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Review

Tuberculosis vaccine candidates based on mycobacterial cell envelope components

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ABSTRACT

Even after decades searching for a new and more effective vaccine against tuberculosis, the scientific community is still pursuing this goal due to the complexity of its causative agent, *Mycobacterium tuberculosis* (Mtb). Mtb is a microorganism with a robust variety of survival mechanisms that allow it to remain in the host for years. The structure and nature of the Mtb envelope play a leading role in its resistance and survival. Mtb has a perfect machinery that allows it to modulate the immune response in its favor and to adapt to the host's environmental conditions in order to remain alive until the moment to reactivate its normal growing state. Mtb cell envelope protein, carbohydrate and lipid components have been the subject of interest for developing new vaccines because most of them are responsible for the pathogenicity and virulence of the bacteria. Many indirect evidences, mainly derived from the use of monoclonal antibodies, support the potential protective role of Mtb envelope components. Subunit and DNA vaccines, lipid extracts, liposomes and membrane vesicle formulations are some examples of technologies used, with encouraging results, to evaluate the potential of these antigens in the protective response against Mtb.

1. Introduction

Despite establishing *Mycobacterium tuberculosis* (Mtb) as the causative agent for tuberculosis (TB) and the availability of a live vaccine for its prevention since the beginning of the past century, the disease continues to claim more than a million lives each year [1,2].

Bacille Calmette Guerin (BCG), the current vaccine against TB protects against miliary and meningeal TB in children but its protection against pulmonary TB (PTB) in adults is questionable. The pulmonary form of the disease is the most common form and is primarily responsible for disease transmission [3–5]. BCG generally show high and consistent efficacy in the developed world, but in contrast, its effect in developing countries has been far from successful [3–5].

Mtb uses diverse strategies to survive in a variety of host environments and to evade the host's immune response (IR) [2]. The nature of Mtb envelope confers to the bacilli strong resistance to degradation by host enzymes, impermeability to toxic macromolecules and extreme hydrophobicity [6,7]. Molecules expressed on the mycobacterial cell envelope (CE) mediate the interactions between Mtb and the host, its recognition by host cell receptors is crucial to influence the type of the ensuing innate immune response, which will in turn determine the subsequent specific immune response against the bacteria [6,7].

Considering the relevant role of the CE in the infection process and its outcome, the use of its components as adjuvants and/or targets for vaccine development has dominated the efforts for the development of new generation vaccines against TB [8]. In this review, we will discuss on Mtb CE components (CEC) and their potential for the development of new vaccine candidates (VCs).

2. Mtb CE. Structural organization and components

Mtb CE is a complex structure, formed by three main layers: 1)

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capsule, 2) cell wall (CW) and, 3) plasmatic membrane [9].

About 40% of the CE dry mass is represented by lipids [10], and 90% of the subclasses of mycobacterial lipids are molecularly distinct from humans and other prokaryotes [7,11]. This unique CE composition and organization is believed to render mycobacteria less susceptible than other bacterial pathogens to various antibiotic classes, and provide protection against oxidative radicals and desiccation resistance, in addition to the possibility to manipulate the host immune system [12–14].

2.1. Capsule

Mtb capsule is an external carbohydrate-enriched layer that contains proteins, polysaccharides and low quantity of lipids [9]. It confers protection to mycobacteria against several external factors, such as antimicrobial agents, and has a direct interaction with the elements of the IR [6,7,9–15]. The proteins embedded in the capsule are involved in the synthesis and maintenance of the CE, and together with some of the capsular glycans, are responsible for adhesion, penetration, infection and survival of mycobacteria in the host cells [16]. The capsule also serves as a passive barrier by impeding the diffusion of macromolecules towards the inner parts of the envelope [16]. Additionally, secreted enzymes are identified which are potentially associated with the detoxification of reactive oxygen intermediates such as catalase/peroxidase and superoxide dismutase, related with the active resistance of the mycobacteria to the host's microbicidal mechanisms [16]. In addition, some toxic lipids, lytic substances and capsular constituents causes immunopathology during Mtb infection by inhibiting both macrophage-priming and lymphoproliferation [16].

Stokes and colleagues demonstrated that the capsule of the Mtb family members could limit the interaction of the bacteria with macrophages in the absence of serum opsonins, thereby reducing and/or regulating the uptake of bacteria by the phagocytes [17]. A previous study with a Mtb strain mutated in the polyketide synthase gene *msl3* with deficit in lipoglycans diacyltrehalose and polyacyltrehalose, showed alteration in the attachment of the capsule of the mutant [18].

