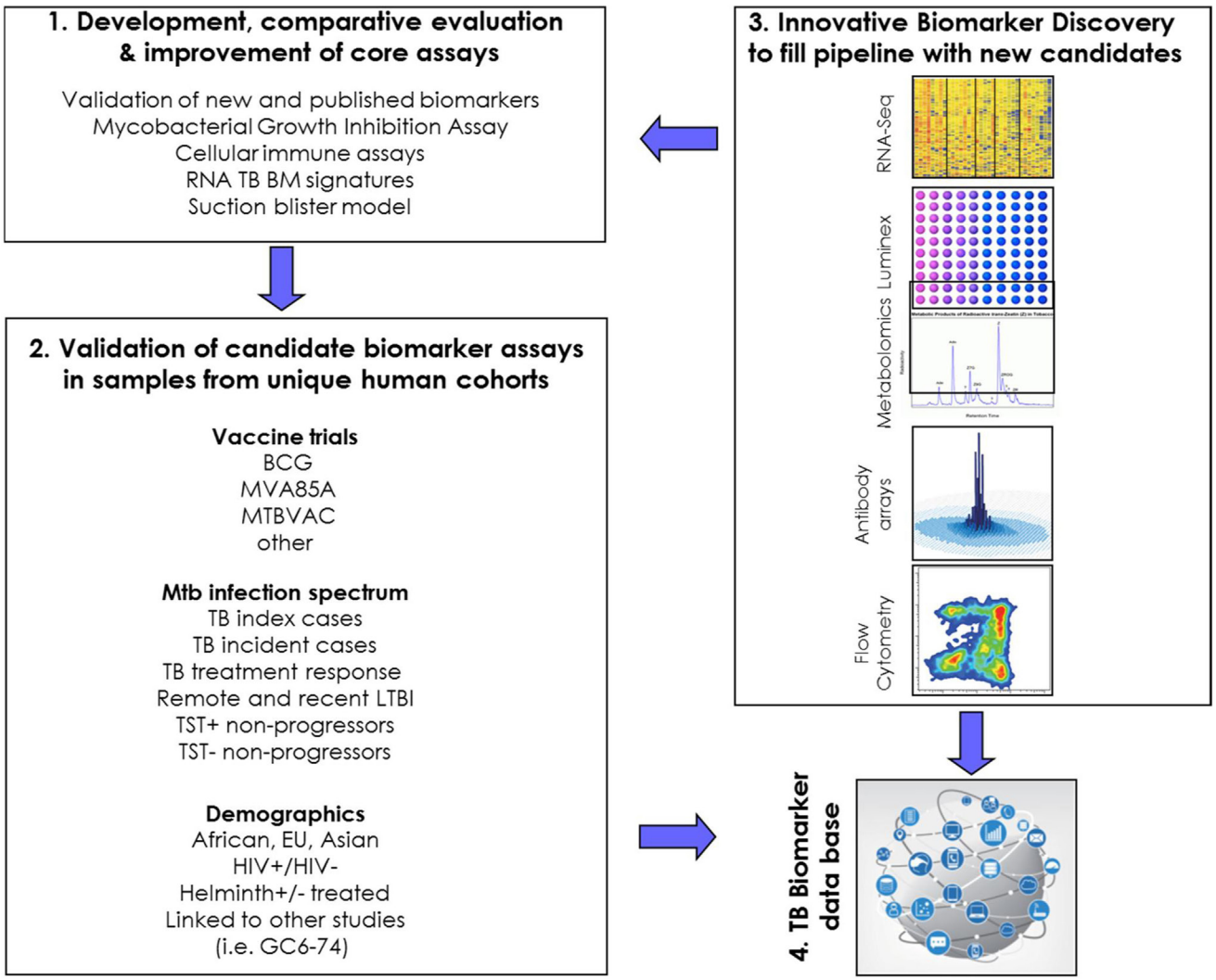

FIGURE 2 | Schematic representation of research priorities and connectivity in WorkPackage 5.

vaccine-induced CoP a protective vaccine is needed, but with the exception of BCG in settings where it does induce protection, this is not yet available. As BCG is able to induce partial protection, studies dissecting BCG-induced BMs are part of WP5. Moreover, a number of candidate correlates have recently emerged: BCG-vaccinated TB-protected South African infants had higher numbers of IFNγ-producing cells, and antibodies to *Mtb* antigen Ag85A also correlated with reduced risk of developing TB (40, 41). Other candidate CoR have been emerging (71), including myeloid/lymphoid (M/L) ratios (72), inflammation signaled by activated $CD4^+$ T cells (40, 41), an IL13/AIRE ratio in HIV *Mtb* co-infected individuals (73), the lack of IFNγ responses to HBHA (74, 75), and a 16-gene based transcriptomic GC6/ACS CoR signature (76) found in large cohort studies in Africa. In other joint work by WP5 partners, diagnostic transcriptomic- or serum host protein signature-based algorithms were discovered that have powerful differential diagnostic value in diagnosing TB in both adults and children in Africa (77–79). Several research groups in WP5 are involved in efforts to further validate these correlates in larger, independent cohort studies.

In parallel to attempts identifying CoP, WP5 participants are developing unbiased, functional measures of human protective responses. One example of such an assay is the *in vitro* MGIA (68). In BCG-vaccinated infants in the UK, there was an increased ability to control the growth of BCG following BCG vaccination (43). Other "unbiased" assays include global immune-, gene expression-, and proteomic profiling-assays, which are being evaluated against outcome of infection or vaccination in clinical cohorts. We anticipate that the development and optimization of novel functional read-outs of anti-mycobacterial immunity will help identifying novel correlates, and may lead to deeper insights into potential mechanisms that control mycobacterial outgrowth. Collaborative efforts are ongoing with other EC projects, such as the FP7 EURIPRED Infrastructure project, as well as with EDCTP-funded work that focuses on TB BMs (PREDICT TB and SCREEN TB projects).

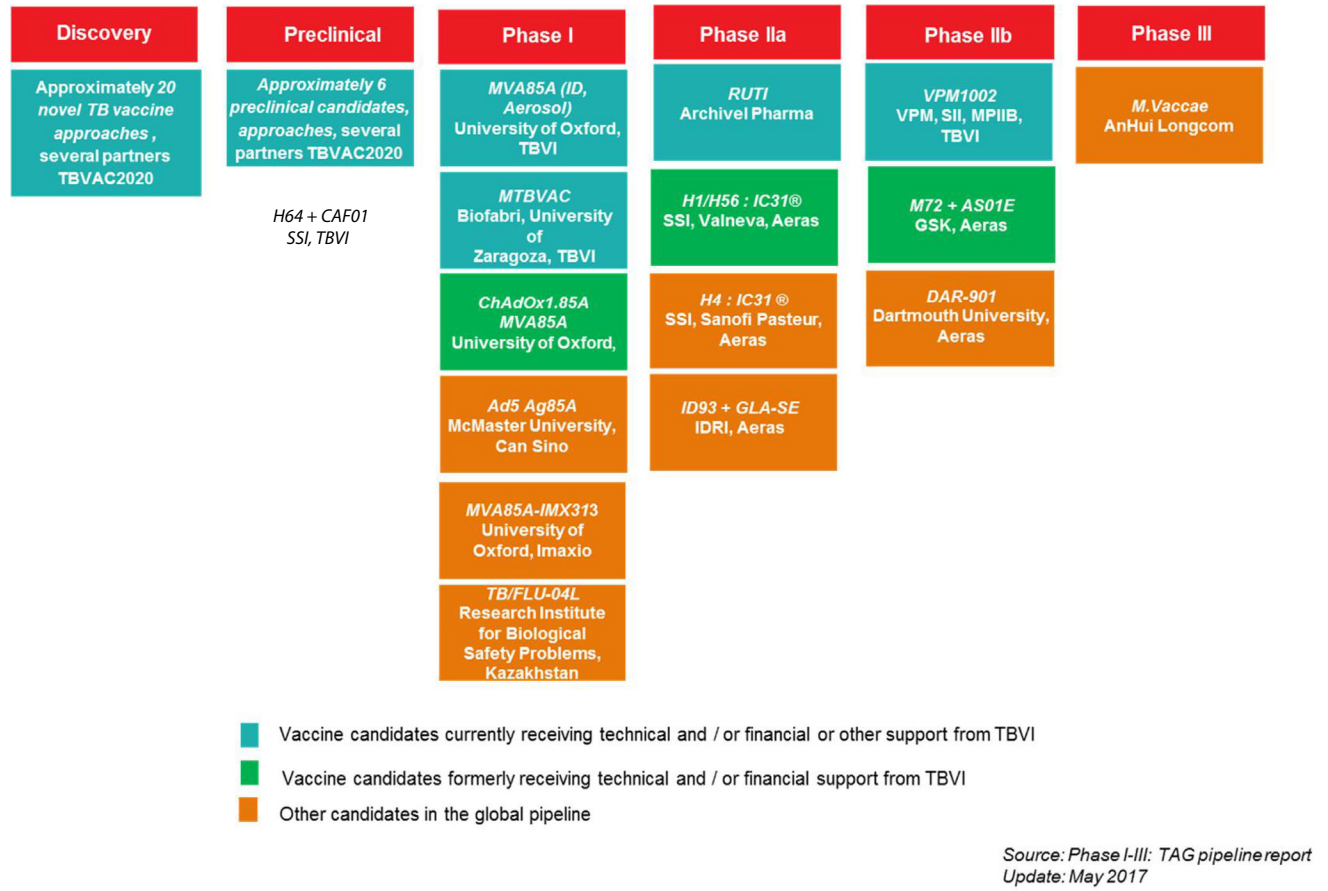

FIGURE 3 | TBVI vaccine pipeline, overview of the current tuberculosis (TB) vaccine candidates in the development pipeline, per text box: acronym vaccine candidate, development partners.

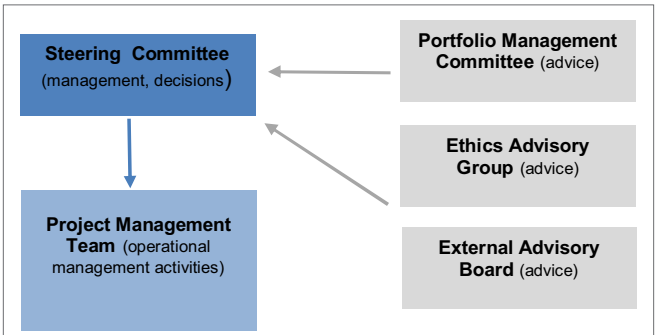

FIGURE 4 | TBVAC2020 management infrastructure.

Together, critical assays and assay variables are being identified to enable the development of improved, quantitative second generation assays. MGIA assays are also being studied for utility in analyzing cellular activity in human Purified Protein Derivative of *Mtb* suction blister models, in broncho-alveolar lavage (BAL) samples from aerosol BCG-infected volunteers and in the NHP model (80).

The second objective of WP5 is the evaluation of these core assays in samples from clinical cohorts. Such cohorts that have been collected include:

1. samples from BCG-vaccinated individuals (adults, children, neonates) from settings where BCG gives good protection against TB (northern Europe);
2. samples from trials with new candidate TB vaccines [including the MVA85 efficacy trial (81) and a recent MTBVAC trial (82)];
3. samples from TB patients with active disease (pre, during, and post treatment);
4. samples from latently TB infected individuals (LTBI), including long-term tuberculin skin test (TST) positive- as well as TST-negative- non-progressors;
5. samples from LTBI subjects co-infected with helminths pre- and post- anti-helminthic treatment;
6. samples from HIV-infected (immune-suppressed) *Mtb* co-infected subjects;
7. samples from BCG challenge models; and
8. samples and data from the unique longitudinal GC6-74 TB-progressor cohort (76).

The unique cohorts and sample banks suitable for the measurement and evaluation of correlates are available for study in WP5 and are being further expanded, including cohorts with long-term longitudinal follow-up. Multiple cohorts have been included to represent geographical, ethnic, and epidemiological diversity, with sites from South Africa, Malawi, The Gambia, Uganda, South Korea, Western/Southern Europe (UK, Netherlands, Belgium, Italy) and Eastern Europe (Belarus). This will also make it possible to test whether TB BMs, including CoR and CoP, show variation by population due to specific epidemiological settings.

The third objective of the WP5 is the discovery of new TB candidate BM using innovative approaches. Given the lack of CoP, BM discovery is needed to fill the TB BMs pipeline with new candidates. It is likely that multi- rather than single parameter signatures, or complex functional assays will be needed to capture the complexity and dynamics of human protective immunity to *Mtb*. Discovery approaches are focusing on both host and pathogen response during the different phases of infection. These include global transcriptomics, proteomics, metabolomics, hematopoietic (myeloid) progenitor analyses, epigenetic, and cellular approaches. In other projects partners are developing intradermal as well as aerosol BCG controlled human infection models (CHIM). Such studies will allow for identifying and measuring immune correlates, and help with correlating *in vivo* with *in vitro* results, e.g., results from CHIM with corresponding MGIA studies. To date, significant progress has been made in transcriptomic marker identification in several cohorts, with further data analysis and validation ongoing.

Assays have also been selected to survey the wider global space in the immune response to *Mtb* rather than the traditional focus on T cells and cytokines, similar to approaches in HIV correlates research. Different compartments of the "immune space" being investigated include adaptive immunity (T cells, B cells, immune regulatory cells), innate immunity [innate lymphocyte-subsets, DC-subsets, macrophages (83), tolerogenic monocytes, myeloid-derived suppressor cells (84)], NK cell subsets, dysregulation of apoptosis or autophagic flux (85), the role of particular immunometabolomic and proteomic markers (86) (in blood or other body fluids) etc. Recent findings point to an important role for: antibodies and B cells in TB (40, 41, 87, 88); a role for HLA-E restricted *Mtb* specific $CD8^+$ T cells (89, 90); and polycytotoxic LAM-specific T cells (91).

The longer-term strategy of WP5 is to select the most promising markers and assays for further development toward user friendly, preferably point of care, tests (92). Partners have expertise in downstream assay development such as lateral flow assays (86) and simplified transcriptomic assays (71). Recent novel work on mathematical modeling of vaccine immune responses has shown that models can predict dose–response relationships (93).

An important point is that TB BM assays may need to be adapted to specific types of TB vaccines (e.g., live vs. subunit) or depending on the antigen and delivery system selected. This may necessitate "tailor made" sets of BMs, for which TBVI is setting up a BM Development Team (BDT).

Finally, WP5 is building a TB transcriptomic biomarker database, to be a helpful resource for the TB BM community to provide a basis for "tailor made" TB vaccine biomarker selection, and to complement a TB diagnostic BM database as a valuable resource for future research (94).

WorkPackage 5 also links to other activities in TBVAC2020, including animal models (mice, NHPs) and human clinical vaccine trial related TB BM assays; and some of WP5 partners actively participate in these activities.

CONCLUDING REMARKS

WorkPackage 5 aims to discover new TB BM (of protection and TB risk) using innovative approaches including omics studies; to develop and improve key functional assays; and, importantly, to initially validate and then cross-validate candidate TB BMs in human cohorts (95). The long-term goal is to test and provide data on the biological validity of the BMs discovered and the assays developed using cohorts in whom protection will be demonstrable and to evaluate them against clinical endpoints (including incident TB and *Mtb* infection). The unique combination of researchers, expertise, technologies and human cohorts for validation studies is key to the success of this ambitious task.

WP6 PORTFOLIO MANAGEMENT

Introduction

Globally, TB vaccine R&D has made tremendous progress over the last 10–15 years, from only a few TB vaccine candidates in the pipeline 15 years ago to currently more than 25 candidates in early development and 13 candidates being evaluated in clinical stages.[4] Despite this progress, significant gaps remain that hamper efficient and effective TB vaccine development. Challenges include the establishment of:

(i) a more diverse pipeline to support successful delivery of new TB vaccines for all populations;
(ii) validated pre-clinical models predicting efficacy in humans;
(iii) validated humanCoP;
(iv) sufficient efficacy trial capacity in TB endemic areas.

TBVAC2020 directly addresses most of these challenges by supporting activities which will diversify and innovate the TB vaccine pipeline (WP1); by developing standardized animal models allowing head-to-head comparison of candidate vaccines for different disease targets (WP2); by accelerating promising preclinical vaccine candidates (WP3); by innovating clinical design and conduct phase I experimental medicine studies (WP4); and by developing CoP (WP5). The progress that TBVAC2020 is making in addressing these challenges shows that new TB vaccines are feasible. The continuation of scientific progress will require a sustained commitment over the long term for funding TB vaccine R&D by the EU and its member states. It is furthermore imperative that the TB vaccine R&D community, in turn, manages the global TB vaccine pipeline as efficiently and as coherently as possible and conducts a rationale selection of TB vaccine R&D efforts through a portfolio management approach.

[4]http://www.tbvi.eu/what-we-do/pipeline-of-vaccines/.

Objective

It is the objective of WP6 of the TBVAC2020 project to contribute to establishing a diverse and innovative portfolio of new TB vaccine candidates through a portfolio management approach: an objective and transparent process to prioritize and accelerate vaccine candidates in this project (**Box 1**).

Portfolio Management

Portfolio management is an efficient and effective mechanism to advance the vaccine pipeline. It is a quality decision-making process seeking to maximize probability of success against acceptable cost and risk. Portfolio management ensures alignment of vaccine candidates and resources, and ensures an optimal

BOX 1 | Role of TBVI in accelerating the discovery and development of new tuberculosis (TB) vaccines in TBVAC2020.

TBVI

TBVI is an innovation partnership that works to discover and develop new safe, effective, and affordable TB vaccines.

TBVI consist of two parts:

1. The TBVI R&D partners including more than 50 partners from academia, research institutes, and private industry in the TB vaccine field from about 20 countries in Europe, North America, Africa, Asia, and Australasia.
2. The TBVI organization that is a support structure to vaccine researchers and developers to facilitate the discovery and development of new, safe, and effective TB vaccines and biomarkers (BMs) for global use. TBVI brings together R&D organizations, scientists and industry partners in one network in order to facilitate optimal development of vaccine candidates in clinical settings. The organization provides independent technical and scientific advice to its R&D partners and funders.

TBVI provides a range of essential services to support its R&D partners as well as funders, technical agencies, and other stakeholders. These services include:

1. Technical support,
2. Knowledge development, exchange, and networking,
3. Project identification, design, development, and management,
4. Resource mobilization.

Contribution to the global TB vaccine pipeline

The support activities of TBVI to its TB R&D partners have been instrumental in the development of the current global TB vaccine pipeline. By facilitating discovery, preclinical, and early clinical development, TBVI has contributed to moving 50% of candidates globally to clinical stages of development (**Figure 3**).

1. *Technical* support services
   TBVI provides technical support for product and clinical development through its independent Product and Clinical Development Teams (P&CDT). This team helps manage the development of the candidates prioritized by the portfolio management process (see chapter WP6) while researchers and developers move their candidates from concept to clinical development. The P&CDT also provides advice on the experimental design of preclinical studies conducted in WorkPackage (WP) 3 and on the clinical design of the phase I trials conducted in WP4 (For more information on P&CDT see: http://www.tbvi.eu/about-us/organisation/product-and-clinical-development-team/).
2. Knowledge development, exchange, and networking.
   To optimize the discovery and development of new TB vaccines and BMs, TBVI facilitates and supports the generation of new knowledge and exchange among R&D partners by promoting knowledge-sharing through scientific meetings and workshops, publication in scientific and non-scientific journals, formal and informal networking. Through joint collaborative working with open sharing of data prior to publication, a network of trust and respect has been established between TB vaccine R&D groups throughout Europe and the world. The annual project meetings are a key example of this trust and open data sharing within the network. The annual project meeting in 2017 of the TBVAC2020 consortium organized in combination with the TBVI annual symposium brought together 163 participants from 19 countries worldwide. Beside the project R&D partners and linked industry partners, the representatives from many partner organizations, like EC, Aeras, European & Developing Countries Clinical Trials Partnership, Bill & Melinda Gates Foundation (BMGF), World Health Organization, European Research Infrastructure for Translational Medicine and European Vaccine Initiative attended this meeting.
3. Project identification, design, development, and management.
   TBVI initiates new projects when new funding opportunities arise. In line with its R&D strategy, TBVI applies a bottom-up process working closely with its partners to develop project proposals. An example of this process was the creation of the TBVAC2020 project proposal. TBVI identified the European Commission (EC) Horizon 2020 call on new TB vaccines. In consultation with leading experts in the field, TBVI identified the areas and scope of work to be included in the project proposal. To enable the involvement of new partners and to receive the best new ideas, TBVI launched an open call for Expression of Interest (EoI). Over 100 EoI letters were received. A project selection committee selected the best project proposals based on predetermined selection criteria. This finally resulted in EC awarding €18.2 million for the TBVAC2020 project. Additional funds from the Swiss, Korean, and Australian governments complemented the project budget to a total of €24.6 million (http://horizon2020projects.com/sc-health/tb-consortium-awarded-e24m-international-grant/).
   TBVI is the coordinator of the TBVAC2020 project and provides essential services to contribute to successful completion of the pre-set project deliverables and milestones, to increase the overall impact of the project, and to ensure coordination and linkages between TBVAC2020 and other TB vaccine R&D initiatives. To ensure efficient and effective management of the project, TBVI has established and implemented the management infrastructure (**Figure 4**). The project infrastructure is composed of the Steering Committee, the Project Management Team (PMT led by the Coordinator) and the following three advisory groups: Portfolio Management Committee, Ethics advisory group, and External Advisory Committee.
4. Resource mobilization.
   TBVI mobilizes resources for its R&D partners. TBVI was able to leverage funding from this project with other funders including Norwegian Agency for Development Cooperation, Department of International Development and BMGF. This enabled additional and accelerated development of two preclinical and three clinical stage candidates, of two novel and refined preclinical models, and made it possible to continue key research and development activities on correlates of protection of 7 partners.

balance between them (96). The aim of WP6 is to contribute to the establishment of a balanced and diverse portfolio of preclinical and early clinical TB vaccine candidates with prospective applications in infants, adolescents, and adults that focus on either preventing active TB disease or improving treatment of TB disease.

The portfolio management approach applied in TBVAC2020 is based on three sets of criteria:

1. Stage-gating criteria. Stage-gating criteria provide a linear assessment of each individual candidate to determine if there is sufficient robust evidence to support advancement to the next stage of development (34, 97).
2. Portfolio assessment. Priority-setting assessment criteria allow comparison of similar vaccine candidates in a portfolio to select and deselect candidate(s), especially those at the same developmental stage.
3. Entry of new candidates. Entry criteria allow assessment of new candidates into the portfolio to create more diversity and balance in the portfolio and to increase chance of success.

An independent PMC uses these three sets of criteria to monitor and provide advice to the Steering Committee of TBVAC2020 on the vaccine pipeline of the project. It monitors discovery activities in WP1 and WP2, provides advice on entrance of new candidates, advises on selection and priority of vaccine candidates for evaluation in preclinical models or to support their preclinical development (WP3), and for selection and evaluation of candidates in clinical trials (WP4). During the first 30 months of the TBVAC2020 project, PMC advised on the selection of 21 candidate vaccines for evaluation in 6 head-to-head preclinical models, 4 candidates for receiving support for preclinical development among others formulation, Good Manufacturing Practice (GMP) and developing a product development plan, and two candidates for evaluation in a phase I clinical trial. During this period, PMC deselected six vaccine candidates for evaluation in the head-to-head preclinical models and 1 candidate was deselected for evaluation in a phase I clinical trial. It is the intention that PMC of WP6 will integrate with the Global Portfolio Review Committee (GPRC) for the Global TB vaccine Partnership (GTBVP) once this entity is established. The GPRC aims to provide advice from a global perspective, with regard to selection or deselection of vaccine candidates for support to the next step in development.

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REVIEW
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Polyfunctional CD4+ T Cells As Targets for Tuberculosis Vaccination

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Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), remains a leading cause of morbidity and mortality worldwide, despite the widespread use of the only licensed vaccine, Bacille Calmette Guerin (BCG). Eradication of TB will require a more effective vaccine, yet evaluation of new vaccine candidates is hampered by lack of defined correlates of protection. Animal and human studies of intracellular pathogens have extensively evaluated polyfunctional CD4+ T cells producing multiple pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-2) as a possible correlate of protection from infection and disease. In this study, we review the published literature that evaluates whether or not BCG and/or novel TB vaccine candidates induce polyfunctional CD4+ T cells and if these T cell responses correlate with vaccine-mediated protection. Ample evidence suggests that BCG and several novel vaccine candidates evaluated in animal models and humans induce polyfunctional CD4+ T cells. However, while a number of studies utilizing the mouse TB model support that polyfunctional CD4+ T cells are associated with vaccine-induced protection, other studies in mouse and human infants demonstrate no correlation between these T cell responses and protection. We conclude that induction of polyfunctional CD4+ T cells is certainly not sufficient and may not even be necessary to mediate protection and suggest that other functional attributes, such as additional effector functions, T cell differentiation state, tissue homing potential, or long-term survival capacity of the T cell may be equally or more important to promote protection. Thus, a correlate of protection for TB vaccine development remains elusive. Future studies should address polyfunctional CD4+ T cells within the context of more comprehensive immunological signatures of protection that include other functions and phenotypes of T cells as well as the full spectrum of immune cells and mediators that participate in the immune response against Mtb.

Keywords: T-cell immunity, CD4+ T cells, vaccine-induced immunity, tuberculosis, vaccine, protective immunity, BCG, polyfunctional T cells

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INTRODUCTION

Despite the widespread global use of the only licensed tuberculosis (TB) vaccine, Bacille Calmette Guerin (BCG), TB remains a significant cause of morbidity and mortality, with 10.4 million cases and 1.8 million deaths each year (1). The WHO End TB strategy cites that a new and more efficacious vaccine is one of the interventions required for worldwide elimination of TB by 2035 (2).

