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REVIEW

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A review of the BCG vaccine and other approaches toward tuberculosis eradication

Thomas Cho[a], Christopher Khatchadourian[a], Huy Nguyen[a], Yash Dara[a], Shuna Jung[a], and Vishwanath Venketaraman [a,b]

[a]College of Osteopathic Medicine of the Pacific, Western University of Health Sciences, Pomona, CA, USA; [b]Graduate College of Biomedical Sciences, Western University of Health Sciences, Pomona, CA, USA

ABSTRACT

Despite aggressive eradication efforts, Tuberculosis (TB) remains a global health burden, one that disproportionally affects poorer, less developed nations. The only vaccine approved for TB, the Bacillus of Calmette and Guérin (BCG) vaccine remains controversial because it's stated efficacy has been cited as anywhere from 0 to 80%. Nevertheless, there have been exciting discoveries about the mechanism of action of the BCG vaccine that suggests it has a role in immunization schedules today. We review recent data suggesting the vaccine imparts protection against both tuberculosis and non-tuberculosis pathogens via a newly discovered immune system called trained immunity. BCG's efficacy also appears to be tied to its affect on granulocytes at the epigenetic and hematopoietic stem cell levels, which we discuss in this article at length. We also write about how the different strains of the BCG vaccine elicit different immune responses, suggesting that certain BCG strains are more immunogenic than others. Finally, our review delves into how the current vaccine is being reformulated to be more efficacious, and track the development of the next generation vaccines against TB.

Introduction

Although nearly eradicated in developed countries, tuberculosis (TB), the disease caused by the bacteria *Mycobacterium tuberculosis (M. tb)*, is estimated to have infected over a quarter of the world's population and remains the global leading cause of death by a single pathogen.[1] Despite the existence of a standardized antibiotic regimen against TB, issues with access to medicine, the rise of Multidrug Resistance TB (MDR-TB) and Extreme Multidrug-resistant TB (XMDR-TB) continue to make TB a global public health burden.[1] In areas where TB burden is low, disease prevention is mainly controlled with proper hygiene and screening, vaccination being neither required nor recommended. It is only in areas with a high TB burden where the BCG vaccine is given regularly. The discrepancy in recommendations is due in part to large inconsistencies in the efficacy of the vaccine, which ranges from 0% to 80% effective in preventing for the prevention of TB.[2]

This review paper gives a brief overview of the pathophysiology of TB and the historical background of the development of the BCG vaccine. We also discuss a possible mechanism of action of the vaccine, which involves its ability to attenuate a lesser-known immune system called trained immunity. The vaccine appears to also invoke epigenetic reprogramming in hematopoietic stem cells. Finally, we discuss the work being done to change the route of administration of the vaccine, and briefly introduce the numerous approaches toward either augmenting the efficacy of the current vaccine or develop a new vaccine to supersede BCG.

Pathophysiology of tuberculosis

TB is an airborne infection spread by aerosolized particles harboring tubercle bacilli, the etiological agent of the disease.[3] Tubercle bacilli most commonly infects the airways and lungs of exposed individuals.[3,4] Following inoculation in the new host, tubercle bacilli are phagocytosed primarily by macrophages and dendritic cells which aggregate into structures called granulomas.[3] *M. tb*, the primary causative agent of TB disease, persists within immune cells by evading multiple cellular pathways and organelles, particularly with the endocytic and autophagic pathways. The ability to disrupt autophagy allows intracellular persistence of *M. tb*.[3] Patient immunocompetency is crucial to the development of TB disease as immunocompromised groups have more severe disease manifestations.[4]

Current BCG immunization protocol

The BCG vaccine is the only vaccine currently recommended for the prevention of TB. Vaccination recommendations vary between countries, with endemicity of TB often being the basis for issuing recommendations.[5–7] Universal recommendation of the BCG vaccine is commonplace in most countries.[5,6] These countries either recommend single dose administration, or utilize schedules encompassing multiple doses.[1,5,7] The World Health Organization (WHO) recommends vaccinating neonates residing in high-incidence TB and/or Leprosy settings with a single dose of the BCG vaccine at birth.[1] Furthermore, neonates residing in low-incidence settings and at high risk of contracting TB disease and/or Leprosy should

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CONTACT Vishwanath Venketaraman ✉ venketvi@gmail.com 🔵 Microbiology/Immunology, Department of Basic Medical Sciences, College of Osteopathic Medicine of the Pacific, Western University of Health Sciences, 309 E Second Street, Pomona, CA 91766-1854, USA.

also be considered for vaccination.[6] High-risk neonates, defined as those with close contact to currently or previously infected individuals. Repeat BCG vaccination is not currently recommended by the WHO as evidence arguing its efficacy is insufficient.[4] Despite its wide range of purported efficacy in preventing primary TB, with many studies predicting efficacy anywhere between 0 and 80%, the BCG vaccine continues to be a mandatory vaccine for much of the world.[4,6] The vaccine's strong safety profile, measured as one major side effect from vaccine administration per one million doses administered to immunocompetent individuals, may also be a reason for its continued use in TB endemic countries.[4]

Vaccine efficacy may be dependent on vaccine strain

One hypothesis for the wide efficacy range is the genotypic differences within the BCG vaccine. The vaccine, was first produced by passing a pathogenic *Mycobacterium bovis* (*M. bovis*) strain over 230 times throughout a period of 10 years, which eventually led to its attenuation.[2] Once the vaccine's efficacy was established, Calmette and Guerin distributed their strain globally in 1924 to regions that requested their newfound vaccine. Eventually, geographical isolation permitted independent mutations among samples, promoting genotypic variation between once identical strains.[8]

More recent advancements in genomic sequencing laid light to these genotypic changes, termed regions of differentiation (RD). From these studies emerged at least 16 different RDs in the world's supply of BCG vaccines.[8] Further studies into the strains found an additional 14 different sub-strains, each named after the region in which they were distributed to.[8] Six of the most researched sub-strains are BCG Tokyo (BCG Japan), BCG TICE, BCG Danish, BCG Pasteur, BCG China, and BCG Prague. BCG strain distribution tends to be regional, and international vaccine distribution is mainly controlled by UNICEF, who receives its BCG vaccine from four suppliers.[8] These four suppliers produce three vaccine strains: BCG Denmark, BCG Russia, and BCG Japan.[1,8]

Early research identifying variation in efficacy between individual strains is sparse, conflicting, and in many cases, poorly designed: either demographics were uncontrolled, studies were not randomized, nonpopular strains were used, or endpoints/immune markers were not equivalently analyzed.[9–11] Nevertheless there is some preliminary data that suggests different strains produce differing immunological responses.[8]

A randomized trial in Australia on 288 infants assigned to one of three treatment groups (BCG Denmark, BCG Japan, BCG Russia) showed infants vaccinated with BCG Denmark and BCG Japan had a larger polyclonal CD4 T cell response accompanied by a greater increase in associated cytokines when compared to infants vaccinated with BCG Russia.[8] A more current study found some association between administration of BCG Beijing with higher levels of TB drug resistance later on in life.[11] A very interesting case out of Orizaba, Mexico the BCG strain given affects the immune response. Peripheral Blood Mononuclear Cells (PBMCs) were harvested from neonates who had received one of three different BCG strains, which were then infected in vitro by *M. tb*.[12] Subsequent analysis found different BCG strains elicited very different cytokine expression profiles.[12] Most importantly, they found that vaccination with BCG-Brazil or BCG-Denmark induced cytokines involved in the adaptive immune system, while BCG-Japan strain-induced proinflammatory response and memory formation.[12] The relationship between BCG strain and immune response was also seen in a study conducted in Nigeria and South Africa, where CD4 T cell responses following BCG vaccination were more robust and durable following inoculation with BCG-Denmark over BCG-Russia or BCG-Bulgaria.[13] Finally, variation in immune response between vaccine strains was also noted among murine model studies,[14] and dosage/route of administration also seems to affect the immune response.[15] Although vaccine efficacy cannot be extrapolated from these findings, it is safe to say that different strains induce different immunological response profiles. Further research into the immunological response to the BCG vaccine has led to another theory of how the BCG vaccine might protect against TB known as trained immunity.

