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REVIEW ARTICLE

Novel Vaccine Candidates against Tuberculosis

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Abstract: Ranking above AIDS, Tuberculosis (TB) is the ninth leading cause of death affecting and killing many individuals every year. Drugs' efficacy is limited by a series of problems such as Multi-Drug Resistance (MDR) and Extensively-Drug Resistance (XDR). Meanwhile, the only licensed vaccine BCG (Bacillus Calmette-Guérin) existing for over 90 years is not effective enough. Consequently, it is essential to develop novel vaccines for TB prevention and immunotherapy. This paper provides an overall review of the TB prevalence, immune system response against TB and recent progress of TB vaccine research and development. Several vaccines in clinical trials are described as well as LAM-based candidates.

Keywords: Tuberculosis, vaccine, Lipoarabinomannan, immunotherapy, response, LAM-based candidates.

1. INTRODUCTION

1.1. TB - A Global Health Problem

Tuberculosis (TB) is the ninth leading cause of death in the world because of late diagnosis, lack of access to treatment and associated infections such as HIV, although great efforts have been made to prevent and treat this disease. In 2016, 10.4 million people were infected with TB and there were an estimated 1.3 million TB deaths, with more than 90% of cases occurring in developing countries [1, 2]. According to WHO, this disease killed 1.6 million people in 2017. In addition, it is reported that nearly 2 billion people have been exposed to the tuberculosis bacillus and are at risk of developing active disease [3]. Those whose immune system are damaged by diseases like AIDS, malnutrition or diabetes and long-term smokers are more likely to get TB. Although traditional antibiotic therapy is

overall successful for TB treatment, two existing obstacles impede its development. First, it usually needs 6 months or longer time to take the TB drugs, like isoniazid, rifampicin, pyrazinamide and streptomycin. During the time of therapy, Directly Observed Therapy Short Course (DOTS) strategy should be undertaken [2], which costs amounts of money. Second, drug resistance is a serious problem because 6% of new TB cases and 20% of retreatment cases are MDR TB (Fig. 1). For example, in 2016, there were 600.000 new cases with resistance to the most effective first-line drug rifampicin and 490.000 individuals had MDR TB [2]. Generally, it needs a combination of three specific drugs to avoid drug resistance [4, 5]. So, instead of using medicine to treat the patients, it is better to prevent it by using vaccines.

1.2. History and Drawbacks of BCG Vaccine

Currently, *Mycobacterium bovis* (*M. bovis*) BCG is the only licensed vaccine against TB, which is received by more than 3 billion people worldwide. It is an attenuated strain of *M. bovis* derived from a virulent strain after more than 13 years of continuous *in vitro* passage in the beginning of 1900s [6]. Now the BCG vaccine maintains its position as the world's most

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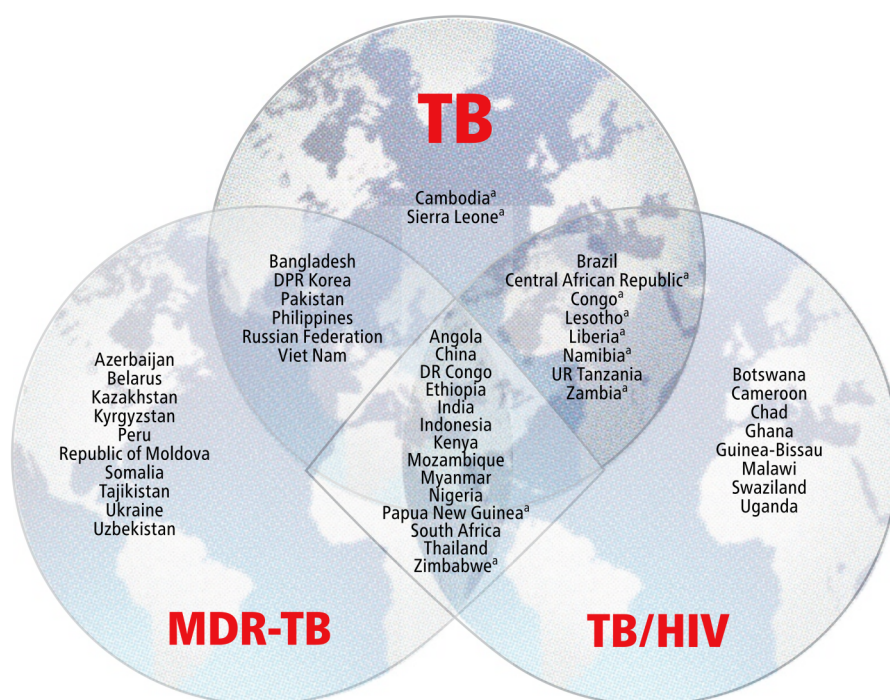


Fig. (1). Main countries suffering from TB; TB/HIV; MDR-TB, and their overlap during the period 2016-2020 [2].

^aTop 30 countries that suffer from TB according to incidence. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

widely used vaccine, however, it is not effective and safe to satisfy today's needs. Many studies showed that BCG vaccine has a decent effect on children, with a meta-analysis showing that the protective efficacy of BCG on children's tuberculous meningitis and miliary tuberculosis is 73% and 77%, respectively [7]. However, when it comes to teenagers and adults, the protective efficacy is controversial due to the fact that efficacy can vary from 0 to 80% in different countries and areas [8]. Generally, countries in North America can be well protected by BCG, while BCG's efficacy is particularly poor in tropical and subtropical regions [9]. Lalor *et al.* argued that the environmental factors, maturity of the body's immune system, T-cell response, mother-to-child transmission related diseases, and other vaccination effects may lead to the difference [10]. Besides, the effect of BCG vaccine for HIV-infected individuals is problematic. Mansoor N suggested that HIV infection may seriously affect BCG-induced specific cellular responses and increase the risk of BCG-osis in HIV-infected infants. Infants infected with HIV have a risk of disseminated BCG-related diseases after vaccination against BCG, which is hundreds of times more than uninfected HIV infants [11]. Currently, TB is the leading cause of death in HIV-infected people because of the high incidence of dual infections and accompanying inhibition of the immune system by the two dis-

eases. It was reported that co-infection with HIV and *mycobacterium tuberculosis* (*M. tuberculosis*) increases the risk of developing TB 30-fold [12]. Lastly, an adverse reaction is another affair that needs to be well considered. In general, BCG vaccine is relatively safe; the incidence of inflammation is less than 1% and the incidence of life-threatening BCG-related diseases is less than 2 per 1 million. However, with the increase in the diagnosis of immune-deficient diseases recently, the increase in disseminated pulmonary tuberculosis and severe disease-deficient disease, BCG has received much attention in the vaccination of immunodeficient infants [13].

1.3. Immune Response to *M. tuberculosis* Infection

M. tuberculosis is an intracellular parasite, and thus cellular immunity plays a major role in the prevention of infection. When *M. tuberculosis* invades, the host initiates the first line of defense by the natural immune system to fight against the infection. In the system, macrophages, natural killer cells, and neutrophils are important to control TB infection [14-16]. Macrophages can phagocytose and kill the invading *M. tuberculosis* in the body and the phospholipase D released by macrophage can promote phagocytic lysosome maturation and alveolar epithelial type II cells to kill intracellular *M. tuberculosis*. Moreover, it was reported

that antigen carried by exosome from macrophage can protect the mouse from being infected and exosome can induce antigen-specific CD4 and CD8 T cells to secrete Interferon (IFN)- γ and Interleukin (IL)-2. The killing effect of natural killer cells against *M. tuberculosis* does not depend on the release of IFN- γ and cytotoxic particles, but on cell-cell interactions. IL-2 secreted by natural killer cells can effectively enhance phagocytic lysosomal fusion and help cells clear intracellular *M. tuberculosis*. Neutrophils can fight against *M. tuberculosis* in two ways. Firstly, the myeloperoxidase in neutrophil kills *M. tuberculosis* depending on chloride ion and hydrogen peroxide. Neutrophils can also form neutrophil extracellular traps to trap the invading microorganism when it is activated [17]. Not only innate immune cell is essential to fight against *M. tuberculosis*, but T-cell plays an important role. T-cell cytokines including IFN- γ and other T helper 1 (Th1) cytokines can activate antimicrobial activities in macrophages. Then, T cells kill *M. tuberculosis* directly and *Mycobacteria*-reactive T cells can infect macrophages, which seems to be a prerequisite for killing by T cells of microbes residing inside macrophages [12, 18, 19].