Yet a new vaccine that improves upon the partial protection provided by BCG remains elusive (3). One of the significant challenges to rational TB vaccine development is lack of identified immune correlates of protection. The absence of bone fide correlates hampers both pre-clinical research as well as clinical trials (4). The Food and Drug Administration defines a correlate of protection as a laboratory parameter, which is associated with protection from the occurrence of clinical disease as shown after sufficient and controlled trials (5). Correlates of protection can be further divided into ones that are causally responsible for protection (mechanistic correlates of protection) or ones that are significantly correlated with, though not the cause of protection (non-mechanistic correlates of protection) (6). The search for correlates of protection in the TB vaccine field has generally focused on mechanistic correlates and in this regard, polyfunctional $CD4^+$ T cells, defined by the simultaneous co-expression of multiple pro-inflammatory cytokines (e.g., IFN-γ, TNF-α, IL-2) on a single cell level, have garnered much attention (4). While both polyfunctional $CD4^+$ and $CD8^+$ T cells have been defined, the TB vaccine literature investigating polyfunctional T cells as a possible correlate of protection, has focused almost exclusively on $CD4^+$ T cells. Therefore, we limited our review to articles that referred to induction of IFN-γ^+TNF-α^+IL-2^+ polyfunctional $CD4^+$ T cells in the context of TB vaccination and vaccine immunogenicity in pre-clinical and clinical studies and focused on reviewing the published evidence that polyfunctional $CD4^+$ T cells represent a mechanistic correlate of protection for TB vaccines.

With advances in multi-parameter flow cytometry to include seven or more parameters, in combination with intracellular cytokine staining (ICS) techniques, it became possible to evaluate the production of multiple cytokines simultaneously on a single cell basis. Polyfunctional $CD4^+$ T cells were defined as those cells producing two or more cytokines, and were first defined within the context of examination of vaccine-induced T cell responses (7). De Rosa et al. showed that vaccines to Hepatitis B virus, tetanus, and HIV induced antigen-specific T cell responses, which were functionally complex, differed from the antigen-specific T cell responses elicited by natural infection, and were underestimated when measured by IFN-γ production alone. Furthermore, Bansal et al. demonstrated with an HIV vaccine that vaccines can elicit a more diverse array of T cells than natural infection and that vaccine dose and route can alter the cytokine profile and polyfunctionality of the T cells elicited (8). Furthermore, by varying the administration of the same vaccine, these authors illustrated that polyfunctional T cells possess a dynamic range that could be evaluated for correlates of protection in vaccine models.

Darrah et al. provided the first definitive evidence that the magnitude of the vaccine-induced polyfunctional $CD4^+$ T-cell response was highly correlated with protection from infection (9). Using a mouse model of *Leishmania* disease, various vaccines induced $CD4^+$ T cells displaying distinct cytokine profiles and different degrees of protection against disease upon *L. major* challenge. In this study, frequencies of MML-specific polyfunctional $CD4^+$ T cells co-expressing IFN-γ, TNF-α, and IL-2 were correlated closely with the various degrees of protection elicited by a panel of vaccines. By comparison, the total number of IFN-γ-producing $CD4^+$ T cells, $CD4^+$ T cells producing IL-4 or IL-13, or the T regulatory cell response did not correlate with vaccine-induced protection. This study was also the first to show that BCG elicits polyfunctional $CD4^+$ T cells in both the murine TB model, in which BCG mediates a degree of control of bacterial replication after *Mycobacterium tuberculosis* (Mtb) challenge, and in humans (9), as discussed further below.

In humans, polyfunctional $CD4^+$ T cells have been studied with reference to severity of disease due to some intracellular infections [reviewed in Ref. (10)]. For example, slower progression to AIDS with HIV-2 than HIV-1 infection (11) and control of HIV-1 without anti-retroviral medications (12) are associated with high frequency polyfunctional HIV Gag-specific $CD4^+$ T cells. By comparison, studies of polyfunctional $CD4^+$ T cells in relationship to host containment of Mtb infection are contradictory. On the one hand, stronger mycobacteria-specific polyfunctional $CD4^+$ T cell responses are found in adults with sputum smears negative for acid fast bacilli (AFB) than those with AFB smear positive TB (13), and in adults with latent Mtb infection (LTBI) than in those with TB (14, 15). Moreover, successful TB treatment, which rapidly reduces the bacterial load, is associated with marked increases in proportions of polyfunctional $CD4^+$ T cells (13). On the other hand, other studies demonstrate that polyfunctional $CD4^+$ T cell responses positively correlate with increased bacillary load. For example, there are also studies that showed stronger mycobacteria-specific polyfunctional $CD4^+$ T cell responses in adults with TB than those with LTBI (16, 17) and in adults with TB than in those in healthy household contacts of adults with TB (18). These contradictory results highlight an important limitation of such correlative studies, which is that it is not possible to discern whether or not polyfunctionality of $CD4^+$ T cells plays a causal role in immune control of the pathogen, or simply reflects the underlying bacterial burden.

The mechanism(s) by which polyfunctional $CD4^+$ T cells induced by vaccines or natural infection may be associated with protection from infection and/or disease have not been defined. It is certainly conceivable that cells expressing multiple effector functions may be more effective in controlling infection than those producing a single pro-inflammatory cytokine. For example, IFN-γ and TNF-α act synergistically to enhance the ability of macrophages to contain *L. major* infection (19, 20), which in turn is associated with enhanced control of disease by the combination of IFN-γ and TNF-α in the murine model (20). Similarly, IFN-γ and TNF-α synergistically inhibit Mtb replication within murine macrophage cell lines (21). As first defined in the murine *Leishmania* model, among vaccine-induced $CD4^+$ T cells producing IFN-γ, TNF-α, and/or IL-2, cells producing all three cytokines (3^+ cells) produce more cytokine on a per cell basis [as defined by mean fluorescence intensity (MFI)], than do those that produce two cytokines (2^+ cells), which in turn produce more cytokine than cells producing a single cytokine (1^+ cells) (9). Moreover, Darrah et al. defined integrated MFI (iMFI), a metric that combines the frequency of each cytokine-producing $CD4^+$ T cell response with its associated MFI and showed that the iMFI for each of IFN-γ, TNF-α, or IL-2, also correlated with the degree of vaccine-induced protection. This analysis is consistent with the

interpretation that it may be the high potency of multifunctional T cells to produce cytokines on a per cell basis that is associated with vaccine-induced protection. Finally, Seder et al. proposed a linear differentiation model for $CD4^+$ T cells based upon their cytokine profile (22). In this model, naïve $CD4^+$ T cells, upon activation with antigen, first acquire the capacity to secrete TNF-α or IL-2, followed by the capacity to produce both cytokines and finally, upon further differentiation, additionally produce IFN-γ as well. These early lineage T cells also express CCR7, consistent with central memory T cells (T_{CM} cells). Subsequently, with continued antigenic stimulation these polyfunctional T cells lose CCR7 expression and capacity to secrete TNF-α or IL-2, and finally produce only IFN-γ in the highly differentiated, terminal effector stage. Thus, according to this model, protective potential of a polyfunctional $CD4^+$ T cell producing IFN-γ, TNF-α, and IL-2 may be associated with its degree of differentiation and simultaneous capacity for memory and for effector function.

POLYFUNCTIONAL T CELLS AS A BIOLOGICALLY PLAUSIBLE CANDIDATE FOR A MECHANISTIC CORRELATE OF PROTECTION IN TB

The literature regarding the possible role of polyfunctional $CD4^+$ T cells in mediating vaccine-induced protection for TB has primarily investigated those cells co-producing IFN-γ, TNF-α, and IL-2, hereafter, we will refer to as "polyfunctional $CD4^+$ T cells." There is substantial evidence derived from study of murine TB models and humans that all three of these cytokines are necessary for the control of Mtb infection.

Essential roles in host defense for IFN-γ, and for $CD4^+$ T cells that produce IFN-γ, were initially defined in the murine TB model using IFN-γ-deficient mice (23, 24), $CD4^+$ T cell deficient mice, and adoptive transfer of $CD4^+$ T cells in the murine TB model [reviewed in Ref. (25)]. More recently, Green et al. showed that $CD4^+$ T cells are important as a source of IFN-γ mediating protection from Mtb, by demonstrating that IFN-γ production from all other cellular sources than $CD4^+$ T cells was insufficient in controlling chronic infection and maintaining survival and that $CD4^+$ T cell derived IFN-γ promoted $CD8^+$ T cell responses (26). Consistent with this, humans deficient in IL-12 receptor expression (27), IFN-γ receptor expression, or IFN-γ signaling (28) are more susceptible to mycobacterial disease. However, it is important to note that these deficiencies are not T cell-specific and thus the precise role of T cell-derived IFN-γ vs other sources of IFN-γ in humans remains unresolved. At least in the murine TB model, a mechanism by which IFN-γ may mediate its effector function is through activation of macrophages, which in turn inhibit Mtb growth *via* induction of iNOS and autophagy [reviewed in Ref. (29)].

TNF-α is also essential for TB host defense. Mice deficient in TNF receptor or TNF-α are highly susceptible to Mtb infection (30, 31). There are multiple cell types that produce TNF-α, including innate immune cells, epithelial cells, endothelial cells, and fibroblasts. Early control of Mtb infection in the mouse model, prior to the acquisition of adaptive immunity, is primarily mediated by TNF-α derived from macrophages (32). Conversely, mice deficient for TNF-α expression in T cells poorly control chronic infection (32). In humans, pharmacological blockade of TNF-α, used for treatment of rheumatologic disorders, increases susceptibility to mycobacterial disease (33, 34). As demonstrated in both the murine TB model and in humans, TNF-α promotes the formation of mature granulomas and like IFN-γ, also activates infected macrophages, which, at least in mice, contain infection *via* induction of iNOS and autophagy [reviewed in Ref. (29)].

IL-2 induces proliferation, promotes the survival of TCR-activated T cells [reviewed in Ref. (35)] and promotes the development of fully competent memory T cells during primary infection (36). Therefore, IL-2 is generally assumed to aid TB host defense through supporting the expansion and maintenance of the T cell response [reviewed in Ref. (35)]. IL-2 also has a role in T cell tolerance, through its role in maintaining $CD4^+CD25^+$ regulatory T cells. In support of this, IL-2 and IL-2R deficient mice develop early severe autoimmune disease [reviewed in Ref. (35)]. Consequently, these mice cannot be utilized to determine the role of IL-2 in TB host defense as has been done with IFN-γ and TNF-α. Nonetheless, in the murine TB model, induction of IL-2-producing $CD4^+$ T cells is associated with vaccine-induced protection to Mtb and loss of IL-2-producing $CD4^+$ T cells is associated with loss of protection [reviewed in Ref. (37)]. In humans, decreased proportions of polyfunctional $CD4^+$ T cells in individuals with AFB smear positive TB compared to those with AFB smear negative TB, or in those with TB disease as compared to those with LTBI, was associated with decreases in proportions of total IL-2 producing T cells (13, 14).

TB VACCINES INDUCE POLYFUNCTIONAL T CELLS IN ANIMAL MODELS

BCG and novel TB vaccine candidates utilizing various antigens and vaccine platforms induce polyfunctional $CD4^+$ T cells in murine, bovine, and non-human primate (NHP) TB models. These T cell populations possess attributes, which are critical for mediating vaccine-mediated immunity in that they can traffic to the lung and include memory T cell populations that persist in the vaccinated host.

BCG and Other Live Mycobacterial Vaccines Elicit Polyfunctional $CD4^+$ T Cells

As the only licensed TB vaccine and in light of its demonstrated record of partial efficacy, BCG is often used as a control vaccine in the mouse model of TB vaccination (**Table 1**). Several studies have shown that BCG induces polyfunctional mycobacteria-specific $CD4^+$ T cells detected among lung (9, 38) and splenic (9, 39–44) lymphocyte populations (**Table 1**). Darrah et al. found that polyfunctional mycobacterial $CD4^+$ T cells produced more cytokine on a per cell basis than do those producing two or one cytokines, similar to *Leishmania*-specific polyfunctional $CD4^+$ T cells (9). In the murine TB model, these T cell responses constitute minor (38, 39, 42, 44), predominant (43), or major (9) subsets within the first 2–4 months after immunization.

TABLE 1 | BCG induces polyfunctional $CD4^+$ T cells in animal models of TB.

BCG strain	Study design	IA	Conclusions	Reference
SSI	C57BL/6 mice BCG i.m. (+0) *IA*: (+4 months)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: spleen and lung *Antigen(s)*: PPD	BCG induced a major subset in lung and spleen at 4 months	Darrah et al. (9)
China	C57BL/6 mice BCG s.c. (+0) *IA*: (+12, +32 weeks)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: spleen *Antigen(s)*: Ag85B (p.p.); HspX (p.p.); PPD	BCG induced a minor subset in spleen at 12 and 32 weeks	Yuan et al. (44)
SSI	Balb/c mice BCG s.c. (+0) *IA*: (+13, +22 weeks)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: spleen *Antigen(s)*: PPD; Ag85A (p.p.)	BCG induced a minor subset in spleen at 13 and 22 weeks	Tchilian et al. (42)
Pasteur	C57BL/6 mice BCG s.c. (+0) *IA*: (+2, +8, +14 months)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: spleen *Antigen(s)*: BCG	BCG induced a predominant subset in spleen at 2 and 8 months, not detected at 14 months	Derrick et al. (43)
Pasteur	Balb/c mice BCG s.c. (+0) *IA*: (+14 weeks)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: spleen and lung *Antigen(s)*: PPD; Ag85A (p.p.)	BCG induced a minor subset in spleen at 14 weeks	Forbes et al. (39)
SSI	CB6F1 BCG s.c. (+0) *IA*: (+3 weeks)	*Cell measured*: $CD4^+CD44^{hi}$ 3^+ cells *Cellular source*: spleen *Antigen(s)*: rTB10.4; rAg85B	BCG induced TB10.4- but not Ag85B-specific memory T cells in spleen after 3 weeks	Elvang et al. (40)
SSI	C57BL/6 mice BCG s.c. (+0) *IA*: (+10 weeks)	*Cell measured*: $CD4^+CD44^{hi}$ 3^+ cells *Cellular source*: lung and spleen *Antigen(s)*: PPD	BCG induced a minor subset of memory T cells in lung and spleen at 10 weeks	Lindenstrom et al. (38)
SSI	C57BL/6 mice BCG s.c. (+0) *IA*: (>40 weeks)	*Cell measured*: $CD4^+CD44^{hi}$ 3^+ cells *Cellular source*: spleen *Antigen(s)*: PPD	BCG induced a major subset of memory T cells in spleen after 40 weeks	Lindenstrom et al. (41)
SSI	Holstein steers BCG s.c. (+0) *IA*: (+6 weeks)	*Cell measured*: $CD45RO^+$ $CCR7^+$ $CD4^+$ 3^+ cells after 2 weeks *in vitro* culture *Cellular source*: PBMC *Antigen(s)*: rAg85A; rTB10.4	After 2 weeks of *in vitro* culture, a major subset of memory T cells was identified 6 weeks after BCG	Maggioli et al. (45)
SSI	*Macaca mulatta* BCG aerosol (+0) *IA*: (+8 weeks)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: PBMC; BAL *Antigen(s)*: PPD	BCG induced a major subset of T cells in PBMC and lung (BAL) after 8 weeks	White et al. (46)

SSI, Staten Serum Institute; i.m., intramuscular; s.c., subcutaneous; i.d., intradermal; CB6F1, C57BL/6 X Balb/c; 3^+, IFN-γ^+TNF-α^+IL-2^+; PBMC, peripheral blood mononuclear cells; BAL, bronchoalveolar lavage; p.p., peptide pool; BCG, Bacille Calmette Guerin; TB, tuberculosis; IA, immune assay.
Major subset = subset that constitutes >50% of the total cytokine-producing cells; minor subset = subset that constitutes <20% of the total cytokine-producing cells; predominant subset = subset that constitutes 20–50% of the total cytokine-producing cells.

In addition, polyfunctional $CD4^+$ T cells have been detected among $CD44^+$ memory T cell populations as early as 3 weeks after vaccination (40) and persist as a major subset more than 40 weeks after vaccination (38). By contrast, though detecting polyfunctional $CD4^+$ T cells as a predominant T cell population 2 and 8 months after BCG, Derrick et al. were unable to detect these cells 14 months after immunization, suggesting that this subset may not persist long-term (43). These differences in persistence of polyfunctional $CD4^+$ T cells and wide phenotypic spectrum in cytokine-producing $CD4^+$ T cells are not readily reconciled between studies. However, differences in the experimental protocols utilized, such as differences in BCG strain and/or delivery method, mouse strain and/or cell populations studied and the nature and dose of the Mtb challenge, may underlie the inconsistency in findings. Finally, BCG induces polyfunctional T cells in cattle (45) and rhesus macaque monkeys of Chinese origin (*Macaca mulatta*) (46). Maggioli et al. showed that BCG induced mycobacteria-specific polyfunctional $CD4^+$ T cells as a major cytokine expressing subset in peripheral blood mononuclear cells (PBMC) obtained from calves 6 weeks after BCG and cultured with antigen for 2 weeks *in vitro* (45). These T cells expressed phenotypic markers consistent with memory T cells. Finally, White et al. showed that BCG delivered by the aerosol route induced PPD-specific polyfunctional $CD4^+$ T cells as a major subset in both PBMC and bronchoalveolar lavage (BAL), fluid of Chinese rhesus macaque monkeys 8 weeks after BCG administration (46).

Similarly, recombinant BCG constructs (42, 44) induce polyfunctional $CD4^+$ T cell responses in mice (**Table 2**). Yuan et al. compared BCG overexpressing Ag85B and HspX (rBCG:XB) to non-recombinant BCG and demonstrated that rBCG:XB elicited comparable PPD- and Ag85B-specific and stronger HspX-specific polyfunctional T cell responses 12 and 32 weeks after vaccination (44). Comparing a recombinant BCG expressing membrane-perforating listerolysin (with the rationale of promoting $CD8^+$ T cell responses from cytosolic antigens)

and deficient in urease C (ΔureC *hly*$^+$, VPM1002), Tchilian et al. demonstrated low-frequency splenic PPD- and Ag85A-specific polyfunctional CD4$^+$ T cells, comparable to those induced by BCG, 13 and 22 weeks after immunization (42). Maggioli et al. showed that vaccination with a cocktail of four distinct BCG deletion mutants (Δfdr8, ΔleuCD, Δpks16, ΔmmaA2, and ΔmetA) induced mycobacteria-specific polyfunctional CD4$^+$ T cells in 2 weeks cultures of PBMC from bovine calves 6 weeks after vaccination. These responses were comparable to those induced by non-recombinant BCG (45).

Vaccine Candidates Utilizing Viral Vectors Can Elicit Polyfunctional CD4$^+$ T Cells

Adenoviral vectors expressing Mtb proteins also elicit polyfunctional CD4$^+$ T cells in murine and NHP models of TB (**Table 2**). Derrick et al. showed that a replication deficient adenoviral vector, Ad5, expressing ESAT-6 fused to the n-terminus of Ag85B (E6-85) induced BCG-specific polyfunctional CD4$^+$ T cells, detected in spleen of mice 2 and 8 months after vaccination, but no longer detectable 14 months after vaccination (43). Forbes et al. used an Ad5 vector expressing Ag85A as a boost to BCG and compared intradermal (i.d.) to intranasal (i.n.) delivery of the adenovirus (39). Boosting i.d. but not i.n. induced Ag85A-specific polyfunctional CD4$^+$ T cells, as a predominant subset in spleen 4 weeks after the adenoviral boost vaccination. By contrast, when lung T cells were examined, this adenoviral vector only induced Ag85A-specific polyfunctional CD4$^+$ T cells when delivered i.n. but not i.d. Elevang et al. showed that an Ad5 vector expressing H4, a recombinant Ag85B/TB10.4 fusion protein (Ad-H4), induced Ag85A and TB10.4-specific polyfunctional CD4$^+$ T cells, representing a predominant subset of CD44$^+$ memory T cells in spleen 3 weeks following the vaccination and also when administered as a boost to recombinant H4 delivered in CAF01 (H4:CAF01) 1 week following this vaccination (40). Magalhaes et al. showed that an adenoviral vector, Ad35, expressing Ag85A, Ag85b, and TB10.4 (AERAS-402) delivered i.m. as a boost to either BCG or recombinant BCG expressing perfringolysin (AERAS-401), elicited transient Ag85A/B and TB10.4-specific polyfunctional CD4$^+$ T cell responses in PBMC of rhesus macaque monkeys of Chinese origin (*M. mulatta*) 1 week after the first boost (47). Hokey et al. went on to show that AERAS-402 delivered *via* aerosol to Chinese rhesus macaques induced weakly detectable Ag85A/B-specific polyfunctional CD4$^+$ T cells in PBMC 3 days after and in BAL 28 days after completion of immunization (48). Therefore, at least in the short-term, adenoviral vectors expressing Mtb proteins elicit polyfunctional CD4$^+$ T cells when delivered with alone or as a boost to BCG in mice and NHPs.

Two additional viral vector systems have also been assessed in the mouse TB model (**Table 2**). A vesicular stomatitis virus (VSV) construct expressing Rv3615c, Mtb10.4, and Rv2660c elicited dual-cytokine-producing (IFN-γ$^+$TNF-α$^+$; TNF-α$^+$IL-2$^+$; and IFN-γ$^+$IL-2$^+$) but not triple-cytokine producing (IFN-γ$^+$TNF-α$^+$IL-2$^+$) polyfunctional CD4$^+$ T cell responses when delivered i.n. either alone or as a boost to BCG (49). By contrast, a modified vaccinia Ankara (MVA) expressing Ag85A (MVA85A), when delivered as a boost to BCG, elicited predominantly Ag85A-specific polyfunctional CD4$^+$ T cells 3 and 12 weeks following MVA immunization (42). The capacity for MVA85A to elicit polyfunctional CD4$^+$ T cell responses in humans has been more extensively evaluated and is discussed below.

Recombinant Antigen Vaccines Elicit Polyfunctional CD4$^+$ T Cells

Recombinant Mtb antigen subunit vaccines, typically comprising proteins formulated in adjuvant, have been extensively evaluated in the mouse TB model (**Table 2**). Several adjuvanted recombinant Mtb proteins or fusions of multiple Mtb proteins have been shown to elicit polyfunctional CD4$^+$ T cells in mice, including recombinant Ag85B (50), and MT1721 (51) and fusion proteins comprised of ESAT-6 fused to the n-terminus of Ag85B (E6-85) (43), Ag85B/TB10.4 (H4) (40, 52), Ag85B/ESAT-6 (H1) (38, 41, 53), and gene products of Rv2608, Rv3619, Rv3620, and Rv1813 (ID93) (54). These recombinant protein vaccines have successfully utilized several different adjuvant formulations based upon immunostimulatory lipids (38, 40, 41, 43, 53, 54) or nucleotides (50–52) to elicit these T cell responses. In addition, adjuvanted recombinant protein vaccines induce polyfunctional CD4$^+$ T cell responses when used alone (40, 41, 43, 50, 52–54) and when administered as a boost after BCG prime (38, 43) or a DNA vaccine prime (51). Moreover, one study examined antigen dose and found that a lower dose of H4:IC31 [recombinant H4 delivered in IC31 (cationic peptide and oligodeoxynucleotide), 0.5 μg] induced greater frequencies of polyfunctional CD4$^+$ T cells than a higher dose (5 μg) (52). Finally, recombinant protein vaccines induce polyfunctional CD4$^+$ T cells detected among CD44$^+$ memory T cell populations (38, 40, 41) as early as 1 week following completed immunization (40) and persisted 56 weeks when recombinant H1 delivered in CAF01 (H1:CAF01) was used (41) and as long as 14 months when recombinant E6-85 delivered in DD/MPL (liposomal monophosphoryl lipid A, E6-85: DDA/MPL) was used to boost BCG (43). Therefore, polyfunctional CD4$^+$ T cells are induced with several distinct adjuvanted recombinant Mtb protein vaccines, when used alone or as a boost to BCG or DNA vaccine, and are included among memory CD4$^+$ T cell populations.

TB Vaccines Promote Polyfunctional CD4$^+$ T Cells in the Lung

Recent studies in the mouse TB demonstrate the importance of tissue location for protective immune responses against *M. tuberculosis* and suggest that lung resident CD4$^+$ T cells mediate control of Mtb infection better than CD4$^+$ T cells that reside in the pulmonary vasculature but do not enter the parenchyma [reviewed in Ref. (37)]. Protective parenchymal CD4$^+$ T cells express activation markers such as PD-1 and CD69, do not express the terminal differentiation marker KLRG1, and produce less IFN-γ than intravascular T cells. Therefore, the ability of TB vaccines to promote the accumulation of lung resident polyfunctional CD4$^+$ T cells may be an additional important component of vaccine-induced protection by these cells.

TABLE 2 | TB vaccines induce polyfunctional CD4+ T cells in animal models of TB.

