

REVIEW



## The current status, challenges, and future developments of new tuberculosis vaccines

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### ABSTRACT

*Mycobacterium tuberculosis* complex causes tuberculosis (TB), one of the top 10 causes of death worldwide. TB results in more fatalities than multi-drug resistant (MDR) HIV strain related coinfection. Vaccines play a key role in the prevention and control of infectious diseases. Unfortunately, the only licensed preventive vaccine against TB, bacilli Calmette-Guérin (BCG), is ineffective for prevention of pulmonary TB in adults. Therefore, it is very important to develop novel vaccines for TB prevention and control. This literature review provides an overview of the innate and adaptive immune response during *M. tuberculosis* infection, and presents current developments and challenges to novel TB vaccines. A comprehensive understanding of vaccines in preclinical and clinical studies provides extensive insight for the development of safer and more efficient vaccines, and may inspire new ideas for TB prevention and treatment.

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### Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* complex (MTBC) whose natural history traces back 70,000 years ago.<sup>1</sup> In 2016, there were an estimated 10.4 million new TB cases globally, 1.3 million TB-associated deaths, and an additional 374,000 deaths from TB patients with co-infection of HIV-MTBC.<sup>2</sup> Although the number of TB deaths decreased, TB remained one of the top 10 causes of death worldwide. To address TB challenges, the World Health Organization (WHO) has introduced the “End TB Strategy”, which indicates that the phased strategy has progressed from controlling the prevalence of TB (before 2015) to ending the prevalence of TB (2016–2035). The target for 2035 is a 95% reduction in TB deaths and a 90% reduction in the TB incidence rate compared to levels in 2015, and the target for 2050 is less than one TB patient per million people each year.<sup>2</sup> However, challenges of controlling TB infection and developing more effective vaccines remain, and concerted effort will be required to achieve the global TB control strategy formulated by WHO.

Vaccination is the most effective way to prevent and control TB. As early as 1890, Robert Koch proposed the first immunotherapy against TB.<sup>3</sup> However, bacilli Calmette-Guérin (BCG) is the only licensed preventive vaccine against TB and has existed for 96 years.<sup>4</sup> Although BCG vaccination can effectively protect infants and young children from TB infection, and prevent severe diseases such as disseminated TB and tuberculous meningitis, it has variable efficacy against pulmonary TB, particularly in adults.<sup>5</sup> Clinical trials conducted on adults in the United Kingdom (UK) have shown that the protective effect of BCG was 60 to 80%. However, studies performed with South

African infants have shown that BCG had no protective effect.<sup>6</sup> Reasons for this variability could be explained by several factors, including genetic differences, environmental factors, coinfection, production methods, the diversity of BCG strains, and the impact of poverty and nutrition.<sup>7,8</sup>

The BCG vaccine does not effectively stimulate the T cell mixed population (especially for CD8<sup>+</sup> T cells), and the immuno-protective effect of BCG vaccination only persists for 10 to 15 years. Researchers worldwide have reached consensus that the development of more effective vaccines is necessary to compensate for the limitations of the BCG vaccine. With rapid developments in immunology and molecular biology, some novel TB vaccines have become available, including inactivated vaccines, recombinant live vaccines, attenuated live vaccines, subunit vaccines, and DNA vaccines. At present, there are 25 new TB vaccines in clinical trials, of which three vaccines (Vaccae in patients with latent TB infection (LTBI), *Mycobacterium indicus pranii* (MIP)/Mw, and VPM1002) have reached Phase III clinical trials.<sup>9,10</sup> Three vaccines (Vaccae, Utilins, and BCG-PSN) have obtained registration certificates from the China Food and Drug Administration (CFDA, <http://eng.sfda.gov.cn/WS03/CL0755/>) and have been widely used to clinically treat TB in China. However, three vaccines (rBCG30, AERAS-422, and H1:LTK63) have been terminated for their disappointing issues after phase I clinical trials.<sup>11–13</sup> Herein, we review the developmental progress and challenges of these new TB vaccines, and we will also introduce five novel TB vaccines (Utilising, *M. smegmatis*, AEC/BC02, BCG-PSN, and GX-70) for the first time, which may give a fresh perception into the TB vaccine research field.

## TB infection and immunology

According to the WHO report, almost one third of the world-wide population has been infected by *M. tuberculosis*. However, only 10% of infected individuals develop an active disease state with the appearance of clinical symptoms, suggesting that the immune system can control the infection and prevent active disease in the majority of the population.<sup>8</sup> Although the interactions between the host and *M. tuberculosis* are still unclear, it is generally believed that innate immunity and adaptive immunity play critical roles in controlling *M. tuberculosis* infection in humans.<sup>14</sup>

### Innate immunity

The innate immune cell types associated with *M. tuberculosis* infection are macrophages, dendritic cells (DCs), neutrophils, and natural killer (NK) cells.<sup>15</sup> At the portal of entry, *M. tuberculosis* is first recognized and controlled by macrophages and DCs through pattern recognition receptors (PRR) such as Toll-like receptor (TLR), nucleotide-binding oligomerization domain (NOD)-like receptor (NLR), C-type lectin receptor (CLR), and retinoic acid-inducible gene I (RIG-I)-like helicases receptor (RLR).<sup>16</sup> Once recognized, *M. tuberculosis* will be killed by macrophages via several mechanisms, including phagocytosis, inflammasome activation, reactive oxygen species (ROS), autophagy, and apoptosis.<sup>17</sup> However, instead of being digested like other bacteria, *M. tuberculosis* escapes from the killing of macrophages by interrupting the autophagy signaling pathway, inhibiting the fusion of phagosomes and lysosomes.<sup>18</sup> DCs have the ability to present antigens of *M. tuberculosis* to prime naïve T cells, bridging innate and adaptive immunity. Khan N et al. suggested that activated DCs showed a strong liberation of cytokines and nitric oxide, autophagy, and improved migration towards the lymph nodes, which consequently inhibited the intracellular survival of *M. tuberculosis*.<sup>19</sup> Nevertheless, monocyte-derived DCs are not supportive of *M. tuberculosis* dissemination and reproduction.<sup>15</sup>

Besides macrophages and DCs, neutrophils and NK cells are also involved in innate responses at the early stage of *M. tuberculosis* infection through production of nonspecific cytokines and chemokines.<sup>20,21</sup> In the past, neutrophils had been considered as short-lived cells that were essential to eliminate extracellular pathogens.<sup>22</sup> This outdated viewpoint has been revised recently by mounting evidence, which demonstrates that neutrophils can secrete cytokines and effector molecules.<sup>22</sup> Numerous studies have suggested that interleukin-17 (IL-17) and chemokines produced by neutrophils play a vital role in inhibiting *M. tuberculosis* H37Rv strain growth by mediating ROS production and the migration of neutrophils in the early stages of infection.<sup>20,23</sup> NK cells belong to lymphocytes of the innate immune system, and have a beneficial effect on the initial defense against *M. tuberculosis* infection by producing cytokines, chemokines, and perforin.<sup>24</sup> NK cells have an innate memory ability that is associated with the effort to develop therapies and vaccines to improve the initial phases of the immune response against TB.<sup>25</sup>

In addition, a growing number of studies have indicated that pathways initiated by TB vaccines were critical for TB vaccine-

related immunity. It has been shown that vaccination with BCG led to non-specific protective effects against unrelated infections and mortality. BCG vaccination can instruct innate immune cells through the Akt/mTOR (mammalian target of rapamycin)/HIF1 $\alpha$  (hypoxia-inducible factor 1 $\alpha$ ) pathway,<sup>26</sup> cellular metabolism pathway,<sup>27</sup> and NOD1 signaling pathway.<sup>28</sup>

### Adaptive immunity

Adaptive immunity includes both cellular and humoral immunity. Antigens of *M. tuberculosis* are mainly presented by class II major histocompatibility complex (MHCII) to CD4<sup>+</sup> T cells such as T-helper 1 (Th1), Th2, Th17, and T regulatory cells (Tregs). MHCII molecules provide an opportunity for these cells to activate adaptive immune responses. Th1 and Th17 cells are the key effector CD4<sup>+</sup> T cells during TB infection.<sup>29</sup> Our previous studies have demonstrated that Th1 cells were critical for controlling intracellular pathogens including *M. tuberculosis* by secreting interferon gamma (IFN- $\gamma$ ) and activating antibacterial action in macrophages.<sup>30-36</sup> Previous studies have indicated that Th1 cells can secrete IFN- $\gamma$  and tumor necrosis factor alpha (TNF- $\alpha$ ) to activate macrophages to control *M. tuberculosis* infection. The mechanism of action includes reactive oxygen and nitrogen intermediates, lysosomal enzyme attack, antimicrobial peptides, and autophagy, as well as activating downstream antimicrobial effector pathways.<sup>37,38</sup> However, the classical paradigm of IFN- $\gamma$  responses has been challenged by recent studies, which indicate that an increasing IFN- $\gamma$  response in the lungs of mice was more damaging to the host than to the pathogen.<sup>29,39</sup> Th17 cells, a distinct lineage of T cells, secrete several effector cytokines such as IL-17, IL-17F, IL-21, and IL-22.<sup>40</sup> Th17 cells are associated with *M. tuberculosis* infection, but the role of the Th17/IL-17 responses in the human TB protection response remains to be understood. It has recently been documented that Th17 controlled TB infection by secreting IL-17 to recruit neutrophils and IFN- $\gamma$  positive CD4<sup>+</sup> T cells. This synergized the function of the Th1 immune response in the host defense against *M. tuberculosis* by downregulating IL-10 and upregulating IL-12 production in DCs.<sup>41,42</sup> In addition, studies have shown that Th17 was also associated with neutrophilic inflammation and histopathological lesions.<sup>43,44</sup> Although Th1 and Th17 cells play a central role in host protection, additional strategies are needed to improve protective immune responses in vaccine development. Accumulating data have shown that inhibition of immune responses of Th2 and Treg cells could dramatically enhance *M. tuberculosis* clearance and induce superior Th1 responses in mice and humans,<sup>45-48</sup> which suggests that enhancing Th1 immune responses while inhibiting both Th2 and Treg immune responses should be a useful method for developing more effective TB vaccines.