It was reported that CD4 T cells are mainly in Th1 type. These cells are judged the most crucial mediators of protection which can produce IFN- γ ; Tumor Necrosis Factor α (TNF α); and IL-2 [20-22]. Recent studies showed that CD4 T cell-mediated immune protection against tuberculosis involves controlling the spread of early tuberculosis infection to the lungs, preventing the development of tuberculosis, and assisting the killing of CD8 T cells and natural killer cells against *M. tuberculosis* (two types of antibody-mediated protection mechanism are shown in Figs. (2) and (3)) [22]. Besides, CD8 T cells are important for protection, because they are an extra producer of cytokines of Th1 type, which can promote the production of opsonization antibodies and block the spread of pathogens from cheese-like necrotic sites to distant tissues. It can also secrete perforin and granzyme to degrade target cells. $\gamma\delta$ T cells and CD1-restricted $\alpha\beta$ T cells may recognize mycobacterial components and be participants in the protection against tuberculosis, although their precise role is not clear [23].

2. CURRENT PROGRESS OF TB VACCINE

Depending on the time of administration, there are two potential types of vaccines against TB nowadays, those given before and after exposure to the pathogen. Vaccines given before exposure are called pre-exposure or prophylactic vaccines, while those given

after exposure are called post-exposure or therapeutic vaccines [24]. Two strategies are commonly considered to develop the pre-exposure vaccine. The first strategy is to design a longer-lasting and more protective vaccine to replace BCG using novel recombinant BCG or attenuated *M. tuberculosis* vaccine. The other strategy is hammering at boosting and prolonging BCG's immunity in individuals who have already been BCG vaccinated [25]. So, using BCG for priming and a subunit vaccine as a booster may provide a direction for pre-exposure of vaccine development. Post-exposure vaccination is targeting to latently infected individuals accounting for over one-third of the world's population. Since tuberculin was once used as a therapeutic vaccine against active disease and caused many deaths from treatment, antigens need to be well considered before using it. However, now it is commonly thought that carefully selected antigens will not cause safety problems as a therapeutic vaccination in latently infected individuals [25, 26].

The reverse vaccinology approach allowed the development of protein-based vaccines and new BCG vaccines over expressing selected antigens [27]. Furthermore, characterization of the epitopes of these proteins inspired the rational design of new vaccine products including chimeric proteins obtained by genetic recombination of different antigens, as well as DNA or RNA based vaccines. The subunit vaccines are commonly composed of one or more antigens selected among the pool of proteins secreted by *M. tuberculosis*. In fact, *M. tuberculosis* secretes more than 30 different proteins [28]. The most abundant proteins-antigen secreted by *M. tuberculosis* is the protein-complex Antigen (Ag) 85, but other predominant antigens are proteins belonging to the ESAT6 family and Mpt64. The Ag85 complex is a 30-32 kDa family of three mycolyl transferases (Ag85 A, B and C) involved in the coupling of mycolic acids with the arabinogalactan in the cell wall [29]. Ag85B is the most powerful *M. tuberculosis* antigen and induces both humoral and cell-mediated immune response. For this reason, it has been considered for the development of most of the new vaccines products under clinical investigation.

Various studies were performed for the characterization of Ag85B epitopes and in some cases, homologs such as Ag85B from *M. Bovis* or *M. Smegmatis* were considered [29-33]. As reported in Table 1, for T-cell activity, human subjects and different animal models have been investigated, while for B-cell activity, have been indicated as a putative T-cell epitope studies were carried out in the mouse. Different sequences

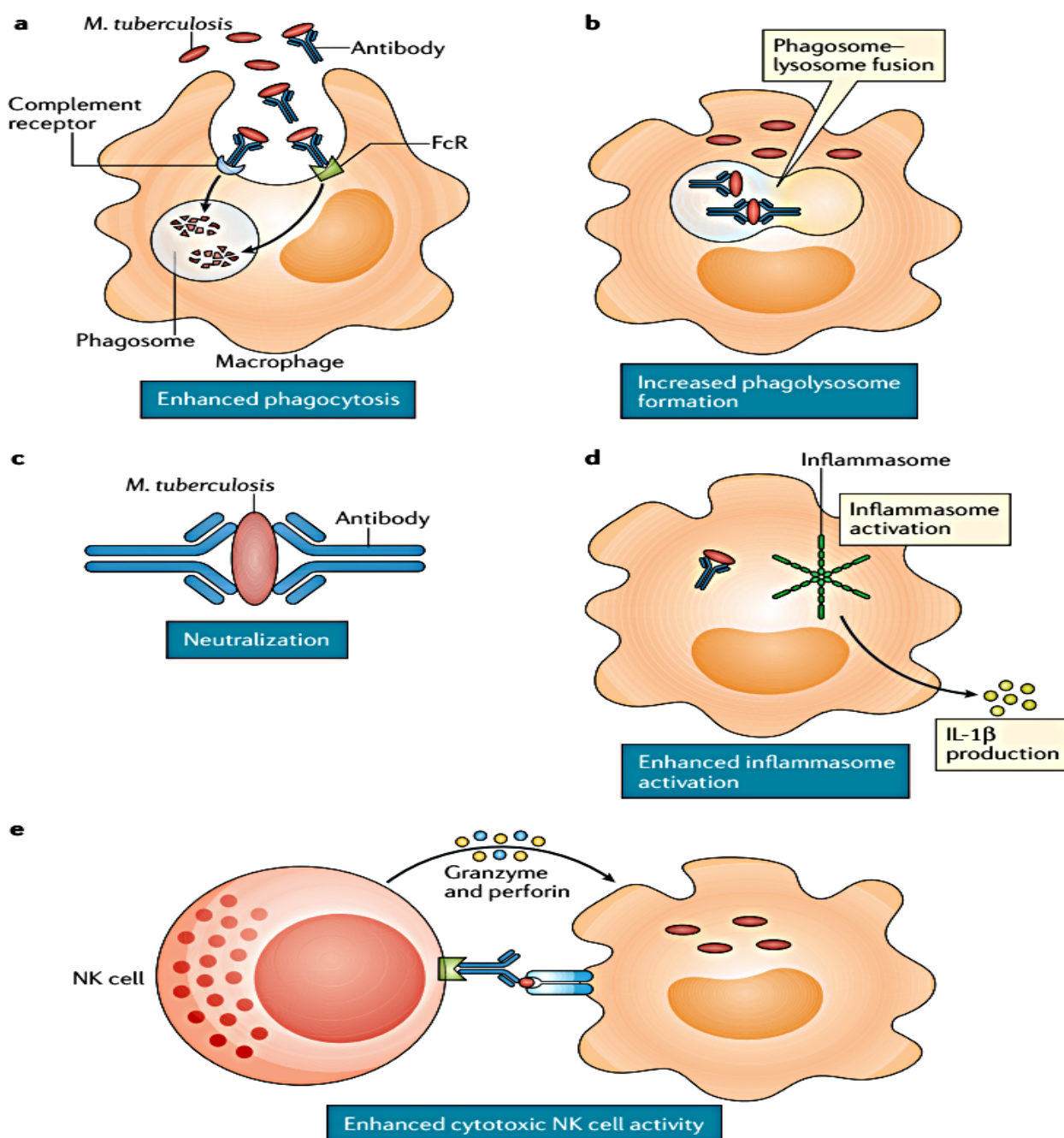


Fig. (2). Potential Mechanisms of Antibody-mediated protection against *M. tuberculosis* [24]. (a) The complexes of *M. tuberculosis* and antibody can be phagocytosed by macrophages through Crystallizable Fragment (Fc) Receptors (FcRs) and complement receptors; (b) Antibodies against *M. tuberculosis* can increase lysosome-phagosome fusion, in which *M. tuberculosis* usually interferes with and thereby restricts *M. tuberculosis*' growth; (c) *M. tuberculosis* antibodies may direct microbicidal or neutralizing activity, or they may prevent the uptake of the bacteria to promote *M. tuberculosis* killing; (d) Antibodies against *M. tuberculosis* can promote inflammasome activation in macrophages, which is associated with ASC speck formation and IL-1 β secretion; (e) Antibodies against *M. tuberculosis* may stimulate killing of infected cells through natural killer (NK) cell mediated antibody dependent cell cytotoxicity [24]. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