TB vaccine	Study design	IA	3+ cells/protection[a]	Conclusions	Reference
BCG (SSI)	*Mouse strain*: C57BL/6 *Immunization*: BCG i.m: (+0) *Challenge*: Mtb Erdman aerosol (+3 months) *Post-challenge*: lung and spleen CFU (+1 month)	*Cell measured*: CD4+ 3+ cells *Cellular source*: spleen and lung *Antigen(s)*: PPD *Pre-challenge*: (+4 months)	+ BCG induced 3+ memory T cells and protection in lung and spleen as compared to naïve mice	BCG induced a major subset in lung and spleen at 4 months. Correlation of pre-challenge 3+ T cell frequencies with protection with BCG vs naïve mice	Darrah et al. (9)
rBCG:XB BCG (China)	*Mouse strain*: C57BL/6 mice *Immunization*: rBCG:XB s.c. vs BCG s.c: (+0) *Challenge*: Mtb H37Rv i.n. (+12 weeks) *Post-challenge*: lung and spleen CFU (+4, +10, +20 weeks)	*Cell measured*: CD4+ 3+ cells *Cellular source*: spleen *Antigen(s)*: Ag85 (p.p.); HspX (p.p.); PPD *Pre-challenge*: (+12, +32 weeks)	++ rBCG:XB vs BCG: 1 log protection in spleen and lung	rBCG:XB induced more HspX-specific 3+ T cells than BCG, which correlated with greater protection in lung and spleen	Yuan et al. (44)
VPM1002 BCG (SSI) MVA85A	*Mouse strain*: Balb/c mice *Immunization*: BCG s.c. (+0) vs VPM1002 s.c. (+0) vs BCG s.c. (+0)/MVA85A i.d. (+10 weeks) vs VPM1002 s.c. (+0)/MVA85A i.d. (+10 weeks) *Challenge*: Mtb H37Rv aerosol (+14 weeks) *Post-challenge*: lung and Spleen CFU (+12 weeks)	*Cell measured*: CD4+ 3+ cells *Cellular source*: spleen *Antigen(s)*: PPD; Ag85A (p.p.) *Pre-challenge*: (+13, +22 weeks)	– 3+ *T cell response*: BCG/MVA85A > VPM1002/MVA85A >> BCG or VPM1002 *Protection*: VPM1002 or VPM1002/MVA85A > BCG or BCG/MVA85A	BCG and VPM1002 induced a minor subset of 3+ T cells. MVA85A boost to either BCG or VPM1002 induced 3+ T cells. The 3+ T cell response did not correlate with protection	Tchilian et al. (42)
E6-85: DDA/MPL AdE6-85 pVax6-85 BCG (Pasteur)	*Mouse strain*: C57BL/6 *Immunization*: E6-85 in DDA/MPL s.c. (+0, +2, +4 weeks) vs AdE6-85 i.m. (+0, +1 month) vs pVaxE6-85 i.m. (+0, +3, +6 weeks) vs BCG s.c. (+0); vs BCG mixed with E6-85 in DDA/MPL s.c. (+0, +2, +4 weeks) *Challenge*: Mtb Erdman aerosol (+2, +8, +14 months) *Post-Challenge*: lung and spleen CFU (+1 month)	*Cell measured*: CD4+ 3+ cells *Cellular source*: spleen *Antigen(s)*: BCG *Pre-challenge*: (+2, +8, +14 months)	+++ The hierarchy of 3+ T cell frequencies correlated with that of protection in lung > spleen at 14 months	Strong correlation between the 3+ T cell frequency and degree of protection induced by several vaccine regimens	Derrick et al. (43)
BCG (Pasteur) Ad85A	*Mouse strain*: Balb/c *Immunization*: BCG s.c. (+0)/Ad85A i.d. (+10 weeks); vs BCG s.c. (+0)/Ad85A i.n. (+10 weeks) *Challenge*: Mtb Erdman aerosol (+14 weeks) *Post-Challenge*: lung and spleen CFU (+6 weeks)	*Cell measured*: CD4+ 3+ cells *Cellular source*: spleen and lung *Antigen(s)*: PPD; Ag85A (p.p.) *Pre-challenge*: (+14 weeks)	+/– Ad85A i.n. boost induced more 3+ T cells and more protection in lung than Ad85A i.d. boost. However, Ad85A i.d. boost induced more 3+ T cells in spleen than Ad85A boost	Boosting i.d. but not i.n. induced Ag85A-specific 3+ T cells as a predominant subset in spleen. Boosting i.n. but not i.d. induced Ag85A-specific 3+ T cells in lung. Lung, not splenic 3+ T cells correlated with protection in the lung	Forbes et al. (39)
H4:CAF01 Ad-H4 BCG (SSI)	*Mouse strain*: CB6F1 *Immunization*: Ad-H4 s.c. (+0) vs H4 in CAF01 s.c. (+0, +2 weeks) vs H4 in CAF01 s.c. (+0)/Ad-H4 (+2 weeks) vs BCG s.c. (+0) *Challenge*: Mtb Erdman aerosol (+8 weeks) *Post-Challenge*: lung and spleen CFU (+6 weeks)	*Cell measured*: CD4+CD44hi 3+ cells *Cellular source*: spleen *Antigen(s)*: TB10.4 (p.p.); Ag85B (p.p.) *Pre-challenge*: (+3 weeks)	++ H4:CAF01/Ad-H4 induced more memory 3+ CD4+ T cells, and was more protective in lung than H4:CAF01 or Ad-H4 alone	Correlation between the 3+ T cell frequency and degree of protection induced by three different vaccine regimens	Elvang et al. (40)

(*Continued*)

TABLE 2 | Continued

TB vaccine	Study design	IA	3+ cells/protection[a]	Conclusions	Reference
VSV-836 BCG (SSI)	*Mouse strain*: Balb/c *Immunization*: VSV i.n. (+0) vs BCG i.m. (+0) vs BCG i.m. (+0)/VSV i.n. (+12 weeks)	*Cell measured*: CD4+ 3+ cells *Cellular source*: spleen *Antigen(s)*: TFP846 *Immunogenicity*: (+18 weeks)	N/A	Neither VSV nor BCG/VSV induced 3+ T cells	Zhang et al. (49)
rAg85B:CpG rAg85B:CpG in NP	*Mouse strain*: C57BL/6 *Immunization*: rAg85B with CpG i.n. vs rAg85B with CpG i.d. vs rAg85B with CpG in NP i.n. vs rAg85B with CpG in NP i.d. (+0, +7, +21 days) *Challenge*: Mtb Erdman aerosol (+49 days) *Post-Challenge*: lung CFU (+1 month)	*Cell measured*: CD4+ 3+ cells *Cellular source*: spleen *Antigen(s)*: rAg85B *Pre-challenge*: (+28, +49 days)	++ rAg85B/CpG/NP i.n. induced more 3+ T cells and more protection in lung than did rAg85B/CpG	rAg85B in CpG ± NP induced 3+ T cells as a predominant subset Correlation between the 3+ T cell frequency and degree of protection induced by two different vaccine regimens	Ballester et al. (50)
rMT1721:GLA DNA-MT1721	*Mouse strain*: C57BL/6 *Immunization*: rMT1721 in GLA s.c (0)/DNA-MT1721 i.m. (+4, +8 weeks) vs DNA-MT1721 i.m. (0)/rMT1721 in GLA s.c (+4, +8 weeks)	*Cell measured*: CD4+ 3+ cells *Cellular source*: spleen *Antigen(s)*: rMT1721 *Immunogenicity*: (+10 weeks)	N/A	rMT1721 as either a prime or a boost to DNA-MT1721 induced 3+ T cells as a minor subset in splenocytes	Cayabyab et al. (51)
H4:IC31	*Mouse strain*: CB6F1 *Immunization*: H4 in IC31 s.c. (+0, +2, +4 weeks) comparing 0.5 μg vs 5 μg *Challenge*: Mtb Erdman aerosol (+10 weeks) *Post-Challenge*: lung CFU (+6 weeks)	*Cell measured*: CD4+ 3+ cells *Cellular source*: PBMC *Antigen(s)*: rAg85B; rTB10.4; H4 *Pre-challenge*: (+5 weeks)	++ H4:IC31 induced stronger 3+ T cells at a lower dose (0.5 μg) vs a higher dose (5.0 μg), which correlated with better protection in the lung	H4:IC31 induced 3+ T cells, as a major subset in PBMC Correlation of 3+ T cell frequencies with protection with H4:IC31 delivered at lower vs higher doses	Aagaard et al. (52)
H1:CAF01 BCG (SSI)	*Mouse strain*: C57BL/6 *Immunization*: BCG s.c. (+0) vs BCG s.c. (+0)/H1 in CAF01 s.c. (+4 weeks) *Challenge*: Mtb Erdman aerosol (+10 weeks) *Post-Challenge*: lung CFU (+7, +26, +50 weeks)	*Cell measured*: CD4+CD44hi 3+ cells *Cellular source*: spleen and lung *Antigen(s)*: PPD *Pre-challenge*: (+10 weeks)	+ BCG/H1:CAF01 induced more memory 3+ memory T cells and protection in lungs as compared to naïve mice	BCG/H1:CAF01 and BCG induced a minor subset of memory 3+ T cells in lung and spleen Correlation of pre-challenge 3+ T cell frequencies with protection with BCG/H1:CAF01 vs naïve mice	Lindenstrom et al. (38)
H1:CAF01 BCG (SSI)	*Mouse strain*: C57BL/6 *Immunization*: BCG s.c. (+0) vs H1 in CAF01 s.c. (+0, +2, +4 weeks) *Challenge*: Mtb Erdman aerosol (+14 months) *Post-Challenge*: lung CFU (+6 weeks)	*Cell measured*: CD4+CD44hi 3+ cells *Cellular source*: spleen *Antigen(s)*: H1; PPD *Pre-challenge*: (+52 weeks)	+ H1:CAF01 and BCG induced comparable levels of 3+ memory CD4+ T cells and protection in lung as compared to naïve mice.	H1:CAF01 induced a major subset of memory 3+ T cells after 52 weeks Correlation of pre-challenge 3+ T cell frequencies with protection with H1:CAF01 or BCG vs naïve mice.	Lindenstrom et al. (41)
H1:MMG/DDA H1:M_{32}MMG/DDA	*Mouse strain*: C57BL/6 *Immunization*: H1 in MMG/DDA s.c. (+0, +2, +4 weeks) vs H1 in M_{32}MMG/DDA s.c. (+0, +2, +4 weeks)	*Cell measured*: CD4+ 3+ cells *Cellular source*: spleen *Antigen(s)*: H1 *Immunogenicity*: (+6 weeks)	NA	H1:MMG/DDA or H1:M_{32}MMG/DDA induced 3+ T cells as a major subset in splenocytes	Andersen et al. (53)

(*Continued*)

TABLE 2 | Continued

TB vaccine	Study design	IA	3+ cells/protection[a]	Conclusions	Reference
ID93:GLA-SE	*Mouse strain*: C57BL/6 *Immunization*: ID93 in GLA-SE s.c. (+0, +3, +6 weeks) *Challenge*: Mtb H37Rv aerosol (+9–10 weeks) *Post-Challenge*: lung and spleen CFU (+4 weeks)	*Cell measured*: $CD4^+$ $CD44^+3^+$ cells *Cellular source*: spleen *Antigen(s)*: ID93 *Pre-challenge*: (+9 weeks)	+ ID93:GLA-SE induced more memory 3^+ memory T cells and protection in lungs as compared to control mice	ID93:GLA-SE induced memory 3^+ T cells as a minor subset in splenocytes, which correlated with protection in the lung vs control mice	Bertholet et al. (54)
BCG mutant cocktail (SSI)	*Holstein steers*: newborn calves *Immunization*: BCG mutant cocktail s.c. (+0)	*Cell measured*: $CD45RO^+$ $CCR7^+$ $CD4^+$ 3^+ cells after 2 weeks *in vitro* culture *Cellular source*: PBMC *Antigen(s)*: rAg85A; rTB10.4 *Immunogenicity*: (+6 weeks)	N/A	After 2 weeks of *in vitro* culture, a major subset of memory T cells was identified 6 weeks after BCG mutants	Maggioli et al. (45)
BCG (SSI) AERAS-401 AERAS-402	*Monkeys*: *Macaca mulatta* *Immunization*: BCG i.d. (+0)/AERAS-402 i.m. (+15, +27 weeks) vs AERAS-401 i.d. (+0)/AERAS-402 i.m. (+15, +27 weeks)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: PBMC *Antigen(s)*: Ag85A (p.p.); Ag85B (p.p.); TB10.4 (p.p.) *Immunogenicity*: (+1, +4, +8, +15, +16, +19, +20, +24, +27, +28, +31, +37 weeks)	N/A	AERAS-402 delivered as a boost to either BCG or AERAS-401 elicited transient 3^+ T cell responses in PBMC 1 week after the first boost	Magalhaes et al. (47)
AERAS-402	*Monkeys*: *M. mulatta* *Immunization*: AERAS-402 *via* aerosol (+1, +8, +15 days)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: PBMC and BAL *Antigen(s)*: Ag85A/B (p.p.) *Immunogenicity*: (+18, +43 days)	N/A	AERAS-402 delivered *via* aerosol induced weak, detectable 3^+ T cells in PBMC 3 days and in BAL 28 days after completing immunization	Hokey et al. (48)

*SSI, Staten Serum Institute; rBCG:XB, Recombinant BCG overexpressing Ag85B and HspX; VPM1002, Recombinant BCG which is urease C deficient and expressing membrane-perforating listerolysin (L. monocytogenes); MVA85A, Modified Vaccinia Ankara expressing Ag85A; E6-85, recombinant ESAT-6 fused to the n-terminus of Ag85B without the signal sequence; DDA, dimethyl dioctadecyl ammonium bromide; MPL, monophosphoryl lipid A; AdE6-85, adenoviral vector (Ad5) expressing E6-85; pVax6-85, DNA construct expressing E6-85; Ad85A, human adenoviral vector expressing Ag85A; H4, Recombinant Ag85B/TB10.4 fusion protein; CAF01, cationic liposomes formulated with synthetic mycobacterial cord factor; Ad-H4, Adenoviral (Ad5) vector expressing H4; VSV-836, Vesicular Stomatitis Virus (VSV) expressing a fusion of Rv3615c, Mtb10.4, Rv2660c (TFP846); rAg85B, recombinant Ag85B; NP, polypropylene sulfide nanoparticles; rMT1721, recombinant MT1721; GLA, glucopyranosyl lipid A; IC31, cationic peptide and oligodeoxynucleotide (ODN1); H1, Recombinant Ag85B/ESAT-6 fusion protein; MMG, monomycoloyl glycerol: M_{32}MMG, synthetic analog of MMG; ID93, Recombinant Rv2608, Rv3619, Rv3620, and Rv1813 fusion protein; GLA-SE, synthetic MPL in stable oil-in-water nanoemulsion; BCG mutant cocktail, BCG Δ*fdr8*, Δ*leuCD*, Δ*pks16*, Δ*mmaA2*, and Δ*metA*; AERAS-401, Recombinant BCG expressing perfringolysin; AERAS-402, Adenoviral vector (Ad35) expressing Ag85A, Ag85B, and TB10.4; CB6F1, C57BL/6 X Balb/c; i.m., intramuscular; s.c., subcutaneous; i.d., intradermal; i.n., intranasal; CFU, colony forming units; 3^+, IFN-γ^+TNF-α^+IL-2^+; PBMC, peripheral blood mononuclear cells; BAL, bronchoalveolar lavage; p.p., peptide pool; BCG, Bacille Calmette Guerin; TB, tuberculosis, Mtb, M. tuberculosis; IA, immune assay.*

+ = weak evidence for 3^+ cells as a correlate of protection (positive correlation between 3^+ T cell frequency and one or two similarly performing vaccines vs control); ++ = moderate evidence for 3^+ cells as a correlate of protection (positive correlation between 3^+ T cell frequency and at least two differently performing vaccines ± control); – = evidence against 3^+ cells as a correlate of protection (no correlation between 3^+ T cell frequency and four vaccines); +++ = strongest evidence for 3^+ cells as a correlate of protection (positive correlation between 3^+ T cell frequency and five vaccines); +/– = equivocal evidence for 3^+ cells as a correlate of protection; N/A, Not applicable.
Major subset = subset that constitutes >50% of the total cytokine-producing cells; minor subset = subset that constitutes <20% of the total cytokine-producing cells; predominant subset = subset that constitutes 20–50% of the total cytokine-producing cells.

[a]*Vaccine-induced 3^+ T cells correlate with protection from Mtb challenge. Protection is defined as control of Mtb replication.*

TABLE 3 | Lung resident polyfunctional $CD4^+$ T cells before and after TB vaccination.

TB vaccine	Study design	IA	Conclusions	Reference
BCG (SSI)	*Mouse strain*: C57BL/6 *Immunization*: BCG i.m: (+0)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: lung *Antigen(s)*: PPD *Immunogenicity*: (+4 months)	BCG induced a major subset in lung at 4 months after vaccination	Darrah et al. (9)
BCG (SSI) H1:CAF01	*Mouse strain*: C57BL/6 *Immunization*: BCG s.c. (+0) vs BCG s.c. (+0)/rH1 in CAF01 s.c. (+4 weeks) *Challenge*: Mtb Erdman aerosol (+10 weeks) *Post-Challenge*: lung CFU (+7, +26, +50 weeks)	*Cell measured*: $CD4^+CD44^{hi}$ 3^+ cells *Cellular source*: lung *Antigen(s)*: PPD; Ag85A, ESAT-6, TB10.4 (p.p.) *Pre-challenge*: (+10 weeks) *Post-challenge*: (+7, +26, +50 weeks)	BCG/H1:CAF01 and BCG induced a minor subset of memory 3^+ T cells in lung pre-challenge For BCG/H1:CAF01, both an increased 3^+ T cell response and decreased CFU in the lung relative to control mice were observed 26 weeks after infection. However, when comparing BCG/H1:CAF01 with BCG post-infection, BCG/H1:CAF01 was associated with an increased 3^+ T cell response but not a statistically significant decrease in lung CFU	Lindenstrom et al. (38)
BCG (Pasteur) Ad85A	*Mouse strain*: Balb/c *Immunization*: BCG s.c. (+0)/Ad85A i.d. (+10 weeks); vs BCG s.c. (+0)/Ad85A i.n. (+10 weeks)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: spleen and lung *Antigen(s)*: Ag85A (p.p.) *Immunogenicity*: (+14 weeks)	Boosting BCG with Ad85A i.n. but not i.d. induced Ag85A-specific 3^+ T cells in lung	Forbes et al. (39)
rESAT-6: CAF01	*Mouse strain*: CB6F1 *Immunization*: rESAT-6 s.c. in CAF01 (+0, +2, +4 weeks) *Challenge*: Mtb Erdman aerosol (+10 weeks) *Post-Challenge*: lung CFU (+4, +6, +10 weeks)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: lung *Antigen(s)*: ESAT-$6_{1–15}$ peptide *Post-challenge*: (+2, +6, +24 weeks)	For ESAT-6:CAF01, both an increased 3^+ T cell response and decreased CFU in the lung relative to control mice were observed at all time points measured	Aagaard et al. (55)
H56:CAF01 H1:CAF01 BCG (SSI)	*Mouse strain*: CB6F1 *Immunization*: H56 s.c. in CAF01 (+0, +2, +4 weeks); H1 s.c. in CAF01 (+0, +2, +4 weeks) BCG (+0) *Challenge*: Mtb Erdman aerosol (+10 weeks) *Post-Challenge*: lung CFU (+6 weeks)	*Cell measured*: $CD4^+CD44^{hi}$ 3^+ cells *Cellular source*: lung *Antigen(s)*: rAg85B *Post-challenge*: (+6, +12, +24 weeks)	For H56:CAF01 relative to control, both an increased 3^+ T cell response and decreased CFU in the lung were observed at all time points measured For H56:CAF01 relative to H1:CAF01, both an increased 3^+ T cell response and decreased CFU in the lung were observed at +12 and +24 weeks For H56:CAF01 relative to BCG, both an increased 3^+ T cell response and decreased CFU in the lung were observed at +24 weeks only	Aagaard et al. (56)
H56:CAF01	*Mouse strain*: CB6F1 *Immunization*: H56 s.c. in CAF01 (+0, +2, +4 weeks) *Challenge*: Mtb Erdman aerosol (+10 weeks) *Post-Challenge*: lung CFU (+42 days)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: lung *Antigen(s)*: rESAT-6 *Post-challenge*: (+42 days)	For H56:CAF01 relative to control, both an increased 3^+ T cell response and decreased CFU in the lung were observed at +42 days	Woodworth et al. (57)
BCG (SSI) VPM1002	*Mouse strain*: Balb/c *Immunization*: BCG s.c. (+0); VPM1002 (+0) *Challenge*: Mtb H37Rv aerosol *Post-Challenge*: lung CFU (+90 days)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: lung *Antigen(s)*: PPD *Post-challenge*: (+7, +90 days)	VPM1002 vaccination prior to challenge resulted in greater frequencies of 3^+ T cells in the lung as compared to BCG-immunized or control mice 7 days after challenge. Ninety days after challenge, frequencies of 3^+ T cells in the lung were comparable in VMP1002-immunized, BCG-immunized and control mice. At this same time point, VMP1002-immunized mice controlled infection better than BCG-immunized mice	Desel et al. (58)

(*Continued*)

TABLE 3 | Continued

TB vaccine	Study design	IA	Conclusions	Reference
H4:CAF01 Ad-H4 BCG (SSI)	*Mouse strain*: CB6F1 *Immunization*: Ad-H4 s.c. (+0) vs H4 in CAF01 s.c. (+0, +2 weeks) vs H4 in CAF01 s.c. (+0)/Ad-H4 (+2 weeks) vs BCG s.c. (+0) *Challenge*: Mtb Erdman aerosol (+8 weeks) *Post-Challenge*: lung CFU (+6 weeks)	*Cell measured*: $CD4^+CD44^{hi}$ 3^+ cells *Cellular source*: lung *Antigen(s)*: TB10.4; Ag85B (p.p.) *Post-challenge*: (+2 weeks)	For H4:/Ad-H4 relative to control, both an increased 3^+ T cell response (+2 weeks) and decreased CFU in the lung (+6 weeks) were observed	Elvang et al. (40)
BCG (SSI)	*Monkeys*: *Macaca mulatta* *Immunization*: BCG aerosol (+0)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: BAL *Antigen(s)*: PPD *Immunogenicity*: (+4, +8, +13 weeks)	BCG induced 3^+ T cells as a major subset in BAL fluid 8 weeks after vaccination	White et al. (46)
AERAS-402	*Monkeys*: *M. mulatta* *Immunization*: AERAS-402 *via* aerosol (+1, +8, +15 days)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: BAL *Antigen(s)*: Ag85A/B (p.p.) *Immunogenicity*: (+18, +43 days)	AERAS-402 delivered *via* aerosol induced weak, detectable 3^+ T cells in BAL 28 days after completing immunization	Hokey et al. (48)

SSI, Staten Serum Institute; H1, Recombinant Ag85B/ESAT-6 fusion protein; CAF01, cationic liposomes formulated with synthetic mycobacterial cord factor; Ad85A, human adenoviral vector expressing Ag85A; rESAT-6, recombinant ESAT-6; H56, Recombinant Ag85B/ESAT-6/Rv2660c; VPM1002, Recombinant BCG which is urease C deficient and expressing membrane-perforating listerolysin (L. monocytogenes); H4, Recombinant Ag85B/TB10.4 fusion protein; Ad-H4, adenoviral vector expressing H4; AERAS-402, Adenoviral vector (Ad35) expressing Ag85A, Ag85B, and TB10.4; CB6F1, C57BL/6 X Balb/c; i.m., intramuscular; s.c., subcutaneous; i.d., intradermal; i.n., intranasal; CFU, colony forming units; 3^+, IFN-γ^+TNF-α^+IL-2^+; BAL, bronchoalveolar lavage; p.p., peptide pool; rAg85B, recombinant Ag85B; rESAT-6, recombinant ESAT-6; BCG, Bacille Calmette Guerin; TB, Tuberculosis; Mtb, M. tuberculosis; IA, immune assay.

Major subset = subset that constitutes >50% of the total cytokine-producing cells; minor subset = subset that constitutes <20% of the total cytokine-producing cells.

Tuberculosis vaccine candidates can elicit polyfunctional $CD4^+$ T cells in the lung (**Table 3**). Darrah et al. showed that BCG delivered i.m. induced PPD-specific polyfunctional $CD4^+$ T cells, as a major subset, in the lung 4 months after vaccination (9). Lindenstrom et al. showed that BCG and BCG boosted with Ag85B-ESAT-6 fusion protein in CAF01 (H1:CAF01) induced PPD-specific polyfunctional $CD4^+$ T cells, as a minor subset in lung 10 weeks after the completed immunization (38). By contrast, Forbes et al. showed that an Ag85A-expressing adenoviral vaccine-induced polyfunctional $CD4^+$ T cells in the lung after i.n. but not i.d. administration of the vaccine (39). In NHP's, polyfunctional $CD4^+$ T cells are detected in BAL after aerosol delivery of BCG (46) or adenoviral vaccine, AERAS-402 (48).

Studies utilizing the mouse TB model have also investigated the effect of prior vaccine administration on the magnitude of the polyfunctional $CD4^+$ T cell response in the lung following Mtb challenge (**Table 3**). Vaccination with recombinant ESAT-6 in CAF01 (rESAT-6:CAF01) (55) or Ag85B/ESAT-6/Rv2660c fusion protein in CAF01 (H56:CAF01) (56, 57), or VPM1002 (58), recombinant Ag85B/TB10.4 fusion protein in CAF01 (H4:CAF01), boosted with an Ad5 vector expressing recombinant H4 (Ad-H4) (40), or BCG boosted with Ag85B-ESAT-6 fusion protein in CAF01 (H1:CAF01) (38) all resulted in increased Mtb antigen-specific polyfunctional $CD4^+$ T cell responses in lung relative to control mice 2 weeks (40, 55) up to 26 weeks (38) after Mtb challenge. In most of these studies both an increased polyfunctional $CD4^+$ T cell response and vaccine-induced protection in the lung, as measured by decreased CFU in the lung relative to control mice, were observed at the same time point after infection. However, Desel et al. showed that 90 days after challenge, frequencies of polyfunctional T cells in the lung were comparable in VMP1002-immunized, BCG-immunized and control mice (58). Despite that, at this same time point, VMP1002-immunized mice controlled infection better than BCG-immunized mice and BCG-immunized mice controlled infection better than naïve mice. Also, Lindenstrom et al. showed that vaccination with BCG boosted with H1:CAF01 resulted in increased PPD-specific polyfunctional $CD4^+$ T cell responses in lung relative to control mice and BCG-vaccinated mice, yet differences in lung CFU in BCG/H1:CAF01 vs BCG-vaccinated mice were not statistically significant (38). In another study, vaccination with recombinant Ag85B/ESAT-6/Rv2660c fusion protein (H56:CAF01) resulted in Ag85B-specific polyfunctional $CD4^+$ T cells in the lungs, which were not detected in BCG-immunized or control mice, yet both BCG and H56-vaccinated mice demonstrated a similar reduction in lung CFU as compared to control mice at 6 and 12 weeks after challenge (56). In conclusion, in animal models, TB vaccines induce polyfunctional $CD4^+$ T cells that are present in lung after immunization and in some, but not all studies, an increase in polyfunctional $CD4^+$ T cells in the lung following Mtb challenge is temporally associated with vaccine-induced control of bacterial replication in the lung relative to control mice.