It is well known that CD4<sup>+</sup> T cells are a crucial component of protective immunity against TB. However, an essential role of CD8<sup>+</sup> T cells and B cells should be considered in the design of new vaccines against TB infection. CD8<sup>+</sup> T cells recognize the antigens presented by MHC Class I molecules, which control *M. tuberculosis* infection by releasing cytokines, causing cytotoxicity, and inducing direct microbicidal activity.<sup>49</sup> Although previous studies had an ingrained bias that CD8<sup>+</sup> T

cells were dispensable and not essential for the control of *M. tuberculosis* infection,<sup>49</sup> an increasing number of studies have indicated that CD8<sup>+</sup> T cells contribute to protective immune responses against *M. tuberculosis* infection in animal models and humans.<sup>50,51</sup> Just as there was prejudice against the importance of CD8<sup>+</sup> T cells, the role of humoral immune responses in host defense against TB has been considered marginal, mostly due to the view that antibodies had a miniscule role in eliminating intracellular pathogens. This assumption has been changing in recent years.<sup>38,52</sup> Growing evidence has demonstrated that intradermal BCG vaccination can induce secretion of IgG and IgM recognizing several mycobacterial antigens.<sup>53-55</sup> Further, some of these antibodies could enhance both cellular and humoral immunity against *M. tuberculosis* infection based on various mechanisms, including accelerating phagolysosomal fusion, promoting the clearance of immunomodulatory antigens, and influencing the outcome of mycobacterial infection through their ability to modulate inflammation.<sup>55</sup>

Recently, it has been shown that nontraditional T cells such as mucosal-associated invariant T (MAIT) cells and CD1<sup>+</sup> T cells were potentially important for TB vaccine-induced immunity. MAIT cells were recently identified as a non-classical CD8<sup>+</sup> T cell subset. They have Th1 effector capacity positioning them to play a critical role in the early immune response to *M. tuberculosis*.<sup>56</sup> Furthermore, CD1-restricted T cells can recognize mycobacterial lipids and glycolipid antigens derived from *M. tuberculosis*. Previous studies have demonstrated that CD1-restricted T cells from peripheral blood could be stimulated by autologous immature CD1<sup>+</sup> DCs and respond at a significant magnitude and frequency in asymptomatic *M. tuberculosis*-infected donors.<sup>57</sup>

### New TB vaccines

Since the BCG vaccine was first used, scientific advances have provided understanding of the mycobacterial genetic system, proteomics, and immunology, which have accelerated the development of safer and more effective TB vaccines.<sup>58</sup> TB vaccines were divided into therapeutic vaccines and preventive vaccines based on function. The therapeutic vaccines were used to treat TB patients, while the preventive vaccines were used to prevent MTBC infection and TB development in healthy person or those with LTBI. Currently, there are four strategies for developing a new generation of TB vaccines: (1) Immunotherapeutic vaccine: develop therapeutic vaccines that might synergize with chemotherapy to shorten a treatment course for active TB and prevent TB recurrence; (2) Immunopreventive vaccine: prevent MTBC infection in healthy patients and those with LTBI endogenous activation, as well as exogenous reinfection; (3) Prime-boosting vaccine: boost the limited immunity conferred by BCG to produce stronger and more persistent protection; (4) Priming vaccine: replace BCG with either live recombinant BCG (rBCG) or genetically attenuated MTB vaccines that confer greater safety and protective efficacy.<sup>59</sup> Common to all of these strategies is the cellular immune response, requiring induction of Th1 cells as well as cytotoxic T lymphocyte (CTL) immune responses. BCG, inactivated vaccine, and subunit vaccine tend to stimulate CD4<sup>+</sup> T cell immune responses rather than CD8<sup>+</sup> T cell immune responses, but live

vaccine and DNA vaccine can induce both CD4<sup>+</sup> and CD8<sup>+</sup> T cell immune responses. At present, 25 new TB vaccines have been licensed or are being evaluated in clinical trials (Table 1), and more TB vaccines are still in basic or preclinical research. These TB vaccines can be divided into five categories: inactivated vaccines, recombinant live vaccines (recombinant mycobacterium vaccines, recombinant bacterium vaccines, live virus vaccines), attenuated live vaccines, subunit TB vaccines, and DNA vaccines. The following sections will provide a perspective of the latest progress of new TB vaccines licensed or in clinical trials as well as an overview of vaccine candidates in preclinical studies.

### Inactivated TB vaccines

Inactivated TB vaccines have long been used to prevent *M. tuberculosis* infection and treat TB. The inactivated TB vaccines are composed of whole bacteria that have been inactivated, or their cleavage fragments prepared by physical or chemical methods. These vaccines can induce humoral and Th1-type cellular immune responses to defend against extracellular pathogen infection, and have a better immunotherapeutic effect at controlling TB development.<sup>8,60,61</sup> Inactivated vaccines have several shortcomings, including weaker preventive protection, inability to induce a cytotoxic T lymphocyte response, short immunity period, multiple required inoculations, and high dose. However, inactivated vaccines have advantages in terms of safety, production, and administration, which has led to extensive and rapid development of this type of vaccines. At present, two inactivated TB vaccines (Utilins and Vaccae for TB) have been approved for clinical use, and five inactivated TB vaccines are in clinical trials, including *M. smegmatis* vaccine, Vaccae for LTBI, MIP/Mw, RUTI<sup>®</sup>, and DAR-901.

### Utilins/Mycobacterium phlei F.U.36

*Mycobacterium phlei* is a fast-growing mycobacterium that flourishes at temperatures ranging from 28°C to 52°C. Heat-killed *M. phlei* (termed Utilins or *M. phlei* F.U.36) vaccine was produced first by Chengdu Jinxing Jiankang Pharmaceutical Co., Ltd. (China), and currently by Chongqing Lummy Pharmaceutical Co., Ltd. (China). Utilins was given a new drug certificate from the CFDA (Approval No: S20040068), and was widely used as a therapeutic vaccine or immunomodulator in China. After deep intramuscular injections, Utilins stimulates T lymphocytes to release a variety of cytokines such as IL-2, IL-4, TNF- $\alpha$ , IFN- $\gamma$ , macrophage activating factor (MAF), migration inhibitory factor (MIF), and macrophage cytotoxicity factor (MCF). These cytokines induce and activate macrophages, NK cells, and B lymphocytes to clear pathogens.<sup>62</sup> Previous studies suggested that Utilins could increase TLR4 expression on CD4<sup>+</sup>CD25<sup>+</sup> cells, promote IL-10 release from CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, and inhibit IL-17 secretion from Th17 cells in asthmatic mice.<sup>63,64</sup> To date, evidence has shown that Utilins produced efficacious therapeutic effects in the treatment of asthma,<sup>65</sup> atopic dermatitis,<sup>62</sup> non-small cell lung cancer,<sup>66</sup> reiterative respiratory tract infections,<sup>67</sup> malignant pleural effusion,<sup>68</sup> verruca vulgaris,<sup>69</sup> and condyloma acuminatum,<sup>70</sup> as well as pulmonary TB.<sup>71</sup> Several studies also indicated that the sputum negative conversion rate, foci

Table 1. Vaccine candidates in clinical development.

Vaccine type	Vaccine name	Vaccine composition	S&C <sup>a</sup>	Strategy <sup>b</sup>	Phase	CTR Number <sup>c</sup>	Status <sup>d</sup>
Inactivated TB vaccines	Utilins	Heat-killed <i>M. phlei</i>	FAHGMU	IT	III	ChiCTR-TRC-11001189	Completed
	<i>M. smegmatis</i>	An acellular <i>M. smegmatis</i> vaccine	NICBPB	IT	I	Unknown	Completed
	Vaccae <sup>TM</sup>	Heat-killed whole <i>M. vaccae</i>	AZLBP	IT	III	NCT01979900	Completed
	MIP/Mw	Heat-killed <i>M. indicus pranii</i>	DBT	IT	III	NCT00265226	Completed
Recombinant live vaccines	RUT <sup>®</sup>	Detoxified liposomal fragments of <i>M. tuberculosis</i>	AFSL, Parexel	IT	Ila	NCT00341328	Completed
	DAR-901	Heat-killed <i>M. tuberculosis</i>	DMHC, Aeras, NIAID	B	Ilb	NCT01136161	Not yet recruiting
	VPM1002	rBCG expressing listeriolysin and lacking urease gene	SIIP, SGCCR, VPMG	P	Ilb/III	NCT02711735	Active, not recruiting
	rBCG30	rBCG over-expressing Ag85B	Aeras	P	I	NCT02391415	Active, not recruiting
	AERAS-422	rBCG over-expressing Ag85A, Ag85B, and Rv3407	Aeras, AI, DIM, DPID, SB	P	I	NCT03152903	Not yet recruiting
	MVA85A/	A recombinant strain of modified MVA expressing Ag85B from <i>M. tuberculosis</i>	Aeras, UOXF, EDCTP, UCT, MRC	B	Ilb	NCT01340820	Completed and stopped
	AERAS-485					NCT01151189	Completed and stopped
	Ad35/AERAS-402	A replication-deficient adenovirus type 35 expressing Ag85A, Ag85B, and TB10.4.	Aeras, EDCTP, CHBV	B	II	NCT00953927	Completed
	Ad5Ag85A	A human adenovirus serotype 5 expressing Ag85A.	MU, CIHR	B	I	NCT02178748	Completed
	ChAdOx1.85A TB/FLU-04L	A recombinant simian adenovirus expressing Ag85A. A live recombinant influenza vector expressing Ag85A and ESAT-6.	UOXF, UB RIBSP, RII	B B	I Ila	NCT02414828 NCT01017536 NCT01198366 NCT00800670 NCT02337270 NCT01829490	Terminated Recruiting Completed Completed
Attenuated live vaccines	MTBVAC	Genetically attenuated phoP-fadD26-deletion mutant of <i>M. tuberculosis</i>	P	Ib/Ila	NCT02501421	Completed	
TB subunit vaccines	AEC/BC02	Fusion protein Ag85b-ESAT6-CFP10 in BC02 adjuvant	BLS, Aeras, UZ, CHUV, TBVI	B	Ib/Ila	NCT02729571	Active, not recruiting
	BCG-PSN	Polysaccharide and nucleic acid extracted from BCG		B	I	NCT02933281	Not yet recruiting
	Mtb72F	Fusion protein Mtb32a-Mtb39a in AS01E or AS02A adjuvant	AZLBP, NIFDC	IT	I	NCT03026972	Not yet recruiting
	H1:IC31	Fusion protein Ag85B-ESAT-6 in IC31 adjuvant	JZT	B	I	Unknown	Unknown
	H1:CAF01	Fusion protein Ag85B-ESAT-6 in CAF01 adjuvant	GSK, Aeras	B	Ilb	NCT02097095	Completed
	H1:LTk63	Fusion protein Ag85B-ESAT-6 in LTK63 adjuvant	SSI, TBVI, EDCTP	B	II	NCT01755598	Active, not recruiting
	H4:IC31/	Fusion protein Ag85B-TB10.4 in IC31 adjuvant	SSI	B	I	PACTR201105000289276	Completed
	AERAS-404	Fusion protein Ag85B-ESAT-6-Rv2660c in IC31 adjuvant	SGUL, SSI	B	I	NCT00922363	Completed
	H56:IC31/	Fusion protein Ag85B-ESAT-6-Rv2660c in IC31 adjuvant	Aeras, SSI, SP	B	Ila	NCT00440544	Terminated
	AERAS-456	Fusion protein Rv2608-Rv3619-Rv3620-Rv1813 in GLA-SE adjuvant	SSI, Aeras, SP	B	Ila	NCT02075203	Completed
DNA vaccines	ID93+GLA-SE	Four-antigen plasmids from MTB together with Fit3 ligand.	IDRI, WT, SATVI	B	Ila	NCT01861730	Active, not recruiting
	GX-70		YU	IT	I	NCT01865487	Completed
						NCT03265977	Not yet recruiting