Two of the most abundant components of the Mtb capsule are the 19-kDa glycoprotein and the antigen 85 complex [19]. The 19-kDa secreted lipoglycoprotein (Rv3763; LpqH) is an abundantly expressed CE-associated and secreted glycolipoprotein [20]. Henao-Tamayo et al., showed that the 19-kDa lipoprotein is essential for the replication of Mtb in the lungs of normal and immunocompromised mice in an aerosol infection model, while mutant Mtb, which lacks the protein, allows the bacilli to persist as a low-grade chronic infection [21]. On the other hand, the Ag85 complex which includes three proteins: Ag85A (31-kDa), Ag85B or α-antigen (30-kDa), and Ag85C (31.5-kDa), represent the most common Mtb proteins secreted into culture fluids [22]. The role of the Ag85 complex in the pathogenesis and virulence of Mtb is widely studied and the main described mechanisms include binding to fibronectin and inhibition of phagosome maturation in macrophages [22].

2.2. Cell wall

Mtb CW is different from other prokaryotes as it is composed of two segments, i.e. upper (outer membrane) and lower (CW core). The lower segment functions as a central axis, integrated by peptidoglycans covalently attached to arabinogalactan via phosphoryl-N-acetyl-glucosaminosyl-rhamnosyl linkage, which in turn esterified to mycolic acids (α-alkyl, β-hydroxy long chain fatty acids) and formed mycolyl arabinogalactan-peptidoglycan (mAGP), better known as the CW core [6]. While the upper segment (outer membrane) comprises free lipids, proteins, phosphatidylinositol mannosides (PIMs), phthiocerol-containing lipids, lipomannan (LM) and lipoarabinomannan (LAM) [6], which is the major component of the outer membrane [23,24].

Previous studies showed that PIMs, LM and LAM have potent immunomodulatory and immunopathogenic activities during mycobacterial phagocytosis, macrophage activation and macrophage microbicidal mechanisms via regulation of cytokine production and secretion. Due to its high solubility, these lipids and proteins are important molecules for signaling during disease, while the insoluble CW core is important to maintain cell viability and a robust basal structure supporting the outer “myco-membrane” (outer membrane) [6,25]. This outer membrane is especially hydrophobic since it is rich in mycolic acids, phospholipids and cord-factor [12]. The so-called “cord-factors” are the best-known mycolic acids esters in mycobacteria, principally trehalose-6,6-dimycolate (TDM) and trehalose monomycolate (TMM) [26].

TDM is the most abundant lipid released by Mtb, which have multiple functions in the pathogenesis of primary, secondary and cavitary TB [27]. In primary TB, TDM interacts with lipids within granulomas to form caseating granulomas and in secondary TB, the accumulation of mycobacterial Ags and host lipids in alveoli promotes the activation of toxicity and antigenicity of TDM which rapidly leads to caseation necrosis and formation of cavities [27]. TDM hinder the elicitation of an effective IR through the promotion of a detrimental pro-inflammatory cytokine production, the persistence of the mycobacteria inside macrophages and retarding the phagosome maturation [21]. Phthiocerol dimycocerosates (PDIMs) are the most abundant mycobacterial lipids, which is non-covalently attached to the CW skeleton [10]. They are the only non-amphipathic lipids produced by this organism and they play an important structural role in providing a stable base for insertion of other lipids [10]. They could also act as a fluidity modifier, modulating the CW viscosity [28]. Thus, PDIMs may affect the organization of the host membrane which favor receptor-mediated-phagocytosis of Mtb and prevent the phagosomal acidification which enable Mtb to survive in a protective niche [29]. The escape of Mtb from the phagosome has been postulated to be linked to the presence of PDIMs which induce cell necrosis and Mtb dissemination [30]. Across the CW there are embedded proteins which are abundantly expressed in Mtb and associated with the other components of this structure, e.g. the 19 kDa lipoprotein and 71 kDa protein which have been studied as potential VCs for TB [8,31]. Overall, the unusually high mycolic acid content, together with a variety of cell surface polysaccharides and other intercalated lipids such as sulfolipids (SLs) contribute to the wall's limited permeability, it's virulence and resistance to therapeutic agents [7,28].

The CW of Mtb is a well-equipped frame that protects this pathogen from unfavorable environments [32]. Peptidoglycan is a complex polymer described as essential component of the bacterial CW [33]. Recently, the role of two enzymes, RodA and PbpA, which are required for the structural shape of Mtb peptidoglycan were elucidated [33]. Both enzymes are required for regulating cell length, without affecting mycobacterial growth and in the guinea pig infection model, RodA and PbpA are also essential for bacterial survival and formation of granuloma, suggesting that these proteins may be involved in virulence and as a consequence, in the survival of Mtb inside the host [33].