BCG vaccine response may trigger "trained immunity" in the innate immune system

Human immunity is classically parsed into two distinct systems, the more nonspecific but broad spectrum coverage innate system and the highly specific and memory driven adaptive system, which is also termed cell-mediated immunity and humoral immunity.[16] Classical teachings in immunology suggests that innate immunity, though highly effective, produces no "memory" after an attack and instead relies on chemokine gradients and physiologic changes to clear infections.[16] Recent discoveries by Dr Netea Mihai suggest a memory component in the innate immune system, which she has coined "trained immunity".[17] The immunological background of trained immunity is based on the resistance mechanisms of plants. First postulated by Kenneth Chester in 1933 and later coined "systemic acquired resistance" (SAR), plants inoculated with a specific pathogen gained immunity to a number of pathogens, including those which the plant was never exposed to.[17]

While the exact mechanisms of trained immunity remain unknown, early data suggests that cross protection may trigger "heightened" alertness in the innate immune system .[18] Garly et. al discovered that children with a BCG scar and a positive tuberculin reaction residing in high mortality areas of Guinea Bissau had overall lower mortality than children with no history of receiving the BCG vaccine. No similar trend was observed in children who had received previous diphtheria or tetanus immunization.[18] Roth et. al discovered that children who received the BCG vaccine had lower mortality rates than their non-BCG vaccinated peers against malaria, further suggesting that BCG vaccination confers some form of protection against non-tuberculous diseases.[19] Conferred protection spans mycobacterial pathogens like *M. leprae*,[20] viruses, like Respiratory Syncytial Virus (RSV),[21] intestinal nematodes,[22] and yeast, like Candida.[18] Further *in vitro* studies revealed human adult monocytes receiving the BCG vaccine had increased IFN-γ production two weeks and three months following *S aureus* and *C albicans* inoculation.[23] More importantly, these

augmentations to the immune system were intact one year after initial vaccination.[23] A persistent increase in pro-inflammatory cytokine production post *in vitro* LPS-mediated challenge remained. Additionally, increased concentrations of pattern recognition receptors (PRRs), specifically Toll-Like Receptor 4 (TLR4), TLR2, and C type lectins on monocytes, were also observed.[23]

This idea of using the BCG vaccine as a broad-spectrum immunization was first tested in the elderly. The ACTIVATE (a randomized clinical trial for enhanced trained immune responses through BCG vaccination to prevent infection of the elderly) trial concluded its phase 3 randomized control trial in Greece in 2019 after administering either the BCG vaccine or a placebo in 202 patients on the last day of hospitalization.[24] As this population is very susceptible to infection, efficacy of the vaccination was measured via time to first infection between those receiving BCG against placebo, and blood samples were drawn in 57 patients (31 placebo and 26 BCG vaccinated) to assess cytokine levels and PBMC activation. Overall, BCG vaccinated individuals had a significantly increased "time to first infection time "over the placebo cohort – 16 weeks vs 11 weeks .[24] In addition, analysis of the PBMCs showed enhanced cytokine response to potential pathogens, specifically IL-6 and TNF-alpha.

Finally, using the yellow fever vaccine (YFV) as a model for an in vivo viral infection, BCG vaccination was shown to provide cross immunization against non-tuberculosis infections.[25] YFV is a live-attenuated viral vaccine, and YFV viremia peaks on the fifth day following vaccination.[26] Analysis of YFV concentrations in subjects who were administered a BCG vaccine 1 month prior to a YFV vaccine found lower concentrations of YFV in samples, as well as a lower circulating concentration of proinflammatory cytokines.[25] Notably, *ex vivo* infection of PBMCs gathered from the YFV and BCG vaccinated subjected with *C. albicans* showed higher levels of IL-1b expression and subsequent lower YFV viremia concentrations. IL-1b has been shown to be an important marker of trained immunity activation,[27] suggesting that BCG vaccination offers some sort of cross immune protection against non-tuberculosis infections.[25]

Activation of natural killer cells might influence immune response to BCG vaccine

Another emerging hypothesis on the mechanism of trained immunity focuses on the actions of Natural Killer (NK) cells.[28] As part of the innate immune system, these should not harbor memory. Recent studies, however, have shown that murine and human NK cells augment IFN-y release upon reinfection.[27,29] NK cells promote phagolysosome fusion within antigen-presenting cells (APCs) infected by *M. tb*, thus increasing *M. tb* killing.[30] According to Dhiman *et. al*, this is accomplished by the release of IL-22, a cytokine also released by memory CD4 + T cells in response to infections.[30] IL-22 was thought to trigger the release of antimicrobial peptides via direct induction.[30,31] Experiments performed by Dhiman showed that the release of IL-22 by CD4 T cells caused intracellular mycobacterial growth arrest, thus supporting previous predictions.[30]

NK cells may also induce the production of proinflammatory cytokines in response to unrelated pathogens following periods of 2 weeks and 3-months post BCG vaccination.[29] In a series of animal and human trials, Kleinnijenhuis *et. al* demonstrated that proinflammatory markers such as IFN-y and IL1 in humans increased post BCG vaccination[23,27,29,32] following an in vitro challenge of blood samples against *Candida albicans* and *Staphylococcus aureus*.[29] A similar increase in proinflammatory markers was seen among mice vaccinated with BCG followed by infection with *C. albicans*.[29] The importance of the NK cell response in BCG vaccine-induced cross protection against unrelated pathogens was further studied in mice with severe immunodeficiency (SCID).[32] SCID mice vaccinated with BCG prior to challenge with lethal *candida* had a 100% survival rate compared to the 30% survival rate seen in control mice.[29] The importance of NK cells in BCG-induced immunity was discovered when the same test was administered among NOD/SCID/IL2Ry (NSG) mice.[29] These mice not only lacked the B and T cell activation seen in SCID mice, but also lacked functional NK cells.[23] Following intravenous *candida* infection, all normal mice vaccinated with BCG survived, but the survival rate for NSG mice fell to about 70% despite receiving the vaccine. This suggested that NK cells have a crucial role in cross protection, and that BCG vaccination was necessary to induce cross protection.[23]

The BCG vaccine and its epigenetic effects

The upregulation of PRRs seen after BCG vaccination also seems to be mediated by epigenetics.[23] Expression of PRRs like TLR4 and MR on monocytes were elevated in BCG vaccinated subjects when compared to unvaccinated subjects,[23] remaining so even one year post-vaccination.[23] Therefore, investigations into how a more reactive immune response to *M. tb* infections is activated have led to findings that suggest epigenetic programming is involved.