<sup>a</sup>: Sponsors and collaborators: AFSL: Archival Farma S.L. (Spain); AI: Aurum Institute (South Africa); AZLBP: Anhui Zhifei Longcom Biologic Pharmacy Co, Ltd (China); BSL: Biofabri, S.L. (Spain); CHBV: Crucell Holland BV (Netherlands); CHUV: Centre Hospitalier Universitaire Vaudois (Switzerland); CIHR: Canadian Institutes of Health Research (Canada); DPID: Division of Pediatric Infectious Diseases, Columbia University; DBT: Department of Biotechnology (India); DIM: Department of Internal Medicine, Saint Louis University (USA); DHMC: Dartmouth-Hitchcock Medical Center (USA); EDCTP: European & Developing Countries Clinical Trials Partnership; FAHGMU: First Affiliate Hospital of Guangxi Medical University (China); GSK: Glaxo-SmithKline Biologicals (UK); IDRI: Infectious Disease Research Institute; IP: Institute Pasteur (France); JZT: Jiuzhitang Co., Ltd (China); MRC: MRC/UVRI Uganda Research Unit on Aids; MU: McMaster University (Canada); NIAID: National Institutes of Allergy and Infectious Diseases (USA); RIBSP: Research Institute for Biological Safety Problems (Kazakhstan); NICPBP: National Institute for the Control of Pharmaceutical and Biological Products (China); NIFDC: National Institutes for Food and Drug Control (China); RII: Research Institute on Influenza (Russia); SATVI: South African Tuberculosis Vaccine Initiative (South Africa); SB: Seattle BioMed (USA); SGCCR: Swiss Group for Clinical Cancer Research (Germany); SGUL: St. George's, University of London (UK); SIIP: Serum Institute of India Pvt. Ltd (India); SSI: Statens Serum Institute (Denmark); SP: Sanofi Pasteur (Canada); TBVI: TB Vaccine Initiative (Netherlands); UB: University of Birmingham; UCT: University of Cape Town; UOXF: University of Oxford; UZ: University of Zaragoza (Spain); VPMG: Vakzine Projekt Management GmbH (Germany); WT: Wellcome Trust (UK); YU: Yonsei University (Korea).

<sup>b</sup>: B: Boosting vaccine; IT: Therapeutic vaccine; P: Priming vaccine.

<sup>c</sup>: CTR number: Clinical trial registration number; NCT number: ClinicalTrials.gov Identifier; PACTR number: Pan African Clinical Trials Registry; ChiCTR-TRC number: Chinese Clinical Trial Registry. Only the latest clinical trials were listed here.

<sup>d</sup>: The status of each trial was offered by ClinicalTrials.gov data bank (<https://clinicaltrials.gov/c2/home>), Pan African Clinical Trials Registry (<http://www.pactr.org/>), or Chinese Clinical Trial Registry (<http://www.chictr.org.cn/index.aspx>). The data were obtained at October 31, 2017.

absorption rate, and cavity closure rate in elderly pulmonary TB or new smear positive pulmonary TB patients immunized with Utilins vaccine combined with anti-TB drugs were significantly higher than that in the control group immunized with anti-TB drugs alone.<sup>72,73</sup> Additionally, a Phase III trial has been conducted by First Affiliate Hospital of Guangxi Medical University (#6 Shuangyong Road, Nanning 530021, China) to evaluate the effect of inhaled Utilins vaccine on prevention and treatment of moderate bronchial asthma in China (Chinese Clinical Trial Registry No: ChiCTR-TRC-11001189).

### ***Mycobacterium smegmatis* vaccine**

*Mycobacterium smegmatis* is also a non-pathogenic fast-growing mycobacterium. *M. smegmatis* shares more than 2000 homologous genes and a peculiar cell wall structure with *M. tuberculosis*.<sup>74,75</sup> Furthermore, compared with pathogenic mycobacterial species as well as BCG, *M. smegmatis* can induce higher levels of cytokines by macrophages, activate the maturation of DCs by upregulating MHC class I molecules, and present mycobacterial antigens more efficiently via the MHC class I pathway.<sup>75</sup> In a previous study, an acellular *M. smegmatis* vaccine was successfully prepared and produced a protective effect on guinea pigs infected with *M. tuberculosis*.<sup>76</sup> To evaluate the safety, tolerance, and PPD skin reactions of this vaccine, a Phase I clinical study was performed with 55 healthy volunteers in China.<sup>77</sup> The results showed that mild side effects were observed in 14 volunteers, but all volunteers tolerated this vaccine well, and skin reactions appeared PPD-strong.<sup>77</sup>

### ***Vaccae*<sup>TM</sup>**

*Mycobacterium vaccae* was first isolated and obtained from the mammary glands of cows by Boenickse R and Juhasz E in 1964.<sup>78</sup> Four years later, Tsukamura M et al. found that this bacterium was a fast-growing mycobacterium without pathogenicity to humans and animals, and contained many protective antigens with immunomodulating effects.<sup>79</sup> In 1990, Stanford J L used irradiation-killed *M. vaccae* as an immunotherapeutic adjunct to chemotherapy in the treatment of pulmonary TB.<sup>80</sup> In recent studies, heat-killed preparations of *M. vaccae* (MV or SRL-172) also showed preliminary evidence of activity as an adjunct to anti-TB chemotherapy.<sup>81-83</sup> Whole inactivated MV was shown to be safe and immunogenic in Phase I and II clinical trials in HIV-infected adults with prior BCG vaccination in both Finland and Zambia.<sup>84,85</sup> A double-blind placebo-controlled Phase III clinical trial in Tanzania demonstrated that MV vaccination was safe, well-tolerated, and provided significant protection against TB infection.<sup>10</sup>

In 1999, MV was improved by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) in collaboration with the 309th Hospital of Chinese PLA (Beijing, China) using high-pressure air flow shearing technology (<https://www.clinicaltrials.gov/ct2/home>). The improved MV was a non-cell *Mycobacterium vaccae* vaccine termed *Vaccae*<sup>TM</sup> for TB immunotherapy.<sup>86</sup> *Vaccae*<sup>TM</sup> was given a Chinese new drug certificate in 1999 (Certificate No: (1999) S-03), and was later approved by the CFDA (Approval No: S20010003) for the adjuvant treatment of TB. *Vaccae*<sup>TM</sup> was first produced by Anhui Zhifei Longcom Biologic Pharmacy Co., Ltd (now by Anhui Zhifei Biological Pharmaceutical

Co., Ltd.) (China), and played an important role in improving immunity, promoting phagocytosis, regulating bidirectional immunoreaction, and reducing pathological damage (ClinicalTrials.gov Identifier: NCT01979900). It has been used for adjuvant treatment of TB in the clinic.<sup>87,88</sup> In addition, Sun Z. P. et al. used *Vaccae*<sup>TM</sup> to treat purified protein derivative (PPD)-strong positive patients, who were followed for 4 years.<sup>89</sup> The results suggested that TB incidence in the *Vaccae*<sup>TM</sup> treatment group (0.30%, 2/660) or isoniazid treatment group (0.61%, 4/660) was significantly lower than that in the control group (3.48%, 23/660). Furthermore, the adverse reaction rate of the *Vaccae*<sup>TM</sup> treatment group was significantly lower than that of isoniazid treatment group. These results suggested that *Vaccae*<sup>TM</sup> may have an immunoprophylactic effect on patients with LTBI. Currently, a Phase III trial, including 10,000 people aged 15–65 years with a tuberculin skin test (TST) >15 mm, was implemented to assess *Vaccae*<sup>TM</sup> efficacy and safety in preventing TB disease in people with LTBI in Guangxi province in China.<sup>2</sup> The trial was scheduled to be completed by June 2017, but the latest results have not been published (ClinicalTrials.gov Identifier: NCT01979900). *Vaccae*<sup>TM</sup> is currently the only recommended drug in TB immunotherapy by the WHO,<sup>2</sup> although this vaccine may produce localized rashes, induration, or fever in very few individuals.<sup>90</sup>