Mtb is a pathogen characterized by the export of large quantities of proteins during its growing process. One of the major extracellular proteins is the glutamine synthetase, an enzyme related with the presence of a poly-L-glutamate component in the CW [34]. Since poly-L-glutamate is absent in non-pathogenic mycobacteria [35], the presence of glutamine synthetase has been suggested to be important in mycobacterial virulence. [34],

The α/β hydrolases constitute a powerful family of enzymes in Mtb associated with lipid metabolism, with their key role in the biosynthesis and maintenance of the pathogen's CE. They have also been associated with Mtb evasion and modulation of the host IR, as well as, with mycobacterial growth, response to hostile environments and latency [36]. A cholesterol ring-cleaving hydrolase, IpdAB, is an important virulence factor implicated in Mtb pathogenesis [37] since the virulence and persistence of this pathogen is related to its ability to degrade host-derived lipids, including cholesterol [37]. IpdAB is also essential for

Mtb growth in macrophages based on transposon mapping [38] and through using deletion mutants of the enzyme [39].

LytR-CpsA-Psr (LCP) is a protein domain with an important role on bacterial CW synthesis, specifically related to the transference of arabinogalactan to peptidoglycan. In a previous study using single mutants in the genes encoding these Mtb proteins, it was demonstrated that these genes are important for mycobacterial growth and antibiotic susceptibility [40].

Hydrolase Important for Pathogenesis-1 (Hip1) is another CW related protein with an important function on Mtb virulence [41]. The catalytic activity of Hip1 on the host IR was demonstrated using a Hip1-knockout strain, which induced an increased proinflammatory response in both macrophages and neutrophils compared to the wild type [42].

2.3. Plasmatic membrane

Plasmatic membrane, also known as cytoplasmic membrane, is composed of a phospholipid bilayer containing cardiolipin, phosphatidylethanolamine and phosphatidylinositol, which is similar to the rest of prokaryotes. However, in mycobacteria the phospholipid derivatives are highly glycosylated [9]. Mtb can be distinguished by a genus-specific, C19 fatty acid, known as tuberculostearic acid [15]. It appears that the mycobacterial plasmatic membrane plays a limited role in pathogenicity and its main function is maintenance of the influx-efflux equilibrium [11,16].

3. Role of Mtb CE in virulence

Mtb CEC constitute the major determinants of mycobacterial virulence. Being present at the interface between the microorganism and the host, the components of the mycobacterial CE are responsible in targeting host–pathogen interactions [43]. The expression of genetic determinants involved in the interaction between the microorganism and the host, have been demonstrated to influence the ability of a bacterial pathogen to survive inside the host [44]. This mechanism results in the possibility of pathogens to resist physiological and environmental stress [44]. The virulence of Mtb also depends on the genes responsible for the processes of biosynthesis, degradation and transport of the CE [45].

There are many virulence factors which have evolved in the Mtb complex members as a response to the IR [45]. The CE contains unique lipids and glycolipids that render extreme hydrophobicity to the outer surface. These lipids which include mycolic acids, phosphatidyl inositol mannosides, PDIMs and lipoglycans such as LM and LAM play important roles in maintaining integrity of the CE and are involved in the pathogenicity of mycobacteria [46,47].

Mycolic acids are the hallmark of the CE of Mtb which create a special lipid barrier with their perpendicular orientation relative to the plane of the membrane [6]. These components affect the permeability of the CE and the ability of Mtb to form biofilms [48]. Furthermore, it is essential for the survival of mycobacteria and promote the pathogenicity during infection [49]. Different studies have reported that the disruption of the mycolic acids synthesis pathway or alteration of their structure, affect the virulence of mycobacteria [50].

LAM is a major virulence factor associated with Mtb since it allows the mycobacteria to survive in the host cell environment by altering host resistance and IR [51]. LAM inhibits phagosomal maturation in the host cell and contributes to the inhibition of macrophage functions [52–55].

TDM or cord factor is another virulence factor produced abundantly in virulent strains of Mtb [27,56,57]. TDM blocks the phagosome-lysosome fusion and migration of polymorphonuclear neutrophils [27,56,57]. It contributes to the maintenance of the granulomatous response and the long term survival of Mtb in host cells [27,56,57]. Accumulation of TDM causes weight loss in the host, resulting in the condition known as cachexia [58]. The cyclopropane modification of TDM in virulent Mtb strains increase the inflammatory activity upon Mtb recognition by effectors of the innate IR, promoting the Mtb virulence through the manipulation of immune activation [59].