When BCG was used as a replacement for mycobacterial infection in mice, researchers discovered an increase in IL-15 production from phagocytes infected with *M. bovis*, the mycobacterial strain of the BCG vaccine.[33] IL-15 has been shown to stimulate NK cell production of IFN-y.[33] Thus, its release in the face of mycobacterial challenge is of great interest. IL-15 production at the mRNA and protein levels was detected in mice following in vitro BCG inoculation,[33] noteworthy support of BCG's ability to affect immunity at the epigenetic level. Furthermore, levels of IFN-y in serum were significantly higher in mice with upregulated Il-15 expression than at baseline.[33]

Further research into the composition of BCG vaccinated vs. unvaccinated monocytes showed differences in key receptors.[32] Monocytes from humans who were vaccine-naïve showed significant increase in TLR4 expression following BCG vaccinatio .[32] Alongside CD14 and CD11b expression, TLR4 expression remained elevated 3 months post immunization.[32] This suggests that BCG vaccination caused some form of monocyte "training", via one or several signaling pathways. Kleinnijenhuis, *et. al.* inhibited receptors on monocytes exposed to BCG *in vitro*, specifically TLR2, TLR4, and NOD2.[32] Successful education was determined by proinflammatory cytokine expression levels following training and

subsequent exposure to a non-mycobacterial challenge.[32] Of the three receptors, only monocytes from NOD2 deficient patients failed to mount a noticeable cytokine response to the challenge,[32] suggesting that monocyte education is an epigenetic process that is influenced by the NOD2 pathway.

These experiments strongly suggest BCG vaccination's ability to incite epigenetic reprogramming of monocytes via changes in the expression of certain receptors.[27,32,33] A more recent paper suggests that epigenetic modification affects hematopoietic stem cell (HSC) differentiation in the bone marrow,[34] leading to the development of monocytes specifically programmed to recognize *M. tb*. Compared to B and T cells, monocytes and macrophages have very short lifespans,[34] therefore, hypotheses suggest that any memory component the vaccine imparts begins at the stem cell level.[32,34] Kaufman *et. al* administered BCG-TICE via IV into mice and found the attenuated strain in bone marrow[34] where it remained detectable for up to 7 months post vaccination.[34] This persistence of the vaccine in close proximity to HSCs, supported the idea that BCG may cause HSC expansion. Indeed, after tracking bone marrow (BM) expansion via the HSC progenitor lineage LKS+ population in both BCG vaccinated and non-vaccinated mice, it was only seen in the vaccinated group.[34] Transcriptomic data from this experiment showed BCG immunized HSCs favored myeloid lineage lymphocyte proliferation, upregulation of IFN-dependent gene expression, and increased production of "trained" monocytes/macrophages which were more effective in producing antimycobacterial immunity via elevated expression of IFN-y, TNF-α, and IL-10.[34] These effects were established by the different histone modifications seen between BCG primed and non-primed monocytes,[34] specifically H3K27 acetylation and H3K4 trimethylation.[32,34] These findings were noted prior to subsequent *M. tb* challenge, further suggesting that BCG's efficacy as a vaccine might begin via epigenetic modifications. Notably, H3K27 acetylation was also found in PMBCs studied in the aforementioned ACTIVATE trial, though the authors conceded that their sample size was too small to draw a direct correlation between their findings and any further implication of epigenetic changes.[24]

The aforementioned study on YFV viremia also showed epigenetic changes that attenuated specific proinflammatory pathways following vaccination.[25] Monocytes that had been primed with BCG had enhanced IL-8 and IL-1b expression following YFV introduction, both in cytokine and mRNA concentrations.[25] Interestingly, these expression levels remained low if the monocytes were also treated with a histone methyltransferase inhibitor, suggesting the expression is correlated to histone/epigenetic modifications.[25] The epigenetic changes evident in the murine model were later confirmed to occur in humans *in vivo*. After administering the BCG vaccine in 15 healthy but BCG naïve individuals, Cirovic, et. al analyzed blood count, PBMCs, immune activation markers, and bone marrow aspirates immediately following vaccination, then 14- and 90-days post vaccination.[35] While there were no changes in the number of mature myeloid or myeloid progenitor populations in either the peripheral blood or bone marrow, there was evidence of transcriptional and epigenetic changes as long as 90 days post vaccination.[35] Transcriptome analysis completed on the BM aspirates and

PBMCs revealed increased upregulation of genes associated with myeloid and granulocyte activation pathways, with additional Gene Ontology Enrichment Analysis (GOEA) of the enriched pathways finding genes associated with neutrophil activation (such as those from the SERPINA family) and transcription factor (TF) activity being affected the most.[35] Furthermore, the transcriptomic changes in the epigenome of CD14+ macrophages in BCG vaccinated individuals were different compared to the nonvaccinated population.[35] These upregulated pathways were consistent with the same pathways found in HSCs, suggesting that the epigenetic changes caused by vaccination was imprinted into the progenitor cell line and passed into the succeeding individual cells.

Different routes of BCG vaccination

There has also been a considerable push to reformulate the current BCG vaccine, which is administered intradermally (ID), into an oral vaccine.[36] Oral administration is cheaper, requires limited medical skill to administer, easier to distribute, and is a familiar route to most people. Direct mucosal exposure to the vaccine is considered highly immunogenic[37] but formulating a vaccine into a form that can withstand the degrading properties of mucosa, especially the stomach, has limited its use.[36]

One possible means around this problem is to coat the dry powder form of the vaccine in Eudragit copolymers.[38] Already used in enteric coating formulations of ibuprofen and other oral medications, this coating is acid-resistant and protects against early degradation of the drug.[38] From a materials science perspective, this study was a success in creating a modern day oral tablet form of this vaccine, but the authors found that the immense pressure needed to compact the BCG powder to a digestible size lead to decreased efficacy via death of the live attenuated bacteria that make the vaccine.[38] Future oral BCG vaccines therefore need to take into consideration not only the size of an oral tablet, but also the number of viable colonies that might exist per administration.

Oral administration of the vaccine is also being meticulously studied in animal sciences. European badgers (*meles meles*) are a major reservoir host for bovine tuberculosis, a TB-like illness that endangers cattle herds.[39] Today, bovine TB control is focused on vaccinating badgers with BCG via an intramuscular formulary (BadgerBCG),[40] as the culprit is a pathogenic strain of *M. bovis*.[39] As this effort is costly, dangerous, and not sustainable,[41] considerable effort has gone into formulating a sustainable oral form of the BCG vaccine[40]. BCG vaccine in the oral form is formulated with badger bait and left in the wild, with the idea that eventually, a large enough population will be inoculated to maintain herd immunity.[39] Notably, initiatives to mass inoculate wild badger populations have run into a myriad of problems, chief among them producing enough of the vaccine to be viable in an oral formulary.[41] While some of this quantity issue has been resolved with a new method of culturing the vaccine stain in bioreactors,[41] the more pressing problem arises in the fact that the oral form of BCG is most effective when taken up in the oral mucosa compared to the ileal mucosa.[41] From these animal studies, a viable human oral BCG vaccine could be created

using a bioreactor to create enough viable colonies to survive a pill mold, then instructing recipients to allow the tablet to dissolve in their mouths to allow for oral mucosa uptake of the vaccine. However, such a method has yet to be tested, and more studies need to be done on both the production and administration side before moving forward. Fortunately, other routes of administration are currently being explored today with considerably more success.