CORRELATION OF POLYFUNCTIONAL T CELL RESPONSES AND PROTECTION IN THE MOUSE MODEL

Whether or not vaccine-induced polyfunctional CD4+ T cells represent a correlate of protective immunity from Mtb infection has been addressed in several mouse studies investigating the correlation between the magnitude of the polyfunctional CD4+ T cell response present before Mtb challenge and vaccine-induced control of bacterial replication, the most common measure of vaccine protection against TB in the murine model (**Table 2**). The strongest correlative evidence for polyfunctional CD4+ T cells as a correlate of protective immunity comes from studies in which a correlation between control of bacterial replication and the magnitude of the CD4+ T cell response was established from experiments including multiple distinct vaccine candidates that elicit a range of protective responses. Conversely, the weakest correlative evidence comes from studies in which a single vaccine candidate both induces a polyfunctional CD4+ T cell response and demonstrates protection compared to unimmunized control mice. For example, BCG (9, 41), H1:CAF01 (38, 41), and ID93:GLA-SE (54), all induced mycobacteria-specific polyfunctional CD4+ T cell responses at the time of Mtb challenge, which were associated with protection as compared to naïve or control mice, who lack these T cell responses. Somewhat better correlative evidence comes from studies comparing two or more vaccine candidates to one another. Comparing rBCG:XB to BCG, Yuan et al. showed that rBCG:XB elicited stronger HspX-specific polyfunctional T cell responses than BCG, which was associated with greater protection than was observed in BCG-vaccinated mice (44). Ballester et al. showed that recombinant Ag85B delivered in CpG (Ag85B:CpG) with polypropylene sulfide nanoparticles (NP) delivered i.n. induced more polyfunctional Ag85B-specific CD4+ T cells in spleen pre-challenge than did Ag85B:CpG without NP and this correlated with better protection in the lung (50). Also, comparing priming alone with prime/boost strategies, Elevang et al. demonstrated that recombinant Ag85B/TB10.4 fusion protein in CAF01 (H4:CAF01), boosted with an Ad5 vector expressing recombinant H4 (Ad-H4) induced more Ag85A- and TB10.4-specific polyfunctional memory CD4+ T cells, pre-challenge than did either recombinant H4 or Ad-H4 alone, which in turn correlated with better protection in lung after challenge (40). In addition, comparing different dosages of recombinant antigen, Aagaard et al. showed that H4:IC31 induced stronger Ag85B- and TB10.4-specific polyfunctional CD4+ T cells in PBMC pre-challenge at a lower dose (0.5 μg) vs a higher dose (5.0 μg), and this correlated with better protection in the lung at the lower dose (52). Finally, the strongest degree of correlation was demonstrated using several different vaccine regimens inducing various levels of protection (43). Derrick et al. showed that both the frequency of polyfunctional CD4+ T cells and the iMFI for TNF-α and IFN-γ at the time of Mtb challenge correlated with vaccine-induced protection in the lung and spleen over a 14-month period. For example, the magnitude of BCG-specific CD4+ polyfunctional T cell responses at the time of challenge 8 months after immunization with BCG or BCG mixed with recombinant E6-85, were greater than those induced by a DNA construct expressing recombinant E6-85 and correlated with greater protection afforded by either of these vaccine BCG regimens than the DNA construct. Moreover, using Pearson correlation analysis of iMFI values at all the challenge time points, these investigators showed that IFN-γ iMFI values were highly correlated with protection in the lung and spleen. Yet, of note, these investigators only reported measurements of the polyfunctional CD4+ T cell response, while other immune parameters that may also be mediators of protection, such as antibody responses and CD8+ T cell responses, were not evaluated for correlation with control of bacterial replication.

By contrast, two other studies show either equivocal evidence for or evidence against a correlation between pre-challenge polyfunctional CD4+ T cells and vaccine-induced protection. For example, boosting BCG with an Ad5 vector expressing Ag85A, induced Ag85A-specific polyfunctional CD4+ T cells pre-challenge in the lung only when delivered i.n. but not i.d. and this correlated with improved protection in the lung in i.n. vaccinated mice (39). However, i.d. rather than i.n. delivery induced polyfunctional CD4+ T cells in the spleen, such that only lung, not splenic polyfunctional CD4+ T cells correlated with the degree of vaccine-induced protection. In another study, both BCG and recombinant BCG (VPM1002) required boosting with MVA85A to elicit PPD- and Ag85A-specific polyfunctional CD4+ T cells, with BCG boosted with MVA85A eliciting greater T cell responses than VPM1002 boosted with MVA85A (42). Yet VPM1002 provided better protection in lung compared to BCG and the MVA85A boost, which induced high levels of polyfunctional CD4+ T cells, did not augment protection obtained with either BCG or VPM1002. Differences in these two studies as compared to numerous studies that support a correlation between polyfunctional CD4+ T cells are difficult to resolve, but may simply reflect differences in the vaccination protocol, or how polyfunctional T cells or protection was measured. In addition, two studies showed a stronger correlation between dual IL-2 and TNF-α producing CD4+ T cells and vaccine-induced protection than with triple IL-2, TNF-α, and IFN-γ-producing CD4+ T cells (38, 41). Finally, Yuan et al. showed a stronger correlation between the magnitude of dual TNF-α and IFN-γ-producing CD4+ T cells and vaccine-induced protection than with triple IL-2, TNF-α, and IFN-γ-producing CD4+ T cells (44). In summary, studies of the role of polyfunctional CD4+ T cells in mediating vaccine-induced protection in the mouse TB vaccine model provide evidence that these T cell responses represent at best an imperfect correlate of protection. In addition, these data can neither confirm nor refute CD4+ polyfunctional T cells as a mechanistic correlate of protection—i.e., a causal relationship between the vaccine-induced polyfunctional CD4+ T cell responses and control of bacterial replication following challenge is uncertain and cannot be ruled in or ruled out based upon these studies.

TB VACCINES INDUCE POLYFUNCTIONAL T CELLS IN HUMANS

Bacille Calmette Guerin and novel TB vaccine candidates utilizing various antigens and vaccine platforms induce polyfunctional CD4+ T cells in infants, older children and adults. These T cell

populations include memory T cell populations that persist for months to years after vaccination.

BCG and Other Live Mycobacterial Vaccines Elicit Polyfunctional T Cells

Several studies have demonstrated that BCG induces $CD4^+$ polyfunctional T cells in infants (**Table 4**). BCG induces mycobacteria-specific polyfunctional $CD4^+$ T cells, in healthy infants from South Africa (59–62), Uganda (63), Australia (64, 65), and the UK (66). These T cell responses represented the predominant subset among cytokine-producing T cells (59, 60, 66) peaked between 6 and 10 weeks of age and persisted for at least 1 year (60, 66). Comparison to unimmunized infants demonstrated that the polyfunctional T cell response was elicited by BCG and not by exposure to environmental mycobacteria (60, 66). Studies of Australian infants (65), South African infants (60), and Ugandan infants (63) compared BCG immunization at birth to delayed immunization at 6–10 weeks. These studies in general demonstrated no differences in polyfunctional $CD4^+$ T cell responses during the first few weeks following immunization. However, South African infants who received delayed BCG had higher polyfunctional $CD4^+$ T cell responses at 1 year of age than did those receiving BCG at birth (60). Also, Ritz et al. showed that BCG (Denmark, SSI) and BCG (Japan, Tokyo-172) induced higher polyfunctional $CD4^+$ T cell responses than did BCG (Russian, SL-222), which correlated with more severe local reactions to the vaccine (64). BCG at birth also induced polyfunctional $CD4^+$ T cell responses in HIV-infected infants, though at decreased numbers relative to HIV-exposed/uninfected infants and HIV-unexposed infants (61). Finally, Loxton et al. compared the immunogenicity of BCG to recombinant BCG, VPM1002, in healthy South African newborns and demonstrated comparable frequencies of polyfunctional $CD4^+$ T cell responses 6 weeks, 18 weeks and 6 months after vaccination (67). Thus, ample evidence demonstrates that BCG induces polyfunctional $CD4^+$ T cells in human infants.

Fewer studies have evaluated polyfunctional $CD4^+$ T cells in BCG-immunized adults (**Table 5**). Two small studies of adults with a remote history of BCG immunization demonstrated mycobacteria-specific polyfunctional $CD4^+$ T cell responses in these individuals, which represented the predominant subset of cytokine-producing cells (9, 71) and displayed a memory phenotype (71). In a small study of BCG naïve, Mtb-uninfected adults immunized with BCG, BCG-specific polyfunctional $CD4^+$ T cell responses were variably detected and when present, peaked 8 weeks following vaccination, demonstrated an effector phenotype and correlated with local inflammation at the vaccination site (72). These responses waned by 1 year after vaccination. Consistent with these results, no polyfunctional $CD4^+$ T cell responses were observed in British adolescents 1 year after BCG vaccination (73). Finally, Spertini et al. compared the immunogenicity of BCG with MTBVAC, a live attenuated strain of Mtb, in a randomized double-blind Phase I trial of BCG naïve, Mtb-uninfected Swiss adults (74). Both BCG and MTBVAC induced MTBVAC- and BCG-specific polyfunctional $CD4^+$ T cell responses that persisted up to 210 days after vaccination. Therefore, although the evidence is less abundant than for infants, live mycobacterial vaccines also induce polyfunctional $CD4^+$ T cell responses in adults.

Recombinant Protein Subunit Vaccines Elicit Polyfunctional T Cells

Several recombinant antigen TB vaccines have been studied in adults for their capacity to induce polyfunctional $CD4^+$ T cell responses (**Table 5**). Recombinant Ag85B/TB10.4 fusion protein formulated with IC31 adjuvant (H4:IC31), induced H4-specific polyfunctional $CD4^+$ T cells in previously BCG-vaccinated, Mtb-uninfected South African adults (75). While all doses induced these T cell responses, lower doses (5 or 15 μg) induced higher magnitudes of $CD4^+$ T cells than higher doses (50 or 150 μg), which could be detected up to 182 days after vaccination. Recombinant Ag85B/ESAT-6/Rv2660c fusion protein formulated with IC31 (H56:IC31) also induced polyfunctional $CD4^+$ T cells in both previously BCG-immunized, Mtb-uninfected and Mtb-infected South African adults (76). Frequencies of these polyfunctional $CD4^+$ T cells were higher in Mtb-infected than uninfected individuals and comprised the predominant proportion of the cytokine-producing cells 70 days after vaccination. These cells persisted, expressing markers of central memory cells, up to 210 days after immunization. As in the H4:IC31 study, a lower dose of H56:IC31 resulted in higher proportions of polyfunctional $CD4^+$ T cells while in recipients of a high 50 μg H56 dose, $CD4^+$ T cells expressing only IFN-γ predominated. Recombinant Ag85B/ESAT-6 formulated in IC31 (H1:IC31) induced H1-specific polyfunctional $CD4^+$ T cells in Mtb-uninfected and infected South African adolescents which persisted at least 70 days in Mtb-infected and 224 days in Mtb-uninfected individuals (77), and in HIV-infected Tanzanian adults, which peaked at 70 days and persisted for 182 days after vaccination (78). Finally, a recombinant Mtb32A/Mtb39A fusion protein formulated in $AS02_A$ (Mtb72F:$AS02_A$) induced polyfunctional $CD4^+$ T cells in Mtb-infected and previously BCG-immunized Swiss adults (79), a recombinant Mtb32A/Mtb39A modified to increase stability formulated in $ASO1_B$ (M72:$ASO1_B$) or in $AS02_A$ (M72:$AS02_A$) induced polyfunctional $CD4^+$ T cells in BCG naïve, Mtb-uninfected Belgian adults (80), and M72 formulated in $AS01_E$ (M72: $AS01_E$) induced polyfunctional $CD4^+$ T cells in Mtb-infected and uninfected South African adults and adolescents (81, 82), HIV-infected and uninfected Indian adults (83), and HIV-infected Swiss adults on anti-retroviral therapy (84). In the latter study, polyfunctional $CD4^+$ T cell populations also co-expressed CD40L and persisted from 7 months to 3 years after immunization. Thus, adjuvanted recombinant protein TB vaccine candidates are good inducers of $CD4^+$ polyfunctional T cell responses in humans.

Viral Vector Vaccines Elicit Polyfunctional T Cells

Adenoviral TB vaccine candidates have been studied in a few trials of infants and adults (**Tables 4** and **5**). In a Phase I double-blinded randomized placebo-controlled trial of BCG-immunized, Mtb

TABLE 4 | TB vaccines induce polyfunctional $CD4^+$ T cells in human infants.

Vaccine	Study design	IA	Conclusions	Reference
BCG (SSI)	*Cohort(s)*: healthy South African infants born at term; HIV-uninfected/unexposed, TB uninfected/unexposed *Immunization*: BCG i.d. (+0) *Randomized*: birth ($n = 25$) vs 10 weeks ($n = 21$)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular Source*: whole blood *Antigen(s)*: BCG *Immunogenicity*: (+10, +20, +50 weeks)	3^+ T cells were readily detected in both cohorts, which peaked 10 weeks after vaccination. No 3^+ T cells were detected pre-vaccination in the 10-weeks delayed cohort. 3^+ T cells persisted at 1 year and the magnitude was greater in the BCG delayed than in the BCG at birth cohort	Kagina et al. (60)
BCG (Japanese)	*Cohort(s)*: 2-year follow-up of healthy South African infants born at term HIV-uninfected/unexposed TB (Cx^+; $n = 29$) Healthy Mtb exposed ($n = 55$) Healthy Mtb unexposed ($n = 55$) *Immunization*: BCG (+0)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: BCG *Immunogenicity*: (+10 weeks)	3^+ T cells were equivalent in the TB, TB exposed, and TB unexposed cohorts. There was no correlation between 3^+ T cells at 10 weeks and development of TB within 2 years	Kagina et al. (62)
BCG (SSI)	*Cohort(s)*: South African infants born at term HIV-infected (ART naïve, $n = 20$) HIV-exposed ($n = 25$) HIV-unexposed ($n = 25$) HIV was ART naïve *Immunization*: BCG i.d. (+0)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: BCG *Immunogenicity*: (+3, +6, +9, +12 months)	3^+ T cells were equivalent in the HIV-exposed and HIV-unexposed cohorts and decreased in the HIV-infected, ART naïve cohort. 3^+ T cells peaked at 3 months in all cohorts	Mansoor et al. (61)
BCG (SSI)	*Cohort(s)*: healthy South African infants born at term; HIV-uninfected/unexposed, TB uninfected/unexposed ($n = 29$) *Immunization*: BCG i.d. (+0)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: BCG *Immunogenicity*: (+10 weeks)	3^+ T cells were readily detected at 10 weeks after immunization	Soares et al. (59)
BCG	*Cohort(s)*: healthy BCG-immunized Ugandan infants, 9 months old; HIV-uninfected/unexposed, Mtb unexposed *Immunization*: BCG at birth ($n = 50$) BCG at 6 weeks of age: ($n = 42$)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: BCG *Immunogenicity*: once at 9 months of age	3^+ T cells were readily detected in both cohorts and were of comparable magnitude in infants immunized at birth and at 6 weeks of age	Lutwama et al. (63)
BCG Danish (SSI) Japan (Tokyo-172) Russia (SL-222)	*Cohort(s)*: healthy Australian infants born at term; HIV-uninfected/unexposed, TB uninfected/unexposed *Immunization*: BCG i.d. (+0) Denmark ($n = 54$) Japan ($n = 54$) Russia ($n = 57$)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: BCG; PPD; heat-killed Mtb (H37Rv) *Immunogenicity*: (+10 weeks)	3^+ T cells were equivalent in infants immunized with the Danish and Japanese BCG strains. BCG- and PPD-specific 3^+ T cells, as well as the local reaction sizes, were greater in infants immunized with the Danish and Japanese BCG strains, than in those immunized with the Russian BCG strain	Ritz et al. (64)
BCG (SSI)	*Cohort(s)*: healthy Australian infants born at term; HIV-uninfected/unexposed *Immunization*: BCG i.d. (+0) Birth ($n = 54$) 2 months ($n = 44$)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: BCG; PPD; heat-killed Mtb (H37Rv) *Immunogenicity*: (+10 weeks)	3^+ T cells were a minor subset and equivalent in infants immunized at birth and at 2 months of age	Ritz et al. (65)
BCG (SSI)	*Cohort(s)*: healthy term infants born in the UK; HIV-uninfected/unexposed *Immunization*: BCG i.d. at 5.6 (4.3–8 weeks; $n = 24$) BCG naïve ($n = 15$)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: PPD *Immunogenicity*: (+4 months and +1 year of age)	3^+ T cells were a major subset at 4 months and 1 year of age. No 3^+ T cells were detected in BCG naïve infants	Smith et al. (66)
BCG (SSI) VPM1002	*Cohort(s)*: open-label, randomized Phase II study of healthy South African infants born at term; HIV-uninfected/unexposed *Immunization*: BCG i.d. (+0; $n = 12$) VPM1002 i.d. (+0; $n = 36$)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: PPD, BCG *Immunogenicity*: (+6, +18 weeks, +6 months)	3^+ T cells were equivalent in infants immunized with BCG or VPM1002, 6 weeks, 18 weeks, and 6 months after vaccination	Loxton et al. (67)

(*Continued*)

TABLE 4 | Continued

Vaccine	Study design	IA	Conclusions	Reference
BCG AERAS-402	*Cohort(s)*: double-blinded, randomized placebo-controlled trial in South Africa, Kenya, and Mozambique Healthy BCG-immunized infants, 16–26 weeks-old; HIV-uninfected; Mtb-uninfected *Immunization*: AERAS-402 i.m. (+0, +28, +280 days; *n* = 60) Placebo (vaccine buffer) i.m. (+0, +28, +280 days; *n* = 55)	*Cell measured*: CD4$^+$ 3$^+$ cells *Cellular source*: PBMC *Antigen(s)*: Ag85A; Ag85B; TB10.4 (p.p.) *Immunogenicity*: [+308, study end (+448–664 days)]	3$^+$ T cells were detected 308 days after the first immunization and at the end of the study (448–664 days). These responses were lower than had been observed in BCG-immunized adults in a prior study	Tameris et al. (68)
BCG MVA85A	*Cohort(s)*: open-label Phase 2a trial of healthy BCG-immunized South African infants, 5–12 months old; HIV-uninfected, Mtb-uninfected *Immunization*: MVA85A i.d. (+0; *n* = 18) Prevnar i.m. (*n* = 12)	*Cell measured*: CD4$^+$ 3$^+$ cells; GM-CSF; IL-17 *Cellular source*: whole blood *Antigen(s)*: Ag85A (p.p.) *Immunogenicity*: [+0, +28, +168 days; and 3.3 (3.2–3.5 years)]	MVA85A boost to BCG induced a major subset of 3$^+$ T cells, the majority of which co-expressed GM-CSF, and a minority of which also co-expressed IL-17. These responses peaked at 28 days and persisted over 3 years following immunization. 3$^+$ T cells at 3 years displayed predominantly an effector memory phenotype (CD45RA^{neg}CCR7^{neg})	Scriba et al. (69) and Tameris et al. (70)
BCG (SSI) MVA85A	*Cohort(s)*: double-blind Phase 2b trial in healthy BCG-immunized South African infants 4–6 months old; HIV-uninfected, Mtb-uninfected/unexposed *Immunization*: MVA85A i.d. (+0; *n* = 17) Candin i.d. (+0; *n* = 19)	*Cell measured*: CD4$^+$ 3$^+$ cells *Cellular source*: whole blood *Antigen(s)*: Ag85A (p.p.) *Immunogenicity*: (+0, +28 days)	MVA85A boost to BCG elicited 3$^+$ T cells 28 days after immunization, which were not present prior to vaccination or in placebo recipients. No significant efficacy against Mtb infection or TB disease as compared to BCG alone was observed. These 3$^+$ T cell responses were lower than had been observed in BCG-immunized adults in a prior study	Tameris et al. (3)

SSI, Staten Serum Institute; VPM1002, recombinant BCG which is urease C deficient and expressing membrane-perforating listerolysin (L. monocytogenes); AERAS-402, adenoviral vector (Ad35) expressing Ag85A, Ag85B, and TB10.4; MVA85A, modified vaccinia Ankara expressing Ag85A. ART, anti-retroviral therapy; Prevnar, pneumococcal 7-valent conjugate vaccine (Wyeth); Candin, Candida skin test antigen; i.m., intramuscular; i.d., intradermal; 3$^+$, IFN-γ$^+$TNF-α$^+$IL-2$^+$; PBMC, peripheral blood mononuclear cells; p.p., peptide pool; BCG, Bacille Calmette Guerin; TB, tuberculosis; Mtb, M. tuberculosis; IA, immune assay.

Minor subset = subset that constitutes <20% of the total cytokine-producing cells; major subset = subset that constitutes >50% of the total cytokine-producing cells.

and HIV-uninfected South African adults, an Ad35 expressing Ag85A, Ag85B, and TB10.4 (AERAS-402), induced polyfunctional CD4$^+$ T cells that constituted the predominant CD4$^+$ T cell subset 28 days after immunization, but this subset was not detectable 182 days after immunization (85). AERAS-402 also induced polyfunctional CD4$^+$ T cells in BCG-immunized, HIV-infected South African adults, which peaked 42 days after the first vaccination (86). By contrast, in a double-blinded randomized trial of healthy BCG-immunized HIV-uninfected infants performed in South Africa, Kenya, and Mozambique, AERAS-402 induced detectable but low level CD4$^+$ polyfunctional T cell responses, at frequencies lower than had been observed in BCG-vaccinated adults in the afore mentioned study (68). Finally, an Ad5 expressing Ag85A vaccine candidate induced polyfunctional CD4$^+$ T cell responses in both BCG naïve and previously BCG-vaccinated, Mtb and HIV-uninfected Canadian adults (87). Polyfunctional CD4$^+$ T cells comprised the predominant subset of cytokine-producing T cells and peaked at 2–4 weeks after vaccination. Therefore, adenoviral TB vaccines induce polyfunctional CD4$^+$ T cell responses in humans that do not persist as long as those induced by protein subunit TB vaccines.

The capacity for MVA expressing A85A (MVA85A) to elicit polyfunctional CD4$^+$ T cells, as a boost to previous BCG vaccination, has been extensively evaluated in several studies of infants, children, adolescents, and adults (**Tables 4** and **5**). MVA85A induced polyfunctional CD4$^+$ T cell responses in previously BCG-vaccinated Mtb- and HIV-uninfected South African adults (88), Mtb- and HIV-uninfected British adults (89, 90), Mtb-infected HIV-uninfected South African adults (91), Mtb-infected HIV-uninfected British adults (92), and Mtb- and HIV-uninfected infants (3, 69), young children (69) and adolescents (69). In these studies, polyfunctional CD4$^+$ T cells represented the predominant subset of cytokine-producing T cells and persisted at least up to 168 days after vaccination. Moreover, in a follow-up study of the South Africa cohorts (69, 88, 91), Tameris et al. demonstrated that MVA85A induced polyfunctional CD4$^+$ T cell responses persisted 3–5 years after immunization (70). Of note, these T cell responses were of lower magnitude in infants (3) than observed previously in adults (89). Thus, MVA85A is a potent inducer of polyfunctional CD4$^+$ T cells responses, which persist for 3–5 years after vaccination.

TABLE 5 | TB vaccines induce polyfunctional $CD4^+$ T cells in human children, adolescents, and adults.