### ***MIP/Mw***

*Mycobacterium indicus pranii* (MIP) or *Mycobacterium w* (Mw) is a non-pathogenic, rapidly growing strain of non-tuberculous mycobacteria (NTM) classifiable in Runyons Group IV.<sup>91</sup> The killed MIP vaccine originally used for leprosy has also been found to be useful in the prevention of TB in mice<sup>92</sup> and guinea pigs.<sup>93</sup> MIP treatment was able to activate NF- $\kappa$ B via involvement of TLR-4 signaling, leading to enhanced pro-inflammatory cytokine and NO generation in infected macrophages and generation of a protective immune response.<sup>94</sup> The protective efficacy of MIP was evaluated in a rural population of 28,948 people belonging to 272 villages in Ghatampur, Kanpur (India).<sup>95</sup> Recently, to evaluate the efficacy and safety of MIP in TB patients, two Phase III, multi-centric clinical trials in Category-II pulmonary TB patients were conducted by the Department of Biotechnology, Ministry of Science and Technology (Govt. of India), and Cadila Pharmaceuticals Ltd., India (ClinicalTrials.gov Identifier: NCT00265226 and NCT00341328). Both studies indicated that MIP was safe with no adverse effects and played a role in clearance of the mycobacterium.<sup>9</sup>

### ***RUTI*<sup>®</sup>**

*RUTI*<sup>®</sup> vaccine, developed by Archivel Farma S.L. (Spain) in collaboration with Parexel (USA), is a non-live therapeutic vaccine based on fragmented and detoxified *M. tuberculosis*.<sup>96</sup> This vaccine provides a strong humoral and cellular immune response against antigens from actively growing and latent bacilli.<sup>97</sup> It has been demonstrated that *RUTI*<sup>®</sup> vaccine had efficacy in controlling LTBI in experimental models of mice, guinea-pigs, goats, and mini-pigs after a short period of chemotherapy.<sup>98</sup> Based on these encouraging results, a double-blind, randomized, and placebo-controlled Phase I clinical trial was performed to determine the tolerability, immunogenicity, and

safe dosage range of RUTI<sup>®</sup> vaccine in healthy volunteers (ClinicalTrials.gov Identifier: NCT00546273). The results supported the feasibility of future evaluation on subjects with LTBI.<sup>99</sup> In 2010, a Phase II clinical trial was performed to assess the safety, tolerability, and immunogenicity of two doses of RUTI<sup>®</sup> vaccine in 96 subjects (48 HIV- and 48 HIV+ subjects) in South Africa (ClinicalTrials.gov Identifier: NCT01136161).<sup>98</sup> Currently, a Phase IIa clinical trial is being conducted by University Medical Center Groningen (UMCG, Hanzeplein 1, Groningen 9713 GZ, Netherlands) in collaboration with Archivel Farma S.L. to investigate the safety and immunogenicity of RUTI therapeutic vaccination in patients with multidrug-resistant TB (MDR-TB) after successful intensive-phase treatment (ClinicalTrials.gov Identifier: NCT02711735). So far, this study has not been opened for participant recruitment.

### DAR-901 booster vaccine

The DAR-901 booster vaccine, a whole-cell, heat inactivated, NTM vaccine, is generated from detoxified and liposomed *M. obuense* cell fragments by Dartmouth-Hitchcock Medical Center and Aeras (USA).<sup>100</sup> The vaccine induces a Th1 immune response as well as a quicker and stronger specific immunity against structural and growth-related antigens that reduced both *M. tuberculosis* load and pulmonary pathological lesions.<sup>101</sup> To determine the safety, tolerability, and immunogenicity of the vaccine at different doses, a Phase I clinical trial has been completed in BCG immunized adults with and without HIV infection in the United States (ClinicalTrials.gov Identifier: NCT02063555). The results showed that DAR-901 induced cellular and humoral immunity and boosted protection from *M. tuberculosis* compared to a homologous BCG boost.<sup>102</sup> In April 2016, a randomized, placebo-controlled, double-blind Phase II clinical trial was initiated among adolescents who received BCG previously in the United Republic of Tanzania (ClinicalTrials.gov Identifier: NCT02712424). At present, this study is ongoing, but not recruiting participants.

### Recombinant live vaccines

For this type of vaccine, live mycobacteria (such as *M. bovis* BCG, *M. smegmatis*, and *M. vaccae*), live bacteria (such as *Listeria monocytogenes*, *Lactobacillus*, *Streptococcus mitis*, and *Salmonella*), and live virus (such as *Vaccinia virus Ankara*, *Adenovirus*, *Sendai virus*, and *Influenza virus*) were used as vectors to express *M. tuberculosis* protective antigens, human cytokines (e.g., IFN- $\gamma$ , IL-2, Granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-15, IL-18),<sup>103-105</sup> or apoptosis-promoting factors (Bcl-2 protein family BAX gene)<sup>106</sup> by replicating bacteria or virus in the hosts. These live vaccines not only have adjuvant and carrier functions, but also have the properties of exogenous antigens and live vaccines. A single inoculation can induce strong and persistent specific humoral and cellular immune responses against TB.

### Recombinant mycobacterial vaccines

*M. bovis* BCG, one of the strongest known immune adjuvants, was widely used as an expression vector due to its advantages in safety, cost, stability, and persistent nonspecific immune stimulation. Recombinant BCG (rBCG) vaccines were designed as

priming vaccines, which were derived from different BCG strains via expression and secretion of a foreign virus, bacterial antigens, mycobacterial protective antigens, human cytokines, and pro-apoptotic factors, or by transforming mycobacteria through stable site-specific integration of plasmids into bacterial genomes.<sup>107</sup> Currently, several rBCG vaccines have been developed and their protective efficacies as well as humoral and cellular immune responses were evaluated in animal models and humans. There are three rBCG vaccines in clinical trials (VPM1002, rBCG30, and AERAS-422), but two of them (rBCG30 and AERAS-422) have been stopped because of unsatisfactory results.<sup>11,12</sup> Other novel rBCG vaccines have been constructed and their safety and immunogenicity were tested in animal models, including rBCG-AE (rBCG::Ag85B-ESAT6),<sup>108</sup> rBCG-AEI (rBCG::Ag85B-ESAT6-IFN- $\gamma$ ),<sup>103</sup> rBCG::RD1-2F9,<sup>109</sup> rBCG::Ag85B-ESAT6-TNF- $\alpha$ ,<sup>110</sup> rBCG::Ag85B-ESAT6-Rv2608,<sup>111</sup> and rBCG::Ag85B-ESAT6-Rv3620c.<sup>112</sup> It was found that these vaccines could induce higher antibody titers and elicit stronger and more enduring Th1-type cellular immune responses than the parental BCG strain, which itself conferred similar or even better protective efficacy against *M. tuberculosis* infection than the BCG vaccine.<sup>103,108-112</sup> In addition, some other recombinant mycobacterial vaccines were also constructed to protect against *M. tuberculosis* infection. *M. smegmatis* has been engineered as a recombinant vaccine vector expressing major *M. tuberculosis*-specific antigenic proteins, such as Ag85C-MPT51-HspX,<sup>113</sup> Ag85B epitopes,<sup>114</sup> esx-3,<sup>115</sup> ESAT6-CFP10,<sup>116</sup> Ag85B-ESAT-6,<sup>117</sup> HBHA, and hIL-12,<sup>118</sup> to evaluate the immunogenicity as well as protective efficiency in animal models. This immunological vaccine not only induced specific Th1 responses against *M. tuberculosis*, but also was not distinctly harmful to the mice or cattle hosts.<sup>113-117</sup> This provided experimental evidence for the development of novel *M. smegmatis*-based vaccines against TB. *M. vaccae* was also used as a recombinant vaccine vector expressing *M. tuberculosis*-specific MPT64 protein. The results showed that it could induce high levels of specific IgG antibody, Th1-type cytokines, and a CTL effect, which resulted in an ideal protective efficacy against TB in mice.<sup>119</sup>

**VPM1002 (rBCG  $\Delta$ ureC::hly).** Recombinant BCG  $\Delta$ ureC::hly vaccine (BCG Danish parental strain), licensed to Vakzine Projekt Management GmbH (Mellendorfer Str. 9, Hannover 30625, Germany) and named "VPM1002", is manufactured by submerged fermentation in minimal medium, and the final product is a lyophilized cake of live bacteria.<sup>120</sup> VPM1002 is a rBCG vaccine in which the urease C gene has been substituted by membrane perforating listeriolysin O (LLO) encoding gene (hly) from *Listeria monocytogenes*.<sup>121</sup> This vaccine can secrete LLO to promote mycobacterial antigens and DNA into the cytosol. This enhances the production of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells and induces autophagy, inflammatory activation, and apoptosis.<sup>120</sup> Originally, VPM1002 was developed to enhance MHC-I-related immune responses by cytosolic egression of mycobacterial proteins to improve induction of CD8<sup>+</sup> T cells.<sup>122</sup> Previous studies have demonstrated that VPM1002 vaccine could induce Th1- and Th17-type immune responses, and was more effective and safer than BCG in *M. tuberculosis* aerosol-challenged mice, immune-deficient mice, guinea pigs, rabbits, and non-human primates (NHP).<sup>120</sup>