Previous studies showed that PDIM of Mtb are involved in macrophage invasion, inducing changes in the organization of plasma membrane lipids [6,29,30]. Regarding other components of the Mtb CE, the production of phenolic glycolipids in Mtb is associated with the hypervirulent phenotype displayed by a subset of Mtb isolates. There is also a clear correlation between the presence of sulfolipids in Mtb isolates and virulence in guinea pigs [60,61].

4. Indirect evidences of the protective role of Mtb CEC

Studies with polyclonal (pAbs) and monoclonal antibodies (mAbs), which challenged the traditional dogma of the exclusive role of cellular immunity in the defense against Mtb, had been very important in providing indirect evidence of the potential role of Mtb CEC in the protection against Mtb [62].

The first study describing a beneficial effect of the administration of mAbs directed to mycobacterial CEC on the course of Mtb infection was conducted with the mAb 9d8 (IgG3) that recognizes AM exclusively [63]. This mAb, increased the survival of intratracheally-infected mice when the Mtb Erdman strain was pre-coated with it. In this study, the positive effect on survival was associated with an enhanced granulomatous response in the lungs as compared to controls receiving an isotype-specific non-related mAb [63].

Another mAb, MBS43 (IgG2b) directed to MPB83, a surface lipoglycoprotein, prolonged the survival of intravenously infected mice associated with reduced granuloma size and decreased necrosis in the lung [64].

Enhanced survival has also been observed in experiments using the mAb SMITB14 (IgG1) directed against the AM portion of LAM. Passive immunization of BALB/c mice with SMITB14 and its corresponding F (ab') have been shown to provide protection against Mtb infection in BALB/c mice, as determined by dose-dependent reduction in bacterial load in lungs and spleens, reduced weight loss and increased long-term survival [65].

In another study, mice receiving intravenous mAb 5c11 (IgM), that recognizes other mycobacterial arabinose-containing carbohydrates in addition to AM, prior to mannosylated lipoarabinomannan (ManLAM) administration, showed a significant clearance of ManLAM and redirection of this Mtb product to the hepatobiliary system [66]. This study provided evidence that Abs can affect the fate of free mycobacterial polysaccharides. In addition, it was suggested that the liver and bile salts may have a role in the defense against mycobacterial infection, especially in the presence of specific Abs [66].

Heparin-binding haemagglutinin (HBHA) is a surface exposed protein which has been involved in mycobacterial dissemination [67]. Two mAbs against HBHA: 3921E4 (IgG2a), and 4057D2 (IgG3), were used to coat mycobacteria before administration to mice. In mice receiving mycobacteria pre-coated with either mAb, spleen CFUs were reduced while lung CFUs were comparable to those of control [67]. These results suggested that anti-HBHA Abs interfered with mycobacterial dissemination.

The Mtb 16 kDa protein (also called Acr antigen or HspX) has been identified as a major membrane protein and its expression is increased in Mtb growing inside infected macrophages [68–70]. IgA mAbs against this protein reached the respiratory fluids after its administration by different routes [71]. Intranasal administration of mAb TBA61 (IgA), directed against 16-kDa antigen of Mtb resulted in lung CFU reduction 9 days after intranasal or aerosol challenge with Mtb [72]. These results suggested that Abs could affect the early stages of infection. In another series of studies, López and colleagues evaluated the efficacy of TBA61 in the control of pulmonary infection [73]. Using an intratracheal model of pulmonary infection with Mtb H37Rv, they evaluated bacterial load and morphometric and histological changes in the lungs of

infected mice treated with the mAb [73]. The results showed a significant reduction in bacterial load and morphometric and histopathological changes in lungs of mice treated with TBA61, compared to control groups. The reduction of CFU in lungs of the treated group was associated with a better organization of the granulomas and less pneumonic area [73].

Balu and colleagues evaluated the properties of a new human mAb recognizing the 16 kDa protein [74]. The mAb 2E9 (IgA1) was constructed using a single-chain variable fragment clone selected from an Ab phage library [74]. The intranasal co-inoculation of 2E9 (IgA1) with recombinant murine IFN-γ significantly inhibited lung infection in transgenic mice for human myeloid IgA Fc receptor, CD89 [74]. Inhibition of the infection by the Ab was synergistic with human rIFN-γ in cultures of purified human monocytes [74]. This study demonstrated the feasibility of generating human mAbs to mycobacterial Ags, and their efficacy in mouse models adapted to human immune system [74].