An aerosolized formulation of the BCG vaccine is also being studied for as an improved delivery mechanism. Murine experiments have shown that aerosolized BCG elicits a stronger CD4 and CD8 T cell response to *M. tb* infection following vaccination, especially in lung vasculature.[37,42] Mice were then challenged with *M. bovis*, and 8 weeks later were sacrificed, after which the amount of bacterial infection in various organs were quantified.[42] Notably, the *M. bovis* burden in the lungs was significantly reduced in mice who had received the aerosolized vaccine, while there was no reduction in bacterial burden in those that received the ID formulation.[42] Unfortunately, histological analysis of the lungs showed a stronger granulomatous inflammation reaction in mice who received BCG via aerosol versus the traditional route, suggesting there may have been more damage incurred in the lungs when BCG is aerosolized.[42] This result, if found to be true in subsequent experiments, would warrant further research into the safety of an aerosolized BCG vaccine.

Of all the available routes of administration, the intravenous (IV) route has elicited the strongest immune response.[43] When BCG was given IV compared to oral or ID administration into Macaques Monkeys, the IV formulation promoted a five-fold increase in total cells, especially T cells, in bronchoalveolar lavage (BAL) samples.[43] IV administration also elicited a 100-fold increase from the ID response in cytokine concentrations classically associated with TB infection (IFNγ, IL-2, TNF, and IL-17), and these levels remained elevated for longer than compared to ID administration.[43] Titer levels of *M. tb* specific IgG, IgA, and IgM also peaked higher and longer following IV administration compared to ID, suggesting that the immune response elicited may be more effective in combating TB.[43] While IV administration of BCG may seem attractive, its efficacy is limited to infrastructure issues, as it requires even more medical knowledge and healthcare professional experience than ID or oral administration. IV bags are more cumbersome to store and ship, and patients may be turned off by the idea of receiving a drip. Larger needles also mean higher risk of complications, so further work must be done to quantify how much more efficacious IV administration is over the current ID formulary before placing the necessary investments into IV vaccinations.

Finally, an even more novel approach to vaccine administration was tested in 2017, when the non-reconstituted vaccine was directly loaded into the hollow tips of a dissolvable microneedle array (MNA).[44] Tested on mice, the MNA was first pressed through the epidermis, where the sharp tips of the MNAs dissolved to release the vaccine into the epidermis and dermis.[44] Initial results showed limited site infection and evidence of cytokine production and T cell activation resembling post-BCG inoculation. This method has multiple advantages over the current route of administration. It does not require reconstitution, decreasing error, and need for skilled practitioners. It can be stored like dry powder, increasing shelf life .[44] More studies need to be done to test vaccine efficacy and affordability, but the technique is certainly promising.

The future of tuberculosis vaccination – development of a novel vaccine

Given the wide range of reported efficacy of the BCG vaccine, considerable research has also gone into developing the next generation of TB preventative vaccinations. Going beyond BCG reformulation, there appear to be three different approaches to vaccine development: development of an entirely new TB vaccine, creation of a novel recombinant vaccine derived from the existing BCG vaccine, and development of a booster vaccination to reinforce an existing BCG vaccination. The three approaches will now be discussed. A summary of the next generation vaccine candidates is listed in Table 1.

Method 1 – development of a new TB vaccine

The first approach involves the advent of a novel vaccine via the exploitation of various mycobacterial species, viral vectors, or the construction of fusion proteins (Table 1).

A novel TB vaccine based on a different mycobacterial species

Mycobacterium indicus pranii (M. pranii)[124] is a nonpathogenic atypical mycobacterium historically exploited for its efficacy against leprotic infections.[125] However, it shares highly antigenic forms of the Proline-Glutamate/Proline-Proline-Glutamate (PE/PPE) family of proteins with *M. tb*, thus suggesting efficacy in granting immunity against *M. tb*.[126] Animal models have demonstrated the safety and preventative effects of heat-killed *M. pranii*.[45,127] In fact, dynamic Th1 responses with greater secretion of IL-12 and IFN-y cytokines in concomitance with the influx of CD4+ and CD8 + T cells in the lungs were observed in animals subcutaneously injected with heat-killed *M. pranii*.[45,127] Furthermore, the apoptotic process and autophagy of infected macrophages was significantly accelerated; thereby, facilitating antigen presentation in the *M. pranii* -vaccinated group .[128] As a result, the *M. pranii* -vaccinated group portrayed markedly reduced lung pathology and bacterial burden as well as enhanced survivability.[45] Interestingly, *M. pranii* can be administered via the mucosal route, inducing more powerful Th1 immune responses, increased localization of CD4+ and CD8 + T cells in the lungs, activation and maturation of dendritic cells (DCs), and enhancing migration of bone marrow-derived dendritic cells (BMDCs) via upregulation of CCR7.[45,129,130] Further studies were conducted to explore *M. pranii*'s immunotherapeutic role, suggesting enhanced bacterial clearance and improved lung pathology among guinea pig models[131]. Finally, Sharman et al demonstrated the efficacy of intra-dermal injections of *M. pranii* in conversion of sputum culture in patients with advanced pulmonary TB.[46]

Table 1. Summary of current anti-TB vaccine candidates according to tuberculosis vaccine initiative.