Furthermore, this vaccine has been used as an immunotherapy alternative to BCG in treating non-muscle invasive bladder cancer.<sup>123</sup> In several Phase I open-label randomized clinical trials conducted by Vakzine Projekt Management GmbH, the safety and immunogenicity of VPM1002 were assessed in adults and infants in South Africa (ClinicalTrials.gov Identifier: NCT01113281) and Germany (ClinicalTrials.gov Identifier: NCT00749034). The results indicated that VPM1002 could stimulate multifunctional T cells producing IFN- $\gamma$  or B cells producing antibodies.<sup>124</sup> This conclusion is being confirmed in two Phase II clinical trials. One was carried out by Serum Institute of India Pvt. Ltd in infants in South Africa (ClinicalTrials.gov Identifier: NCT02391415),<sup>125</sup> and the other is being performed by Swiss Group for Clinical Cancer Research in adults in Germany (ClinicalTrials.gov Identifier: NCT02371447). In May 2017, the Serum Institute of India Pvt. Ltd designed a phase II/III clinical trial in pulmonary TB patients to calculate the efficacy of this vaccine against TB recurrence (ClinicalTrials.gov Identifier: NCT03152903). This trial is not yet open for participant recruitment. Based on VPM1002, several novel rBCG  $\Delta ureC::hly+$  vaccines were developed to improve the protective efficacy or safety by enhancing specific T cell responses or reducing colony forming units (CFUs). These include rBCG  $\Delta ureC::hly_{hIL7}$  or rBCG  $\Delta ureC::hly_{hIL18}$ ,<sup>126</sup> rBCG  $\Delta ureC::hly+\Delta secA2$ ,<sup>127</sup> rBCG  $\Delta ureC::hly+\Delta nuoG$ ,<sup>127</sup> rBCG  $\Delta ureC::hly+Rv2659c-Rv3407-Rv1733c$ ,<sup>128</sup> and rBCG  $\Delta ureC::hly+\Delta pdx1$ .<sup>129</sup>

### rBCG30

rBCG30 (BCG Tice or Conn parental strain) overexpresses the *M. tuberculosis* 30-kDa major secretory protein antigen 85B (Ag85B). It was the first rBCG vaccine shown to induce significantly greater protection against TB in animals.<sup>130</sup> At present, rBCG30 has completed Phase I clinical trials. The results indicated that this vaccine improved the immunogenicity of healthy adults, and no serious adverse reactions were observed.<sup>131</sup> However, the vaccine has been restricted and stopped due to government regulations, because the vaccine contains an antibiotic resistance gene.<sup>11</sup>

### AERAS-422 (rBCG::Ag85A-Ag85B-Rv3407)

AERAS-422 is a rBCG derived from the Danish 1331 strain of BCG.<sup>132</sup> It contains a plasmid encoding three selected *M. tuberculosis* immunodominant antigens (Ag85A, Ag85B, and Rv3407), and carries a modified pfoA gene coding for the protein perfringolysin O (PFO) from *Clostridium perfringens*.<sup>121</sup> The vaccine induced a better immune response in CD8<sup>+</sup> T cells in humans, which could inhibit the growth of the TB pathogen.<sup>12</sup> A randomized, active-controlled, Phase I clinical trial was performed in healthy BCG-naïve adults to assess the safety and immunogenicity of AERAS-422 (ClinicalTrials.gov Identifier: NCT01340820). Although this vaccine could induce antigen-specific lymphoproliferative responses and reduce mycobacterial activity, the development of AERAS-422 vaccine has been discontinued due to very painful skin herpes that appeared in two adult volunteers.<sup>12</sup> This may have been caused by latently infected herpes zoster virus that was activated by PfoA lysozyme.

### Recombinant bacterial vaccines

Recently, some other recombinant bacterial vaccines were also constructed to protect against *M. tuberculosis* infection. Pnz8149-ag85a/NZ3900 vaccine is a live recombinant *Lactococcus lactis* vaccine expressing *M. tuberculosis* antigen Ag85A.<sup>133</sup> After immunization of mice with intragastric administration, this vaccine induced a local mucosa immune response, resulting in a higher level of specific SIgA antibody.<sup>133</sup> Daifalla N et al. constructed a stable recombinant *Streptococcus mitis* vaccine expressing *M. tuberculosis* protein Ag85B by homologous recombination, which resulted in efficient oral colonization and production of oral and systemic anti-Ag85B specific IgA and IgG antibodies in gnotobiotic piglets.<sup>134</sup>

### Recombinant live virus vaccines

Recombinant live virus vaccine uses a chemically weakened virus to transport target genes of the pathogen to stimulate an immune response. Compared with other genetic engineering vaccines, virus-vectored vaccines can carry large gene fragments and has advantages in safety, ease of production, and cost. However, virus-vectored vaccines have some shortcomings, such as virulence recovery and foreign gene expression instability.<sup>135</sup> At present, the main viral vectors for TB vaccine are modified *vaccinia virus* Ankara (MVA), *Adenovirus-Ad5*, *Ad35*, *simian adenovirus*, *Influenza virus*, *Hemagglutinating virus*, and *Sendai virus*. Recently, Hansen. et al. reported a novel cytomegalovirus-based vaccine termed RhCMV/TB that could induce CD4<sup>+</sup> and CD8<sup>+</sup> memory T cell responses, and reduce the load of *M. tuberculosis* in pulmonary and extrapulmonary TB.<sup>136</sup>

### MVA85A (AERAS-485)

MVA85A vaccine, created by Aeras and Oxford University, is a recombinant strain of modified MVA expressing antigen 85A from *M. tuberculosis*.<sup>137</sup> Previous studies suggested that MVA85A could stimulate humoral and cell-mediated immune responses in animal models,<sup>138,139</sup> and induce protection against *M. tuberculosis* in mice, guinea pigs, cattle, and rhesus macaques.<sup>140</sup> Further, its protective efficacy and tolerability were evaluated by several phase I and II trials in healthy volunteers in South Africa,<sup>137</sup> healthy BCG-vaccinated adults in the UK,<sup>141,142</sup> HIV-infected healthy adults in the UK (ClinicalTrials.gov Identifier: NCT00395720), adult volunteers latently infected with TB in the UK,<sup>143</sup> and BCG-vaccinated African adolescents (ClinicalTrials.gov Identifier: NCT02178748). These trials demonstrated that MVA85A was a safe and feasible vaccine that produced a strong CD4<sup>+</sup> T cell response.<sup>137,141,142,144</sup> In a randomized, placebo-controlled Phase IIb trial in two-month-old infants who had been vaccinated with BCG, the results suggested that MVA85A was well tolerated and modestly immunogenic. However, adverse events and efficacy against TB or *M. tuberculosis* infection after two years of enhanced immunization showed no significant difference between the BCG+MVA85A immunization group and the single BCG immunization group.<sup>145</sup> The possible reasons for MVA85A lacking protective efficacy in this clinical trial are: (1) The protective effect of BCG vaccine was better and could last 10–15 years, such that the boosted protection of MVA85A in two-month old newborns could not be observed in a short 2-

year period; (2) Vaccinia virus vector vaccine is only effective for a single inoculation, and is not amenable to booster immunization. In this case, the immunogenicity induced by MVA85A was not sufficient to produce significant protective efficiency in infants; 3) Due to immature immune systems in two-month-old newborns, their immune responses induced by MVA85A vaccine were not the same as those of adults; 4) The case size of this clinical trial was too small to meet the statistical requirements, because *M. tuberculosis* infection rates and TB incidences in the infants and children were much lower than those of adults. Therefore, the main reasons for the failure of this clinical trial are unreasonable design, inappropriate study subjects, and too short an observation time. It is suggested that a strengthened vaccine should be administered during the adolescent period with BCG-induced immunity in obvious decline, which might result in more significant protective efficacy. This speculation may be confirmed in a forthcoming clinical study in latently infected healthy adult volunteers in the UK (ClinicalTrials.gov Identifier: NCT02532036).

#### Ad35/AERAS-402

AERAS-402 is a replication-defective recombinant adenovirus (rAd) type 35 vaccine. It expresses a fusion protein of Ag85A, Ag85B, and TB10.4.<sup>100</sup> Darrah PA et al. used an aerosol vaccination strategy to administer AERAS-402 in BCG-primed or unprimed rhesus macaques. They found that this vaccine could induce robust IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 expression, rather than protect against high-dose *M. tuberculosis* challenge.<sup>146</sup> Several Phase I studies have been conducted in HIV-negative, BCG-vaccinated healthy adults in India,<sup>147</sup> BCG-vaccinated healthy adults in the UK,<sup>142</sup> healthy adult volunteers in the USA (ClinicalTrials.gov Identifier: NCT02375256), and BCG-vaccinated healthy adults in Kenya (ClinicalTrials.gov Identifier: NCT02430506). These Phase I clinical trials have come to a consistent conclusion that AERAS-402 could significantly enhance both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses after BCG priming, and had acceptable safety parameters.<sup>142,147</sup> This conclusion has been further validated by two Phase II trials of AERAS-402 conducted in healthy infants in sub-Saharan Africa<sup>148</sup> and HIV-infected South African adults.<sup>149</sup> A Phase II trial in adults recently treated for pulmonary TB suggested that AERAS-402 vaccine induced a strong CD8<sup>+</sup> and moderate CD4<sup>+</sup> T-cell response, and was not associated with clinically significant pulmonary complications.<sup>150</sup>

#### Ad5Ag85A

Ad5Ag85A, developed by McMaster University, is a human adenovirus serotype 5 vaccine expressing the mycobacterial secreted antigen Ag85A.<sup>100</sup> In previous studies, the safety and immunogenicity have been assessed in animal models.<sup>151-153</sup> An open-label Phase I clinical trial for this vaccine was conducted by McMaster University Medical Centre (MUMC, 1200 Main St. West, Hamilton L8N 3Z5, Canada), and the results showed that it was safe, well tolerated, and immunogenic in HIV-negative healthy adults.<sup>154</sup> Furthermore, another Phase I clinical trial is being performed by McMaster University to determine the safety and immune responses of Ad5Ag85A in healthy volunteers. This study is currently recruiting participants (ClinicalTrials.gov Identifier: NCT02337270).

#### ChAdOx1.85A

ChAdOx1.85A is a simian adenovirus vaccine expressing *M. tuberculosis* protein Ag85A, which was studied at the University of Oxford (England).<sup>155</sup> In a preclinical study, its immunogenicity and protective efficacy against *M. tuberculosis* challenge were assessed in a mouse model, which demonstrated that intranasally administered ChAdOx1.85A induced stronger CD8<sup>+</sup> than CD4<sup>+</sup> T cell immune responses in both lungs and spleens, but failed to protect the mice against aerosol *M. tuberculosis* infection.<sup>155</sup> In contrast, a further boost with MVA85A could potentially improve its immunogenicity and protective efficacy.<sup>155</sup> Currently, this vaccine is being evaluated in a Phase I clinical trial in BCG-vaccinated adults (ClinicalTrials.gov Identifier: NCT01829490).