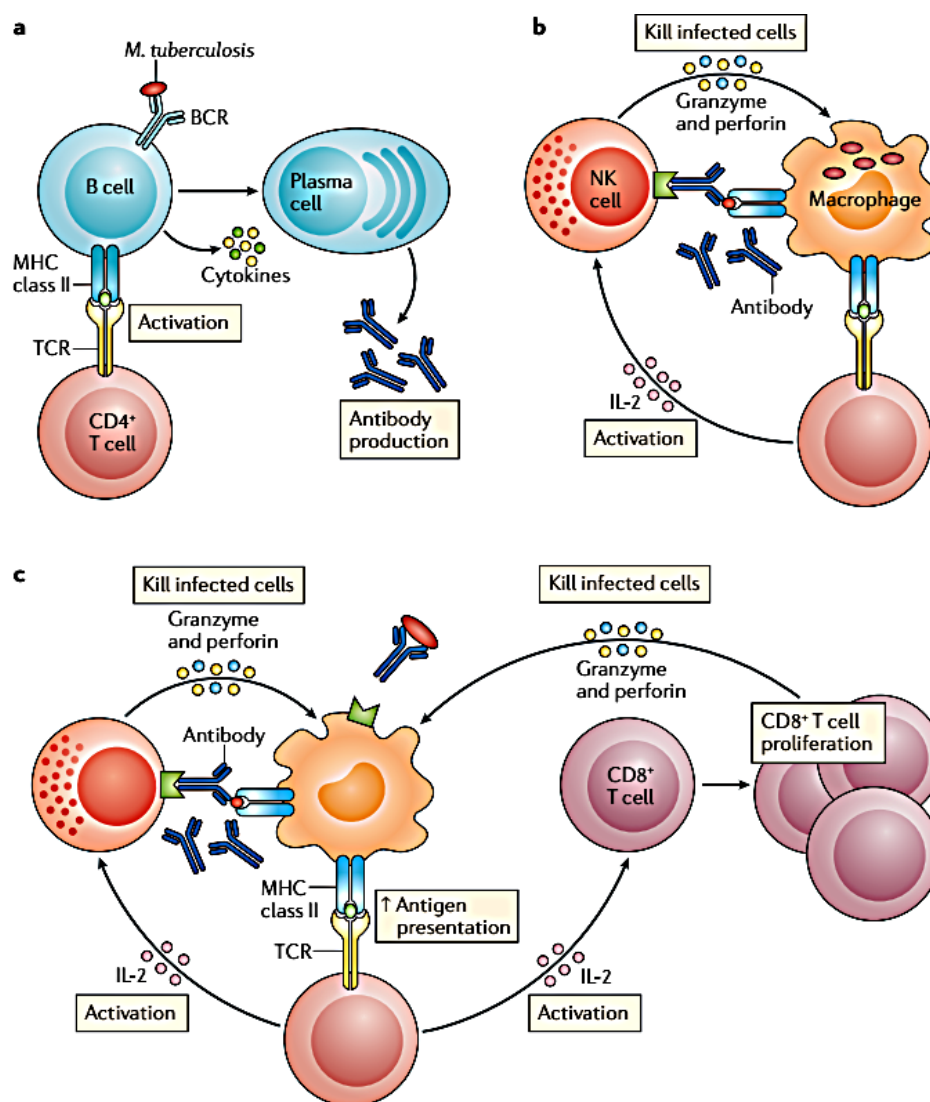


Fig. (3). Potential CD4+ T cell-dependent mechanisms of antibody-mediated protection [24]. (a) CD4 T cells may help B cells stimulate the maturation of antibody responses; (b) CD4 T cells may stimulate antibody-mediated to produce cytokines which can activate Natural Killer (NK) cells and enhance antibody dependent cell cytotoxicity responses to kill *M. tuberculosis* infected cells; (c) Complexes of *M. tuberculosis* and antibody may lead to increased display and presentation of *M. tuberculosis* antigens to CD4 T cells through professional phagocytes, such as macrophages or dendritic cells [24]. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

(Table 1) in the region between positions Leu11-Ala35 and Ala90-Arg113 while two sequences have been indicated to be able to stimulate both the T and B cell antigenic activity against Ag85B (Ser126-Pro140 and Thr261-lys275).

The ESAT6 family includes some low-molecular-mass proteins such as ESAT-6, TB10.4 and CFP10 strongly recognized by T cell [34]. About ESAT-6, nine synthetic 20AA peptides were prepared and tested [35], allowing the identification of two sequences with T-cell activity: ESAT-6₅₁₋₇₀ (YQGVQKWDATATE LNNALQN), and ESAT-6₁₋₂₀ (MTEQQWNFAGIEA

AASAIQ). It was also discovered that the ESAT-6₁₋₂₀ sequence also included an epitope for B-cells [36].

Recently, in the case of TB10.4, two *in silico* methods indicated the sequences Met2-Met5, His14-Tyr21 and Gly13-Thr24 as putative epitopes in the N-terminal region, Gln37-Gl41 in the central part of this protein and one epitope near the C-terminal region (Val64-Thr88) [37]. Finally, in the ESAT6 family, CFP10 is an important protein because it is not present in BCG and consequently, can be associated with BCG or used as a boost for a subject already vaccinated with BCG. This protein induces both CD4 and CD8 T-cell immunity, and Li *et al.* synthesized and tested 26 peptides [38]

Table 1. Epitopes T and B of Ag85B already reported in the literature.

Epitope	Sequence in Ag85B of MTB	<i>Mycobacterium</i>	Host
T-Cell (CD4)	LQVPSPSMGRDIKVQFQ (11-27)	<i>M. tuberculosis</i>	Human
	LQVPSPSMGRDIKVQFQSGG (11-30)		
	GRDIKVQFQSGGNNSPA (19-35)		
	AGCQTYKWETFLTSE (90-104)		
	CQTYKWETFLTSELPQW (92-108)		
	LTSELPQWLSANR (101-113)	<i>M. tuberculosis</i>	Mouse
	SMAGSSAMILAAYHP (126-140)		
	THSWEYWGAQLNAMK (261-275)		
	YYQSGLSIVM (61-70)		
	MPVGGQSSFY (70-79)	<i>M. bovis</i>	Human
B-Cell	LTSELPQWLSANR (101-113)	<i>M. smegmatis</i>	Mouse
	SMAGSSAMILAAYHP (126-140)		
	THSWEYWGAQLNAMK (261-275)		

allowing the identification of four epitopes: CFP10₃₅₋₄₃ (TAGSLQGQW), CFP10₇₅₋₈₃ (NIRQAGV-QY), CFP10₃₋₁₁ (EMKTDAATL) and CFP10₁₃₋₂₁ (QE-AGNFERI).

There are several studies also for the characterization of T-cell [39] and B-cell epitopes [40] of Mpt64, including in *silico* predictions [41], while the research of other MTB protein-antigens has been reported [42] and is the object of continuous investigation.

Many antigenic proteins have been considered for the development of new vaccine products against MTB. Nowadays, although no vaccine can totally replace BCG's role in vaccination, several vaccine candidates are evaluated in phase I, II or III trials and some of them are likely to be substitutions in the future (Table 2).

2.1. Prophylactic Vaccines

2.1.1. Live *Mycobacterium* Vaccines

Live mycobacterium vaccines play important roles in TB vaccines' development. Recombinant BCG vaccines and live attenuated vaccines are the two main types.

2.1.1.1. Recombinant BCG (rBCG) Vaccine

Since BCG has been widely used in clinical practice, it has its own defects, thus the establishment of new vaccines based on BCG is a promising direction.

VPM1002 and rBCG30 are two examples of recombinant BCG candidates.

rBCG30

The first recombinant BCG vaccine is rBCG30, which was constructed by recombining the *M. tuberculosis* secretory antigen Ag85B (a 30 kDa enzyme). This protein is involved in outer cell-wall synthesis and it is a key component in several TB vaccines that increase immunity responses [43]. It was reported that rBCG30 vaccine shows better protection efficacy in a highly susceptible guinea pig model than BCG [44]. Although this vaccine has completed phase I trial without serious adverse events and could increase vaccine immunogenicity in healthy adults, it was stopped for further development because it contained an antibiotic resistance marker [45].

VPM1002

Another representative vaccine is VPM1002 (Δ ureCHly⁺rBCG), which was developed to enhance major histocompatibility complex class (MHC)-I-related immune responses [46]. It is a rBCG vaccine developed by Max Planck Institute of Germany in which the urease C gene has been replaced by the Listeriolysin O (LLO) encoding gene hly from *Listeria monocytogenes* [47]. Compared with the parental BCG vaccine, it showed better protection efficacy against TB by stimulating CD4 and CD8 T-cell responses as well as type 1 and type 17 cytokines [48-51]. VPM1002

showed excellent result in Trial I in Germany and South Africa in adults and it was reported that VPM1002 vaccine is safe, well-tolerated, and immunogenic vaccine in newborn infants [50]. It has successfully passed two phase I trials and completed the phase IIa randomized clinical trial in healthy infants in South Africa. Now a phase II/III trial for prevention of TB recurrence in adults is undergoing in India [2].

2.1.1.2. Live attenuation of *M. tuberculosis*

Live attenuated vaccines are derived from *M. tuberculosis*. Its strain is well-tolerated and it is more immunogenic than BCG vaccine [52]. The main advantage of attenuated live *M. tuberculosis* vaccine is that it contains numerous genetic regions encoding important immunodominant *M. tuberculosis* antigens which do not exist in BCG while safety and genetic stability are induced by chromosomal deletions of virulence genes [53, 54].