Vaccine	Components	Development Status	Studies
Novel anti-TB vaccine			
Mycobacterium incidus pranii (MIP)	A nonpathogenic atypical mycobacterium	Phase III	• Safe and induced prominent Th1 responses with heat-killed MIP in mice and guinea pigs[45] • Enhanced bacterial clearance and improved lung pathology in guinea pig model[46] and advanced pulmonary TB patients[46]
Mycobacterium vaccae (M. vaccae)	Heat-killed *M. vaccae*, a nonpathogenic mycobacterial species	Phase III	• Effectively control *M. tb* infection in animal models[47,48] • Failed to provide protective benefits as a single-dose regimen in human trials[49,50] • Well-tolerated and immunogenic in three-dose and five-dose regimen in healthy[51] and HIV-infected human[52,53]
RhMCV/TB	Recombinant rhesus macaques Cytomegalovirus vectors expressing *M. tb* antigens	Preclinical	• Effective in TB prevention in rhesus macaque model[54]
ChadOx1/PPE15	Recombinant chimpanzee Adenoviral vector expressing *M. tb* antigens	Preclinical	• Intranasal administration induced differentiation of lung parenchymal naïve CD4+ and CD8 + T cells to protective phenotype CXCR3+ KLRG1- in mice[55]
AEC/BC02	Fusion of Ag85B and ESAT-6/CFP-10 antigens in CpG/ aluminum salt-based adjuvant	Phase I	• Reduced bacterial load in guinea pig model[56] • Dose-dependent potency in stimulation of IFN-γ response and effective in latent or active disease[57]
H1:IC31	Fusion of ESAT6 and Ag85B antigens in IC31 adjuvant system	Phase I	• Safe and immunogenic in murine models[58] • Safe in human adults[59,60] and HIV-infected individuals[61] • Two low-dose regimens optimally induced polyfunctional CD4 + T cells in healthy adolescents[62]
M72/AS01E	Fusion of *M. tb*32A and *M. tb*39A antigens in AS01 adjuvant system	Phase II	• Safe and immunogenic in BCG-vaccinated infants,[63] healthy HIV-infected[64] and *M. tb*-infected adults[65] • Render 54% protection against reactivation in TB-latent adults[66]
RUTI	Polyantigenic liposomal expressing mycobacterial latent antigens	Phase II	• Reduce bacterial burden and macrophage infiltration in granulomas, promote strong IFN-γ secretion in Th1 immune responses in murine models, and produce a balanced Th1/Th2 response in addition to M. tb antigen-specific IgG antibodies[67–70] • Safe and immunogenic in healthy adults[71] • Safe and immunogenic with a low dose vaccine in latent TB adults[72]
Recombinant BCG strain			
BCG-Zmp1	Deletion of zmp1 gene in M. bovis BCG strain	Preclinical	• More intense immune responses against *M. tb* compared to wild-type BCG in mice[73] • Safe and immunogenic in guinea pig model[74]
SapM:TnBCG	Deletion of SapM gene from parental M. bovis BCG strain	Preclinical	• Robust Th1 immune response, decline in bacterial load, and improve long-term survival compared to parental BCG in mice[75]
CysVac2	Recombinant BCG expressing Ag85B-CysD fusion protein	Preclinical	• Steady reduction in bacterial load in heterologous prime-boost with BCG-CysVac2[76] • Robust immunity prior to and after *M. Tb* exposure with induction of polyfunctional CD4 + T cells[77] • Profound Th1 response when CysVac2 formulated with Advax adjuvant[78]
VPM1002	Recombinant BCG replacing urease C gene with listeriolysin O	Phase III	• Acceptable safety profile in animal models and SCID mice[79,80] • Highly efficacious induction of Th1 responses and bacterial burden reduction compared to BCG-control[80–83] • Safe and efficacious in prevention of TB in newborns[84]
BCG Booster Vaccines			
BCG revaccination	Wild-type M. bovis BCG strain	Phase II	• Increase magnitude of a robust CD4 + T cells response with no significant affect to the response rate of CD4 + T cells in homologous BCG prime-boost regimen[85] • Confer 45.4% efficacy against *M. tb* infection in BCG-primed group[86] • No additional benefits upon homologous BCG prime boost in two large-scale randomized trials[87,88]
Ad5Ag85A	Adenovirus type 5 expressing Ag85A antigen	Phase I	• Superior efficacy of heterologous BCG-prime Ad5Ag85A-boost regimen among mice,[89] cattle,[89] and goat[90] • Greater degree of protection against TB seen in bovine model[91] • Administered intranasally, elicited significant T cells responses in the lung with better protection following pulmonary challenge[92]
Ad5-CEAB	Adenovirus type 5 expressing CFP10, ESAT6, Ag85A, and Ag85B antigen in a mixture rather a fusion protein	Unknown	• Antigen-specific T cell responses were significantly amplified in heterologous prime-boost regimen[93]

Table 1. (Continued).

Vaccine	Components	Development Status	Studies
Ad35-TBS (AERAS-402)	Adenovirus type 35 expressing Ag85A, Ag85B, and TB10.4 antigens	Unknown	• Strong CD8+ and CD4 + T-cell response in a dose-dependent manner in murine model[94] • Heterologous prime-boost regimen greatly induces multifunctional CD4+ and CD8 + T cells[95]
Ga*M. tb*Vac	Fusion of Ag85A and ESAt6-CFp10 with dextran-binding domain fixated on dextran and mixed with a dextran core/CpG ODN adjuvant system	Phase I	• Heterologous prime-boost regimen considerably enhanced antigen-specific responses and reduced bacterial burden compared to BCG alone and homologous Ga*M. tb*Vac-Ga*M. tb*vac regimen[96] • Safe and efficacious as a heterologous prime-boost regimen in human adults[97]
H4:IC31 (AERAS-404)	Fusion of TB10.4 and Ag85B in IC31 adjuvant system	Unknown	• Safe and protective against *M. tb* challenge in murine model[98] • Safe and optimal responses at low dose in human model[99,100]
H56:IC31	Fusion of Ag85B, ESAT-6, and Rv2660c in IC31 adjuvant system	Phase II	• Safe and effectively reduce bacterial burden in BCG-primed mice[101] and nonhuman primates[102,103] • Induce polyfunctional CD4 + T cell responses at low dose[104] • Induce robust immune responses with three doses of H56:IC31 in QFT-negative individuals whereas no additional benefits were seen from the third dose in QFT-positive individuals[105]
ID93/GLA-SE	Fusion of Rv1813, Rv2608, Rv3619, and Rv3620, combined with glucopyranosyl lipid adjuvant in oil-in-water stable emulsion	Phase I	• Heterologous BCG-prime ID93/GLA-SE-boost regimen enhanced survival in guinea pig model[106,107] and against a virulent *MTb* strain in the mouse model[108] • Induced polyfunctional CD4 + T cells double expressing either cytokines CD154+ IFN-y+ or CD154+ TNF-a+ in mice[109] and TB naïve human[110] • Tuberculin Skin Test is not compromised in ID93/GLA-SE vaccinated animals[111]
DAR-901	Inactivated *M. tb* SRL172 strain	Phase II	• Safe and immunogenic with superior protection compared to BCG alone in BCG-DAR-901-vaccinated mice[112] and human adults[113,114]
***MTB*VAC**	Live-attenuated *M. tb* with deletion of virulence factors, fadD26 and phoP	Phase II	• Safe in SCID mice and guinea pigs[115] • No effect on growth and development in newborn mice[116] • Similar safety profile to BCG in adults and infants[117] • Reduce bacterial burden and enhance survival in mice,[118] goats,[119] and nonhuman primate[120] • Greater multifunctional CD4 + T cell responses with acceptable safety profile in humans[121] • BCG-prime *M. TB*VAC-boost regimen confers greater protection in guinea pigs compared to BCG alone[122] • Intranasal administration of heat-killed *M. TB*VAC induce profound humoral and cellular responses systemically and locally in BCG-prime animals[123]

Another mycobacterium species that has been exploited for therapeutic purposes is *Mycobacterium vaccae* (*M. vaccae*).[47,132] In numerous studies, *M. vaccae* has consistently demonstrated its efficacy against *M. tb*, possibly by inducing a Th1-biased response while suppressing Th2.[47,48] In subsequent human trials, *M. vaccae* failed to provide protective benefits as a single dose regimen,[49,50] but three and five-dose regimens of *M. vaccae* were well-tolerated with minimal adverse reactions and conferred protection against *M. tb* among healthy[51] and HIV-infected subjects.[52,53] Notably, PPD skin test conversion and alteration of HIV viral load were not observed in either regimen.[51–53] *M. vaccae* vaccines are available in either injectable or oral form (Table 1). The injectable form was investigated as a single therapeutic vaccine agent with protective capacity against pulmonary TB in mice.[133] Nevertheless, it is frequently used in conjunction with immunotherapy in human trials, substantially enhancing TB immunotherapy with 68% clearance of sputum smear compared to 23.1% in placebo.[134] Therefore, *M. vaccae* is an anti-TB vaccine currently in phase III of clinical trials which has produced phenomenal results.