#### TB/FLU-04L

TB/FLU-04L, developed by the Research Institute for Biological Safety Problems (RIBSP, Kazakhstan) in collaboration with Research Institute on Influenza (RII, Russia), is a live recombinant influenza A virus (A/Puerto Rico/8/34(H1N1)) vaccine expressing *M. tuberculosis* antigens Ag85A and ESAT-6.<sup>2</sup> Protective efficacy of TB/FLU-04L has been investigated in mice, and the results suggested that the protective efficacy induced by BCG was significantly improved by one intranasal booster immunization with this vaccine.<sup>156</sup> Additionally, a single center, Phase I, double-blind, randomized, placebo-controlled clinical trial was performed in BCG-vaccinated healthy adults to explore the safety and immunogenicity of this vaccine (ClinicalTrials.gov Identifier: NCT02501421). This Phase I trial was completed in February 2015, but the results were not disclosed. According to a WHO report in 2017, a Phase IIa clinical trial is being implemented in patients with LTBI.<sup>2</sup>

#### Attenuated live vaccines

The development of attenuated live *M. tuberculosis* mutants provides a possibility for discovering novel potential vaccine candidates against TB. In previous studies, some amino acid biosynthesis-related genes (e.g. *cysH*,<sup>157</sup> *panC*,<sup>158</sup> and *panD*<sup>158</sup>), virulence-related genes (e.g. *lpqS*,<sup>159</sup> *sapM*,<sup>160</sup> *mptpA*,<sup>160</sup> and *mptpB*<sup>160</sup>), and long-term survival in macrophages-related genes (e.g. *bioA*,<sup>161</sup> *phoP*,<sup>162</sup> and *sigE*<sup>163</sup>) of *M. tuberculosis* were knocked out, mutated by random mutagenesis, or targeted for homologous recombination to construct live attenuated *M. tuberculosis* vaccine that could reduce the virulence but maintain viability of the mycobacterium. Compared with inactivated TB vaccines, attenuated TB vaccines have several significant benefits, including a broad immune response, low cost, and ease of transport and administration. On the contrary, attenuated live vaccines also suffer from a number of drawbacks, including the potential risk of virulence recovery and complications for immunocompromised patients.<sup>164</sup>

To date, only one attenuated TB vaccine (MTBVAC) is in a clinical trial. There are others in preclinical research in animal models, including *M. tuberculosis* MT103 *phoP* strain (replicating *in vivo*),<sup>162</sup> *M. tuberculosis* H37Rv  $\Delta$ *leuD*  $\Delta$ *panCD* strain,<sup>165</sup> *M. tuberculosis* H37Rv  $\Delta$ *lysA*  $\Delta$ *panCD* strain (mc<sup>2</sup>6020, non-replicating *in vivo*) as well as *M. tuberculosis* H37Rv  $\Delta$ *RD1*  $\Delta$ *panCD* strain (mc<sup>2</sup>6030, replicating *in*



*vivo*).<sup>158,166,167</sup> The safety and efficacy of mc<sup>2</sup>6020 vaccine were better than those of mc<sup>2</sup>6030 vaccine.

MTBVAC is a new live TB vaccine based on a genetically attenuated *phoP-fadD26*-deletion mutant of *M. tuberculosis*.<sup>168</sup> *phoP* is essential for the growth of *M. tuberculosis* isolate MT103 in macrophages. It encodes transcription factors that can regulate a variety of virulence factors (such as ESAT-6). *fadD26* is necessary for the synthesis of *M. tuberculosis* phthiocerol dimycocerosates, which is a main component of the cell wall and a virulence factor that protects *M. tuberculosis* from host defenses. This vaccine was developed by the Biofabri, S.L (Spain) and Aeras (USA) in collaboration with the University of Zaragoza (Spain), Centre Hospitalier Universitaire Vaudois (Switzerland), and the TB Vaccine Initiative (Netherlands) according to the ClinicalTrials.gov database. The primary target population is neonates (BCG replacement vaccine), with a secondary target being adolescents and adults (booster vaccine). MTBVAC is the first live-attenuated *M. tuberculosis* vaccine to be in clinical trials, and to date has shown a safety profile comparable to BCG. As early as 2013, a Phase I clinical trial was conducted by Biofabri, S.L and other collaborators to evaluate the safety and immunogenicity of MTBVAC in comparison with BCG in HIV-negative volunteers in Switzerland (ClinicalTrials.gov Identifier: NCT02013245). The results of this Phase I clinical trial showed that the safety of vaccination with MTBVAC at all doses was similar to that of BCG vaccine, and vaccination did not induce any serious adverse events.<sup>169</sup> Recently, two Phase Ib/IIa clinical trials were performed by Aeras and Biofabri, S.L in adults and/or infants in South Africa (ClinicalTrials.gov Identifier: NCT02933281 and NCT02729571, respectively). Both trials are in progress.

### TB subunit vaccines

TB subunit vaccines usually consist of some immunoactive ingredients (e.g. proteins, polypeptides, myolic acids, glycolipids, etc.) isolated and purified from *M. tuberculosis*.<sup>170</sup> This vaccine can induce immune protection or immunotherapy with the help of an adjuvant. Therefore, the subunit vaccine is usually used as a therapeutic vaccine or an enhanced vaccine. The features of the TB subunit vaccine include low cost, easy preparation, high yield, high purity, safety, repeated use, and persistent immune memory of effector T cells by enhanced vaccination. These make it an ideal vaccine for defending people against TB infection. However, compared with BCG vaccine, TB subunit vaccine faces some challenges, including shorter duration of immunogenicity, poor memory immunity, and requiring the assistance of adjuvants. Fortunately, some progress has been made to solve these drawbacks. Previous studies have shown that TB subunit vaccines composed of multiple protein mixtures, recombinant fusion protein, chimeric protein, or epitope-tandem protein of dominant antigens could induce stronger CD4<sup>+</sup> T cell responses and more protective efficacy than single protein subunit vaccines.<sup>8,61,171-176</sup> Furthermore, several novel adjuvants or delivery systems, such as biodegradable polymer microspheres,<sup>177</sup> liposomes,<sup>178</sup> emulsions, and virosomes<sup>179</sup> were developed to enhance the immunogenicity and protective efficacy of TB subunit vaccine. At present, although plenty of subunit vaccines have been identified and

studied in animal models, only seven candidates are being evaluated in clinical trials.

### AEC/BC02

AEC/BC02 vaccine is composed of a recombinant fusion protein Ag85B-ESAT6-CFP10 (AEC) and adjuvant BC02 that is based on BCG-derived cytosine-phosphate-guanine (CpG) and aluminum salt.<sup>180</sup> This vaccine was developed by the National Institutes for Food and Drug Control (Beijing, China) and manufactured by Anhui Zhifei Longcom Biologic Pharmacy Co., Ltd. (Anhui, China). Previous studies have demonstrated that this vaccine could induce long-term antigen-specific cellular immune responses in mice, produce a therapeutic effect, and reduce the risk of causing Koch phenomenon in a MTB latent infection guinea pig LTBI model.<sup>181</sup> Moreover, an increasing number of studies noted that the vaccine induced type I hypersensitivity in guinea pigs.<sup>180, 181</sup> Currently, a Phase I clinical trial of AEC/BC02 in healthy volunteers is in progress in Shanghai, China (ClinicalTrials.gov Identifier: NCT03026972), but this study is not yet open for participant recruitment.

### BCG polysaccharide and nucleic acid injection (BCG-PSN)

BCG Polysaccharide and Nucleic Acid Injection (BCG-PSN, alternative name SIQIKANG) is an immunomodulator produced by Jiuzhitang Co., Ltd. (China) and approved by the CFDA (Approval No: S20020019). It was prepared from the BCG Danish strain using hot-phenol to remove bacterial proteins and ethanol to extract polysaccharide as well as nucleic acid.<sup>182</sup> BCG-PSN can protect against TB infection by regulating cellular and humoral immunity, stimulating the reticuloendothelial system, and activating monocytes and macrophages.<sup>183</sup> A recent study suggested that BCG-PSN could induce high levels of IFN- $\gamma$  and TNF- $\alpha$  in peripheral blood CD4<sup>+</sup> T cells from mice receiving BCG-PSN powder delivered via microneedle patch. Treatment improved pathological changes in their lungs and spleens compared to the control group.<sup>182</sup> With a growing number of BCG-PSN applications in the clinic, this immunomodulator has been widely used for adjuvant chemotherapy for TB as well as prevention and therapy for chronic bronchitis, colds, erosive oral lichen planus, nasopharyngeal carcinoma cells, and asthma for years in China.<sup>184</sup> However, some defects of BCG-PSN should not be ignored, such as long treatment cycles, nodules, rash, and slight fever.<sup>185</sup>

### Mtb72F

Mtb72F vaccine (alternative names GSK-M72, M72: AS01E/AS02A, GSK-692342, and Mtb72F/AS02A) was originally developed by Glaxo-SmithKline Biologicals (GSK, USA).<sup>100</sup> This vaccine is a 72 kDa chimeric protein composed of two highly immunogenic proteins (Mtb32 and Mtb39, encoded by *rv0125* and *rv1996*, respectively), which is delivered with GSK adjuvants AS01E or AS02A.<sup>186</sup> It has been determined that Mtb72F could induce more efficient immune responses in animal models than either Mtb32 or Mtb39 alone,<sup>187</sup> and more efficient protection than BCG alone in mice, guinea pigs, and NHP animal models.<sup>186, 187</sup> Further, Mtb72F has also been shown to stimulate T cell proliferation and IFN- $\gamma$  secretion in

PPD positive healthy individuals.<sup>187,188</sup> Currently, the safety and immunogenicity of Mtb72F vaccine have been evaluated in some Phase II clinical studies performed in healthy PPD-positive adults in the Philippines,<sup>189</sup> in healthy HIV-negative adolescents in South Africa,<sup>190</sup> and in adults in Taiwan and Estonia.<sup>191</sup> All three Phase II clinical trials showed similar safety profiles and antibody responses, and indicated that this vaccine could induce strong CD4<sup>+</sup> T cell immune responses, rather than CD8<sup>+</sup> T cell responses.