Vaccine	Study design	IA	Conclusions	Reference
BCG	*Cohort(s)*: BCG-immunized adults from the United States; Mtb-uninfected (RD-1 ELISPOTneg), no history of TB ($n = 20$) *Immunization*: BCG (remote)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: PBMC *Antigen(s)*: Mtb cell wall *Immunogenicity*: once (unknown timing)	3^+ T cells were detected as a major subset in adults with a remote history of BCG immunization	Adekambi et al. (71)
BCG	*Cohort(s)*: BCG-immunized adults ($n = 4$) *Immunization*: BCG (remote)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: PBMC *Antigen(s)*: PPD *Immunogenicity*: once (unknown timing)	3^+ T cells were detected as a major subset in adults with a remote history of BCG immunization	Darrah et al. (9)
BCG (SSI)	*Cohort(s)*: BCG naïve adults from the Netherlands; Mtb-uninfected (TSTneg, QFTneg, $n = 12$) *Immunization*: BCG i.d. (+0)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: PBMC *Antigen(s)*: BCG *Immunogenicity*: (+0, +4, +8, +12, +52 weeks)	BCG induced 3^+ T cells in some individuals with a greater local skin reaction. When observed, 3^+ T cells responses peaked 8 weeks after immunization	Boer et al. (72)
BCG (SSI)	*Cohort(s)*: BCG naïve adolescents from UK ($n = 8$) *Immunization*: BCG (+0)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: PBMC *Antigen(s)*: PPD *Immunogenicity*: (+12 months)	3^+ T cells were not detectable in adolescents 1 year after BCG vaccination	Smith et al. (73)
MTBVAC BCG (SSI)	*Cohort(s)*: randomized double-blind Phase I trial of BCG naive adults from Switzerland; BCG naïve, HIV-uninfected, Mtb-uninfected (RD-1 ELISPOTneg) *Immunization*: MTBVAC i.d. (5×10^3, 5×10^4, 5×10^5 CFU; +0, $n = 9$, each dose) BCG i.d. (+0, $n = 9$)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: MTBVAC; BCG *Immunogenicity*: (+0, +28, +90, +210 days)	BCG and MTBVAC induced 3^+ T cells to both BCG and MTBVAC stimulations. MTBVAC at the highest dose (5×10^5 CFU) induced 3^+ T cells, which were still detectable 210 days after immunization	Spertini et al. (74)
H4:IC31	*Cohort(s)*: double-blind, Phase I trial of South African adults; BCG-immunized (remote), HIV-uninfected, Mtb-uninfected (QFTneg), no TB *Immunization*: H4 in IC31 i.m. (5, 15, 50, 150 μg; +0, +56 days; $n = 8$, each dose) Saline i.m. (+0, +56 days; $n = 8$)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: PBMC *Antigen(s)*: Ag85B; TB10.4 (p.p.) *Immunogenicity*: (+0, +14, +28, +56, +70, +84, +182 days)	H4:IC31 induced 3^+ T cells in Mtb-uninfected, BCG-immunized adults. While all doses elicited 3^+ cells, lower doses induced greater frequency 3^+ T cells than did the highest dose (150 μg) and the highest magnitude response was 84 days after immunization (15 μg)	Geldenhuys et al. (75)
H56:IC31	*Cohort(s)*: open-label Phase I trial of South African adults; BCG-immunized (remote, assumed), HIV-uninfected, no TB disease, Mtb infections defined with QFT *Immunization*: rH56 in IC31 i.m. (+0, +56, +112 days) Mtb-uninfected (50 μg, $n = 8$) Mtb-infected (15 μg, $n = 8$) Mtb-infected (50 μg, $n = 8$)	*Cell measured*: $CD4^+$ 3^+ cells, CD45RA, CCR7 *Cellular source*: whole blood *Antigen(s)*: H56 *Immunogenicity*: (+0, +70, +210 days)	H56:IC31 induced 3^+ T cells in both Mtb-infected and Mtb-uninfected BCG-immunized adults. 3^+ T cell responses were greater in Mtb-infected than in Mtb-uninfected individuals, comprised a predominant subset at +70 days and persisted at least to +210 days. At +210 days, 3^+ T cells displayed a central memory ($CD45RA\text{-}CCR7^+$) or effector memory (CD45RA-CCR7-) phenotype. Among Mtb-infected individuals, the low dose (15 μg) elicited greater frequencies of 3^+ T cells than the high dose (50 μg)	Luabeya et al. (76)
H1:IC31	*Cohort(s)*: observer-blinded Phase II trial of South African adolescents (12–18 years old); BCG-immunized at birth, HIV-uninfected, no TB disease, comparison of Mtb-infected (QFTpos; $n = 25$) to Mtb-infected (QFTneg; $n = 35$) individuals *Immunization*: H1 in IC31 i.m. (15 μg; +0, +56 days)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: Ag85B; ESAT-6 (p.p.) *Immunogenicity*: (+0, +14, +56, +70, +224 days)	H1:IC31 induced 3^+ T cell responses over baseline in both Mtb-infected and Mtb-uninfected adolescents, which comprised a predominant subset in both cohorts, and persisted at least to +70 days in Mtb-infected and to +224 days in Mtb-uninfected individuals	Mearns et al. (77)

(*Continued*)

TABLE 5 | Continued

Vaccine	Study design	IA	Conclusions	Reference
H1:IC31	*Cohort(s)*: randomized double-blind Phase II trial of HIV-infected adults from Tanzania; CD4 counts >350, ARV naïve, no TB disease *Immunization*: H1 in IC31 i.m. (+0, +56 days; $n = 20$) Buffer i.m. (+0, +56 days; $n = 4$)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: H1 *Immunogenicity*: (+0, +14, +56, +70, +182 days)	H1:IC31 induces a predominant subset of 3^+ T cells in HIV-infected adults, which peak 70 days and persist at least 182 days after initiation of immunization	Reither et al. (78)
Mtb72F: $AS02_A$	*Cohort(s)*: randomized observer blind Phase I/II trial of adults from Switzerland; HIV-uninfected, compared BCG-immunized (remote, $n = 20$) to Mtb-infected (TST^+, $n = 18$; included subset with history of TB disease, $n = 5$) *Immunization*: Mtb72F in $AS02_A$ i.m. (+0, +1, +3 months) $AS02_A$ i.m. (+0, +1, +3 months)	*Cell measured*: $CD4^+$ 3^+ cells, CD40L *Cellular source*: PBMC *Antigen(s)*: Mtb32A; Mtb39A (p.p.) *Immunogenicity*: (+0, +60, +90, +240 days)	In both Mtb-infected and BCG-immunized adults, 3^+ T cells were detected before immunization. Also in both cohorts, Mtb72F:$AS02_A$, but not $AS02_A$ alone, resulted in increased 3^+ T cells after the second vaccination, which were not further boosted by the third vaccination, and persisted at least to +240 days. A predominant subset in both cohorts also expressed CD40L	Spertini et al. (79)
M72:$AS01_B$ M72:$AS02_A$	*Cohort(s)*: randomized observer blind Phase I/II trial of adults from Belgium; HIV-uninfected, Mtb-uninfected (TSTneg) *Immunization*: M72 in $AS01_B$ i.m. (+0, +1 month; $n = 40$) M72 in $AS02_A$ i.m. (+0, +1 month; $n = 40$)	*Cell measured*: $CD4^+$ 3^+ cells, CD40L *Cellular Source*: PBMC *Antigen(s)*: M72 (p.p.) *Immunogenicity*: (+0, +1, +2, +12, +24, +36 months)	M72:$AS01_B$ and M72:$AS02_A$ induced 3^+ T cells first detected 1 month after the second immunization (+2 months), which persisted to least 36 months. 3^+ T cell responses were greater in M72:$AS01_B$ than in M72:$AS02_A$ immunized individuals. The majority of the 3^+ T-cell response co-expressed CD40L	Leroux-Roels et al. (80)
M72: $AS01_E$	*Cohort(s)*: open-label, Phase II trial of South African adults; HIV-uninfected; BCG-immunized (remote), Mtb-infected (TSTpos, 67%); no TB disease ($n = 45$) *Immunization*: M72 in $AS01_E$ i.m. (+0, +30 days)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: M72 *Immunogenicity*: (+0, +7, +30, +37, +60, +210 days)	M72:$AS01_E$ induced 3^+ T cell responses over baseline, which comprised a predominant subset, and persisted at least to +210 days	Day et al. (81)
M72:$AS01_E$	*Cohorts*: double-blind, randomized Phase II trial of South African adolescents; HIV-uninfected, BCG-immunized (at birth); Mtb-infected (QFTpos; 53%); no TB disease *Immunization*: M72 in $AS01_E$ i.m. (+0, +30 days; $n = 40$) Saline i.m. (+0, +30 days; $n = 20$)	*Cell measured*: $CD4^+$ 3^+ cells, CD40L *Cellular source*: whole blood and PBMC *Antigen(s)*: M72 (p.p.); M72 *Immunogenicity*: (+0, +7, +30, +37, +60, +120 days)	M72:$AS01_E$ induced 3^+ T cell responses over baseline, which comprised a predominant subset, and persisted at least to +210 days. 3^+ T cells co-expressed CD40L	Penn-Nicholson et al. (82)
M72:$AS01_E$	*Cohort(s)*: randomized, observer blind, Phase II trial of adults from India; no history of TB disease, comparison of HIV-infected on stable ART ($n = 80$) to ART naïve HIV-infected ($n = 80$) to HIV-uninfected ($n = 80$) *Immunization*: M72 in $AS01_E$ i.m. (+0, +1 month; $n = 40$, each cohort) Saline i.m. (+0, +1 month; $n = 40$ each cohort)	*Cell measured*: $CD4^+$ 3^+ cells, CD40L *Cellular source*: PBMC *Antigen(s)*: M72 (p.p.) *Immunogenicity*: (+0, +7, +30, +37, +60 days, +7, +13 months)	In all cohorts, 3^+ T cells were detected before immunization. Also in the cohorts receiving, M72, but not those receiving placebo, M72 resulted in increased 3^+ T cells, which peaked 37 days after immunization and persisted at least 13 months. A predominant subset in both cohorts also expressed CD40L	Kumarasamy et al. (83)
M72: $AS01_E$	*Cohort(s)*: randomized, observer blind, Phase I/II trial of HIV-infected adults from Switzerland on ART; No history of TB disease, CD4 >200, BCG-immunized (73%), Mtb-infected (QFT^+, 3%) *Immunization*: M72 in $AS01_E$ i.m. (+0, +1 month; $n = 22$) $AS01_E$ i.m. (+0, +1 month; $n = 8$) Saline i.m. (+0, +1 month; $n = 7$)	*Cell measured*: $CD4^+$ 3^+ cells, CD40L *Cellular source*: PBMC *Antigen(s)*: M72 (p.p.) *Immunogenicity*: (+0, +30, +60, +201 days)	M72:$AS01_E$ induced 3^+ T cells in HIV-infected adults that peaked at +60 days and persisted at +210 days. All 3^+ T cells co-expressed CD40L	Thacher et al. (84)

(*Continued*)

TABLE 5 | Continued

Vaccine	Study design	IA	Conclusions	Reference
AERAS-402	*Cohort(s)*: randomized, double-blind, Phase I trial of BCG-immunized adults from South Africa; HIV-uninfected, Mtb-uninfected (QFTneg and TSTneg) *Immunization*: AERAS-402 i.m. (3×10^8, 1.3×10^9, 3×10^{10} VP; +0, $n = 7$, each dose) AERAS-402 i.m.(3×10^{10} VP; +0, +56; $n = 7$) Diluent i.m. (+0, ±56, $n = 12$)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: PBMC and whole blood *Antigen(s)*: Ag85A/B, TB10.4 (p.p.) *Immunogenicity*: (0, +7, +28, +84, +182 days)	In BCG-immunized, Mtb-uninfected adults, AERAS-402 induced 3^+ T cells to all vaccine components that were the predominant subset at +28 days, which persisted at +84 days, and were not detected at +182 days. No differences in immunogenicity were noted in the cohorts that received one dose compared to two doses	Abel et al. (85)
AERAS-402	*Cohort(s)*: HIV-infected, BCG-immunized South African adults; CD4 >350, no current ARV, Mtb-infected (QFT^+, 50%) *Immunization*: AERAS-402 i.m. (+0, +1 month; $n = 13$) Buffer i.m. (+0, +1 month; $n = 13$)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: PBMC *Antigen(s)*: Ag85A, Ag85B, TB10.4 (p.p.) *Immunogenicity*: (0, +7, +14, +28, +35, +42, +56, +84, +182 days)	In HIV-infected, BCG-immunized adults, AERAS-402 induced 3^+ T cells to Ag85A/B, but not to TB10.4, that were the predominant subset and which peaked 2 weeks after the second immunization. 3^+ T cell responses were not different in Mtb-infected as compared to Mtb-uninfected individuals	Churchyard et al. (86)
AdHu5Ag85A	*Cohort(s)*: Phase I trial of adults from Canada: HIV-uninfected; Mtb-uninfected (QFTneg), comparison of BCG-immunized ($n = 12$) to BCG naïve ($n = 12$) individuals *Immunization*:AdHu5Ag85A i.m. (+0)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: PBMC *Antigen(s)*: Ag85A (p.p.); Mtb CF *Immunogenicity*: (+0, +2, +4, +8, +24 weeks)	AdHu5Ag85A induced 3^+ T cells in both BCG-immunized and BCG naïve adults, which peaked 2–4 weeks after vaccination and at some time points represented a predominant subset. 3^+ T cell responses where greater in BCG-immunized than BCG naïve adults	Smaill et al. (87)
MVA85A	*Cohort(s)*: open-label Phase I trial of adults from South Africa; HIV-uninfected, Mtb-uninfected (TSTneg), no TB disease, BCG-immunized (50%) *Immunization*: MVA85A i.d. (+0; $n = 24$)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: BCG, Ag85A (p.p.) *Immunogenicity*: [−14, +7, +168 days, +5.7 (5.3–6.1) years]	In Mtb-uninfected adults, 3^+ T cells were detected before immunization. Then MVA85A boosted 3^+ T cells were a predominant subset and persisted for 5–6 years after immunization	Hawkridge et al. (88) and Tameris et al. (70)
MVA85A	*Cohort(s)*: randomized Phase I trial of BCG-immunized adults from the UK; HIV-uninfected, Mtb-uninfected (RD-1 ELISPOTneg) *Immunization*: MVA85A i.m. +0 ($n = 12$) MVA85 i.d. +0 ($n = 12$)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: PBMC *Antigen(s)*: PPD; Ag85A (p.p.) *Immunogenicity*: (+0, +1, +24 weeks)	In BCG-immunized adults, MVA85A, delivered i.m. or i.d. induced 3^+ T cells as a predominant subset equivalently and responses persisted for 24 weeks	Meyer et al. (89)
MVA85A	*Cohort(s)*: BCG-immunized adults from the UK; HIV-uninfected, Mtb-uninfected (TSTneg; $n = 6$) *Immunization*: MVA85A i.d. (+0)	*Cell measured*: $CD4^+$ 3^+ cells, MIP-1β, CD45RA/RO, CD27, CD57 *Cellular source*: PBMC *Antigen(s)*: Ag85A (p.p.) *Immunogenicity*: (+1, 2, 8, 24 weeks)	MVA85A induced 3^+ T cells in BCG-immunized adults, as a predominant subset that persisted at least 24 weeks. Predominant 3^+ T cells subsets co-expressed MIP-1β. 3^+ T cells demonstrated a phenotype consistent with immediate maturity (CD45ROneg/CD27neg/intermediate/CD57neg)	Beveridge et al. (90)
MVA85A	*Cohort(s)*: open-label Phase IIa trial of South African adults; Mtb infection defined by TST and RD-1 ELISPOT, compared HIV-uninfected, Mtb-infected ($n = 12$); HIV-infected, Mtb-uninfected ($n = 12$); HIV-infected, Mtb-infected ($n = 12$); HIV-infected on ARV ($n = 12$) *Immunization*: MVA85A i.d. (+0)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: whole blood *Antigen(s)*: Ag85A (p.p.) *Immunogenicity*: (−7 to 14, +7, +28, +84, +364 days, 3–5 years)	3^+ T cells were present prior to immunization in the Mtb-infected, but not the Mtb-uninfected cohorts. MVA85A induced 3^+ T cells in all cohorts, as a predominant subset, which persisted 3–5 years, except for in the HIV-infected, Mtb-uninfected cohort	Scriba et al. (91) and Tameris et al. (70)

(*Continued*)

TABLE 5 | Continued

Vaccine	Study design	IA	Conclusions	Reference
MVA85A	*Cohort(s)*: open-label Phase I trial of Mtb-infected adults from the UK; HIV-uninfected, Mtb infection defined with TST and RD-1 ELISPOT, no TB disease (n = 12) *Immunization*: MVA85A i.d. (+0)	*Cell measured*: $CD4^+$ 3^+ cells *Cellular source*: PBMC *Antigen(s)*: Ag85A (p.p.) *Immunogenicity*: (+0, +1, +4, +24 weeks)	3^+ T cells were present prior to immunization. MVA85A induced 3^+ T cells as a major subset, which persisted at least 24 weeks	Sander et al. (92)
MVA85A	*Cohort(s)*: open label Phase I/IIa trial of South African, children age 4.3 (1.4–7.7) years (n = 24) and adolescents age (12–14 years, n = 12); HIV-uninfected, Mtb-uninfected; BCG-immunized at birth; no TB disease *Immunization*: MVA85A i.d. (+0)	*Cell measured*: $CD4^+$ 3^+ cells, IL-17, GM-CSF, CD45RA, CCR7 *Cellular source*: whole blood *Antigen(s)*: BCG; Ag85A (p.p.); rAg85A *Immunogenicity*: adolescents: (+7, +28, +168 days, +4.6 [4.4–4.8] years) Children: (+7, +84, +168 days, +3.7 [3.7–3.9] years)	MVA85A induced 3^+ T cells in both children and adolescents, as a predominant subset. 3^+ T cells were greater in adolescents than children, peaked at +28 and +84 days, in adolescents and children, respectively, and persisted 3–5 years in both cohorts. In children, a major subset of 3^+ T cells co-expressed IL-17 and GM-CSF. In adolescents, 3^+ T cells co-expressed IL-17 and displayed an effector memory (CD45RAneg/CCR7neg) phenotype	Scriba et al. (69) and Tameris et al. (70)

SSI, Staten Serum Institute; MTBVAC, a live attenuated strain of Mtb; H4, recombinant Ag85B/TB10.4 fusion protein; IC31, cationic peptide and oligodeoxynucleotide (ODN1); H56, recombinant Ag85B/ESAT-6/Rv2660c fusion protein; H1, recombinant Ag85B/ESAT-6 fusion protein; Mtb72F, recombinant Mtb32A/Mtb39A fusion protein; $AS02_A$, MPL and QS21 in an oil-in-water emulsion; M72, Mtb72F with a point mutation in Mtb32A to improve stability; $AS01_B$ and $AS01_E$, 3-O-desacyl-4′-monophosphoryl lipid A (MPL) and Quillaja saponaria fraction 1 (QS21), combined with liposomes; AERAS-402, Adenoviral vector (Ad35) expressing Ag85A, Ag85B, and TB10.4; AdHu5Ag85A, human adenoviral vector (Ad5) expressing Ag85A; MVA85A, Modified Vaccinia Ankara expressing Ag85A; RD-1 ELISPOT, ELISPOT assay to detect PBMC secreting IFN-γ in response to RD-1 antigens, ESAT-6 and CFP-10 as evidence of Mtb infection; TST, tuberculin skin test; QFT, QuantiFERON assay including QuantiFERON Gold and QuantiFERON Gold in-tube; ARV, anti-retroviral therapy; i.m., intramuscular; i.d., intradermal; CFU, Colony Forming Units; VP, viral particles; 3^+, IFN-γ^+TNF-α^+IL-2^+; PBMC, peripheral blood mononuclear cells; p.p., peptide pools; Mtb CF, Mtb culture filtrate; rAg85A, recombinant Ag85A; BCG, Bacille Calmette Guerin; TB, tuberculosis; Mtb, M. tuberculosis; IA, immune assay.

Major subset = subset that constitutes >50% of the total cytokine-producing cells; predominant subset = subset that constitutes 20–50% of the total cytokine-producing cells.

CORRELATION OF POLYFUNCTIONAL T CELL RESPONSES AND RISK OF TB IN HUMANS

Understandably little is known about the correlation of polyfunctional $CD4^+$ T cells and risk of TB, although two infant vaccine trials have evaluated the relationship of vaccine-induced polyfunctional $CD4^+$ T cells and subsequent development of TB. Kagina et al. performed a subanalysis of a large trial evaluating the immunogenicity of BCG (Japanese) delivered i.d. vs s.c. in South African infants (62). Infants who developed TB over the 2-year follow-up period were compared to healthy, Mtb exposed, and healthy Mtb unexposed infants. There was no correlation between the magnitude of polyfunctional $CD4^+$ T cells and subsequent development of TB during 2 years of follow-up. More recently, Tameris et al. conducted a double-blind Phase IIb trial in South African infants evaluating the immunogenicity and efficacy of MVA85A as a boost after BCG prime at birth, compared with a placebo (3). Although MVA85A-vaccinated infants developed polyfunctional Ag85A-specific $CD4^+$ T cell responses, albeit at lower magnitudes than typically observed in adults, the MVA85A boost provided no additional efficacy against development of TB over BCG alone. By contrast, the majority of mouse TB studies evaluating polyfunctional $CD4^+$ T cells and vaccine-induced protection have noted a correlative relationship. This discrepancy may reflect differences between the mouse TB vaccine model and vaccine-induced protection from human disease, publication bias, or, in the case of the MVA85A study, possibly induction of inadequate levels of polyfunctional $CD4^+$ T cells required for protection. Regarding the latter, Fletcher et al. found that immune activation at the time of MVA85A or placebo vaccination was associated with risk of developing TB, which in turn may be associated with poor BCG vaccine take, i.e., lower frequencies of BCG-specific IFN-γ-expressing cells (93). These data suggest that additional factors over and above the functionality of T cells play a role in protective immunity.

Discrepancies between some mouse TB vaccine studies and others, and between mouse and human TB vaccine studies, could reflect that a correlate of protection may be specific for each particular vaccine platform or may vary for different TB antigens. In this regard, standardization of vaccine study protocols and harmonization between animal and human studies could improve the ability to discern correlates of protection. In addition, discrepant results among animal model and human studies of polyfunctional $CD4^+$ T cells, in general, could reflect that frequencies of polyfunctional $CD4^+$ T cells correlate with a more accurate, yet-to-be determined correlate of protection. In this regard, mouse studies suggest that total IL-2-producing $CD4^+$ T cells may be the best correlate of protection against Mtb infection (38, 41) and in humans, IL-2-producing $CD4^+$ T cells are associated with successful containment of Mtb infection in persons with LTBI (13, 14). Conversely, loss of IL-2 expression

by IFN-γ and/or TNF-expressing Th1 cells is likely indicative of greater T cell differentiation, typically observed in scenarios of high bacterial loads, such as TB disease (13, 14), or following delivery of high doses of vaccine antigens (76). This is consistent with the linear differentiation model of CD4+ T cells derived primarily from acute and chronic viral infections (22). In this model, IL-2-production reflects central memory T cells in early stages of differentiation, with capacity for long-lived memory and proliferation. Loss of IL-2 expression thus reflects more advanced differentiation of CD4+ T cells, such as effector memory or terminal effector cells. This is further supported by a recent study of CD4+ T cell responses to Ag85B and ESAT-6 in mice and humans, which showed that Ag85B-specific T cells were significantly less differentiated than ESAT-6-specific T cells, which correlated with lack of persistent Ag85B antigen expression and persistent ESAT-6 expression by the bacterium during chronic infection in the mouse model (94). Distinct attributes observed between these T cell subsets were higher proportions of polyfunctional and lower proportions of IL-2-expressing ESAT-6-specific T cells, when compared with Ag85B-specific T cells.

CONCLUSION AND PERSPECTIVES

BCG, as well as novel TB vaccine candidates, induce polyfunctional CD4+ T cells in both animal models and humans. These cells possess important functional attributes that may potentially play a role in vaccine-mediated protection including long-lived memory function, persistence in the vaccinated host, and at least in animal models, ability to traffic to and persist in the lung. In the mouse model, the magnitude of vaccine-induced polyfunctional CD4+ T cells often correlates with vaccine-induced protection, making polyfunctional T cells a good candidate for a mechanistic correlate of protection. However, definitive evidence that it is in fact the co-expression of IFN-γ, TNF-α, and IL-2 by these T cells, rather than another functional or phenotypic attribute, that mediates host defense against Mtb remains lacking and would require sophisticated knock-down or adoptive transfer experiments. Some mouse studies, and of note, two human infant studies do not support polyfunctional CD4+ T cells as correlate of protection. Moreover, because these studies focus mainly and often exclusively on defining polyfunctional CD4+ T cells, it is certainly possible that a stronger immunologic correlate was present and not measured. We conclude that induction of polyfunctional T cells is certainly not sufficient and may not even be necessary to mediate protective immunity against Mtb and speculate that the production of multiple pro-inflammatory cytokines by T cells may reflect properties of T cells that may not necessarily be required for protection. Other functional attributes, such as additional effector functions, the differentiation state, tissue homing potential, long-term survival capacity of the T cell, or their ability to recognize the Mtb-infected cell may be equally or more important to promote protection. It is also possible that the induction of polyfunctional CD4+ T cells may be dependent upon the particular antigen or adjuvant utilized. Thus, a correlate of protection for TB vaccine development remains elusive. We propose that studies of protective immunity against Mtb should reach well beyond the measurement of IFNγ, TNF, and IL-2 by investigating other functions, phenotypes and correlates of immunity. Further, in light of the lack of direct causal association between T cell polyfunctionality and protective immunity, care should be taken not to bias studies in favor of the hypothesis that polyfunctional cells are indeed the mediators of protection. The definition of correlates of protection may benefit from standardization of animal TB vaccine studies and harmonization of these protocols with human trials. Finally, future studies should address the full spectrum of CD4+ T cell flavors and colors within the context of more complete immunological signatures of protection, including for example, additional phenotypic and functional attributes of CD4+ T cells, such as IL-17 production, and other major cells, such as classically restricted CD8+ T cells, donor unrestricted T cells, and NK cells, as well as B cells.

AUTHOR CONTRIBUTIONS

DAL performed the literature search and designed the tables. DAL and TS outlined and wrote the manuscript. DAL, DML, and TS discussed and edited the manuscript.

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REVIEW
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Review: Impact of Helminth Infection on Antimycobacterial Immunity—A Focus on the Macrophage

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Successful immune control of *Mycobacterium tuberculosis* (MTB) requires robust CD4+ T cell responses, with IFNγs as the key cytokine promoting killing of intracellular mycobacteria by macrophages. By contrast, helminth infections typically direct the immune system toward a type 2 response, characterized by high levels of the cytokines IL-4 and IL-10, which can antagonize IFNγ production and its biological effects. In many countries with high burden of tuberculosis, helminth infections are endemic and have been associated with increased risk to develop tuberculosis or to inhibit vaccination-induced immunity. Mechanistically, regulation of the antimycobacterial immune response by helminths has been mostly been attributed to the T cell compartment. Here, we review the current status of the literature on the impact of helminths on vaccine-induced and natural immunity to MTB with a focus on the alterations enforced on the capacity of macrophages to function as sensors of mycobacteria and effector cells to control their replication.

Keywords: helminth infection, tuberculosis, vaccination, type 2 immune response, macrophage, IL-4, IL-10

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IMMUNOLOGICAL REQUIREMENTS FOR EFFECTIVE CONTROL OF *Mycobacterium tuberculosis* (MTB) INFECTION

Innate Recognition of MTB

The innate immune system detects incoming mycobacteria during phagocytosis by alveolar macrophages in the lung. The hydrophobic mycobacterial cell wall contains a large number of lipids, glycolipids, and lipoglycans that act as pathogen-associated molecular patterns (PAMPs), which are recognized by several classes of pattern recognition receptors (PRRs) [for review, see Ref. (1)]. Due to the intracellular lifestyle of MTB, which persists and replicates in the phagosome, endosomal PRR have ample opportunity to interact with mycobacterial ligands released into this compartment, e.g., DNA and RNA. With increasing time spent in its host cell, mycobacterial products and even the bugs themselves can enter the cytosol (2), where yet other PRRs sense the presence of intruding microbes.