Δ PhoP Δ fad D26(MTBVAC)

MTBVAC is a vaccine, in which the phoP and fadD26 gene of the clinical strains Mt103 is deleted [53]. Mt103 belongs to the modern *M. tuberculosis* Lineage 4, which represents the most geographically widespread lineages of *Mycobacterium tuberculosis* Complex (MTBC) transmitted by the aerosol route among people with Lineage 2 (Beijing strains) [55]. It was reported that MTBVAC vaccine is as safe as BCG in Severe Combined Immune Deficiency (SCID) mice and it shows same or better efficacy in different animal models comparable to BCG [53, 56-58]. It also showed excellent results by evaluating the safety, local tolerance and immunogenicity of three escalating dose of MTBVAC relative to BCG in healthy, BCG-naïve, HIV-negative adults in phase I trial. Now MTBVAC is the only live attenuated vaccine that has successfully entered clinical trials as a preventive vaccine in the newborn [54]. Furthermore, it was reported that in clinical evaluation, MTBVAC is the only vaccine which can induce CFP10- and ESAT6-specific immune responses and these responses are effective in protecting from pulmonary TB, which has obvious impacts on TB transmission [57]. Now, phase IIa trials in neonates adolescents and adults are ongoing this year [2].

Δ lysA Δ panCD Δ secA2

mc26020 (*M. tuberculosis* H37Rv Δ lysA Δ panCD) and mc26030 (*M. tuberculosis* H37Rv Δ RD1 Δ panCD) are two other attenuated live *M. tuberculosis* vaccine candidates. The safety and efficacy of these candidates

in mice and guinea pigs were tested, with dose 50- fold higher than the recommended human dose for BCG in non-human primates [52, 59, 60]. Mc26020 was cleared in less than 30 days in C57Bl/6 mice (6 to 8 weeks old) while mc26030 could exist for more than 200 days. Both of the candidates can protect the mice model up to 8 months after infection with virulent *M. tuberculosis*. Different studies also demonstrated that vaccine candidates mc26020 and mc26030 are obviously safer than BCG in immunocompromised mice and it was clearly showed that the two candidates are as safe as BCG in non-human primates. mc26020 vaccine showed similar protective efficacy compared with BCG in immunocompetent and CD4-deficient mice in an *M. tuberculosis* Erdman challenge experiment [61], while mc26030 can prolong the survival of wild-type mice and CD4-deficient mice against an aerosol challenge with virulent *M. tuberculosis*. Although both candidates meet Geneva Consensus requirements, more studies are required to warrant a clinical trial.

2.1.2. Subunit Vaccines

The use of subunit protein vaccine is another approach largely studied for the development of efficient immunization strategies. *M. tuberculosis* culture filtrate contains antigens which can stimulate immune protection and the antigens are essential parts of subunit vaccines. Subunit vaccines can be divided into adjuvant recombinant proteins and viral vector systems [62].

2.1.2.1. Recombinant Subunit Fusion Proteins

Among subunit vaccines, proteins have been mostly investigated and significant progress has been made in their construction and testing. Generally, two or more immunodominant agents are used in fusion proteins of the candidate.

H1/IC31

Over 100 proteins are contained in the *M. tuberculosis* culture filtrates and some of them are apparently immunodominant. Ag85 complex and ESAT-6 are two examples, which exist in all mycobacterial species and only in *M. tuberculosis* complex, respectively. H1 is made up of the two secreted antigens Ag85B and ESAT6 [63]. IC31 that can stimulate robust IFN- γ production by CD4 and Th1 cells in humans and maintain long-lasting memory immune responses has been tested in combination with H1 [64]. In phase I clinical trial, H1/IC31 has been proved to be safe in healthy adults with no adverse events reported and can induce strong and lasting Th1 responses [64-66]. In phase IIa trial, the efficacy and safety of H1/IC31 in HIV-infected

adults were tested showing that this chimeric protein can also induce strong and lasting immunologic responses and it is well tolerated. However, the clinical trial has been terminated for safety issues [67].

H4/IC31

H4/IC31 is another vaccine candidate that is similar to H1/IC31, it contains Ag85B and TB10.4 antigens and the antigens are also combined with IC31. TB10.4 is similar to ESAT6, which has decent immunological properties without avoiding the interference with Interferon-Gamma Release Assays (IGRAS) [68, 69]. H4 given in the adjuvant IC31 or DDA/MPL is well tolerated and protective against pulmonary TB in guinea pigs and mice [68] showing a better protection in a guinea pig model than BCG alone when used in a boost regimen [61]. A phase I study showed that H4/IC31 is safe and can introduce CD4 T cell response in healthy and BCG-vaccinated adults. In phase I randomized, placebo-controlled and double-blind trials have been done to invest the vaccine safety and immunogenicity in healthy BCG-vaccinated individuals [70]. Now the candidate is being tested in a pre-proof-of-concept phase II trial of prevention of infection among IGRA-negative, HIV-negative adolescents and it is also in a phase I/II trial in infants [2].

M72/AS01E

M72 contains Mtb32 (Rv1196) and Mtb39 (Rv0125) antigens, which are strong targets for Th1 cells in Purified Protein Derivative (PPD)-positive individuals. AS01 is an adjuvant system with monophosphoryl lipid A and QS-21 in a liposomal formulation, while AS02 is with the same components in oil-in-water formulation [71]. It was reported that candidate Mtb72F/AS02 revealed good safety and immunogenicity profiles in mice, guinea pigs, rabbits and non-human primate models. It is safe and immunogenic in healthy and BCG vaccinated adults [71-75]. Although some adverse events are reported after M72/AS01's vaccination because of adjuvant AS01E, they are not serious and can be resolved in one week. Moreover, compared with AS02-adjuvanted vaccine, M72/AS01E represents longer-lasting multifunctional CD4 T-cell responses among healthy adults as well as BCG vaccinated or *M. tuberculosis* contacted adults [76, 77]. So, AS01-adjuvanted candidates are more suitable for further study. In a phase IIb trial in HIV-negative adults infected with *M. tuberculosis*, M72/AS01E can reduce the development of active TB disease with 54% efficacy successfully [78].

ID93/GLA-SE

Another recent subunit vaccine is ID93/GLA-SE composed of recombinant fusion protein ID93 and adjuvant GLA-SE from the Infectious Disease Research Institute. ID93 contains 4 *M. tuberculosis* antigens (Rv2608, Rv3619, Rv3620 and Rv1813) and GLA-SE is a TLR4L-containing adjuvant that helps to induce Th1 immune responses [79]. ID93/GLA-SE can induce multifunctional IFN- γ , TNF- α , IL-2 and CD4 T cells in mice and guinea pigs with or without BCG vaccination. The candidate was reported to be safe in both mice and monkeys [80] and it can induce Th1 immune responses inhibiting *M. tuberculosis*-induced lung pathology [81]. Moreover, the safety and immunogenicity of ID93/GLA-SE have been completed in TB infected adults by randomized, double-blind, and placebo-controlled clinical trials. Now a phase IIb trial is ongoing to investigate the prevention of recurrence of disease in South Africa [2].

H56/IC31

Another subunit vaccine is H56/IC31 composed of the fusion protein H56 and adjuvant IC31. H56 contains Ag85B, ESAT6 and Rv2660c, which are antigens essential for the survival of *M. tuberculosis*. The Rv2660c antigen is included as a latent-TB antigen which contributes to efficacy [82]. It was reported that H56/IC31 can release multifunctional CD4 T-cell responses in a mouse model and delay TB disease progression in cynomolgus macaques. It is safe and immunogenic in BCG-vaccinated non-human primate models [83, 84]. H56/IC31 was shown to be safe and well-tolerated, and phase I clinical trial has been completed in HIV-negative patients without serious adverse events reported [85]. Now, a phase Ib trial to evaluate its safety and immunogenicity is in process in adolescents [2].

2.1.2.2. Modified Viral Vector Vaccines

Viral vaccine vectors are like their wild-type parental viruses with highly immunogenic, which are able to elicit both CD4 and CD8 T cell responses [86]. Vaccinia and adenovirus are the most clinically advanced which are immunogenic for boosting BCG responses in clinical trials [62, 87, 88]. Three viral vector candidates are in trials now.