In other experiments, the coating of Mtb with human IgG and secretory IgA formulations inhibited the infection in mouse models of progressive TB, which suggest that human Abs, directed to CEC are associated with protection against Mtb [75,76].

The administration to mice of pAbs derived from healthy humans highly exposed to Mtb afforded protection in a Mtb challenge model in mice with the protection being associated with the presence of Abs against the CE [77]. Administration of a commercial human IgG formulation pre-incubated with Mtb, abrogated the initial protection afforded by this formulation, suggesting an important role of Abs in Mtb protection [76].

These experimental evidences suggest a role of Abs in the defense against TB and the importance to consider the potential of mycobacterial CEC to elicit protective Ab responses in TB vaccine development.

5. TB VCs based on mycobacterial CEC

CEC have been used in TB vaccine development as adjuvants, and/or vaccine immunogens, although it is not always possible to establish a clear-cut distinction of these activities due to the overlapping effects of the components in several VCs. In the following sections, we will discuss the use of different CEC as adjuvants and vaccine immunogens.

5.1. Mycobacterial CEC as adjuvants

Vaccines based on individual proteins or fusion proteins are attractive alternatives because they are safe and produce little or no adverse effects. However, with few exceptions, proteins themselves are not detected as signs of danger and therefore do not induce IR in their first encounter with the host. To induce an IR, protein vaccines require pathogen-associated molecular patterns (PAMPs), which are small molecular structures found mainly in microorganisms like bacteria and viruses. The recognition of PAMPs through the pattern recognition receptors (PRR) triggers the induction of innate responses that finally lead to the specific adaptive IR against protein vaccines.

The immune properties of the lipid PAMPs present in the CE of mycobacteria have been extensively investigated. The early studies of Ribi et al. [78] and other studies [79,80] demonstrated the adjuvant capacity of polar and apolar mycobacterial CW extracts in different vaccine formulations against TB and other diseases [81,82], including cancer [83,84]. Further studies have shown that lipids present in the CE of mycobacteria are powerful adjuvant for Th1 IR when delivered as liposomes and that these lipids can improve the protection against TB by themselves [85] or in a formulation with a subunit vaccine [86].

LMs, LAMs, ManLAM, lipoproteins, PDIMs, mycolic acids (MAs) and mycolate esters are among the most important PAMPs on the mycobacterial CE. In recent years, much progress has been made in elucidating the immune molecular mechanisms triggered by mycobacterial glycolipids and the host cellular receptors involved in these processes [87]. However, a few studies have demonstrated the contribution of these glycolipids (or their derivatives) to the protection against Mtb in animal models, when formulated with subunit vaccines.

Below, we describe examples that have been reported on the use of mycobacterial CEC as adjuvants of TB subunit VCs in Mtb-challenge experiments.

5.1.1. Monomycoloyl glycerol (MMG)

The study of Andersen et al. has demonstrated that immunization of mice with Ag85B/ESAT-6 adjuvanted with N,N-dimethyl-N,N-dioctadecylammonium bromide (DDA) liposomes in combination with MMG or synthetic analogues induced a Th1-biased IR that provided significant protection against TB at levels comparable to the protective immunity induced by BCG vaccination [88].

5.1.2. TDM

TDM is one of the most potent immunostimulatory molecules on the mycobacterial CE; this glycolipid formulated with either protein Ags or DNA vaccines has shown powerful adjuvant properties.

The study of de Paula et al., has shown that a single dose of co-encapsulated DNAhsp65 and TDM into biodegradable poly (DL-lactide-*co*-glycolide) (PLGA) microspheres reduced the bacterial burden of Mtb in mice and guinea pigs as efficiently as three doses of naked DNAhsp65 [89]. However, this vaccination scheme did not exceed the protection conferred by BCG. Conversely, boosting BCG-vaccinated mice with DNAapa coencapsulated with TDM in microspheres reduced the bacterial burden in lungs 70 days post Mtb challenge compared to vaccination with BCG alone [90].

Mtb10.4–HspX fusion antigen adjuvated with DDA and TDM induced antigen-specific humoral and cell-mediated immunity. When used as a booster to BCG, this formulation slightly improved the protection conferred by BCG alone against Mtb challenge in mice [91].