This initial interaction between macrophages and MTB is crucial: if the macrophage is able to kill MTB at this stage, no infection occurs and there is no need to call in adaptive immunity (**Figure 1** "innate resistance"). Based on studies on transmission of MTB to household contacts measuring tuberculin skin test or quantiferon responses, this may be the situation in more than 50% of all exposures (3–5). However, since it is difficult to determine the true exposure of household contacts of patients with open tuberculosis to infectious aerosol, the percentage of innate resistance to MTB in humans could also be considerably lower (6). On the other hand, the finding that tuberculin skin test negativity in humans is linked to a chromosomal region overlapping the TNF1 locus provides evidence for genetic control of innate resistance to MTB infection (7). Clearly, the

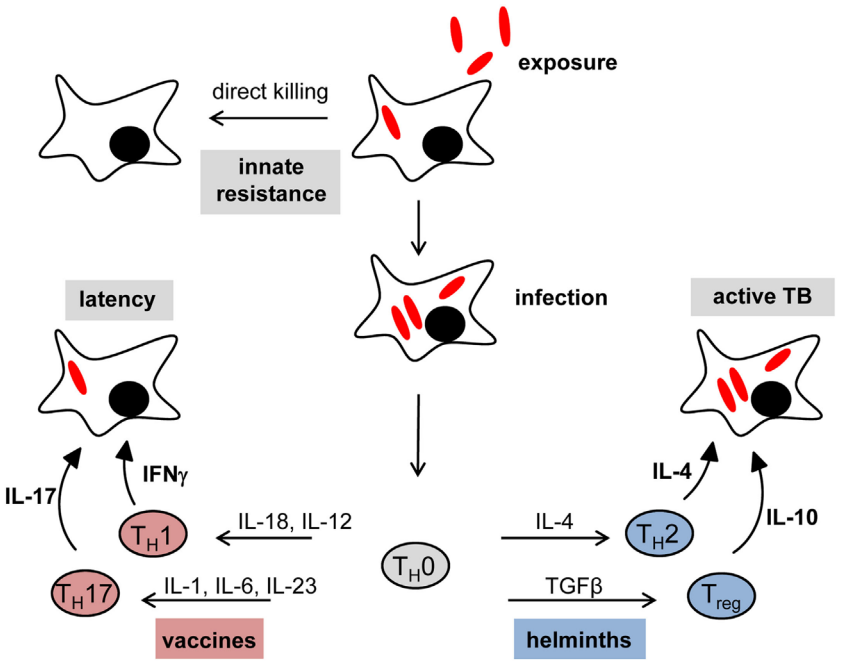

FIGURE 1 | Immune checkpoints in tuberculosis: impact of vaccination and helminth infection. In many cases, the initial exposure to mycobacteria results in direct killing by alveolar macrophages without the need for an adaptive immune response (innate resistance). Failure of initial innate control mechanisms leads to primary infection. The set of chemokines and cytokines produced by innate immune cells are crucial for the shaping of an effective adaptive immune response. The Th1 key cytokine IFNγ is necessary to establish and maintain latent infection. Moreover, IL-17 was found to be important for vaccination-induced protection against tuberculosis. However, concomitant helminth infection shifts the immune system toward a T helper type 2 (Th2)/regulatory T cells (Treg) response rather than a protective Th1/Th17 immune status, which leads to a higher risk to develop active disease and interferes with successful vaccination responses.

factors determining the initial fate of mycobacteria after ingestion by alveolar macrophages are very incompletely understood, and may range from cytokines such as TNF to antimicrobial peptides, the autophagy machinery and control of phagosomal maturation (8). Since all these macrophage functional processes are under the influence of signaling emanating from PRR, it makes sense to assume that the recognition of MTB by different PRR contributes to the initial decision if ingested bacilli survive or are killed. If the mycobacteria manage to establish an intracellular niche in the macrophage, the nature of the innate response (mostly the composition of chemokines and cytokines secreted) depends on PRR pathways and determines the type of adaptive immunity and the swiftness of a protective response characterized by robust Th1 and Th17 T cells.

Toll-like receptors (TLR) have been most intensively studied for their role in the response to mycobacteria. TLR2 and TLR4 bind to mycobacterial cell wall components lipoarabinomannan (LAM) and phosphatidylinositol mannosides (PIM), and lipomannan, respectively (9–12). The 19-kDa lipopeptide of MTB is also a TLR2 ligand (12). The endosomal TLR7 and TLR8 (the later only in humans, but not in mice) sense single-stranded RNA (13), while CpG-rich DNA was initially purified as the immunostimulatory principle of Bacille Calmette–Guerin (BCG) treatment and later explained by activation of TLR9 (14). Independent of their localization on the cell surface or in the phagosome, TLR2, TLR7/8, and TLR9 require the adapter protein Myd88 to activate gene expression. Myd88-dependent signaling is essential for host defense against experimental MTB infection in mice; however, as even the triple knockout of TLR2, TLR4, and TLR9 in mice does not increase mycobacterial load (15), the phenotype of Myd88$^{-/-}$ mice is likely due to a lack of IL-1 receptor signaling rather than TLR activation (16–18).

More recently, several C-type lectins receptor (CLR) have been identified as receptors for PAMPs present in the mycobacterial cell wall [reviewed in Ref. (19)]. Dectin-1 is triggered by an unknown mycobacterial ligand and induces IL-12 production from DC (20) and collaborates with TLR2 in induction of cytokine gene expression (21). The genes for the CLR Mincle (Clec4e), Mcl (Clec4d), and Dectin-2 (Clec4n) are located in close vicinity in the genome and are referred to as the Dectin-2 family (22). Mincle is the receptor for the so-called mycobacterial cord factor trehalose-6,6′-dimycolate (TDM), the most abundant glycolipid in the cell wall of MTB (23, 24). Mincle can form dimers with the related CLR Mcl (25), and both receptors mutually enhance their cell surface protein levels (26). Dectin-2 binds mannose-rich fungal ligands (27, 28), but also the TLR2 ligand mannose-capped LAM (29). Most recently, DCAR (Clec4b1), another FcRγ-coupled CLR, was revealed to bind to PIM of the cell wall, to induce MCP-1 expression by macrophages, and to induce Th1 responses (30). All of these CLR signal *via* the Syk-Card9-Bcl10-Malt1 pathway to activate NFκB and upregulate expression of multiple chemokines, cytokines and inflammatory mediators causing inflammation and directing developing adaptive immune responses (31, 32). Knockout mice for Card9 are

highly susceptible to challenge with MTB, with increased bacterial burden, uncontrolled granulocytic inflammation, and early death (33). The phenotype of mice deficient in individual CLR in MTB infection is much more moderate, as demonstrated for Mincle (34–36), Mcl (37), or shows no difference as in the case of Dectin-1 (38). Thus, similar to the case of TLR, there appears to be considerable redundancy of individual CLR for recognition of mycobacteria, but the combined ablation of the CLR response in Card9-deficient mice is detrimental and suggests an important function of this class of receptors in antimycobacterial defense.

In addition to TLR and CLR localized to the plasma membrane or the phagosome, NOD2 is a cytosolic sensor of mycobacterial muramyl dipeptide, which induces autophagy and activates NFκB-dependent gene expression (39). The ESX-1 secretion system of MTB generates phagosomal perforation and leakage of mycobacterial DNA and RNA. Binding of cytosolic DNA by the sensor cGAS leads to production of cyclic dinucleotides binding and activating STING, which triggers IFNβ expression in an IRF3-dependent manner (40, 41). The activation of the AIM2-NLRP3 inflammasome by cytosolic DNA is an additional pathway triggered by the intracellular pathogen MTB, and causes production of IL-1β (42, 43).

IFNγ As Key Cytokine—Necessary but Not Sufficient

The single most important immunological molecule in defense against mycobacterial infection is the cytokine IFNγ (**Figure 1** "latency"). Produced mostly by Th1 $CD4^+$ T cells but also $CD8^+$ T cells, NK cells and NKT cells, it acts on many different cell types by activating the Jak2-Stat1 signaling pathway that drives the expression of antimicrobial genes such as Nos2 (producing NO), IFNγ-induced GTPase-binding proteins of the 65 and 47 kDa families, bactericidal peptides (e.g., cathelicidin), and many more (44). The inability to upregulate IFNγ expression (e.g., deficiency in the cytokines IL-12 or IL-18, or their receptors), produce it, or respond to it (e.g., deficiency in the IFNγ receptor chains, Jak2 or Stat1), leads to high susceptibility to experimental mycobacterial infection in mouse models and in humans (45) (**Figure 1** "active TB").

While IFNγ clearly is necessary for a successful host response to infection, several studies have shown that it is not sufficient, as increased levels of IFNγ were indicative of disease progression rather than protection in mice and humans (46, 47). Beyond a simple lack of correlation between IFNγ levels and protection in MTB infection, there is even evidence that IFNγ production has to be tightly controlled to prevent damage to the host, particularly the lung tissue, during infection (48).

Vaccination Responses—What Should We Look For?

Despite the nearly 100 years of vaccination with the *Mycobacterium bovis* strain BCG it is still very incompletely understood which vaccine-induced immune responses can be used as biomarkers for protective immunity (49). This applies to IFNγ, which is essentially required for successful immunity, but it is becoming increasingly clear that there is no linear relationship between vaccine-induced IFNγ and immune protection (48). More recently, IL-17-producing Th17 cells have been described as key players in vaccine-induced protection (50). Interestingly, the recombinant BCG vaccine VPM02 (ΔureC; hly^+), which is more effective than the parental BCG in protecting against pulmonary tuberculosis in animal models, induces a balanced Th1/Th17 response and caused earlier recruitment of $CD4^+$ T cells into the lungs after challenge infection (51). In the last years, there has been an increasing appreciation of the important role played by the route of immunization: Aguilo et al. demonstrated that BCG delivered intranasally protects DBA/2 mice against pulmonary tuberculosis much better than after subcutaneous immunization (52). An alternative strategy to improved BCG or novel recombinant MTB live vaccines is the development of subunit vaccines using recombinant MTB proteins. To be effective in generating robust antigen-specific $CD4^+$ T cell responses, these protein vaccines require a carrier (e.g., liposomes) and adjuvants that activate antigen-presenting cells of the innate immune system for provision of costimulation and cytokines directing the desired Th cell differentiation signals. Here, the TLR9 ligands CpG ODN and IC31 are potent inducers of Th1 cells by triggering IL-12 release from DC and macrophages, and have therefore been used experimentally and in clinical studies (53–55). In contrast, ligands for certain CLR are potent inducers of Th17 differentiation: the Dectin-1 ligand Curdlan, a β-glucan, and the Mincle ligand TDB, an analog of the mycobacterial cord factor TDM, trigger production of IL-6, IL-23, and IL-1 from DC and macrophages, thereby providing robust differentiation signals toward a Th17 bias (24, 56, 57). The TDB-containing liposomal adjuvant CAF01 has been used in several experimental infection models, induces long-lived protective CD4 memory T cells in mice, and also appears to be effective in humans (58–61).

IMPACT OF HELMINTH INFECTION ON IMMUNITY TO MYCOBACTERIA

The Epidemiological Evidence

Globally, more than two billion people are infected with parasitic helminths. Intestinal nematodes (*Ascaris lumbricoides*, hookworms, *Trichuris trichiura*, *Strongyloides stercoralis*), filaria (*Wuchereria bancrofti*, *Onchocerca volvulus*, *Loa loa*), and trematodes (*Schistosoma mansoni*) are the most frequent and important human worm infections (62). Similar to tuberculosis, helminth infections are often chronic and not acutely life-threatening. Helminths have complex life cycles and various developmental stages, and elicit distinct host immune reactions. Despite significant differences between specific helminth infections, the immune response to helminths is generally characterized by a T helper type 2 (Th2) pattern with high levels of the cytokines IL-4, IL-5, IL-9, IL-10, and IL-13, as well as eosinophilia, goblet and mast cell hyperplasia, and IgE-biased antibody isotype switching (62). Thus, helminth infection and tuberculosis share the chronic nature, but represent two extremes of immunological bias and immune effector mechanisms. Since tuberculosis and helminth infections are coinciding in many parts of the world, most notably in Africa, South America, and Asia, the possibility that concomitant helminth infection affects antimycobacterial immunity has been investigated by epidemiological studies.

Helminth Infection and Vaccination against Tuberculosis

The efficacy of the BCG vaccine shows large geographic variation, with reduced protection in African and Asian countries, where worm infections are more prevalent than in Europe and North America (63, 64). Deworming of helminth-infected vaccinees before administration of BCG led to increased IFNγ and IL-12 production, yet decreased TGFβ levels, suggesting that intestinal helminths impair the development of a Th1 response to BCG (65). In the mouse model, *S. mansoni* infection decreased protective efficacy of BCG against MTB challenge and the capacity to produce IFNγ by splenocytes (66). As BCG vaccination is usually done in neonates, effects of maternal helminth infection on vaccination responses in the offspring have been investigated in mice and humans. Maternal infection with helminths had a strong negative effect on induction of Th1 immunity in BCG-vaccinated offspring (67). In another study, hookworm-infected mothers had reduced IFNγ responses to MTB proteins, but their children showed rather increased IFNγ production (68). In addition, anthelmintic treatment during pregnancy did not alter the neonatal responses to vaccination including BCG (69). Thus, the literature is at present contradictory whether helminth coinfection is indeed a strong factor in the suboptimal response to BCG and whether anthelmintic treatment can overcome this hurdle.

Impact of Helminth Infection on the Development and Course of Active Tuberculosis

Patients with active tuberculosis were found to be more often coinfected with helminths compared to controls (70, 71). In addition, coinfection with helminths was associated with more advanced disease in tuberculosis patients, coupled to reduced IFNγ but increased production of IL-10 (72). On the other hand, a recent study showed that asymptomatic helminth infection is indeed associated with type 2 immunity in tuberculosis patients, but led to a lower frequency of smear-positive sputum samples, i.e., reduced the risk to develop open cavitary disease (73). Reflecting this ambiguous state of the literature on the impact of helminth infection on the course of active tuberculosis, a treatment study with albendazole showed no change in the clinical course after 2 months of treatment (74).

MECHANISMS OF ANTIMYCOBACTERIAL IMMUNE REGULATION BY HELMINTHS AND HELMINTH-INDUCED TYPE 2 IMMUNITY

Direct Regulation of Macrophage Function by Helminths

Macrophages encounter worm eggs and larval stages in tissues and therefore directly interact with helminths and their products. A number of studies have investigated how different helminth species and their products modulate macrophage activation state and responsiveness to other microbial stimuli. While these studies employed different helminths or helminth-derived products and a variety of readout parameters of macrophage activation, they in general described inhibitory or modulating effects on macrophages. ES-62, a secreted protein of the filarial nematode *Acanthocheilonema viteae* inhibits the production of IL-12, TNF, and IL-6 in response to LPS/IFNγ (75). Secreted filarial cystatins were found to be taken up by macrophages and to induce expression of IL-10, which was sensitive to inhibition of MAPK and was regulated by the phosphatase DUSP1 (76). Microfilariae of *Brugia malayi* induced a regulatory phenotype in human monocytes characterized by expression of IL-10 and PD-L1 (77). A *Fasciola hepatica* fatty acid binding protein exerted suppression of cytokine release and MAPK activation in response to the TLR4 ligand, and was found to bind to the co-receptor for LPS, CD14 (78). Similarly, the excretory/secretory products of the tapeworms *Hymenolepis diminuta* and *Spirometra erinaceieuropaei* inhibited the production of inflammatory cytokines such as TNF by macrophages (79, 80). Recently, Aira et al. observed that antigens prepared from *H. diminuta* and *Trichuris muris* inhibit phagolysosomal maturation and antigen presentation to $CD4^+$ T cells in macrophages infected with MTB, with a direct negative effect on the control of mycobacterial survival in macrophages (81). It should be noted that inhibition of macrophage activation is not uniformly observed upon contact with helminths, e.g., antigens from *S. mansoni* showed the opposite effect, enhancing mycobacterial control and decreasing IL-10 production (81). While inhibiting production of TNF and IL-6 by TLR-triggered dendritic cells, schistosomal egg antigens (SEA) activated the Nlrp3 inflammasome and IL-1β production by activating the Dectin-2-FcRγ-Syk pathway (82).

Helminth-Induced Th2/Regulatory T Cells (Treg) Bias of the T Cell Response

A reduced Th1 and Th17 immunity to mycobacterial antigens in patients with latent or active tuberculosis by coinfection with helminths was described in several studies (72, 83–85). By contrast, the frequency of Treg was consistently increased in coinfected patients (73, 86), together with the production of IL-10 (73), which also contributed to inhibition of Th1 differentiation (84). *In vitro* exposure of human PBMC to SEA increased the expression of IL-10 and IL-4 by $CD4^+$ T cells, which in turn caused a block in phagolysosomal maturation in mycobacteria-infected macrophages (87). The Treg/Th2-biased immune deviation in coinfected patients was corrected by anthelmintic treatment, which restored Th1 cells and diminished Treg numbers (86), decreased the levels of IL-10 (74), or reduced Th2 but increased Th1/Th17 cytokines (88). These data indicate that the bias of antimycobacterial immunity toward Th2/Treg in helminth coinfection is reversible upon treatment.

Type 2 Regulation of Myeloid Cells

Alternative Macrophage Activation by IL-4 Increases Bacterial Burden in MTB Challenge

Experimental infections with filaria, schistosomes (89, 90), and the hookworm *Nippostrongylus brasiliensis* (91) caused

a Th2-biased response to mycobacterial infection and, in part, higher bacterial burden. Potian et al. demonstrated that *Nippostrongylus* infection caused transiently increased mycobacterial burden through the activity of IL-4R-positive alternatively activated macrophages in the lung (91). While this study clearly demonstrated that the transfer of wild-type macrophages into IL-4R-deficient mice was sufficient to increase mycobacterial growth, the mechanistic basis has not been clarified in detail yet. One possible mechanism is upregulation of Arginase-1 expression, which is strongly induced by Stat6 signaling in response to IL-4/IL-13 signals in M2 macrophages and competes with iNOS for Arginine as a substrate (**Figure 2B**). Conditional Arginase-1 knockout mice lacking the enzyme in hematopoietic cells more efficiently clear MTB after pulmonary infection, correlating with enhanced NO production in the lung (92). However, in a coinfection model with MTB and *S. mansoni*, deletion of Arginase-1 did not alter the mycobacterial burden in coinfected mice, although it reversed lung pathology (93). Of note, infection with *N. brasiliensis* can also promote pulmonary Th1 cell responses and activation of alveolar macrophages, which was associated with enhanced control of early infection in a *M. bovis* BCG infection model (94). While these data appear to contradict those of Potian et al. (91), both studies agree in observing a reduced mycobacterial burden early in coinfection of *N. brasiliensis* and mycobacteria. Hence, timing of coinfection appears to be decisive for the impact of helminths on antimycobacterial immune responses.

Downregulation of TLR Expression in Helminth-Infected TB Patients

Babu et al. hypothesized that helminth infection may modulate immune responses by diminishing TLR expression and indeed identified reduced levels of TLR2 and TLR9 in PBMC of patients with filarial infection (95, 96). Cytokine responses to TLR2 and TLR9 ligands were decreased, suggesting that the impaired receptor expression was functionally relevant. Interestingly, anthelmintic treatment restored responsiveness to TLR ligands, indicating the reversibility of macrophage inhibition by filarial infection (95).

Downregulation of CLR Expression by IL-4 and IL-13

CLR are often expressed in cell type-specific and stimulus-dependent manners. Indeed, several CLR are used as markers for certain DC or macrophage cell types, e.g., Clec9a as marker for $CD8^+$ DC. The cord factor receptor Mincle is strongly induced by stimulation of macrophages with the TLR ligands LPS or CpG but also by its ligand TDM itself (97, 98). Interestingly, mRNA expression of Mincle is high in monocytes and neutrophils in both mouse and human. However, during differentiation of human monocytes to DC *in vitro* in culture with GM-CSF

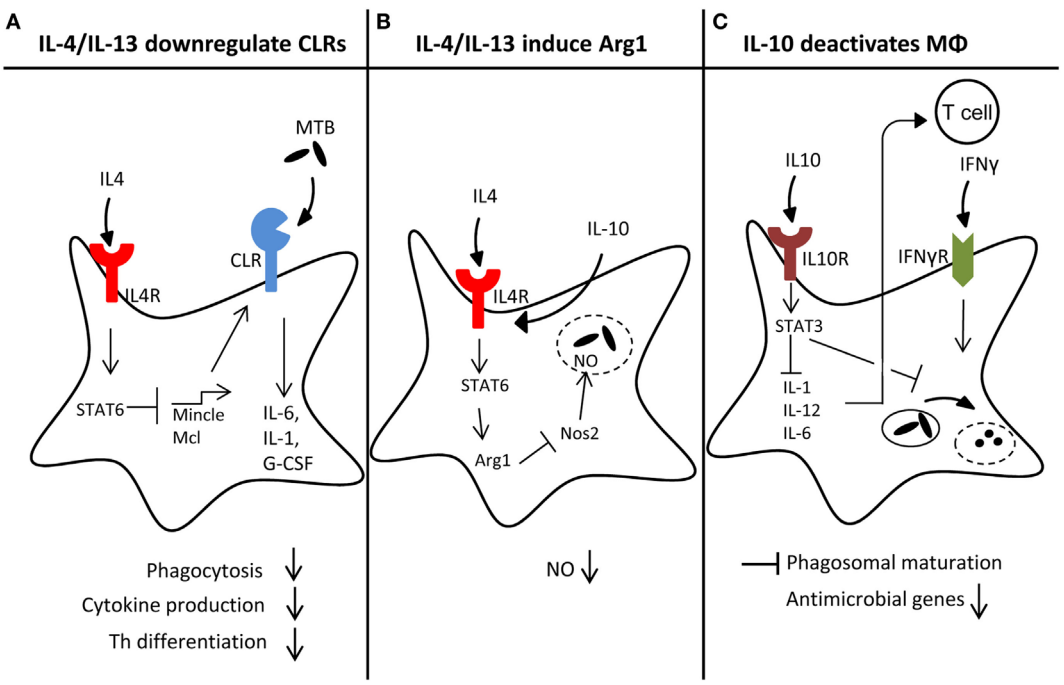

FIGURE 2 | Macrophage reprogramming by helminth-induced type 2 responses. Helminth-induced type 2 responses can act on macrophages on different levels, thereby impeding antimicrobial responses toward *Mycobacterium tuberculosis* (MTB): **(A)** high levels of IL-4/IL-13 downregulate C-type lectin receptor (CLR) expression in a Stat6-dependent manner, which may lead to reduced amounts of pro-inflammatory cytokines like IL-6, IL-1, or G-CSF, impaired phagocytosis and Th1/Th17 differentiation. **(B)** Simultaneously, IL-4/IL-13 signaling through Stat6 strongly induces Arginase1 (Arg1), which counteracts Nos2 through substrate depletion, leading to reduced NO production and less effective killing of MTB inside the phagolysosome. **(C)** The anti-inflammatory cytokine IL-10, produced during helminth infection, can delay phagosomal maturation. Moreover, IL10R signaling through STAT3 inhibits IL-1, IL-12, or IL-6 expression, which in turn inhibits Th1 differentiation.

plus IL-4, pronounced downregulation of Mincle mRNA was observed (99) and could be attributed to the action of IL-4 (100). This effect was also observed for the other Dectin-2 family CLR Mcl and Dectin-2, and was confirmed in mouse macrophages and DC at the mRNA and protein level by flow cytometry (100). By contrast, Dectin-1 expression was not affected negatively by IL-4. Both IL-4 and IL-13 impair expression of Mincle, and do so in a Stat6-dependent manner. Interestingly, the downregulation of Mincle and Mcl was associated with a reduced production of TNF and G-CSF when macrophages were stimulated with the Mincle/Mcl ligand TDM, but not when LPS was used (100). The functional consequence of this targeting of Dectin-2 family CLR expression by IL-4/IL-13 has not been addressed to date, but since Mincle/Mcl bind TDM and Dectin-2 has been identified as a PRR for mannose-capped LAM (29), the lack of receptors for recognition of two major mycobacterial PAMPs can be expected to diminish the response of macrophages to encounter with MTB (**Figure 2A**).

Deactivation of Antimicrobial Capacity by IL-10 in Macrophages

IL-10 by several cell types is upregulated during helminth infection (see also above in Section "Direct Regulation of Macrophage Function by Helminths"). Through its powerful macrophage deactivation properties, it inhibits the production of many cytokines important for development of Th1/Th17 responses, such as IL-12, IL-6, IL-1, and IL-23 (101) (**Figure 2C**). In addition, IL-10 more directly impairs killing of mycobacteria in macrophages by delaying phagosome maturation (102) and inhibiting the expression of IFNγ-induced antimycobacterial effector molecules, such as iNOS and ROS (103). Overexpression of IL-10 in mice makes them more susceptible to mycobacterial infection (104, 105), whereas its deletion or blockade of its receptor lead to enhanced immune control of MTB (106).

OPEN QUESTIONS REGARDING MECHANISMS AND THE IMPACT FOR VACCINATION STRATEGIES

Which PRRs Are Regulated by Type 2 Immune Responses in Humans and in Animal Models?

To date, the information about changes in the expression of TLR and CLR during helminth infection is patchy at best, with the observations on TLR2 and TLR9 made in human patients with filarial disease and the effect of IL-4/IL-13 on Dectin-2 family CLR expression in human and mouse macrophages and DC treated *in vitro*. A comprehensive expression analysis of different cell types (monocytes/macrophages, granulocytes, DC) from humans infected with helminths or not, or before and after anthelmintic treatment, should be performed. For comparison, the same type of expression analysis should be done using myeloid cells isolated from mice infected with helminths experimentally. This expression analysis should also be extended to other PRR families, including cytosolic sensors such as NOD proteins, cGAS, and STING.

Does Impaired Expression of PRR Directly Affect Innate Resistance to MTB Infection?

Reduced expression of receptors for mycobacteria on myeloid cells may impair the antimycobacterial response at several levels. First, cell surface-localized CLR and TLR can be involved in phagocytosis and their absence on the membrane can lead to suboptimal uptake of mycobacteria. While Mincle and Dectin-2 appear to be dispensable for phagocytosis of mycobacteria, Mcl-deficient mice have a delayed clearance of MTB from the airways after experimental infection (37). A lack of TLR-induced signaling during phagocytosis can alter the phagosome maturation process and the capacity for antigen presentation (107, 108), which is also likely in case of CLR deficiency (109, 110). On the other hand, mycobacterial cord factor delays phagosomal acidification in a Mincle-dependent process (111). With regard to adaptive immune responses, the production of Th1- and Th17-inducing cytokines in response to contact with mycobacteria requires TLR-Myd88 and CLR-Syk-Card9 signaling, respectively (23, 24); thus, downregulated expression may alter the quality of the Th cell response toward Th2/Treg and thereby allow mycobacterial growth (**Figure 2A**). Finally, the capacity of macrophages to kill mycobacteria directly and without further activation by T cells is likely dependent on the strength of activation by the different PRR pathways triggered by MTB. Thus, innate resistance to MTB can be hypothesized to be compromised when the macrophages encountering MTB, such as alveolar macrophages, are exposed to type 2 environment and downregulate receptor expression. On the other hand, to demonstrate that diminished cytokine production and killing of mycobacteria is indeed due to abrogated expression of CLR or TLR, and not to other effects of IL-4-Stat6 signaling, will require to reconstitute expression of these PRR, e.g., by retroviral transduction.