ChAdOx185A - MVA85A

As the first new booster tuberculosis vaccine entering into clinical trials in 2002, MVA85A is a recombinant modified vaccinia virus Ankara expressing the

immunodominant *M. tuberculosis* Ag85A [89]. MVA85A is safe and well tolerated in *M. tuberculosis* and HIV infected patients, adolescents, children and infants with no serious adverse events reported and it can boost BCG-induced immune responses and promote releasing of IFN- γ as well as polyfunctional immune responses [90]. However, it cannot induce significant protection against *M. tuberculosis* in 2797 healthy infants who have been previously vaccinated with BCG in South Africa. Now, ChAdOx185A-MVA85A was used to generate a joint-heterologous prime-boost regimen using both systemic and mucosal routes to improve its efficacy [91]. A phase I trial of ChAdOx185A in BCG-vaccinated individuals with or without a prime-boost strategy by MVA85A has been completed in the UK and two studies of aerosol administration of MVA85A have been completed in individuals with BCG-vaccinated. Now, a further study in people with Latent Tuberculosis Infection (LTBI) is ongoing in phase I trial [2].

Ad5 Ag85A

Ad5 Ag85A is a recombinant 5 non-replicating adenovirus expressing the Ag85A protein. It was reported that Ad5 Ag85A can enhance immune protection in animal models [92, 93]. Its safety and immunogenicity have been done in healthy people with and without BCG-immunized in Canada with results showing that the candidate is safe, well-tolerated and immunogenic [2]. Recent studies reported that Ad5 Ag85A may increase the risk of developing acquired immunodeficiency syndrome against HIV [94]. Now a safety and immunogenicity study of aerosol administration is in progress in phase I trial in BCG-vaccinated healthy individuals [2].

TB/FLU-04L

TB/FLU-04L is a recombinant vaccine developed by Research Institutes from Kazakhstan and Russia. It is a mucosal vectored vaccine containing influenza virus with attenuated replication deficiency and expresses

Table 2. TB vaccine candidates in clinical trials.

Name	Target indication	Composition	Type	Clinical trial status	References
VPM1002	Preventative	rBCG vaccine where urease C gene is replaced by the listeriolysin O	Live <i>Mycobacterium</i> vaccines	Phase IIb	[47]
MTBVAC	Preventative	<i>M. tuberculosis</i> MT103 strain without the phoP and fadD26 gene	Live <i>Mycobacterium</i> vaccines	Phase I	[55]
H4/IC31	Preventative	Fusion protein Ag85B and TB10.4 in IC31 adjuvant	Subunit vaccines (recombinant fusion protein vaccines)	Phase IIa	[68, 69]
M72/AS01E	Preventative	Fusion protein Mtb32 (Rv1196) and Mtb39(Rv0125) in AS01E adjuvant	Subunit vaccines (recombinant fusion protein vaccines)	Phase IIb	[71]
ID93/GLA-SE	Preventative	Fusion protein Rv2608, Rv3619, Rv3620 and Rv1813 in GLA-SE adjuvant	Subunit vaccines (recombinant fusion protein vaccines)	Phase IIa	[79]
H56/IC31	Preventative	Fusion protein Ag85B, ESAT6 and Rv2660c in IC31 adjuvant	Subunit vaccines (recombinant fusion protein vaccines)	Phase IIa	[82]
ChAdOx185A - MVA85A	Preventative	Mixture of simian adenovirus and pox virus expressing Ag85B	Subunit vaccines (modified viral vector vaccines)	Phase I	[89, 91]
Ad5 Ag85A	Preventative	5 on-replicating adenovirus expressing the Ag85A protein	Subunit vaccines (modified viral vector vaccines)	Phase I	[92, 93]
TB/FLU-04L	Preventative	Influenza virus vector expressing Ag85A and ESAT-6	Subunit vaccines (modified viral vector vaccines)	Phase IIa	[95]
RUTI	Therapeutic	Detoxified and fragmented <i>M. tuberculosis</i>	Inactivated TB vaccines	Phase IIa	[98]
Vaccae	Therapeutic	Heat-killed whole <i>Mycobacterium vaccae</i>	Inactivated TB vaccines	Phase III	[99, 112]

the Ag85A and ESAT-6 antigens [95]. Protective efficacy of the candidate has been evaluated in mice with results showing that the efficacy induced by BCG was significantly enhanced by a booster immunization with the candidate [96]. The safety and immunogenicity of TB/FLU-04L were explored in healthy adults with BCG vaccination and no serious adverse effects were reported [95, 97]. Now, a phase IIa trial in people with LTBI is being implemented [2].

2.1.3. DNA Vaccines

Due to the disadvantage of some vaccine candidates such as low efficiency and high virulence, researchers pay much attention to DNA vaccines. DNA-based vaccination not only aims at offering cytotoxic T lymphocytes and antibodies, but has engineering of artificial immunogens and co-expression of immunomodulatory proteins [100]. Triccas *et al.* suggested that DNA constructs encoding Ag85 can stimulate substantial humoral and cell-mediated immune responses leading to significant protection against TB [101]. It was reported that injection of naked DNA can stimulate immune responses with high efficiency and long time [102]. Kamath *et al.* studied DNA vaccines efficacy with encoding mycobacterial protein MPT 64, Ag85B and ESAT6 individually and compared their efficacy with the vaccine which contains the same three proteins. They suggested the latter vaccine efficacy is better and multi-subunit DNA vaccination is likely to be a new approach for developing efficient TB vaccines [103]. Accordingly, Yu *et al.* also studied a combined DNA vaccine encoding Ag85B, MPT-64 and MPT-83 and demonstrated that compared to individual antigens, the combined DNA vaccine can stimulate more IFN- γ in mice treated with isoniazid and pyrazinamide [104]. Chauhan *et al.* studied α -crystallin based DNA vaccine (DNAacr) and SodA based DNA vaccine (DNAsod). They suggested that both DNA vaccines increase the production of T_{EM} cells compared with using chemotherapy alone and the overall results showed the DNAacr has a potential role in shortening the duration of TB chemotherapy [105]. Teimourpour *et al.* isolated Mtb32C and heparin-binding haemagglutinin adhesion (HBHA) genes from H37Rv genome and constructed the DNA vaccine encoding these two genes of *M. tuberculosis* [106] allowing efficient expression of Mtb32C-HBHA fusion protein *in vitro*. The immunogenicity of this new DNA vaccine was studied *in-vivo* alone and in combination with BCG and the combination with BCG resulted in better use of DNA vaccine or BCG only, which induced the production of higher amounts of IFN- γ in mice [107]. Ahn *et al.* compared

seven well-known TB antigens delivered by DNA vaccine, and studied their immunogenicities and protective efficacies with Flt3-L in pre- and post-exposure mice models, respectively. Among all the antigens, Mtb32 is the most effective and Flt3L-Mtb32 DNA vaccine can lead to significant protection in both the spleen and lungs against *M. tuberculosis*, comparable with the protection induced by BCG [108]. Although a host of DNA vaccines can elicit cell-mediated and protective immune responses in clinical trials, until now, no Food and Drug Administration (FDA)-approved DNA vaccine is available for human use because of the low immunogenicity observed in humans [109]. So, more studies need to be done to investigate an effective TB DNA vaccine.

2.2. Therapeutic Vaccines

During the past decades, great efforts have been made to develop therapeutic vaccines. Instead of preventing the disease before infection, the target of therapeutic vaccines is to kill *M. tuberculosis*-infected cells by strengthening individual's immune system. Two vaccines are in clinical trials.

2.2.1. RUTI®

RUTI® is an inactivated TB vaccine constituted by detoxified and fragmented *M. tuberculosis*, which is designed to be used in conjunction with a short intensive antibiotic treatment [98]. Preclinical studies showed that RUTI® is safe and can induce Th1-Th2-Th3 responses in animal models [110, 111]. In a phase I trial, RUTI® was reported to be well tolerated in BCG-naïve healthy adults without serious adverse events occurring [99]. In a phase II clinical trial, people with LTBI have demonstrated that RUTI® has a good safety profile and decent immunogenicity at all studied doses. Currently, a phase IIa study in patients with MDR TB is ongoing [2].

2.2.2. Vaccae

Vaccae is a whole heat-inactivated mycobacterium vaccae evaluated as a therapeutic vaccine [112]. Vaccae was reported to induce strong Th1 immune responses in immunized mice and in phase I and II clinical trials, it is safe and immunogenic in BCG-vaccinated, HIV-infected adults [113, 114]. In a phase III clinical trial, vaccae was shown to be safe, well-tolerated, and protective against TB infection in Tanzania [115]. Now vaccae efficacy and safety in preventing TB disease in people with LTBI are being tested in phase III trial [2].