Similarly, Decout et al. found that vaccination of mice with Ag85A and TDM incorporated in DDA liposomes induced strong Th1 and Th17 IRs and conferred protection against Mtb infection [92].

The toxicity of TDM restricts its use as adjuvant for vaccines. Unlike TDM, trehalose dibehenate (TDB), a structural analogue of TDM, in which simpler fatty acids replace the complex mycolic acids, has an acceptable toxicity. Several studies have demonstrated that TDB formulated in cationic liposomes with DDA (formulation called CAF01) and with a fusion protein Ag85B-ESAT-6 or ESAT-6 as single protein induced strong Th1 and Th17 responses and protection against Mtb infection in mice at levels comparable to the protective immunity induced by BCG vaccination [93–95].

The adjuvant properties of CAF01 and two other liposomal adjuvants: Cationic CAF04 (DDA/MMG), and CAF05 [DDA/TDB in/poly (I:C)] have been tested in combination with the fusion protein H56 (Ag85B-ESAT6-Rv2660c) in non-human primates. The results of this study have shown that immunization with formulations of H56 in all adjuvants (used as BCG-boosters) resulted in better survival of the monkeys infected with Mtb and better protection readouts compared to BCG alone, albeit at non-significant levels [96].

5.1.3. Glucose monomycolate

The study of Decout et al., has demonstrated that glucose and mannose esterified at O-6 by a synthetic α-ramified 32-carbon fatty acid are agonists of the C-type lectin receptor Mincle with similar adjuvant activity to that of TDM. One of these structurally simple synthetic

Mincle ligands, GlcC14C18, has been shown to be less toxic than TDB on the host cells. This adjuvant induced protective immunity in a mouse model of Mtb infection when incorporated in DDA and inoculated with Ag85A to a similar extent to that afforded by vaccination with Ag85A/DDA/TDB [92].

5.1.4. Arabinomannan (AM)

The portion of AM of LAM has been used as adjuvant in different vaccine formulations. When AM linked to Ag85B was conjugated in Eurocine™ L3 adjuvant emulsion or in Alum, the resulting conjugates showed good protective efficacy against Mtb challenge in guinea pigs and mice, respectively. However, the protection afforded by these conjugate vaccines in mice was less efficient than BCG vaccination [97]. An AM-tetanus toxoid conjugate (AM-TT), formulated in Eurocine™ L3 adjuvant, was used as intranasal boost to BCG. The bacterial loads in the spleens of Mtb-challenged animals were reduced in boosted animals compared to non-boosted animals. This finding suggests a direct contribution of AM to the protective efficacy of the conjugate vaccine. However, lung protection against Mtb infection was not improved in boosted animals [98].

Finally, Prados-Rosales et al.; have used the native capsular AM in vaccine formulations against tuberculosis infection of mice. They found that vaccination with capsular AM-Ag85b conjugate increased the survival of Mtb infected animals when compared to non-vaccinated or Ag85b-immunized mice. The survival of AM-Ag85b-vaccinated mice was similar to that of BCG-vaccinated animals but the bacterial counts in the lungs and spleen of mice after Mtb challenge was similar between the three groups [99]. Interestingly, the authors of this study used passive immunization of naïve mice with sera from AM-Ag85b vaccinated animals to demonstrate that the protection mechanism induced by AM-Ag85b was antibody (Ab) mediated. They propose that specific Abs to both AM and Ag85b contributed to control bacterial dissemination. This observation is consistent with the modest protection conferred by AM-conjugates linked to Mtb unrelated proteins, such as TT [see above [98]] and PA from *Bacillus anthracis* [99].

5.1.5. Phosphatidylinositol di-mannoside (PIM2)

PIM2 and its derivatives have been formulated with the fusion protein Ag85A-ESAT-6 and tested as anti-bovine TB vaccines in a mouse model. Vaccination of mice with Ag85A-ESAT-6 + PIM2 or BCG conferred a significant reduction in the bacterial load in lungs compared to that for the PBS control, but only BCG vaccination resulted in a significant reduction in the mean spleen bacterial count. Other PIM2 derivatives were not shown to improve the protection induced by the fusion protein Ag85A-ESAT-6 alone. In fact, PIM2ME, a monoether derivative of PIM2, appeared to have a detrimental effect in the control of bacterial replication in lungs and spleen of vaccinated and *Mycobacterium bovis* (Mbo) infected mice [100]. Larrouy-Maumus et al., assayed the adjuvant properties of mycobacterial lipids formulating PIM2 and diacylated sulfoglycolipids (Ac2SGL) in liposomes made of DDA and TDB and used them as vaccines against Mtb in guinea pigs. The results showed that lipid VCs induced reduction of bacterial counts in spleen but not in lungs when compared to the unvaccinated group. However, vaccinated animals showed less pathology and also less lung necrosis [101].