A novel TB vaccine based on insertion of an antigenic protein into a viral vector

Besides attenuating similar pathogens to elicit an immune response, considerable effort has been made to utilize a viral vector in a new vaccine. The rhesus macaque Cytomegalovirus/TB vaccine (RhMCV/Tb) contains vectors that express nine different *M. tb* proteins[54] (Table 1). Among the nine proteins, antigen-85A (Ag85A) was the most immunogenic, capable of eliciting and maintaining high-frequency T cell responses, especially the effector memory phenotype CD8+ and CD4 + T cells.[54] Furthermore, the Ag85A-specific T cell response produced both TNF-α and IFN- γ cytokines, which led to a more robust and longer-lasting immune response compared to one generated by the traditional BCG vaccine.[54] The overall disease and bacterial burden were also significantly

lower in the RhCMV/TB-vaccinated group when compared to the BCG-vaccinated and control group .[54] Intriguingly, the objective data also suggest that BCG-induced inflammation suppressed several protective genes, namely MMP8, CTSG, and CD52, and offset the protective innate immune responses induced by RhCMV/TB alone, thereby curtailing the immune response in RhCMV/TB regimen preceded by BCG vaccination .[54] Therefore, RhCMV/TB vaccination alone suffices in mounting a robust immune response and conferring immunity against *M. tb*.

Like the RhCMV/TB vaccine, ChadOx1/PPE15 is a vaccine comprised a chimpanzee adenovirus expressing a mycobacterial antigen-encoding vector.[55,135,136] Among the expressing antigens, PPE15 was most immunogenic, thus, its presence on ChadOx1 affords significant protective immunity which manifests in reduced *M. tb* bacterial load.[55] However, immunity granted by the ChadOx1/PPE15 vaccine depends on the administration route. Intranasal administration elicited differentiation of lung parenchymal naive CD4+ and CD8 + T cells into the protective CXCR3+ KLRG1- phenotype, while intramuscular administration induced the CX3CR1 + KLRG1+ phenotype, which is predominantly found in blood vessels and incapable of migrating to infected lung tissue.[55] Notably, administration of the ChadOx1/PPE15 vaccine to mice already primed with BCG vaccine elicited a greater immune response than in mice given only BCG, as measured by a larger concentration of CD4+ cells post vaccination.[55] Conversely, a prominent CD8 + T cell response was observed in ChadOx1/PPE15-vaccinated mice without prior BCG vaccination.[55] Nevertheless, both vaccination regimens were able to provide superior protection compared to their respective control group.

Finally, subunit vaccines represent the third class of vaccines that might supplement BCG vaccination (Table 1). This vaccination class isolates immunogenic antigens from bacteria, viruses, or fungi then fuses them to a nonimmunogenic adjuvant. In TB research, the most promising subunit vaccines include AEC/BC02, H1/IC31, M72/AS01E, and RUTI.

A novel TB vaccine based on construction of a fusion protein

A novel vaccine constructed via the fusion of *M. tb*-specific antigens Ag85B and ESAT-6/CFP-10 (AEC), and adjuvanted by BCG CpG and aluminum salt (BC02), the AEC/BC02 vaccine was first introduced and proven effective in reducing the bacterial load in guinea pigs by Chen et al..[56,57] Additionally, Lu et al. uncovered the dose-dependent relationship between AEC/BC02 vaccination and the induction of a highly antigen-specific IFN-γ response.[57] Interestingly, the AEC/BC02 vaccine was inferior to the BCG vaccine in terms of prevention; however, it substantially reduced bacterial burden and gross pathology in latent infection[57] .

Another notable subunit vaccine is H1/IC31, comprised of fusion proteins ESAT6 and Ag85B, formulated in the IC31 adjuvant system which is composed of a leucine-rich peptide and oligodeoxynucleotide known as ODN1a (Table 1).[58] Studies revealed a two-dose regimen of H1/IC31 vaccine was safe in human adults irrespective of their BCG status, prior

M. tb infection,[59,60] or HIV status.[61] These findings are in concordance with the data from a recent phase II trial, in which a two-dose regimen of 15 μg H1-IC31 vaccine optimally evoked vaccine-specific durable polyfunctional CD4 + T cells in healthy *M. tb*-infected and *M. tb*-uninfected adolescents.[62]

Similarly, M72/AS01E is a subunit vaccine comprised a fusion of mycobacterial antigens *M. tb*32A and *M. tb*39A, formulated with the AS01 adjuvant system (Table 1).[137] Many studies described the clinical safety profile of the vaccine and its long-lasting polyfunctional CD4 + T cells expressing IFN- γ, IL-2, and TNF-α among BCG-vaccinated infants,[63] as well as healthy HIV-infected[64] and *M. tb*-infected adults.[65] M72/AS01E has been shown to render 54% protection against disease activation in *M. tb*-infected adults.[66] Furthermore, Kumarasamy et al. observed a greater vaccine-induced seroconversion rate with a steep increase of seroconversion upon administration of a second dose, thereby permitting optimal resistance against *M. tb*.[138]

In contrast with the aforementioned subunit vaccines, which are preventative vaccines, RUTI has been studied for its therapeutic efficacy against TB. RUTI is a poly-antigenic liposomal vaccine comprised antigens corresponding to latency, expressed by *M. tb* under stressful conditions.[139] RUTI has been shown to reduce bacterial burden and macrophage infiltration in granulomas, promote strong IFN-γ secretion during Th1 immune responses in murine models, and produce a balanced Th1/Th2 response in addition to *M. tb* antigen-specific IgG antibodies.[67–70] These preclinical trials suggest that RUTI can successfully induce a well-balanced immune response via promotion of protective cellular immune responses and prevention of excessive inflammation. In human trials, a double-blind, randomized, controlled phase I study has concluded the tolerability and immunogenicity of RUTI in healthy adults.[71] Similarly, a randomized, double-blind, phase II trial demonstrated the safety profile and conferred immunity among adults with latent TB treated with a 1-month isoniazid regimen.[72] Additionally, RUTI vaccine did not impair CD4 + counts and HIV viral loads, thus, the course of HIV progression in HIV-positive subjects remained unaltered.[72]

Method 2 – development of a novel recombinant vaccine derived from an existing BCG strain

Growing evidence supports the efficacy of genetically modified parental BCG strains known as recombinant BCG vaccines. Currently, there are four recombinant BCG vaccines under investigation to replace the parental BCG strain (Table 1).

The first of these vaccines is BCG-Zmp1, a vaccine still in its preclinical phase.[73] The BCG-Zmp1 vaccine is an attenuated *M. bovis* BCG vaccine with a knock-out mutation of the zmp1 gene, which encodes for the zinc metalloprotease Zmp1.[73] Johansen *et al.* discovered that mice immunized with zmp1-deficient BCG strain could mount an intense immune response through the proliferation of antigen-specific T-cells and increased secretion of cytokines, particularly IFN- γ, when compared to mice vaccinated with wild-type BCG.[73] It is worth mentioning that enhancement in the BCG-Zmp1 vaccine's immunogenicity did not come at the expense of diminished persistency or heightened pathology of *M. bovis*.[73]

Likewise, a study conducted on guinea pig models ascertained the vaccine's safety and efficacy compared to BCG-vaccinated and non-vaccinated control groups, as measured via bacterial load in the lungs and spleen.[74] Moreover, survival time was substantially extended among immunocompromised mice vaccinated with BCG-Zmp1 than with BCG alone.[74] Therefore, the BCG-Zmp1 vaccine confers superior protection against *M. tb* due to its high immunogenicity and improved safety profile when compared to traditional BCG vaccination in murine models.