Does Helminth Infection Hinder Vaccine-Induced Immunity *via* Impaired PRR Expression?

Negative effects of concurrent helminth infection on the immune response to vaccination with BCG have been described repeatedly in mice and humans (65, 66). However, it is unclear by which mechanism helminth-induced type 2 immunity interferes with Th1 response induction by BCG (66). Several studies have demonstrated that helminths can inhibit DC activation and costimulatory molecule expression (112, 113). Helminth infection promotes increased induction of Treg (114, 115) and/or IL-10 production (116), leading to suppression of Th1/Th17 immune responses. Downregulation of TLR and CLR by helminths through a type 2 response may be an alternative mechanism: lack of activating signaling reduces production of cytokines driving Th1/Th17 polarization of immune response. New vaccination strategies using recombinant MTB antigens together with different adjuvants as booster vaccines after BCG priming are currently developed. Among the adjuvants employed in these experimental and preclinical vaccines are the TLR ligands IC31 (TLR9) and the CLR ligands TDB (Mincle/Mcl). Since expression of these receptors is downregulated in patients with helminth coinfection

(95, 96) or *in vitro* by type 2 cytokines (100), the adjuvanticity of these vaccines would appear to be particularly vulnerable due to diminished innate activation in helminth-infected patients. To determine whether helminths impair the success of adjuvanted subunit vaccines *via* downregulation of selected PRRs, studies in mouse models should be conducted comparing the efficacy of different adjuvants acting through different innate receptors subject to regulation by helminths or not (e.g., Dectin-1/Curdlan). These studies may also identify optimal adjuvants for use in populations with high rates of helminth infection. Priming of macrophages with the TLR4 ligand LPS can overcome the inhibitory effect of IL-4 on Dectin-2 family CLR expression (100). Thus, the combination of several adjuvants should also be tested because it may circumvent the impaired receptor expression imposed by helminth-induced type 2 responses.

Can Anthelmintic Treatment Increase Vaccination Efficiency?

The connection between helminth infection and the immune response to tuberculosis and the BCG vaccine has triggered attempts to use anthelmintic treatment ("deworming") to enhance protective natural or vaccine-induced antimycobacterial immunity. Elias et al. observed that Albendazole increased the Th1 (IFNγ/IL-12) response in adult helminth-infected BCG vaccinees (65, 117). Since most BCG doses are given to neonates, the finding that maternal helminth infection impairs the vaccine-induced IFNγ response after birth was highly relevant (67, 118) and suggested that treatment of mothers before birth should enhance the immune response to BCG in their offspring; however, this was not confirmed in a larger study performed in Uganda (69). Together, the literature is not conclusive yet and larger studies in different settings will be required. Studies into the effect of anthelmintic treatment will also be required for the newly developed live and subunit TB vaccines, depending on how strongly they are affected by coinfection with helminths.

Can Specific Components of Type 2 Immune Responses Be Targeted to Boost Antimycobacterial Host Responses?

A better understanding of the regulation of protective immunity to TB by helminths at the molecular and cellular level may also contribute to more specific modulation by targeting specific components of the type 2 response. Identification of key factors that suppress Th1/Th17 immunity may allow to specifically antagonize them during vaccination or tuberculostatic treatment without abrogating more beneficial effects of worm-induced immune regulation. It will therefore be important to dissect the contribution of different cell types (e.g., Th2, ILC2, different myeloid cells), cytokines (IL-4/IL-13, IL-10, TGFβ, but also IL-33), and mediators (e.g., Nos2, Arg1), to impaired antimycobacterial resistance and immunity.

CONCLUDING REMARKS

It is now evident that helminth infestations can influence the host response to MTB at multiple levels, from the initial encounter between macrophage and bacilli, to the type of adaptive T cell immunity, and to the development of immunopathology. At the same time, many open questions remain to be answered, both at the clinical–epidemiological level (e.g., regarding the benefit of anthelmintic treatment) and at the fundamental level of immune regulation during coinfection. Clearly, to obtain a mechanistic understanding of observations made in patient cohorts and hypotheses derived from them need to be tested in experimental animal and *in vitro* models. *Vice versa*, it will be exciting to determine whether regulatory effects of type 2 cytokines on macrophages and other cell types identified in laboratory studies hold true also in cohorts of coinfected patients or in vaccination studies in humans.

AUTHOR CONTRIBUTIONS

RL and JS conceived and wrote the manuscript and prepared the figures.

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Strategies to Improve Vaccine Efficacy against Tuberculosis by Targeting Innate Immunity

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The global tuberculosis epidemic is the most common cause of death after infectious disease worldwide. Increasing numbers of infections with multi- and extensively drug-resistant variants of the *Mycobacterium tuberculosis* complex, resistant even to newly discovered and last resort antibiotics, highlight the urgent need for an efficient vaccine. The protective efficacy to pulmonary tuberculosis in adults of the only currently available vaccine, *M. bovis* BCG, is unsatisfactory and geographically diverse. More importantly, recent clinical studies on new vaccine candidates did not prove to be better than BCG, yet. Here, we propose and discuss novel strategies to improve efficacy of existing anti-tuberculosis vaccines. Modulation of innate immune responses upon vaccination already provided promising results in animal models of tuberculosis. For instance, neutrophils have been shown to influence vaccine efficacy, both, positively and negatively, and stimulate specific antibody secretion. Modulating immune regulatory properties after vaccination such as induction of different types of innate immune cell death, myeloid-derived suppressor or regulatory T cells, production of anti-inflammatory cytokines such as IL-10 may have beneficial effects on protection efficacy. Incorporation of lipid antigens presented *via* CD1 molecules to T cells have been discussed as a way to enhance vaccine efficacy. Finally, concepts of dendritic cell-based immunotherapies or training the innate immune memory may be exploitable for future vaccination strategies against tuberculosis. In this review, we put a spotlight on host immune networks as potential targets to boost protection by old and new tuberculosis vaccines.

Keywords: tuberculosis, vaccine, *Mycobacterium tuberculosis*, host-directed therapy, neutrophils, IL-10, CD1, cell death

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INTRODUCTION

With 1.8 million deaths worldwide, the World Health Organization listed tuberculosis among the top 10 causes of death in 2015 (1) and positioned tuberculosis to be the number one killer after infectious disease. Alarmingly high numbers of cases with multidrug- (MDR) and extensively drug-resistant (XDR) variants, prompted the G20 leaders to single out tuberculosis within the emerging problem of antibiotic resistance in their 2017 summit declaration. Nearly, half a million people were identified to be infected with MDR strains of *Mycobacterium tuberculosis* in 2016 but not even half of them were treated successfully (1). A number of XDR tuberculosis cases are even considered untreatable. Those patients have a survival rate of only 30% (1). These figures highlight the global tuberculosis health crisis and emphasize how urgently novel vaccines are needed.

As of today, the widely used attenuated live vaccine *M. bovis* BCG provides only limited protection. It is effective against primary tuberculosis during childhood, which can lead to severe outcomes, including meningitis. However, the protective efficacy of BCG against pulmonary tuberculosis in adults is unsatisfactory and varies tremendously between geographical areas, concretely the greater the distance from the equator the higher the efficacy (2). Consequently, huge scientific and financial efforts have been made to design and develop novel vaccine types, hoping to enhance protective immunity against tuberculosis in adults. The aims are either to sterilely eliminate the mycobacteria or—at least—prevent forms of active disease, such as contagious pulmonary tuberculosis in order to limit transmission. Still, none of the novel vaccine candidates have replaced BCG. A booster vaccine candidate with just one mycobacterial antigen, MVA85A, which was promising in animal models, failed to enhance BCG-primed protection in a recent clinical study in South Africa (3). In this approach, the secreted mycobacterial mycosyl transferase Ag85A, involved in the synthesis of trehalose dimycolate, cell wall maintenance, and *M. tuberculosis* survival (4), was cloned into a recombinant strain of modified Vaccinia Ankara virus (5) to be used as booster vaccine following BCG priming. Despite induction of antigen-specific multifunctional Th1 and Th17 cells in infants that received MVA85A on top of BCG priming (3), 1,399 vaccinees were not better protected from tuberculosis than the placebo controls, raising the question, whether BCG-centric vaccine strategies that aim to elicit potent Th1 cell responses against dominant antigens are still the most promising approaches (6).

A different approach to improve vaccine efficacy is to modulate host immune networks concomitantly or upon vaccination, using old and new vaccines to boost protection. Immune system networks may be exploited to bias immune responses toward protective immunity against tuberculosis. Various host responses following vaccination have been described to interfere with establishment of protective immunity similar as it is seen after natural infection (7). In this review, we discuss novel approaches, which may improve anti-tuberculosis vaccination. These include targeting neutrophils as well as the type of phagocyte cell death, i.e., necroptosis vs. apoptosis during vaccination. We review CD1-binding lipids as antagonists for CD1-restricted T cell responses, which may interfere with proper vaccine-mediated immunity. Additionally, immunoregulatory cytokines such as IL-10 may also affect vaccine efficacy and, thus, are putative targets for *vaccination-accompanying immunomodulation*. Finally, based on promising results in immunotherapy of cancer, we will consider whether induction of immunogenic cell death has the potential to enhance T cell responses.

NEUTROPHILS UPON VACCINATION: THE GOOD, THE BAD, OR THE NEUTRAL FOR VACCINE EFFICACY

Neutrophils are associated with disease in patients with active tuberculosis and susceptible mouse strains developing necrotic granulomas similar to humans. These potent anti-microbial innate immune cells have been shown to represent the main cell population in bronchoalveolar lavage and sputum of patients with active pulmonary tuberculosis and they carry the main mycobacterial load (8). Berry et al. identified a blood mRNA profile, which was dominated by a neutrophil signature and allowed to discriminate between active tuberculosis patients, latently infected ones, and healthy controls (9). Moreover, neutrophil-rich lesions associated with necrotic caseous material were found in resected lungs of patients (10). Thus, active pulmonary tuberculosis is considered to be neutrophil-driven (11). Susceptible mouse models, mimicking the pathology of human tuberculosis, such as C3HeB/FeJ, DBA/2, and I/St, show strong contribution of neutrophils to mycobacterial load, tissue destruction, pathology, and decreased survival rates (12–14). Importantly, not only in active *M. tuberculosis* infection but also after subcutaneous BCG vaccination, neutrophils rapidly enter the site of injection in large numbers (15). Neutrophil influx is also observed in response to vaccination using the synthetic trehalose dimycolate analog, trehalose-6,6-dibehenate (TDB), in a liposome formulation as adjuvant at the site of subcutaneous injection (**Figure 1**). Trehalose dibehenate was proposed as adjuvant to boost efficacy of subunit vaccines against tuberculosis (16). Neutrophils have also been shown to shuttle bacterial antigens to draining lymph nodes for T cell priming (17). However, contradictory roles for neutrophils regarding vaccine efficacy have been described.

Neutrophils have been shown to activate B cells leading to antibody secretion. After reprogramming by IL-10, among other signals, neutrophils entered the marginal zone of the spleen upon mucosal colonization by microbes in murine neonates (18). Here, the neutrophils exhibited a B cell–helper phenotype and induced immunoglobulin diversification and production by T cell-independent B cell activation. However, splenic B cell–helper neutrophils have not been detected in humans so far (19). Nagelkerke et al. identified a homogenous neutrophil population in human spleen that did not exhibit enhanced capabilities over blood neutrophils regarding co-stimulation of B cells and antibody production thereof. Classically, B cell-mediated immunity was thought to contribute only little to protection against tuberculosis. However, more recent studies reveal the potential of specific antibodies to contribute to immune protection against *M. tuberculosis* infection as comprehensively reviewed elsewhere (20, 21). In another study, murine bone marrow-derived neutrophils have been shown to exhibit a neutrophil–dendritic cell hybrid phenotype when cultured with GM-CSF (22). Those cells showed both, typical neutrophil properties, like NET formation and cathelicidin-mediated bacterial killing as well as intrinsic abilities of dendritic cells, like expression of MHC class II and co-stimulatory molecules and IL-12 production. The cells were capable of antigen presentation to T cells. Therefore, shifting the neutrophil phenotype elicited by vaccination toward this unusual subtype, for example, by concomitant administration of rGM-CSF, may strengthen vaccine efficacy. This subtype of antigen-presenting neutrophil, named N_β, was identified to migrate to the site of HIV infection in an NF-kB-dependent manner and activate specific $CD8^+$ T cells (23). However, the function of N_β neutrophils in tuberculosis

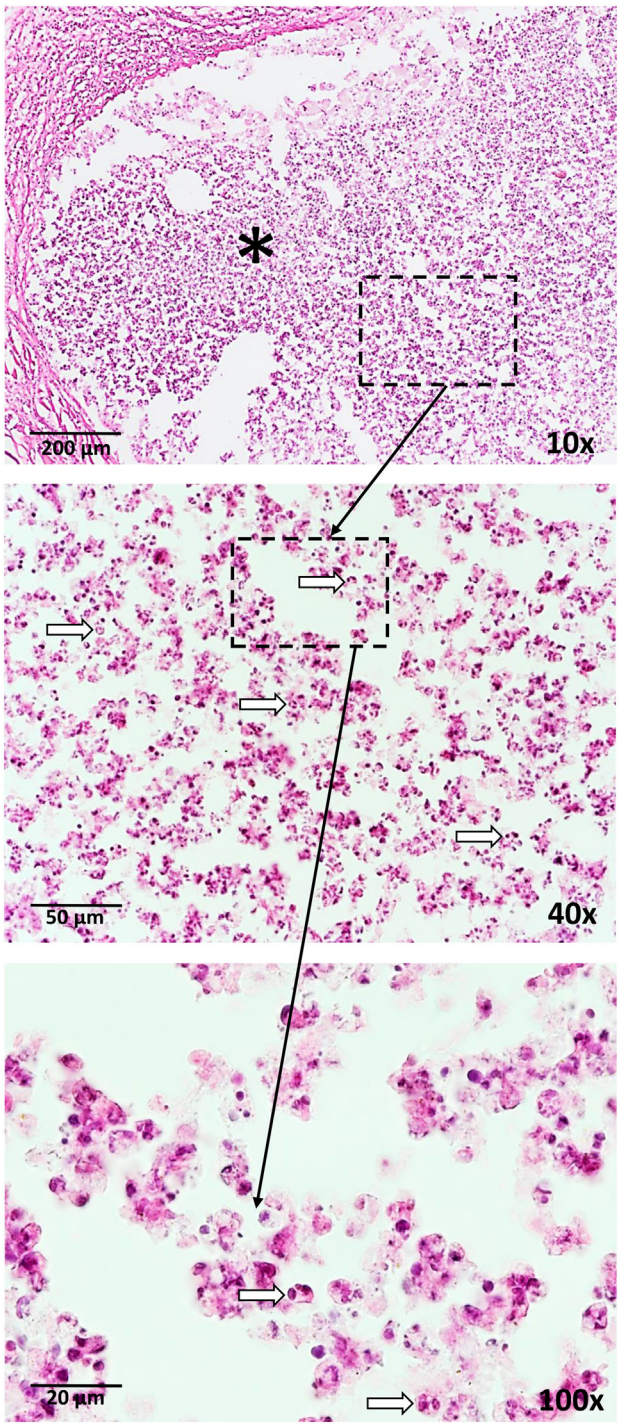

FIGURE 1 | Neutrophil influx (arrows) is a response to vaccination with BCG as well as a trehalose-6,6-dibehenate (TDB)-containing liposome-based adjuvant. The photographs are derived from skin tissue of mice, which were subcutaneously vaccinated with TDB liposomes containing ovalbumin at the tail base. Thirty-one days after vaccination, cryosections were stained with hematoxylin and eosin. * = injection site.

awaits further analysis. After experimental administration of the recombinant *M. smegmatis*-based vaccine mc²-CMX, which expresses fragments of the *M. tuberculosis* antigens Ag85c, MPT51, and HspX, and elicits specific Th1 and Th17 cells, neutrophil-rich lesions with a necrotic centers were observed (24). When neutrophils were depleted during vaccination, Th1- and Th17-specific responses, responsible for reduction of mycobacterial loads were nullified as were the protective efficacy of the mc²-CMX vaccine.

In contrast to immunity-promoting functions of neutrophils, effects detrimental for cellular and humoral immunity have also been described. Neutrophils can interfere with cell-mediated immunity upon adjuvant administration (25). Neutrophil depletion for the first 24 h after immunization restored $CD4^+$ T and B cell responses and improved clustering of antigen-specific T cells with DCs, as shown by intravital microscopy. The strong suppressive effect of neutrophils on T and B cell activation depended on the small lipid mediators prostanoids since neutrophil influx into the lymph nodes was abrogated in cyclooxygenase (COX)-1 and -2 knock-out mice as well as when specific inhibitors for these two enzymes were applied (26). Inhibitors included COX-1-selective SC-560, COX-2-selective NS-398, and Indomethacin. Specifically, the first wave of rapid neutrophil entry was dependent on prostaglandins, while the authors identified the neutrophil-derived eicosanoid thromboxane A_2 to control the extent of T cell responses. Indeed, thromboxane A_2 has been shown to negatively regulate physical contact of DCs and T cells. Treatment by an exogenous thromboxane receptor agonist led to random, not chemokine gradient-guided T cell movement. Thromboxane receptor-deficient mice showed increased immune responses to a model antigen (27).

Taken together, these data demonstrate that neutrophil influxes and cross-talk between neutrophils and professional antigen-presenting cells can negatively influence T cell priming and proliferation upon vaccination. Importantly, neutrophil depletion or neutralization of neutrophil-derived immune mediators restored protective T cell responses upon adjuvants administration. Thus, targeting neutrophil-mediated regulation of the dendritic cell—T cell synapse may improve the establishment of adaptive immunity. Moreover, the neutrophils' short life span of only a few days suggests the type of cell death, e.g., immuno-silent apoptosis vs. immunogenic apoptosis vs. necrotic cell death, as an additional target during immune priming.

IMPACT OF THE TYPE OF IMMUNE CELL DEATH ON VACCINE EFFICACY

For a long time, apoptotic cell death has been thought to be immuno-silent or even anti-inflammatory. This paradigm shifted when it has been shown that the trigger of apoptosis shapes the immunological consequences thereof. For instance, Fas ligand-mediated apoptosis, e.g., of tumor cells, induces strong inflammatory responses through inflammasome activation, i.e., caspase-1 and its IL-1β- and IL-18-activating properties (28). Importantly, mycobacteria-infected macrophages succumbing to apoptosis deliver foreign antigens to dendritic cells and subsequently cross-prime antigen-specific T cells, thereby, establishing protection against experimental tuberculosis in mice (29, 30). Dendritic cells were activated by mycobacterial pathogen-associated molecular patterns carried along by apoptotic vesicles

from infected cells. In contrast to virulent *M. tuberculosis*, BCG induces apoptosis in macrophages, especially upon IFNγ activation (31). These findings are corroborated by us as well as others showing that attenuated *M. tuberculosis* strains as well as BCG primarily induce apoptosis in neutrophils, whereas virulent *M. tuberculosis* strains induce necrotic cell death in both, macrophages and neutrophils (11, 32–34). Moreover, apoptosis has been described as a defense mechanism against *M. tuberculosis* (35). In contrast, Aporta et al. report that BCG as well as the live attenuated vaccine candidate *M. tuberculosis* strain SO2, which lacks phoP, does not induce cell death (apoptosis or necrosis) in murine bone marrow-derived macrophages and the murine macrophage cell line J774, while the virulent parental clinical isolate MT103 induces apoptosis, but not necrosis (36). However, the contradicting findings regarding macrophage cell death induced by *M. tuberculosis* infection, i.e., necrosis vs. apoptosis, may be associated with the different mycobacterial strains (e.g., MT103, H37Rv, and Erdmann) or the macrophage types used (e.g., murine bone-marrow-derived, human monocyte-derived, murine, or human macrophage cell lines), respectively (37–39). Of note, the novel anti-tuberculosis vaccine candidate *M. bovis* BCG VPM that expresses listeriolysin but is deficient in urease, is a better inducer of macrophage apoptosis than the parental BCG strain (40).

The efficacy of whole cell anti-cancer vaccines has been shown to depend on the type of induced cell death prior to administration. In a prophylactic cancer vaccination study, apoptotic vs. necrotic tumor cells were studied for their efficacy to induce tumor antigen-specific T cells (41). Vaccination with γ-irradiated apoptotic tumor cells prevented tumor outgrowth in up to 100% of the mice. In contrast, mice that received necrotic tumor cells, generated by three freeze-thaw cycles, were protected in only 0–30% of the cases. The apoptotic tumor cell vaccine recruited predominantly dendritic cells as well as $CD4^+$ and cytotoxic $CD8^+$ T cells at the site of injection. Injection of necrotic tumor cells elicited strong macrophage influx. Apoptotic tumor cells increased the survival rates of mice. These data suggest that antigens delivered by apoptotic cells to antigen-presenting cells can result in a stronger T cell response than those carried by necrotic cells (42). This notion is corroborated by a study, which combined dendritic cell-based immunotherapy with immunogenic cell death to successfully elicit Th1-mediated immunity to glioma (43). Application of necroptotic tumor cells also proved to be a successful vaccine strategy against cancer in mice. Induction of necroptosis in tumor cells by triggering RIPK3 resulted in the release of damage-associated molecular pattern molecules, subsequent dendritic cell activation, and priming of tumor antigen-specific IFNγ-producing T cell (44). Therefore, necroptotic tumor vaccines may also be employed, which comes in handy since many tumors are resistant to apoptosis induction.

These data indicate that the type of immune cell death upon whole cell anti-tumor vaccination shapes elicited immune responses. However, vaccination strategies, which determine the type of cell death of the antigen-bearing cells, may also improve anti-microbial vaccines. Application of an influenza vaccine with a microneedle following non-ablative fractional laser pretreatment triggered death of antigen-presenting cells and dsDNA release. This activated the cGAS/STING pathway and led to protective immunity (45). The use of this technique for BCG vaccination was effective without generating obvious inflammatory lesions (46). Analysis of the composition of infiltrating immune cells and their fate after tuberculosis vaccine application may improve vaccines by locally applying cell death inhibitors or inducers at the vaccination site. Upon immunization, programed cell death associated with the release of DAMPs promotes Th1-mediated immunity, whereas unregulated necrosis fails to induce $CD4^+$ T cell responses. Certain self-lipid antigens have been shown to antagonize activation of T and natural killer (NK) T cells (47). One might envisage that oxidized lipid antigens, potentially generated during host phagocyte necrosis, interferes with induction of immunity by vaccination as discussed below.

LIPID ANTIGEN ANTAGONISTS TO CONTROL VACCINATION EFFICACY?

The ability of lipid antigens to prime-specific T cells upon presentation by myeloid antigen-presenting cells *via* highly conserved CD1 molecules indicates the possibility of mycobacterial lipid-based vaccinations in an MHC-independent manner. Human antigen-presenting cells can express four CD1 isoforms (CD1a–d) that bind a variety of exogenous and endogenous lipid compounds (48–50). The relevance of CD1-restricted lipid antigens for T cell immunity has been well characterized for autoimmune diseases (51), cancer (52), and infections (53).

Human CD1b can bind and present mycobacterial antigens including mycolic acids and glucose monomycolate to T cells *in vitro* (54). Other CD1b ligands include mycobacterial diacylated sulfoglycolipids (Ac_2SGL) and phosphatidyl-myo-inositol dimannosides (PIM_2) (55). Furthermore, $CD1^+$ dendritic cells have been isolated in great numbers from leprosy skin lesions upon *M. leprae* infection, which was associated with active cellular immune response (56). Importantly, mycobacterial lipid antigen-reactive T cells were identified in blood of patients with latent and active, antibiotic-treated tuberculosis (57).

Emphasizing lipid antigen involvement in mycobacterial immune responses, direct immunization of guinea pigs with a mixture of mycobacterial lipid antigens induced CD4-negative cytotoxic T cells reacting to CD1-expressing, mycobacterial antigen-presenting dendritic cells (58). Another study found that vaccination of guinea pigs with *M. tuberculosis* lipids-containing liposomes in combination with a non-lipid adjuvant, prior to *M. tuberculosis* infection, reduced pathology and mycobacterial loads, presumably due to CD1-restricted T cell responses (59). Similar protection was observed in guinea pigs by a vaccine combining Ac_2SGL and PIM_2 (60). Especially, Ac_2SGL showed promising results, activating human CD1-restricted $CD8^+$ T cells *in vitro* isolated from skin test-positive donors (55). However, long-term memory protection was not detected even though glycolipid-specific immunoglobulin responses can occur after lipid antigen presentation (61, 62). To induce memory B cell responses against lipids, well-established anti-protein vaccine formulations were combined with glycolipids by conjugating

glucose monomycolate to a carrier protein (63). Disappointingly, anti-glucose monomycolate antibody responses were not induced.

Even though indications for protective effects upon immunization with mycobacterial lipid antigens were observed, detailed knowledge of the mechanisms, interaction characteristics, and downstream effects remain largely unknown. Most studies based on mycobacterial lipid vaccination, lack data on long-term memory responses, and prolonged immunity to tuberculosis.