Wedlock et al., used culture filtrate proteins (CFPs) from Mbo with different lipid formulations in DDA as boosts of BCG vaccination in cattle. In this study, PIM2 did not improve the protection induced by BCG alone in Mbo-challenged cattle. Remarkably, only the synthetic lipopeptide and the TLR2 agonist, Pam3Cys-SKKKK, formulated in DDA with CFPs conferred better protection compared to BCG alone [102].

5.1.6. Poly-α-L-glutamine

(PLG) are glutamine-rich self-assembling peptides that are associated with the peptidoglycan layer of mycobacteria through non-covalent interactions. The adjuvant power of these peptides has been recently assessed. PLG improved the protective efficacy of ESAT-6 alone in a mouse model of TB, thus reaching to a protection level equivalent to that conferred by BCG vaccination [103].

5.2. Mycobacterial CEC as immunogens

Vaccines obtained from/or containing CEC can be classified according to the platform strategy: either as subunits, DNA, Mtb Ags expressed in attenuated vectors, CE extracts and natural and artificial membrane vesicles (MVs) (Tables 1–5).

5.2.1. Subunits

Considering the potential for protection of the Ab responses against CE carbohydrates, and the growing evidence of the importance of the lipid components in the elicitation of potent T cell responses against mycobacterial lipids, the breadth of the evaluated VCs as subunits has been expanded beyond the classical protein-based vaccine targets including carbohydrate and lipid components (Table 1) [63,65,104–107].

Various subunit VCs have been evaluated using different adjuvant and delivery systems, as isolated components or as cocktails or fusion proteins, as multi-stage constructions covering different stages of the infection, administered by different routes and in prime-boost regimes, combined with BCG or with other VCs (Table 1).

In general, the VCs evaluated showed good potential as TB vaccines inducing good immunogenic responses and protection against Mtb or Mbo in animal models, some of them are at the advanced stages of clinical evaluation (Table 1).

Table 1
Subunit VCs developed from Mtb CEC.