The second vaccine candidate in preclinical trials is SapM: TnBCG, which contains a SapM gene deletion from the parental *M. bovis* BCG strain (Table 1).[140] SapM gene encodes secreted acid phosphatase, which primarily interrupts host macrophage maturation and lysosome-phagosome fusion, thus playing a critical role in *M. tb*'s pathogenesis.[75] Compared to parental BCG, mice vaccinated with SapM: TnBCG exhibit a more robust Th1 immune response with a decline in bacterial load and increase in long-term survival.[75] Interestingly, while autophagy, maturation, and lysosome-phagosome fusion were not significantly varied between the two strains, a greater degree of DC migration and activation in the lymph nodes was observed among SapM:TnBCG-vaccinated mice.[75]

The third preclinical vaccine candidate is CysVac2, a recombinant BCG vaccine expressing a fusion protein containing the antigen Ag85B and CysD, a protein expressed during persistent infection with *M. tb* (Table 1).[76] CysVac2 vaccination in mice elicited a significant influx of innate immune cells, particularly neutrophils, macrophages, and DCs, at the injection site. Ag85B-specific CD4 + T cell numbers were increased in the draining lymph node and the spleen.[77] Furthermore, a greater number of IFN-γ secreting cells were seen in CysVac2 vaccinated mice when compared to BCG-vaccinated and unvaccinated control groups. Thus, CysVac2 vaccine conferred greater resistance against *M. tb* infection while significantly reducing pulmonary bacterial load.[77] Boosting previously BCG-vaccinated mice with CysVac2 revealed a steady reduction in bacterial load compared to both the unvaccinated group and BCG-group boosted only with an adjuvant.[77] This phenomenon is thought to be the result of increased CysD-specific CD4 + T cell numbers, which secrete IFN-y and TN-α in response to the expression of CysD during late-stage infection. The CysVac2-prime and boost regimen confer sustainable protective immunity both prior to and after *M. tb* exposure.[77]

Lastly, VPM1002 is a recombinant BCG vaccine in which the listeriolysin O encoding gene (hly) of *Listeria monocytogenes* replaces the urease C gene in BCG (Table 1).[141] Hly gene expression in BCG facilitates cytosolic release of antigens and mycobacterial DNA along with consequent activation of autophagy, antigen presentation, immune system activation, and apoptosis.[142] The safety profile of VPM1002 was comparable to BCG in animal models including both SCID and healthy mice, guinea pigs, and newborn rabbits.[79] In fact, the VPM1002 strain is less virulent and never disseminates into the lungs in VPM1002-vaccinated mice.[81] Moreover, VPM1002 vaccination conferred remarkable protective efficacy with significant Th1 response and bacterial load reduction compared to the BCG control group.[80–83] In a phase I trial, both single-dose and three-dose regimens of VPM1002 were well-tolerated, stimulating marked quantities of polyfunctional T cells co-expressing TNF-α, IFN-γ, and IL-2 against *M. tb*.[141] Similarly, a phase II trial concluded the comparable safety and efficacy of VPM1002 to BCG in newborns.[84] Since VPM1002 is also effective in clearing *M. tb* among *M. tb*-exposed mice,[83] a phase III trial of post-exposure vaccination with VPM1002 is currently underway in India.

Method 3 – development of a BCG vaccine augmenting booster vaccine

The concept of BCG revaccination as a booster in BCG-primed populations has been investigated over the past decades with conflicting results. One study demonstrated that BCG revaccination increased the magnitude of the immune response with robust multifunctional BCG-specific CD4 + T cells; however, this did not alter the response rate of CD4 + T cells.[85] Likewise, Nemes *et al.* concluded that BCG revaccination confers 45.4% efficacy against *M. tb* infection.[86] Conversely, two large-scale randomized trials revealed no additional benefits of BCG revaccination against TB.[87,88] These incongruent findings may be due to the geographical variation and mutation of BCG strains, leading to differential responses and efficacies. Nonetheless, priming with BCG produced mild to moderate injection site reactions which were temporary and resolved without any sequelae among adolescents with initial BCG-administration at birth.[85,86] More recent attempts to boost initial vaccination have led to the development of novel booster vaccines derived from either a viral vector, fusion protein, or new bacterial species (Table 1).

Adenovirus type 5 (Ad5), owing to its inherent property of evoking a dynamic immune response, has been used as a vector to express mycobacterial antigen Ag85A in efforts to control *M. tb* infection. Compelling evidence has demonstrated superior effectiveness of the BCG-prime Ad5Ag85A-boost regimen against TB in mice,[89] cattle,[89] and goat compared to BCG alone.[90] Of note, animals who received the heterologous prime-boost regimen consistently showed attenuated bacterial burden as well as attenuated lung and lymph node lesions.[89,90,143] Furthermore, the frequency of Ag85A-specific CD4 + T cells was significantly increased, hence conferring a greater degree of protection against TB in the bovine model.[91] Interestingly, immune responses resulting from Ad5Ag85A vaccination are administration route-dependent (Table 1). In the murine model, the intramuscular route induced robust Ag85A-specific T-cell responses in the spleen and lung interstitial with little to no protection against pulmonary *M. tb*.[92] Conversely, the intranasal route elicited more significant T cell responses in the lungs, thereby promoting better protection following pulmonary challenge[92]

Ad5-CEAB is another recombinant adenovirus vector expressing *M. tb* antigens (Table 1). In the BCG-prime Ad5-CEAB-boost regimen, the antigen-specific T cell responses in mice were significantly potentiated with an elevation of the anti-mycobacterial cytokines IFN-γ, TNF-α, and IL-2 when

compared to BCG alone.[93] Hence, it may be promising in providing resistance against *M. tb* in BCG-vaccinated group.

Like the aforementioned vaccine, the Ad35-TBS vaccine (AERAS-402), which is a recombinant adenovirus 35 vector expressing a different set of *M. tb* antigens, elicited strong CD8 + and CD4 + T-cell responses in a dose-dependent manner among murine models.[94] Furthermore, intramuscular Ad35-TBS produced more efficient and robust T-cell responses than intranasal immunization (Table 1).[95] Nonetheless, both vaccination routes led to improvements in lung histology when compared to non-vaccinated mice.[95] Furthermore, Abel *et al.* revealed the promising results of AERAS-402 vaccination in QuantiFERON Gold (QFT) negative adults through induction of multifunctional CD4+ and CD8 + T cells.[144] Following BCG priming, the AERAS-402 vaccine greatly increased the number of multifunctional CD4 + T cells producing IFN- γ, TNF-α, and IL-2 along with the number of multifunctional CD8 + T cells producing IFN- γ, perforin, and CD107a.[95] Collectively, the immune profile generated by heterologous BCG-prime AREAS-402-boosting confers optimal immunity against TB in human adults when compared to AREAS-402 vaccinated adults alone.[95]

GaMtbvac is a sophisticated vaccine comprised Ag85A and ESAT6-CFP10 fused with a dextran-binding domain fixated on dextran along with an adjuvant system containing a DEAE-dextran core and the TLR9 agonist, CpG oligodeoxynucleotides (Table 1).[96] GaMtbvac portrays powerful immunogenicity and can generate high antigen-specific antibody titers and IFN- γ. GaMtbvac -vaccinated mice were found to effectively control the disease with significantly lower bacterial loads in the lungs and spleen compared to non-vaccinated mice.[96] Furthermore, prime-boost regimens in the murine model were also investigated for their efficacy against TB. A homologous prime-boost regimen using GaMtbvac inadequately reduced bacterial burden in the lungs and spleen compared to BCG vaccination alone.[96] Conversely, heterologous BCG-prime GaMtbvac -boost, considerably enhanced antigen-specific responses while reducing bacterial burden when compared to homologous vaccination with GaMtbvac and BCG alone.[96] The safety and effectiveness of heterologous regimens were further assessed in BCG-vaccinated human adults.[97] Adverse effects associated with GaMtbvac were considered mild and transient, resolving spontaneously. GaMtbvac vaccine-induced Ag85A-specific T-cell responses resulted in the secretion of IL-2 and TNF-α soon after injection, whereas ESAT6-CFP10-specific T-cell responses occurred during later stages with hallmark TNF-α, IL-10, IL-17, and IL-9 elevation among BCG-vaccinated adults.[97] Of note, GaMtbvac also stimulates pronounced secretion of vaccine-specific IgG; its role against *M. tb, however,* is yet to be elucidated.[97]