Besides exogenous lipids, all CD1 molecules can bind and present a broad variety of endogenous, self-derived phospholipids, and glycosphingolipids with differential effects on subsequent T cell activation. Endogenous skin lipids presented by antigen-presenting cells *via* human CD1a molecules have been shown to either inhibit or stimulate T cell responses dependent on the structural properties and charges of the lipid antigen. In contrast to lipids with hydrophilic head groups that protrude into the binding groove of CD1 and, thus, prevent T cell receptor binding and subsequent T cell activation, highly apolar lipids placed deep in the binding groove, facilitated strong T cell receptor binding, and induced an autoreactive phenotype of CD1a-restricted T cells (48). Therefore, autoreactive T cells can become activated upon a shift toward a hydrophobic head group lipid composition, which may occur during certain autoimmune skin diseases (64, 65). Self-derived lipid antigens also indirectly influence the presentation of mycobacterial lipids and downstream signaling. Structural crystallography analysis revealed that CD1b adapts to different sizes and numbers of mycobacterial lipid alkyl chains by capturing small endogenous gangliosides, thus, stabilizing the CD1–lipid complex (66). Huang et al. identified deoxyceramides and diacylglycerols species as scaffolding lipids (47). They were found to nest deeply into the binding groove of CD1 molecules and, thereby, affect binding of exogenous mycobacterial lipid antigens. The efficacy of interference depended on the length of the hydrophobic side domains. Hydrophobic long alkyl side chains, protruding into the binding groove, inhibited T cell activation, whereas those with short hydrophobic domains stabilized the lipid–CD1 complex and facilitated T cell activation (47). These studies strongly suggest that the immune system is able to sense inflammation-induced changes of the lipidome within affected tissues, which in turn modulates immune responses. Inhibition of CD1–T cell receptor interaction by protruding lipids interrupts T cell activation. Therefore, self-derived lipids, serving either as scaffolding molecules or as auto-antigens, may function as agonists or antagonists for downstream T cells activation depending on their molecular structure. Recent studies tried to utilize these findings for development of new therapeutics. CD1d-restricted invariant NK T cells were strongly activated to produce Th1 and Th2 cytokines by recognition of a CD1d–α-galactosylceramide complex (67) indicating α-galactosylceramide as a putative adjuvant for vaccination. Addition of a phenyl group or a shorter phytosphingosine chain, as in the analog OCH, changes CD1d–antigen complexes on antigen-presenting cells and shifts downstream NKT cell responses to either IFNγ or IL-4 production, respectively (67). These modifications can shape immune responses including prevention of autoimmunity, such as experimental autoimmune encephalomyelitis in mice (68) and improved cancer therapy by induction of NKT cell-driven Th1 responses (69, 70) suggesting that CD1–lipid–T cell receptor interactions can be targeted to improve vaccine efficacy.

Upon release of reactive oxygen species, human neutrophils oxidized self-lipids by their own myeloperoxidase (71, 72). Initial oxidation by a single free radical could trigger an oxidization cascade, which proceeds within biological membranes, resulting in accumulation of oxidized lipid species by just one hit (73). Importantly, we have shown that human neutrophils underwent necrotic cell death upon infection with *M. tuberculosis* that was dependent on myeloperoxidase-generated reactive oxygen species (32). Therefore, infected necrotic neutrophils, which are removed by myeloid cells capable to present antigens *via* CD1 molecules, represent a source of oxidized self-lipids that potentially interfere with T cell responses to mycobacteria. Exogenous and endogenous lipid antigens are interesting compounds to control CD1 antigen presentation and, thereby, silencing or activating CD1-restricted T cell responses in order to modulate immunity against mycobacteria during infection or vaccination with whole attenuated mycobacteria. Interestingly, CD1-restricted *M. tuberculosis*—as well as pollen lipid-specific T cells have been shown to produce the anti-inflammatory cytokine IL-10 (74, 75), which is able to interfere with BCG vaccination efficacy (76, 77).

NEUTRALIZING ANTI-INFLAMMATORY IL-10

Immune responses during infection have to be tightly controlled in order to avoid excessive inflammation and subsequent tissue damages. Anti-inflammatory cytokines such as IL-10 prevent immunopathology by restraining both, Th1 and Th17 cell activities (78). Due to its negative effect on protective adaptive immune responses, IL-10 signaling has been associated with detrimental course of infection in experimental tuberculosis in mice (77, 79). Importantly, increased IL-10 production upon vaccination against various pathogens was shown to limit their protective efficacy (80, 81). Several studies reported induction of IL-10 upon BCG challenge in both, humans and mice (82–85). IL-10-deficient mice vaccinated with BCG revealed higher splenocyte numbers generating IFN-γ, IL-17A, and TNF-α when compared with wild-type ones (76, 77). Accordingly, BCG-vaccinated mice were better protected against *M. tuberculosis* infection after treatment with an IL-10-neutralizing antibody (76, 77). Thus, controlled short-term regulation of IL-10 levels during BCG vaccination represents a promising host-directed immune modulation to expedite mycobacterial clearance, establish better T cell-mediated immunity and thereby improve vaccination efficacy.

Agonists for C-type lectin receptors, particularly trehalose dibehenate (TDB), have been highlighted as promising adjuvant candidates for anti-tuberculosis vaccination (86). TDB is the synthetic analog of the mycobacterial cell wall glycolipid trehalose-6,6′-dimycolate (TDM), which binds to the macrophage-inducible C-type lectin, also known as Mincle,

and subsequently induce pro-inflammatory cytokine secretion (16, 87, 88). Interestingly, Lindenstrom et al. demonstrated that CAF01, a liposome preparation containing TDB, could trigger potent and sustained Th1 and Th17 responses (86). Mincle was identified as target for vaccine adjuvants with strong immunogenic properties against *M. tuberculosis* infection in mice (89). We recently showed that Mincle signaling was also critical for IL-10 secretion in response to TDM or BCG challenge *in vitro* (90). Our studies demonstrated that IL-10 secretion down-regulated IL-12 production, a pivotal cytokine for proper induction of Th1 responses (91). Thus, it would be interesting to investigate whether anti-tuberculosis vaccines including those formulated with TDB or other Mincle ligands can be combined with anti-IL-10 antibodies to improve both, efficacy and duration of protection against *M. tuberculosis*. It remains to be elucidated whether this approach can be extended to other anti-inflammatory cytokines such as TGF-β and IL-27. In conclusion, controlled short-term down-regulation of IL-10 levels after vaccination may be a promising host-directed modulation to drive a Th1-/Th17-mediated memory response and promote long-term immunity. In this context, regulation of immune cell populations that interfere with developing Th1-derived protection upon vaccination, e.g., myeloid-derived suppressor cells (MDSC) and regulatory T cells (T_{regs}), have the potential as pharmaceutically controllable switch points to improve long-term vaccine efficacy.

CONTROLLING Th1-SUPRESSING IMMUNE CELL SUBPOPULATIONS

Depletion of immune cell subsets, such as MDSC and T_{regs} by monoclonal antibodies have been shown to improve clinical outcome and vaccination efficacy (92). Increased frequencies of MDSC have been shown in blood samples and at the site of *M. tuberculosis* infection in chronic as well as recently infected patients (93–95). MDSC suppressed $CD4^+$ and $CD8^+$ T cell proliferation and cytokine secretion *ex vivo*. After successful anti-tuberculosis treatment, MDSC frequencies became normalized accompanied by MDSC maturation as indicated by expression of co-stimulatory surface molecule CD80. Importantly, when MDSC were depleted in *M. tuberculosis*-infected mice by administration of all-*trans* retinoic acid, mycobacterial burden, and pathology in lungs were reduced (96). In tumor-bearing mice, researchers found that MDSC migrate along a gradient of the pro-inflammatory alarmin S100A8/9 leading to MDSC accumulation (97). Blockade of S100A8/9 binding to MSDC resulted in decreased frequencies of MDSC. Moreover, mice deficient for S100A9 generated potent tumor rejection responses, while S100A9 overexpression led to accumulation of MDSC and reduced numbers of differentiated dendritic cells and macrophages. Of note, S100A8/9 comprise 45% of all cytosolic proteins in neutrophils, while its amount is 40× lower in monocytes (98). As mentioned above, massive infiltration of neutrophils at the site of BCG vaccination as well as *M. tuberculosis* infection may be the reason for the reported upregulated levels of S100A8/9 during tuberculosis (99, 100), which may result in accumulation of MDSC and, ultimately, suppression of proper Th1 immune responses. Indeed, S100A8/9, specifically derived from neutrophils, was responsible for diabetes-induced thrombocytosis (101). Blockade of S100A8/9 binding to its receptor by administration of Paquinimod was beneficial. Specific inhibition of S100A8/9 by Paquinimod reduced inflammation and pathology under various disease conditions, including leukocyte recruitment during sterile inflammation, experimental systemic sclerosis, and experimental osteoarthritis (102–105). Therefore, MDSC may represent the S100A8/9-driven connection that links massive neutrophil influx to impaired Th1 immune responses and memory phenotypes thereof. Vaccination efficacy may benefit from treatments regulating MDSC influx or their maturation during vaccination-induced immune priming.

Another immune cell subpopulation playing a detrimental role in the successful establishment of Th1-mediated control of *M. tuberculosis* infection are T_{regs}. Increased frequencies of $FoxP3^+$, IL-10-, and TGFβ-releasing T_{regs} have been found in peripheral blood and at the site of infection in tuberculosis patients (106–108). After successful treatment, those frequencies decreased to similar levels as in healthy controls (108). For mice, it has been shown that T_{regs} are responsible for impaired generation of protective immunity against tuberculosis. After aerosol infection of mice, preexisting *M. tuberculosis*-specific T_{regs} expanded in the draining lymph nodes (109). Accumulation of those T_{regs} in the lungs resulted in delayed arrival of pathogen-specific IFNγ-producing $CD4^+$ and $CD8^+$ effector T cells and suppression of protective immunity. Induced expansion of both, T_{regs} and IFNγ-, perforin-producing T effector cells by s.c. administration of rIL-2 led to accumulation of those subsets in the lungs of *M. tuberculosis*-infected macaques (110). Interestingly, infiltrating Th1 effector cells and T_{regs} orchestrated both, control of *M. tuberculosis* burdens as well as resistance to severe inflammation and tissue damage. Targeted inhibition of Th2 and T_{regs} generation by administration of the small compounds suplatast tosylate and D4476 reduced *M. tuberculosis* burden and established a protective Th1 immune response (111). The same research group shows in a follow-up study that the same treatment enhanced BCG efficacy upon vaccination so that *M. tuberculosis* burdens were reduced in lungs and spleens of mice after infection (112). Mycobacteria-specific T_{regs} already occur after administration of commonly used BCG formulations as well as during clinical trials with novel candidate vaccines (113). To enhance vaccine efficacy and Th1-mediated immunity upon BCG vaccination against *M. tuberculosis* infection, T_{regs} have been successfully targeted. Dhiman et al. showed that human $NK1.1^+$ NK cells promoted $CD8^+$ T cell responses and concomitantly reduced frequencies of *M. tuberculosis*-specific T_{regs} in an IL-22-dependent manner (114). BCG vaccination of mice generated those IL-22- and IFNγ- secreting NK cells and their specific depletion upon vaccination led to higher numbers of T_{regs}, increased mycobacterial burden, and reduced T cell responses after *M. tuberculosis* infection. More importantly, concomitant administration of rIL-22 and BCG in $NK1.1^+$ cell-depleted animals restored $CD4^+$ T cell responses and BCG vaccination efficacy, indicating that IL-22 treatment can improve vaccination efficacy. Another study shows that a novel subunit vaccine booster candidate induced strong and

specific Th1-mediated IFNγ and IL-2 production, while T_{regs} were down-regulated (115). This formulation included a fusion protein of Ag85B, Mpt64, and Mtb8.4 formulated in an adjuvant containing dimo-thylidioctyl ammonium bromide and BCG-polysaccharide-nucleic acid, consisting of polysaccharides and nucleic acids extracted from BCG. Finally, subsequent administration of a T_{regs}-depleting anti-CD25 antibody upon BCG vaccination, resulted in an increased IFNγ response, and reduced mycobacterial load upon *M. tuberculosis* infection (116).

Thus, temporal regulation of highly relevant immune subpopulations, such as MDSC and T_{regs} that otherwise modulate immune responses and protect from exacerbated inflammatory tissue destruction, may display a crucial step in shifting the immune memory toward a protective Th1 response upon vaccination with BCG-based vaccines. Besides other T cell populations, which also modulate immune responses upon vaccination, but have been comprehensively reviewed before (117–119), an innate lymphoid cell population, namely NK cells, has recently found its way into the spotlight of tuberculosis research.

NATURAL BORN KILLERS

Because of their ability to rapidly induce cell death without the need of further activation, for instance in response to virus infected cells and tumor cells, NK cells were classified as innate lymphoid cell population (120). However, upon infection, NK cells were found to display a memory-like phenotype providing so-called innate memory (121–123). This phenomenon has also been described upon *M. tuberculosis* infection (124). For instance, NK cells recovered from pleural fluid from tuberculosis patients were a major source of IFN-γ as well as IL-22 production when restimulated with *M. tuberculosis ex vivo* (125, 126). In murine tuberculosis models but also in patients latently infected with *M. tuberculosis*, NK cells expand, mature, and produce IFN-γ upon both, *M. tuberculosis* infection and BCG vaccination (127, 128). Venkatasubramanian et al. found reduced mycobacterial burden in the lungs of *M. tuberculosis*-infected C57 BL/6 mice after adoptive transfer of NK cells that matured and expanded in response to BCG vaccination. This observation is in contrast to findings by Junqueira-Kipnis et al. reporting similar lung CFUs of *M. tuberculosis*-infected C57 BL/6 mice after antibody-mediated depletion of NK cells, suggesting a priming effect of BCG, which induces a memory-like NK cell popuation. Thus, a potential protective role for NK cells during *M. tuberculosis* infection, especially in human individuals, requires further investigations (129). Indeed, a recent phase 1 clinical trial with 72 tuberculin skin test-positive participants from South Africa revealed that re-vaccination with BCG boosted frequencies of IFNγ-secreting NK cells in response to BCG (130). Notably, this was in contrast to BCG-specific CD4, CD8 as well as γδ T cells, which numbers were only transiently enhanced after re-vaccination. Sustained elevated frequencies of NK cells were still observed after 1 year.

The memory-like phenotype of NK cells in response to BCG is based on epigenetic reprogramming, a phenomenon also termed "trained immunity" (131) that has been observed also for myeloid-derived innate immune cells.

TRAINING INNATE IMMUNITY TO BOOST VACCINE EFFICACY

The ability to develop an immunological memory has been attributed exclusively to lymphoid cells of the adaptive immune system, i.e., T and B cells. Recently, memory properties have also been described for prototypical cells of the innate immune system, namely macrophages and monocytes among others (132). Beneficial effects of BCG vaccination on several unrelated diseases, including non-mycobacterial infections, allergies, and cancer, in an antigen-independent manner have been comprehensively reviewed elsewhere with regard to innate memory characteristics (133–135). For instance, monocytes of BCG-vaccinated healthy individuals produced more IFN-γ, TNFα, and IL-1β in response to otherwise unrelated bacterial or fungal pathogens compared with the unvaccinated control group (136). The same study showed that 100% of severe combined immunodeficiency mice were protected from disseminated candidiasis after vaccination with BCG compared with only 30% of non-vaccinated mice. The authors conclude that BCG vaccination can instruct innate immune cells through epigenetic reprogramming based on histone methylation to provide antigen-independent protection in an NOD1 signaling pathway-dependent manner.

It remains to be elucidated whether targeting innate memory can be employed to improve anti-*M. tuberculosis* vaccination. As reviewed by Netea and van Crevel, several studies revealed that BCG-vaccinated household contacts, either juvenile or adults, of active tuberculosis patients were significantly less often interferon-gamma release assay (IGRA)-positive than non-vaccinated individuals, which was even more striking after a second BCG boost (133). The authors suggest that innate immune cells trained by BCG vaccination are able to eliminate *M. tuberculosis* before adaptive immunity kicks in leaving these individuals IGRA-negative, whereas those failing to clear the initial infection turn IGRA-positive. Corroborating these observations, Kagina et al. showed that house hold contacts of tuberculosis patients, which did not develop active tuberculosis, did not have increased mycobacteria-specific T cell responses (137). Thus, the capability of humans to control *M. tuberculosis* is not necessarily correlated with enhanced immune protection by antigen-specific T cells and, therefore, indicates the relevance of vaccination-triggered innate immune activation for clearance of an initial infection or, at least, reduction of the infection dose. Subsequent boost of acquired immune control may lead to latent tuberculosis without pathogen elimination. Identification of the vaccine associated triggers for innate immune cell training may thereby help to design better vaccine formulations to protect against active tuberculosis.

HOST-DIRECTED IMMUNE MODULATION TO IMPROVE VACCINATION SUCCESS

Concomitant administration of immune-modulating factors together with a vaccine has been studied mainly in other

fields than tuberculosis. Especially in experimental cancer therapy, blockade of anti-inflammatory cytokines and T cell inhibitory receptors, such as TGF-β, programmed death-1, and CTLA4, respectively, resulted in increased T cell-mediated tumor regression (138, 139). Interestingly, administration of drugs that systemically suppress certain immune responses, namely the COX-2 inhibitors celecoxib and NS-398, enhanced tumor vaccine efficacies against breast cancer and pancreatic adenocarcinoma (140–142). Treatment of HIV patients with a COX inhibitor rescued T cell functions and humoral memory

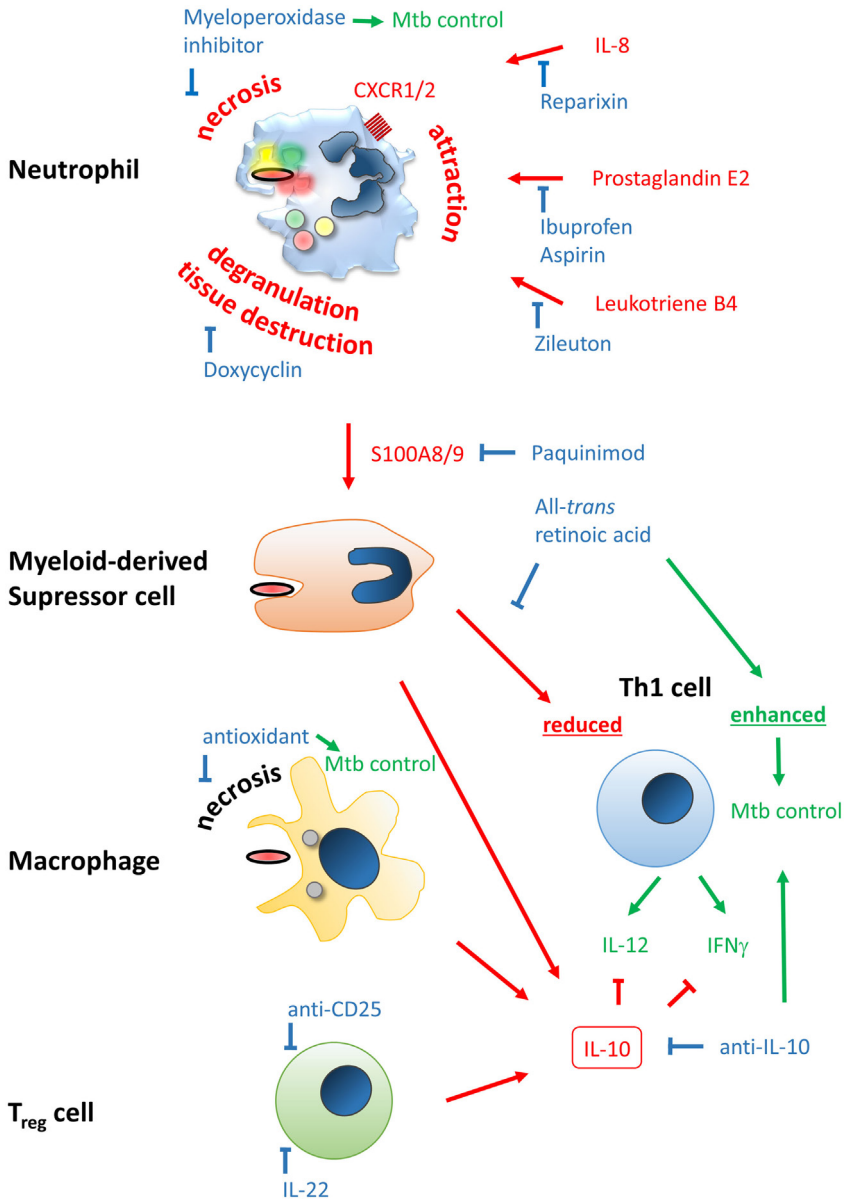

FIGURE 2 | Improving efficacy of classical BCG vaccines by host-directed immune modulation. Potentially detrimental molecules, mechanisms, and outcomes leading to poor vaccine efficacy are color-coded in red, beneficial ones in green, and modulators thereof in blue. Neutrophil infiltration at the site of vaccination may limit vaccine efficacy and generation of a proper and lasting Th1 memory response. Neutrophil attraction is mediated by IL-8, prostaglandin E2, and leukotriene B4, which can be blocked by Reparixin, Zileuton, and Ibuprofen/Aspirin, respectively. The type of cell death upon vaccination may be beneficial or detrimental, i.e., immunogenic apoptosis vs. necrosis, respectively. The latter can be blocked by the myeloperoxidase inhibitor ABAH or anti-oxidants. Degranulation of neutrophils' highly toxic molecules, preventable by Doxycyclin, may lead to tissue destruction and release of the alarmin S100A8/9 that attracts myeloid-derived suppressor cells (MDSC). S100A8/9 can be blocked by Paquinimod. MDSC inherit highly potent T cell-suppressive properties, potentially limiting establishment of Th1-mediated immunity. This mechanism can be counteracted by all-*trans* retinoic acid. Among MDSC, macrophages and regulatory T cells may be attracted to the site of vaccination. Together they shift the immune response to an IL-10-driven Th2 phenotype that inhibits protective Th1 responses shaped by IL-12 and IFNγ, which have been shown to be protective against *Mycobacterium tuberculosis* infection. Spatiotemporal treatment with a blocking anti-IL-10 antibody may rescue successful generation of a Th1 immune signature. Application of rIL-22 or anti-CD25 antibody may limit regulatory T cell (T_{reg}) contribution.

responses to tetanus toxoid, a T cell targeting vaccine (143). Of note, small lipid mediators produced by the cyclooxygenease pathway have been shown to attract neutrophils to the site of *M. tuberculosis* infection (13). Inhibition of neutrophil infiltration by Ibuprofen ameliorated tuberculosis pathogenesis and mycobacterial loads in susceptible C3HeB/FeB mice and, therefore, may be exploitable to improve anti-tuberculosis vaccines including BCG.

Macrophage responses may also serve as targets for host-directed therapies accompanying anti-tuberculosis vaccination. Taus et al. found that pretreatment of THP-1 cells with monosodium urate (MSU) crystals resulted in enhanced ROS production, promotion of phagosome–lysosome fusion, and increased killing of BCG by those cells, likely in an NOD-like receptor-mediated inflammasome-triggering process (144). MSU alone had no effect on BCG viability, indicating its pro-inflammatory properties, and capability to enhance anti-microbial responses. Moreover, BCG vaccination in the presence of MSU resulted in reduced colony-forming units in draining lymph nodes, showing increased killing of BCG *in vivo*. Again, BCG viability within the vaccine formulation was not affected by MSU. Interestingly, when mice were infected with *M. tuberculosis* Erdmann 10 weeks after vaccination, *M. tuberculosis* burden was reduced in the lungs and spleens, when the BCG vaccine was complemented with MSU in comparison with BCG alone. These findings raise the question, if enhanced killing of BCG upon vaccination, which is considered to counteract the maintenance of long-term immunity, may improve vaccine efficacy.

In another approach, the autophagy-inducing drug Rapamycin was used to successfully enhance processing and presentation of Ag85B by murine antigen-presenting cells (145). Immunization with Rapamycin treated dendritic cells led to increased Th1-mediated protection from *M. tuberculosis* infection. Thus, BCG vaccine efficacy may be enhanced by concomitant augmentation of autophagy.

Only a few studies assessed immune-modulatory approaches upon vaccination against tuberculosis. Surprisingly, repeated vaccine injections of BCG in already *M. tuberculosis*-infected BALB/c mice resulted in increased levels of IL-17, neutrophil influx into the lung, and tissue damage (146). This detrimental outcome of vaccination had been prevented by concomitant administration of a blocking anti-IL-17 antibody. Host-directed modulation of IL-17 upon vaccine treatment may have important implications for vaccinations in individuals that are already infected with *M. tuberculosis*.

Although there is only a small amount of data available, few publications show promising first results for increasing BCG vaccine efficacy by adjunct modulation of the host immune system. However, we can probably learn from data derived from tumor vaccination and other non-infectious disease vaccines boosted by concomitant immunotherapy. These results will be instrumental to develop novel strategies by temporarily adjusting the immune network to favor a long-term protective memory immune response against tuberculosis (**Figure 2**). Re-purposing already known host-directed therapeutics for anti-tuberculosis vaccination strategies may improve the protective efficacy, even of the "old" BCG.

CONCLUSION

So far, novel strategies to boost vaccine efficacy against tuberculosis are based on enhancement of the BCG vaccine by addition of antigens that are expressed by *M. tuberculosis*, but not *M. bovis* BCG, e.g., ESX-1 secretion-mediating ESAT-6, TDM-synthesizing Ag85, and ESX-5-associated PE/PPE, or by genetically modifying BCG to express immunity-promoting mediators such as GM-CSF or IL-2 (147, 148). To improve vaccination efficacy against *M. tuberculosis* infection, focusing on more *M. tuberculosis* antigens may enhance the frequency of specific T cells, but may not solve the problem that BCG and next generation anti-TB vaccine types also elicit immunomodulatory functions that hamper immune-priming efficacy. Short-term shapeshift of the local immune responses upon vaccination may result in far-reaching long-term consequences for establishment of protective memory immunity against *M. tuberculosis* infection. These immune modulations may include regulation of neutrophil and MDSC influx to the site of vaccine application, prevention of detrimental kinds of cell death that interfere with generation of a protective memory immune response, and facilitation of other beneficial types of cell death, interference with T cell-inhibiting presentation of (self-)lipids after vaccination-mediated inflammation, temporal regulation of adverse, anti-inflammatory cytokine profiles, e.g., IL-10, and spatiotemporal modulation of immune cell subpopulations that suppress T effector cells, like T_{regs} and MDSC. Promising results of immune modulation during vaccination in other application areas, namely cancer vaccine research, highlight the potential of such host-directed improvements.

AUTHOR CONTRIBUTIONS

LL wrote the chapter "Lipid antigen antagonists to control vaccination efficacy?" EP wrote the chapter "Neutralizing anti-inflammatory IL-10 to improve vaccination success." US and TD wrote all other parts of the manuscript and supervised the process. NR contributed **Figure 1** and its legend. Additionally, TD contributed **Figure 2** and its legend.

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