2.3. Glycoconjugate LAM-based Vaccines

Carbohydrates can be found on the wall of nearly every cell, which is the most complicated and diverse class of biopolymers commonly found in nature [116]. They play important roles in a multitude of biological processes and are essential virulence factors and antigens in most microbial pathogens [117, 118]. Even the simplest monosaccharides can combine in an ocean of ways to form structures more diverse than those formed by naturally occurring amino acids. Besides, carbohydrates can also form antigenic epitopes which can stand high temperatures and other harsh environmental conditions. Thus, polysaccharides have already been successfully considered for the development of efficient vaccines against microbial infections. These products are usually obtained by conjugation of natural or synthetic membrane antigenic sugars with specific immunogenic carrier proteins, required in order to induce a long-term T-cell mediated memory for the antigens [8]. Some of the most successful vaccines based on carbohydrate are pneumococcal vaccines and *H. influenzae* type b [8, 119, 120]. The immunogenic carrier proteins used are Tetanus Toxoid (TT), Diphtheria Toxoid (DT) and a diphtheria toxoid variant protein, Cross-Reactive Material 197 (CRM197) [121-123]. Recently, scientists tried to use glycoconjugates also as vaccine candidates to fight against tuberculosis.

In fact, much progress has been made to study the structure of the mycobacterial cell wall (the structure of *M. tuberculosis* cell wall is given in Fig. (4)). *M. tuberculosis* possesses a unique and complex cell wall containing polysaccharides, proteins and lipids [124, 125]. In the wall, the most abundant constituents are mycolic acids playing important roles in innate and adaptive immunity [126]. It is a kind of long-chain fatty acid containing 60-90 carbons, which is up to 60% of the weight of the cell wall. Lipoarabinomannan (LAM), Lipomannan (LM) and Phosphatidyl-Myo-Inositol Mannoside (PIM) are three major lipoglycans in the mycobacterial cell wall. They are attached to the cell plasma membrane and extend to cell wall's exterior [126, 127]. LAM and LM are based on the PIM; they are two carbohydrates with strong antigenicity [128, 129], but LAM is the major lipopolysaccharide component of the outer cell wall of all mycobacterial species. It is the main carbohydrate antigen and it accounts for over 15% of the bacterial weight [130]. The structure of LAM has been well-established (one kind LAM strain is shown in Fig. (5)). It has a mannan core with 6- and 2,6-linked mannopyranoses containing multiple branched, arabinofura- nosyl side chains [4, 131].

There are two major chemical forms of LAM: those isolated from *M. tuberculosis* or *M. bovis* BCG are called Mannose capped LAM (ManLAM) and those isolated from rapidly growing avirulent strains of mycobacteria are called AraLAM [127, 128, 132]. As an important immuno-modulating compound, LAM plays important roles in mycobacterial infections. It has been reported that LAM can suppress T-cell proliferation, inhibit IFN γ -mediated activation of macrophages and enhance the production of TNF α by mononuclear cells [133-136]. Nowadays, scientists argued that detection of LAM antigens may be used as a way to diagnose tuberculosis with high accuracy [137].

LAM has been investigated for the development of carbohydrate-based tuberculosis vaccines. Hamasur *et al.* isolated Arabinomannan (AM) from LAM of *M. tuberculosis* H37Rv in order to avoid the immunosuppressive effects of the intact liposaccharide molecule. The AM has been then conjugated to TT, CRM197 and DT. Both types of conjugates induced T helper cell-dependent IgG response showing high immunogenicity [138]. They also conjugated AM with Ag85B or a 75 kDa antigenic protein from *M. tuberculosis*. Both AM oligosaccharide (AMOs)-protein conjugates induced significant IgG response in rabbits and guinea-pigs showing a highly immunogenic property. C57BL/6 mice with respective subcutaneous immunization of AMOs-Ag85B and AMOs-TT got significant protection, being higher compared to that obtained without protein-conjugation, against intravenous challenge with 10⁵ H37Rv, which is comparable to BCG vaccine. Mice which received immunization of AMOs-Ag85B in EurocineTM L3 adjuvant showed better survival rate with the infection. In vaccinated guinea-pigs' lungs and spleens, the numbers of viable *M. tuberculosis* are smaller compared with those without immunization against *M. tuberculosis* H37Rv [139]. Haile *et al.* studied the ability of two new TB vaccine candidates, heat-killed BCG (H-kBCG) and AM-TT. Compared to the non-boosted BCG vaccinated mice, a significant reduction of Colony-Forming Unit (CFU) counts was seen in mice boosted with both of the candidates. Although neither of the candidates can induce a significant reduction in bacterial loads in the lungs, granulomatous inflammation was significantly reduced in lungs of mice receiving both of AM-TT and conventional BCG suggesting that AM-TT can improve primary BCG-induced protection [140]. Kallert *et al.* prepared liposomes containing phosphatidylcholine, cholesterol, stearylated octaarginine and LAM via thin layer hydration method (LIPLAM) which can be

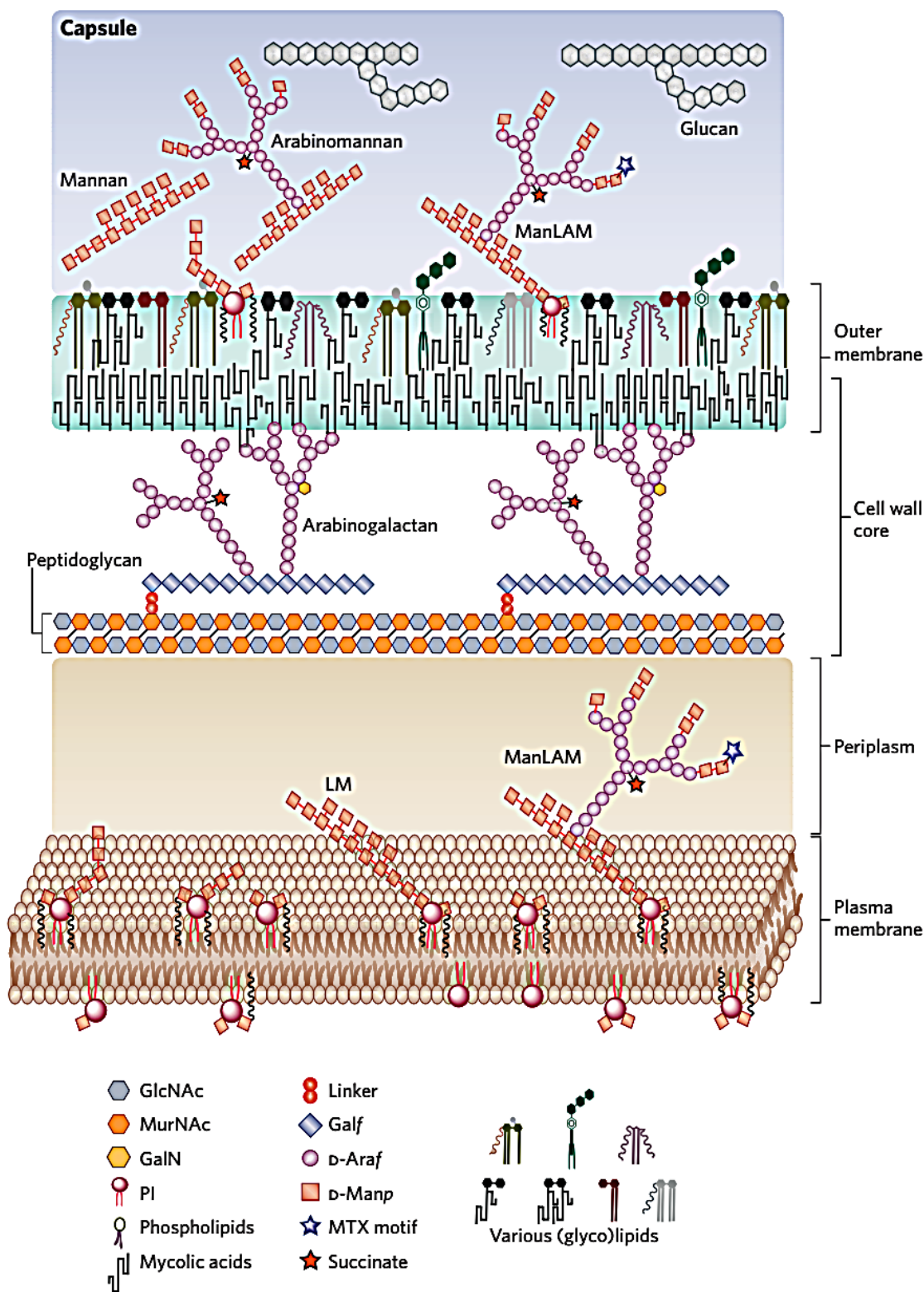


Fig. (4). Structure of Mycobacterial cell wall. The cell wall of *M. tuberculosis* is made up of three parts: plasma membrane, cell wall core and outer membrane. Plasma membrane contains mycobacterium-specific (lipo)proteins, (glyco)lipids and lipoglycans. The cell wall core consists of peptidoglycan covalently attached with arabinogalactan. The outer membrane is made up of an outer membrane consisting of an inner leaflet made of AG-bound mycolic acids, an outer leaflet containing non-covalently bound (glyco)lipids, (lipo)proteins, LM and LAM and an attached capsular-like structure made of proteins, lipids and polysaccharides [124]. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