#### **H1:IC31, H1:CAF01, and H1:LTK63**

Hybrid 1 (H1), developed by Statens Serum Institute (SSI), Denmark, and Valneva (a fully integrated vaccine company), is a subunit vaccine composed of the fusion proteins Ag85B and ESAT-6, both of which were secreted in the acute phase of *M. tuberculosis* infection.<sup>100</sup> H1 was designed to improve the efficacy of BCG, but was not designed to prevent reactivation from LTBI.<sup>192</sup> Previous studies have indicated that the H1 subunit vaccine had the ability to induce protective immune responses in mice,<sup>174</sup> guinea pigs<sup>173</sup> and NHP,<sup>193</sup> suggesting that H1 was a strong candidate for further clinical evaluation. To enhance its protective efficiency, H1 has been tested in combination with various adjuvants such as IC31 (the combination of an immunostimulatory oligodeoxynucleotide containing deoxy-Inosine/deoxy-Cytosine and the cationic polyamine acid KLK), CAF01 (composed of a cationic liposome vehicle dimethyldioctadecyl-ammonium and a glycolipid immunomodulator trehalose 6,6'-dibehenate), and LTK63 (the mutant of *Escherichia coli* heat-labile enterotoxin). A Phase I clinical trial of H1/IC31 showed that this vaccine promoted strong and long-lived *M. tuberculosis* specific T cell responses in naïve human volunteers,<sup>194</sup> which was consistent with a following Phase II clinical trial in HIV-infected adults.<sup>195</sup> An open label, single-center, non-randomized Phase I exploratory trial of H1:CAF01 in mycobacteria-naïve individuals demonstrated that H1:CAF01 was a safe TB vaccine resulting in high levels of IL-2 and TNF- $\alpha$  secretion.<sup>196</sup> Furthermore, a Phase I clinical trial of H1:LTK63 nasal TB vaccine was also conducted by St George's, University of London to determine the safety, as well as cell mediated and humoral immunogenicity profiles in healthy adults (ClinicalTrials.gov Identifier: NCT00440544). However, this clinical trial has been terminated for a safety issue because two healthy subjects experienced transient peripheral facial nerve palsies 44 and 60 days after passive nasal instillation of LTK63.<sup>13</sup>

#### **H4:IC31 (AERAS-404)**

H4:IC31 (AERAS-404) vaccine, developed by SSI, Sanofi Pasteur, and Valneva and Aeras, consists of a recombinant fusion protein Ag85B-TB10.4 (H4) and an IC31 adjuvant.<sup>197</sup> H4-IC31 has been shown to protect animals against pulmonary TB,<sup>198</sup> and induces antigen-specific CD4<sup>+</sup> T cells secreting IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 in humans.<sup>199</sup> Several Phase I clinical trials have been conducted to evaluate the safety and immunogenicity of H4:IC31 in healthy adolescents in South Africa (ClinicalTrials.gov Identifier: NCT02378207), in HIV-negative, TB-negative, BCG-naïve adults in Switzerland (ClinicalTrials.gov Identifier: NCT02420444), in HIV-negative BCG-vaccinated adults in Sweden (ClinicalTrials.gov Identifier: NCT02066428),

and in adults in Finland (ClinicalTrials.gov Identifier: NCT02074956). These clinical trials yielded similar results, in that H4:IC31 had an acceptable safety profile in human beings and induced IFN- $\gamma$  production and a multifunctional CD4<sup>+</sup> Th1 response.<sup>188,197</sup> Additionally, a Phase II clinical trial has been completed by Aeras in healthy adolescents in South Africa (ClinicalTrials.gov Identifier: NCT02075203). The results demonstrated that this vaccine was safe and immunogenic, and indicated that a 15  $\mu$ g dose induced the optimal immune response.<sup>200</sup> At present, the safety, immunogenicity, and dose-range of H4:IC31 are also being evaluated in a Phase I/II study (Desmond Tutu HIV Foundation, Cape Town, South Africa) in HIV-uninfected, HIV-unexposed, and BCG-primed infants (ClinicalTrials.gov Identifier: NCT02378207).

#### **H56:IC31 (AERAS-456)**

H56:IC31 vaccine, developed by the SSI in collaboration with Valneva and Aeras, combines three *M. tuberculosis* antigens (Ag85B, ESAT-6, and Rv2660c) with IC31 adjuvant.<sup>201</sup> H56:IC31 vaccine is based upon the H1:IC31 vaccine with LTBI-related antigen Rv2660c added. It has been demonstrated that H56:IC31 vaccination after exposure could prevent reactivation and significantly decrease the bacterial load compared with adjuvant control group or BCG group in mouse or NHP models with LTBI or active TB.<sup>202</sup> A Phase I clinical trial evaluated its safety and immunogenicity in HIV-negative adults in South Africa. The results showed that H56:IC31 vaccine was safe and induced antigen-specific IgG responses and Th1-type cytokine-expressing CD4<sup>+</sup> T cells.<sup>201</sup> Furthermore, a Phase IIa clinical trial (Aurum Institute, Klerksdorp, South Africa) evaluating the safety, immunogenicity, and efficacy of H56:IC31 in remotely BCG-vaccinated adolescents is currently in progress (ClinicalTrials.gov Identifier: NCT03265977).

#### **ID93+GLA-SE**

ID93+GLA-SE vaccine, created by the Infectious Disease Research Institute in collaboration with Aeras (USA), is a recombinant fusion protein of three *M. tuberculosis* virulence-associated antigens (Rv2608, Rv3619, and Rv3620), one latency-associated antigen (Rv1813), and adjuvant GLA-SE.<sup>203</sup> This vaccine was shown to stimulate CD4<sup>+</sup> T cells secreting high level of Th1 cytokines, which resulted in protection against TB in both BCG-vaccinated and non-BCG-vaccinated mice and guinea pigs.<sup>204,205</sup> Interestingly, this vaccine was able to cause a delayed type hypersensitivity (DTH) response to the vaccine antigen, but did not compromise the PPD reaction, which did not interfere with the auxiliary diagnosis of a PPD skin test.<sup>206</sup> Further study also suggested that ID93+GLA-SE vaccine could elicit protection against W-Beijing strain (*M. tuberculosis* HN878) infection by decreasing bacterial burden, reducing lung pathology, and increasing survival by inducing long-lived Th1 immunity.<sup>203</sup> Two Phase I clinical trials in healthy adults (ClinicalTrials.gov Identifiers: NCT01599897 and NCT01927159) were completed in the United States and South Africa, respectively. The results showed that this vaccine had an acceptable safety profile.<sup>207</sup> Additionally, a Phase IIa clinical trial in HIV-naïve TB patients has also been completed in South Africa to evaluate its safety and immunogenicity

(ClinicalTrials.gov Identifier: NCT02465216), but the results have not yet been published.

### DNA vaccines

DNA vaccines protect against disease by injecting genetically engineered DNA that results in production of a target antigen.<sup>208</sup> It is generally accepted that TB DNA vaccines can express *M. tuberculosis* protective protein antigens that will be recognized by MHC class I or class II molecules. Based on this, DNA vaccines can induce a comprehensive immune response, especially the ability to stimulate specific CTL to recognize and kill infected host cells, and remove intracellular pathogenic *M. tuberculosis*.<sup>35,209-211</sup> TB DNA vaccines, including therapeutic DNA vaccines and enhanced preventive DNA vaccines, have potential merits over conventional vaccines. They are generally safer, more effective, and bear lower production cost.<sup>209,212</sup> However, there are a number of obstacles that DNA vaccines must overcome in clinical trials and subsequent manufacturing applications: (1) The microproteins expressed by DNA vaccines *in vivo* can induce immune responses in vaccinated individuals, but their intensity is usually weaker than that of live vaccine. The possible reason for this might be inefficient conversion or the inability to replicate itself as efficiently as live vaccine; (2) The protective efficiency of DNA vaccines should be optimized and improved as current research showed that it was not superior to BCG; (3) DNA vaccines can induce inoculators to produce anti-DNA IgG, which may increase the risk of developing autoimmune diseases.<sup>2</sup>

Currently, most TB DNA vaccines in preclinical studies are focused on immunogenicity, protective, or therapeutic effects in animal models. These include heat shock protein 65 (*hsp65*) DNA vaccine,<sup>210</sup> *hsp70* DNA vaccine,<sup>213</sup> *ag85a* DNA vaccine,<sup>214</sup> *ag85b* DNA vaccine,<sup>215</sup> *ag85a/TB10.4* chimeric DNA vaccine,<sup>216</sup> phosphate-specific transport system (*pstS*) DNA vaccine,<sup>211</sup> *mpt64* DNA vaccine,<sup>217</sup> and *pIRES-IL-21-ag85a-esat-6* DNA vaccine.<sup>218</sup>