H4:IC31, also known as AERAS-404, is comprised of a TB10.4 and Ag85B fusion protein adjuvanted in a mixture of the leucin-rich peptide and oligodeoxynucleotide, ODN1a (Table 1).[98] HC:IC31 was proven safe and adequately protective against *M. tb* challenges with a significant reduction in bacterial burden among murine models.[98] Protection resulted from antigen-specific polyfunctional CD4 + T cells co-

expressing IFN- γ, TNF-α, and IL-2.[98] Furthermore, H4:IC31 showed an acceptable safety profile in human adults with prior BCG vaccination.[145] Interestingly, the H4:IC31 vaccine induced the highest antigen-specific T cell response among the low-dose group when compared to the placebo and high-dose groups in mouse[145] and human models.[99] Thus, H4:IC31 shows promise as a BCG booster with superior efficacy at a low dose.[99,100]

H56:IC31 is a novel subunit vaccine composed of a Ag85B, ESAT-6, and Rv2660c fusion protein in an IC31 adjuvant system (Table 1).[101] Many studies reveal the immunogenicity and efficacy of this vaccine in reducing bacterial burden and lung pathology among BCG pre-vaccinated mice[101] and non-human primates.[102,103] H56:IC31 has an acceptable safety profile and shows favorable differentiation of antigen-specific polyfunctional CD4 + T cells expressing IFN- γ, TNF-α, and IL-2.[104] Furthermore, H56:IC31 yielded greatest results at lower doses in BCG-vaccinated human adults.[104] Vaccination with H56:IC31 at low doses (either 5 μg:500 nmol or 15 μg:500 nmol), induced high frequency and durable antigen-specific polyfunctional CD4 + T cell responses, irrespective of infection status. Furthermore, the number of vaccinations was dependent on the patient's QFT status. In QFT negative individuals, three doses of H56:IC31 conferred robust and durable polyfunctional CD4 + T cell, whereas no additional benefits were seen from the third immunization in QFT positive individuals.[105]

ID93/GLA-SE is a subunit vaccine comprised a fusion of four mycobacterial antigens combined with glucopyranosyl lipid adjuvant, a TLR4 agonist, and emulsified in an oil-and-water solution (Table 1).[106] Previous studies had demonstrated the safety and efficacy of the vaccine in mice,[107,109] and nonhuman primates.[106] Many studies describe the ability of ID93/GLA-SE to induce differentiation of CD4 + T cells into polyfunctional CD4 + T cells double expressing either CD154 + IFN- γ+ or CD154+ TNF-α+ cytokines in mice[109] and in TB naïve humans.[110] Therefore, ID93/GLA-SE alone can significantly reduce bacterial burden and increase survivability in mice[109] as well as significantly increase antibody responses which mediate NK cell degranulation/activation and THP1 monocyte mediated antibody-dependent phagocytosis in humans.[110] Furthermore, ID93/GLA-SE, when administered after BCG-priming, can enhance survival against TB in the guinea pig model[106,107] and against *M. tb* K, a hyper-virulent strain, in the mouse model.[108] Interestingly, the tuberculin skin test (TST), a delayed-type hypersensitivity (DTH) reaction in response to BCG vaccination and *M. tb* infection, had been uncompromised in ID93/GLA-SE-vaccinated animals. Therefore, in contrast to BCG vaccine, TST's integrity in ID93/GLA-SE vaccinated specimens remained intact, thus preserving its utility in identifying potential exposure to *M. tb.*[111]

DAR-901 is an inactivated whole-cell mycobacterial vaccine manufactured from the SRL172 strain whose use as a booster is under investigation.[112] DAR-901 is comparably safe and immunogenic, conferring superior protection against *M. tb* when compared to BCG alone in both murine models[112] and human adults[113,114] due to stimulation of IFN-γ production (Table 1). Nonetheless, studies establishing the efficacy of the vaccine presented mixed results. DAR-901 induces a smaller

magnitude polyfunctional CD4 + T cell response with no significant differences in T cell cytokine production when compared to the BCG booster vaccine. Furthermore, CD4 + T cells induced by the DAR-901 vaccine were short-lived and nonresponsive to mycobacterial antigens from *M. tb* lysate.[114] Conversely, von Reyn et al. demonstrated that 1 mg of DAR-901 could induce both cellular and humoral responses, accompanied by substantial IFN-γ production in the presence of *M. tb* lysate among healthy adults with prior BCG vaccination.[113] Furthermore, IFN-γ assay remained negative after 3 doses of DAR-901. Thus, the booster can be employed as a preventative vaccine without interrupting *M. tb* screening[114] .

MTBVAC is a live-attenuated *M. tb* vaccine with genetic deletion of two major mycobacterial virulence factors: fadD26 and phoP.[146] MTBVAC was deemed safe when tested in immunocompromised mice and guinea pigs.[115] Congruently, Aguilo *et al.* observed that MTBVAC did not affect growth and development, thus suggesting its safety in newborn mice.[116] Furthermore, *M. TB*VAC had a similar safety profile to BCG when administered subcutaneously in adults and in infants.[117] Many studies among mice,[118] goats,[119] and nonhuman primates[120] have investigated MTBVAC protectivity against *M. tb* as quantified by bacterial load, lung pathology, and survival rate. Overall, *M. TB*VAC confers superior protection in mouse models when compared to BCG vaccination.[116,147] Furthermore, the first phase I trial in humans demonstrated excellent safety, similar immunogenicity, and a greater polyfunctional CD4 + T cell response when compared to BCG vaccination (Table 1).[121] Clark *et al.* demonstrated greater protection against *M. tb* among BCG-primed MTBVAC-boosted guinea pigs when compared to those vaccinated with BCG alone.[122] Surprisingly, heat-killed MTBVAC, when administered intranasally, could also induce profound humoral and cellular responses both systemically and locally in BCG-primed animals.[123] Altogether, evidence suggests that MTBVAC confers greater immunity than BCG when administered alone and even more so in the BCG- MTBVAC prime-boost regimen.

Conclusion

BCG vaccination remains crucial during childhood in much of the world. While its efficacy has been historically challenged, newer research conducted with stronger parameters and controls have shed light into how vaccine strain variations may affect efficacy and the immune system upon administration. As the mechanism of action of the BCG vaccine continues to be discovered, more attention to the strain of BCG vaccine used, as well as the epigenetic changes it may elicit, might allow us to better time and control vaccination-induced immune responses. Subsequent efforts toward full eradication have led to creative ways of reconstituting an old vaccine into a newer, more efficacious form, and the development of the next generation of vaccines and adjuncts. promises to be a growing area of research that might lead to a more effective and consistent vaccine.

ORCID

Vishwanath Venketaraman (iD) http://orcid.org/0000-0002-2586-1160

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