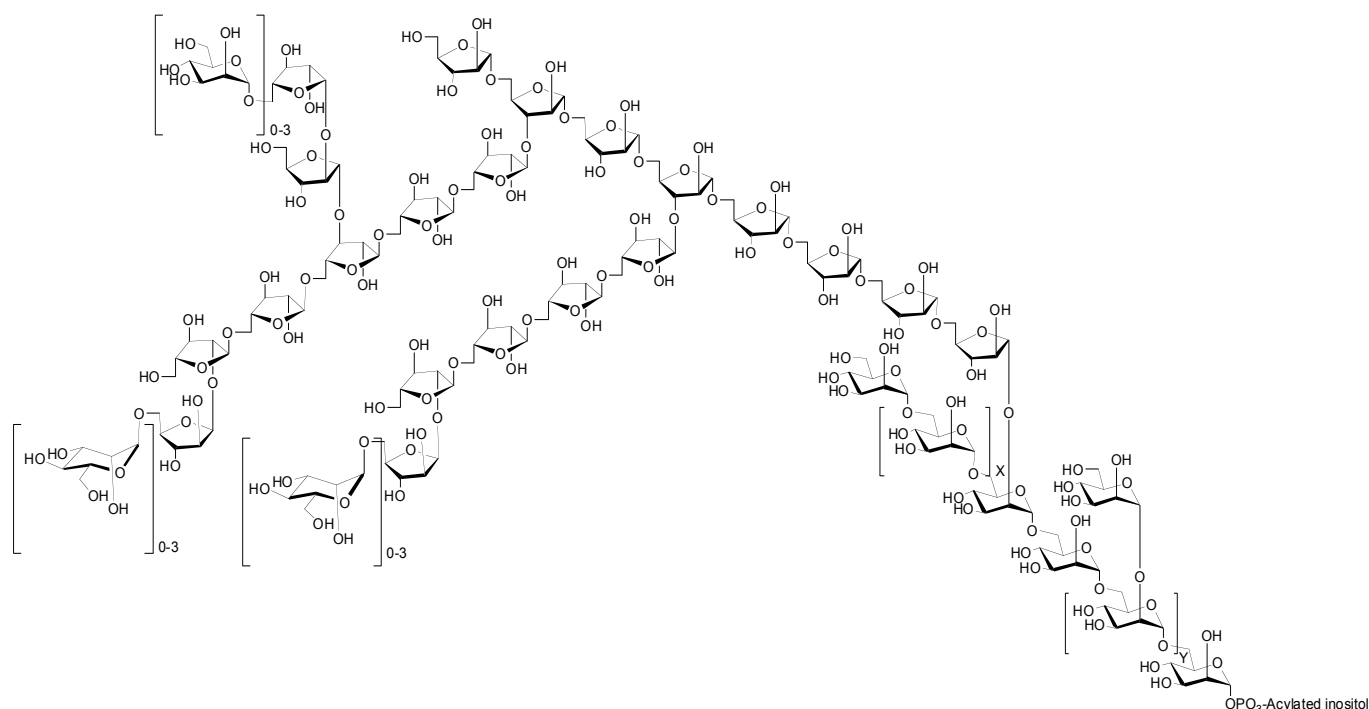


Fig. (5). Structure of LAM. In LAM, phosphatidylinositol moiety is linked to a α -1,6- and α -1,2-linked mannan backbone, which is an arabinan domain containing an α -1,5-linked D-arabinofuranosyl chain. At the end of the arabinose, two types of oligosaccharides are linked to its position 3 and 5. One type is α -1,5 and β -1,2-linked tetraarabinofuranosides, and the other type is α -1,5, α -1,3, and β -1,2-linked hexaarabinofuranosides. The arabinan domains end with different mannopyranoses at the arabinose 5-O-position.

uptaken by antigen presenting cells efficiently. It was reported that higher IFN- γ production from primary human T-lymphocytes was induced by LIPLAM compared with purified LAM or empty liposomes [141]. Glatman-Freedman *et al.* vaccinated AM-*Pseudomonas aeruginosa* Exoprotein A (rEPA) conjugated to mice with an increased antibody response occurring and the number of CFU in their organs reducing in 7 days after infection with *M. tuberculosis* [142]. Prados-Rosales *et al.* developed AM-based conjugates by using AM combined with Ag85B or Protective Antigen (PA) from *Bacillus anthracis*. Both conjugates elicited an AM-specific antibody response in mice stimulating transcriptional changes in *M. tuberculosis*. Lower bacterial numbers were seen in the lungs and spleen of immunized mice with Ag85B-AM conjugate and compared to the control mice they lived longer [143]. The conjugation of AM with recombinant *M. tuberculosis* antigenic proteins could be an innovative strategy for the development of highly efficient vaccines, by combining the activity of two different antigens (sugar and protein). However, this approach could be affected by the glycosylation of the epitopes that may reduce the antigenic activity of the protein [144]. It has been dem-

onstrated that the immunogenic activity of Ag85B decreased after glycosylation with mono and disaccharides activated with the iminomethoxyethyl (IME) thioglycoside reactive linker (that selectively reacts with amino groups of the protein). In fact, a strong reduction of the T-cell activity was observed (Fig. 6) because the two major glycosylation sites, corresponding to K₂₃ and K₂₇₅, are included in two important T-cell epitopes [145]. In order to avoid this problem, Ag85B variants, obtained by site directed mutagenesis replacing the lysine with arginine in position 23 and/or 275, have been proposed as antigenic carriers for the development of efficient AM-AG85B conjugate vaccines. In fact, arginine is not reactive in the glycosylation reaction with the IME-linker and, consequently, the glycosylation of the epitopes in the mutant proteins is avoided. Therefore, these proteins maintained its antigenic activity after glycosylation (Fig. 6) [146].

Although natural polysaccharides can be used to develop vaccines linked with immunogenic or antigenic carrier proteins, their immunodominant features may be damaged during chemical conjugation. Besides, natural polysaccharides may contain toxic components

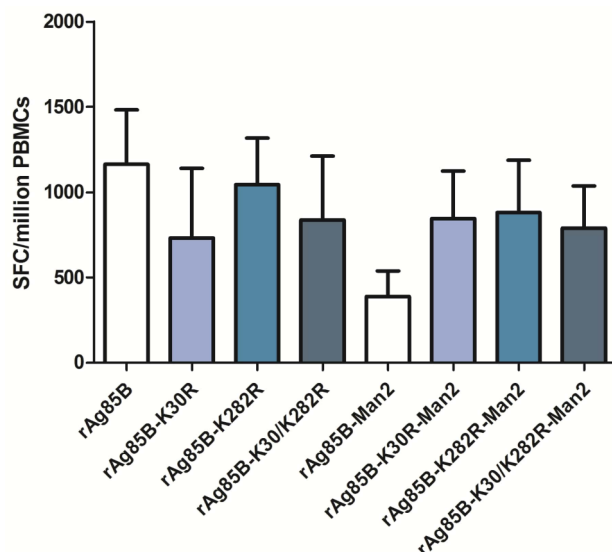


Fig. (6). T-cell responses to rAg85B antigen, its mutants and glycoconjugates. The data are presented as Mean values $\pm$ Standard Error Of The Mean (SEM) of the Spot-Forming Cells (SFC) per million of Peripheral Blood Mononuclear Cells (PBMCs) obtained by elaboration of the ELISPOT data in BCG-vaccinated subjects previously published by Bavaro *et al.* in 2017 [145] and Rinaldi *et al.* in 2018 [146]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article.*)

that are difficult to remove [147, 148] and the structure of natural antigens such as LAM is too complex to think of the development of a synthetic product. Consequently, much work was reported in the fields of synthetic LAM analogs, but only a few of them evaluated

their antigenic activity against tuberculosis [131, 149-152]. Bundle *et al.* synthesized two kinds of β -mannan trisaccharides and an arabinanhexasaccharide, and conjugated them to the polymers by squarate or click chemistry. In ELISA, it was shown that non-protein-based antigens are provided by all the three compounds displaying great characteristics for assay of antibody [153]. Gao *et al.* prepared the analog of mycobacterium tuberculosis which contains the characteristic structures of all three major components (a mannosylated phosphatidylinositol moiety, an oligomannan, and an oligoarabinan). That applied a method to prepare other LAM analogs which can be used as carbohydrate antigen for LAM based vaccine [149]. They synthesized three LAM mimetic compounds **1-3** (Fig. 7) with decent yields and conjugated them effectively with key-hole limpet hemocyanin (KLH) *via* a bifunctional linker. Immunological studies proved that all three compounds are immunogenic upon conjugation with KLH and the structure of compound plays an role on the immunological property [5]. They also synthesized monophosphoryl lipid A (MPLA) derivative **4** (Fig. 8) having the 6-OH group substituted with an NH_2 compound **1** and coupled with compound **1** *via* an amide bond. Compound **1** was also coupled with MPLA at the 1-O-position to get **5** (Fig. 8). Immunological activities of the two synthetic conjugates were evaluated in mice showing that although both conjugates induced IgG antibody responses, the 6'-N-conjugate afforded higher quantity of antibody than the 1-O-conjugate [154, 155]. Wattanasiri *et al.* synthesized LM backbone polysaccharides using rapid synthetic approach and evaluated