In our previous studies, 11 DNA vaccines have been constructed to evaluate their immunogenicity, protective, or immunotherapeutic efficacies in mice, including *esat6* DNA vaccine,<sup>219</sup> *mpt64* DNA vaccine,<sup>220</sup> *hsp65* DNA vaccine,<sup>221</sup> *ag85a* DNA vaccine,<sup>222</sup> *ag85b* DNA vaccine,<sup>219</sup> *ag85a/b* chimeric DNA vaccine,<sup>223</sup> *ag85a/esat6* chimeric DNA vaccine,<sup>224</sup> *rv2190c* DNA vaccine,<sup>209</sup> *rv1419* DNA vaccine,<sup>35</sup> and *IFN- $\gamma$*  and *IL-12* DNA vaccine.<sup>219</sup> After decades of study on these DNA vaccines, we summarize several observations and suggestions that might be quite useful for future DNA vaccine design: (1) These vaccines mainly induced Th1-type immune responses<sup>212,223,224</sup>; (2) The level of specific antibodies produced by these DNA vaccines generally increased after the second immunization, peaked after the third or fourth immunization, and then decreased three months after the last immunization; (3) The *ag85a/b* chimeric DNA vaccine had the best immunoprotective efficacy among these 11 DNA vaccines. Pilot-scale studies for *ag85a/b* chimeric DNA vaccine have been completed, and three batches of samples have been produced to establish a quality control system to evaluate its stability and safety<sup>176,219</sup>; (4) The codelivery of genes encoding cytokines *IFN- $\gamma$*  or *IL-12* could increase the effectiveness of DNA

vaccines, especially *IFN- $\gamma$* <sup>219</sup>; (5) Different DNA vaccines produced different protective effects in a mouse TB model. The lungs in the mice showed various pathological changes, especially from *esat6* DNA vaccine and *ag85a* DNA vaccine, which showed proliferative lesions. Meanwhile, *mpt64* DNA vaccine and *ag85b* DNA vaccine both showed proliferative and exudative damages; (6) The optimal doses of DNA vaccines were 100  $\mu$ g in mice or 1 mg in NHP, as immunizations of less than this dose could induce humoral immune responses rather than enough cellular immune responses to protect the individual against TB<sup>219,223</sup>; (7) The use of electroporation could decrease the dose from 100  $\mu$ g to 50  $\mu$ g without affecting the immunoprotection or immunotherapy of the DNA vaccine, which not only weakened the side effects but also reduced DNA vaccine costs<sup>225</sup>; (8) The effects of DNA vaccines against TB were not affected by MDR-TB,<sup>224</sup> possibly because the DNA vaccine could induce the immune system to kill MDR-MTB. This indicates a potential use as a new adjuvant therapy for MDR-TB; (9) The *ag85a/esat6* chimeric DNA vaccine not only could not improve the therapeutic effect of *ag85a* DNA vaccine, but also caused the death of mice infected by MTBC.<sup>226</sup> A likely reason was that overexpressed ESAT6 could cause a hypersensitivity response or anchor on the pneumocyte cell membrane via its laminin domain to form a channel, leading to dissolution as well as necrosis of pulmonary surface epithelial cells and macrophages.<sup>227</sup> Therefore, we caution against the use of *esat6* DNA or ESAT6 protein as a candidate component of TB therapeutic vaccine. Interestingly, *ag85a/esat6* chimeric DNA combined with effective anti-TB drugs could inhibit the occurrence of hypersensitivity response and decrease the death of mice.

At present, GX-70 is the only DNA vaccine in clinical trial. It consists of the four antigen plasmids (data is not yet available) from *M. tuberculosis* together with recombinant Flt3 ligand according to the ClinicalTrials.gov database. An open-label, dose escalation, Phase I clinical trial was conducted by Yonsei University (South Korea) to evaluate the tolerability, safety, and immunogenicity of GX-70 in pulmonary TB patients with high risk factors for treatment failure or relapse (ClinicalTrials.gov Identifier: NCT03159975). The clinical trial will be divided into two steps. First, GX-70 will be administered in three dose levels (0.26 mg, 1 mg, and 4 mg) by electroporation in the deltoid muscles every four weeks, five times to determine the maximum tolerated dose. Then, antigen-specific *IFN- $\gamma$*  ELISPOT (enzyme-linked immunospot assay) responses and Flt3L concentration will be measured every eight weeks up to 24 weeks. This study is not yet open for participant recruitment, and the estimated study completion date is August 20, 2018.

### Future challenges and conclusion

TB is an infectious chronic respiratory disease which is full of contradictions and challenges in *M. tuberculosis* infection, immunization, prevention, and treatment. Although there has been some progress in the pipeline for new TB vaccine development, these exciting advances are counterbalanced by ongoing challenges and remaining questions, as described below.

1. The pathogenesis and immune protection mechanisms of *M. tuberculosis* need to be further clarified. With

- developments in technology and vaccinology, many new generation TB vaccines have been developed. Their immunogenicity, protection, and therapeutic effects have been evaluated in animal models and humans. However, neither BCG nor any other novel TB vaccines induced a comprehensive immune response to completely clear the pathogen,<sup>2,6</sup> which means that there are still many drawbacks to these vaccines. Puzzlingly, the fact that 90% of people with LTBI have no clinical symptoms indicates that the immune response induced by *M. tuberculosis* infection can provide effective protection for most *M. tuberculosis*-infected people.<sup>2,8</sup> There is no doubt that the confusion comes from our ignorance of the pathogenesis and immunoprotection mechanisms of *M. tuberculosis*. Given a clear understanding of the pathogenesis and biological characteristics of *M. tuberculosis* as well as the transcriptional and metabolic pathways, it may be possible to design an ideal vaccine to prevent *M. tuberculosis* infection and development.
2. The vaccine design and immunization strategies need to be further studied. TB vaccine design should not only consider proliferation-related antigens (such as Ag85A and Ag85B), but also consider the dormant-related antigens. It is urgent that antigens should be chosen to construct a more effective vaccine. It has been suggested that the TB vaccine constructed by both proliferation-related antigens and dormant-related antigens could induce more broad and stronger responses which would provide more effective protection against *M. tuberculosis* infection. In addition, the immune responses induced by subunit vaccines were always weaker than those induced by live vaccines. Therefore, it is crucial to find a suitable immune adjuvant to enhance its immunocompetence. At present, it is not only vaccine design that is suffering from enormous challenges, but vaccination strategies are also beset with difficulties because new vaccine evaluation requires a large population and long-term follow-up observations. Even worse, it is very difficult to choose a large enough sample to conduct a field assessment in some high TB prevalence countries where BCG was widely administered. Based on the above facts, we suggest that future research priorities should be more focused on immunization strategies such as the type of new vaccine, immunization order, immunization interval, and immunization times.
  3. The interaction between human host and *M. tuberculosis* is very complex, but the mechanism is not clear. Therefore, the following questions need to be solved in the development and application of new TB vaccines: (1) Does incidence of TB depend on the virulence of *M. tuberculosis*, the quantity of bacteria, the susceptibility of the individual, the immune system affected, or by other adverse factors? (2) Which criterion should be used to evaluate vaccine effectiveness? General status improvement, sputum bacterium-negative conversion, cellular immune function enhancement, or extended survival? (3) In areas with a high prevalence of TB, most people have been infected by *M. tuberculosis*. If they receive a strong immunogen (new vaccine), will the subsequent host immune response clear the immunized vaccine? Will it trigger latent TB infections and then develop serious disease? (4) Some people have been infected by NTM. How might this affect future vaccine development?
  4. We also need to explore the mechanism of immunotherapy. Immunotherapy using TB vaccine is a new field of exploration. Several inactivated vaccines have been applied in clinic. Their treatment efficacies were disputed, which did not achieve the goal of “ultrashort course chemotherapy”. The possible reasons are: (1) The inactivated bacterial components can only induce a momentary Th1-type cellular immune response, and not the specific CTL response; (2) The clinical application of inactivated vaccines was not standardized for the dosage, application time, treatment course, or immune status of TB patients. The effects of vaccines on the immunity of TB patients still lack in-depth study; (3) Some TB patients improperly administered (such as once a week) may induce immune tolerance. TB is an infectious disease and an immune disease, but the immunoregulatory mechanism of TB has not been fully elucidated. The immune response to TB is a double-edged sword. How can immunoregulatory means and intervention link *M. tuberculosis* antigen sensitization, the host immune response, and the host’s physiological response to inhibit pathological changes? Further studies are necessary to understand and address these relationships.
  5. The emergence of MDR-TB and prevalence of HIV-TB co-infection are new challenges for vaccine research.<sup>2</sup> MDR-TB and HIV-TB co-infection have become a critical threat to TB control and global public health. In 2016, there were an estimated 490,000 new cases of MDR-TB, and 88,200 (7%) of all new TB cases are living with HIV.<sup>2</sup> Without timely diagnosis and treatment, the mortality rate range of patients with MDR-TB and HIV-TB co-infection will be as high as 100%.<sup>2,28</sup> Therefore, both MDR-TB and HIV-TB co-infection should be given sufficient attention with respect to their potential possibility to inhibit the End TB Strategy, and novel multivalent vaccines need to be developed as quickly as possible.
  6. Increasing costs have become an obstacle to the fight against TB in developing countries. According to a WHO report, the cost of prevention and treatment is as high as 9.2 billion dollars worldwide in 2017, and this figure is expected to grow to 12.3 billion in 2020.<sup>2</sup> The main reason may be an increasing number of MDR-TB and HIV-TB co-infection patients, whose cost is ten times than that of a common TB patient.<sup>2</sup> Unfortunately, available funds are not enough to address these needs. The BRICS countries (Brazil, the Russian Federation, India, China, and South Africa) collectively account for about half of the world’s TB cases. However, only 5% of the funding was supported by international donor funding in these countries.<sup>2</sup> In other words, domestic funding for the TB-specific budgets in these countries accounts for the largest single share of funding, which is likely to cause a vicious circle in low-income countries.

In summary, our review shows the fact that the challenges of TB are increasing while the strategies are limited. Although more than 20 vaccines are currently in clinical trials, we urgently need to screen more TB vaccine candidates to develop more effective and safer TB vaccines to defend against TB infection, especially MDR-TB and HIV-TB co-infection. Furthermore, the interaction between immune activation, inflammation, and TB pathogenesis should be understood, which could possibly provide insights into developing a better TB vaccine. There is also a need to further increase funding for TB prevention, diagnosis, and treatment in developing countries, which is necessary to achieve the End TB strategy milestones for reductions in TB cases and deaths set for 2020 and 2025.<sup>229</sup> We should remember the principle that without improved TB vaccines, without controlled TB prevalence.

### Disclosure of potential conflicts of interest

The authors do not have commercial or other associations that might pose a conflict of interest.

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