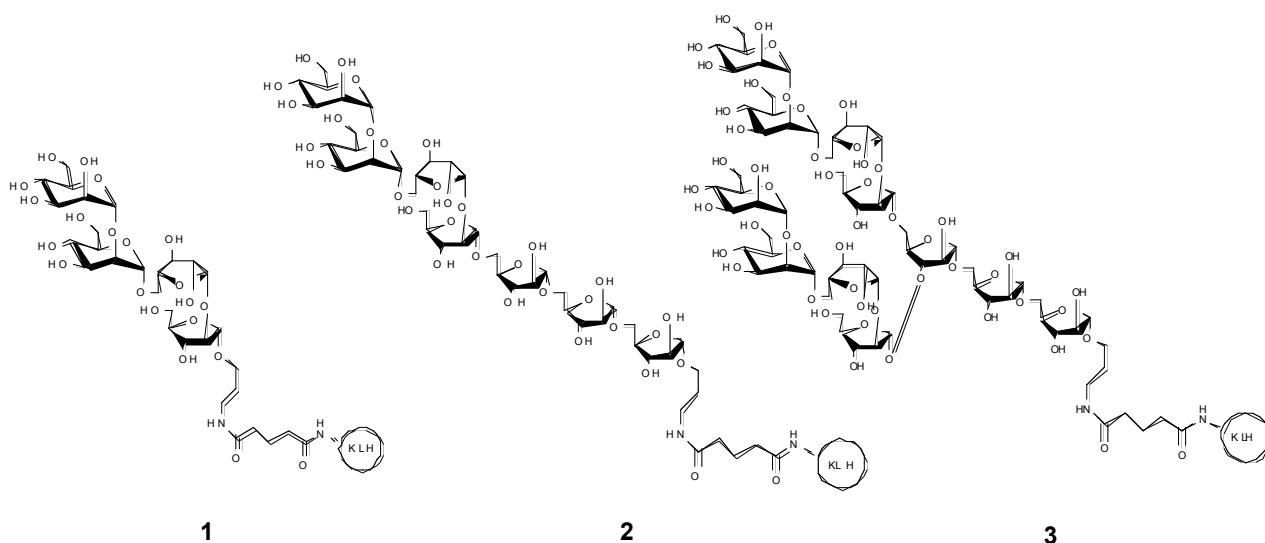


Fig. (7). Structure of LAM mimetic compounds **1-3** [5]. Compounds **1-3** were designed, synthesized and published by Wang *et al.*

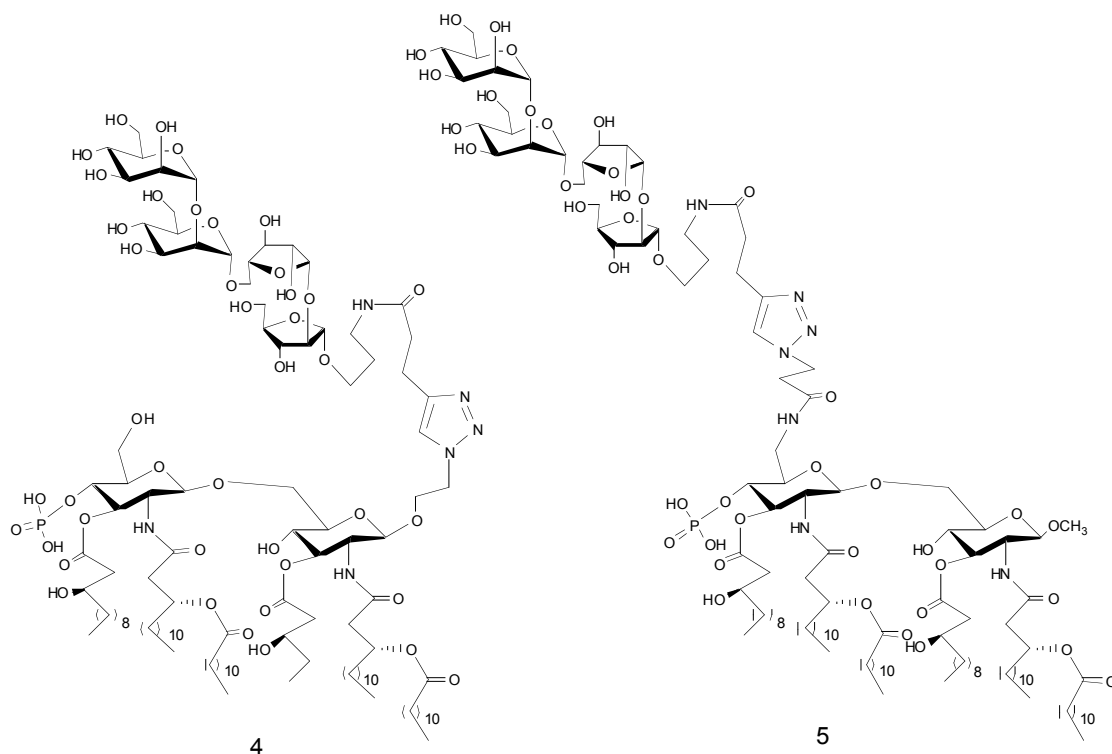


Fig. (8). Structure of LAM mimetic compounds **4**, **5** [154]. Compounds **4** and **5** were designed, synthesized and published by Wang *et al.*

its immunological properties showing that the compound can enhance the secretion of TNF- α , IL-12, IL-6, and IL-1 β . They suggested that the adjuvanticity mechanism of the compound involves the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and inflammasome pathways [156]. So, AM-based conjugates should be regarded as important forthcoming vaccine candidates in the future.

CONCLUSION

In order to fight against the global TB prevalence and replace the only licensed vaccine BCG, several vaccine candidates have been developed and evaluated in different stages, including more than ten candidates being tested in preclinical and clinical trials, either as priming, booster and therapeutic vaccines. Based on the current research, glycoconjugates may play a key role in the development of novel TB vaccine, especially the LAM-based candidates including glycoconjugate products designed using *M. tuberculosis* antigenic proteins carriers. To meet the requirement of WHO's goal of eradicating TB by 2030, introduction of novel diagnostics, drugs, and vaccines will provide the only way. However, in order to achieve the goal, further investment needs to be done on the pathogenesis of *M. tuberculosis* and its interaction with host de-

fense system. Also clinical testing and mass vaccination campaigns are essential for disease eradication.

LIST OF ABBREVIATIONS

Ag	=	Antigen
AIDS	=	Acquired Immune Deficiency Syndrome
AM	=	Arabinomannan
B-cell	=	Bursa-derived Lymphocyte
BCG	=	Bacillus Calmette-Guérin
CD	=	Cluster Designation Antigen
CFU	=	Colony-Forming Unit
CRM 197	=	Cross-Reactive Material 197
DT	=	Diphtheria Toxoid
Fc	=	Crystallizable Fragment
FDA	=	Food And Drug Administration
HIV	=	Human Immunodeficiency Virus Infection
IFN	=	Interferon
IGRAS	=	Interferon-Gamma Release Assays

IME	=	Iminomethoxyethyl
IL	=	Interleukin
KLH	=	Keyhole Limpet Hemocyanin
LAM	=	Lipoarabinomannan
LM	=	Lipomannan
LTBI	=	Latent Tuberculosis Infection
<i>M. bovis</i>	=	<i>Mycobacterium bovis</i>
MDR	=	Multidrug Resistance
MHC	=	Major Histocompatibility Complex
MPLA	=	Monophosphoryl Lipid A
MTB	=	Mycobacterium Tuberculosis
MTBC	=	Mycobacterium Tuberculosis Complex
NK	=	Natural Killer
PIM	=	Phosphatidyl-Myo-Inositol Mannoside
SCID	=	Severe Combined Immune Deficiency
TB	=	Tuberculosis
T-cell	=	Thymus-derived Lymphocyte
Th 1	=	T Helper 1
TNF α	=	Tumor Necrosis Factor A
TT	=	Tetanus Toxoid
WHO	=	World Health Organization
XDR	=	Extensively-Drug Resistance

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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