Vaccine candidates (VC)	Subunit VCs developed from Mtb CEC							Comments	Ref
	Model	Route	Adjuvant	Prime	Boost	Challenge	Protec Y/N		
Proteins									
Ag16 kDa	Mouse	O/IN	Starch microparticles	VC	VCx2	No	–	H&C-IR	[108]
Ag16 kDa-EsxS in PLGA	Mouse	SC	DOTAP	VC	VC	No	–	H&C-IR	[109]
Ag27 kDa	Mouse	SC	Ribi or DDA	BCG	VC	Mtb-**IV**	N-CFU	Strong Th1 (with BCG/Mtb-Ags)	[110]
Ag85A	Mouse	ID	CAF01 or TDB synthetic analogues + DDA	VC	VCx2	Mtb-IN	Y-CFU	H&C-IIR. Protec (+potent: synthetic analogues)	[92]
Ag85B	Mouse	IN	CPG	BCG	VC	BCG-**AER**	Y-CFU	Presence of dendritic in BAL	[111]
Ag85A-Ag85B	Mouse	IN/SC	DDA	VC	VC	Mtb-**IN**	Y-CFU	Mucosal, H&C-IR cells.	[112]
Ag85A-ESAT6	Mouse	SC	Synthetic PIM2+oil + water (Emulsigen™)	VC	VCx2	Mbo-**AER**	Y-CFU	H&C-IR	[100]
Ag85A-Ag85B-CFP20.5-CFP25-CFP32	Mouse	SC	DDA-MPL	VC	VC	Mtb	Y-CFU	H&C-IR	[113]
Ag85B-ESAT6 (**H1**)	Mouse Guinea pig	O/SC	DDA-MPL MPL, CT, LT	VC-O/SC	VC-O/SC	Mtb-**AER**	Y-CFU	C-IR. Oral: no priming effect.	[114]
	Mouse	SC	BCG lipids in cationic liposomes	VC	VCx2	Mtb-**AER**	Y-CFU	H&C-IR LT-Protec > BCG	[86]
	Mouse		PLG				Y-CFU & S	Protec = BCG	[103]
	Mouse	IN	LTK63	BCG	VC	Mtb-**AER**	Y-CFU	C-IR	[115]
	Mouse	SC	MMG in cationic liposomes	VC	VCx2	Mtb-**AER**	Y-CFU	Protec = BCG	[88]
	Mouse	SC	CAF01	VC	VCx2	Mtb-**AER**	Y-CFU	C-IR. LT-Protec.	[93]
	Mouse	IM	CAF01 & Bioneddles	VC	VCx2	Mtb-**AER**	Y-CFU	C-IR. Protec = BCG	[94]
	Mouse-Neonatal/adult	SC	IC31 or Alum	VC		BCG-**IV**	Y-CFU	C-IR.	[116]
	Guinea pig	SC	DDA-MPL or TDB	VC	VC	Mtb-**AER**	Y-S	C-IR.	[117]
	NHP	IM	DDA-MPL or ASO2A	VC		Mtb-**IT**	Y-CFU	H&C-IR	[118]
	HHA-TST-	IM	IC31	VC		No	–	C-IR. AT	[119]
	HHA- BCG-Vac	IM	IC31	VC		No	–	H&C-IR. AT	[120]
	HHA-TST-	IM	CAF01	VC		No	–	C-IR. AT	[121]
	HHIV-Inf-adult	IM	IC31	VC		No	–	Phase II. C-IR (Th1). AT	[122]
	HHAd-TST-/+	IM	IC31	BCG	VC	No	–	Phase II. C-IR. AT	[123]
Ag85B-TB10.4 (**H4**) (**Aeras-404**)	Mouse	SC	CAF01	VC/Ad	Ad/VC	Mtb-**AER**	Y-CFU	C-IR	[124]
	Mouse/Guinea pig	SC	IC31	VC		Mtb-**AER**	Y-CFU	Dose dependent induction Th1.	[125]
	Guinea pig	SC	IC31	BCG	VC	Mtb-**AER**	Y-CFU	C-IR	[126]
	HHA- BCG-Vac	IM	IC31	BCG	VC	No	–	C-IR. AS.	[127]
	HHA-BCG-Vac	IM	IC31	BCG	VC	No	–	Phase I. C-IR. AS&T.	[128]
	HHAd-BCG-Vac	IM	IC31	BCG	BCG/VC	No	–	Phase II. Prevention QFT conversion. AS&T	[129]
Ag85B-ESAT6-Rv2660c (**H56**)	Mouse	SC	CAF01	BCG	VC	Mtb-**AER**	Y-CFU	C-IR. Protec (pre & post exposure)	[130]
	NHP	IM	IC31	BCG	VC	Mtb-**IT**	Y-S	C-IR. Protec (pre & post exposure)	[131]
	NHP	IM	CAF01, CAF04, CAF05, IC31	BCG	VC	Mtb-**IT**	Y-S	C-IR	[96]
	HHA-TST-/+	IM	IC31	BCG	VC	No	–	H&C-IT. AS&T.	[132]
Ag85B-Rv2660c-TB10.4(**H28**)	Mouse	SC	CAF01	VC	VC/MVA28	Mtb-**AER**	Y-CFU	C-IR. Protec > BCG (VC7VC)	[133]
	NHP	IM	IC31	BCG	VC/MVA28	Mtb-**AER**	Y-S	C-IR. Protec (with both schemes)	
Ag85B-HspX-Mpt64-Ag85B -Mpt64-Mtb8.4	Mouse	SC	DDA-BCG PSN	BCG	VC	Mtb-**IV**	Y-CFU	H&C-IR. Protec > BCG Combined vaccines (as BCG-booster)	[134]
	Mouse	SC	DDA-PSN	VC/BCG	VCx2	Mtb-**IV**	Y-CFU	H&C-IR. Protec > BCG (as BCG booster)	[135]
Ag85B-HspX-CFP10-ESAT6-	Mouse	SC	CpG/Alum	VC	VCx2	No	–	H&C-IR	[136]
	Guinea pig	IM	CpG/Alum	BCG	VCx2	Mtb-**SC**	Y-CFU	–	
CFP8-CFP10-CFP15 ESAT6-TB10.4	Mouse	SC	MPL/DDA	VC	VCx2	Mtb-**IV**	Y-CFU	–	[137]
CFP11-CFP21-CFP22.5-CFP31-MPT64									
CFP10-HBHA-TB8.4-TB10.4-Rv3615c-	Mouse	SC	DDA/MPL/TDB (DMT)	VC	VC	Mtb-**AER**	Y-CFU	H&C-IR	[138]
ESAT6	Mouse	SC	CAF01	VC	VCx2	Mtb-**AER**	Y-CFU	C-IR. LT-Protec (subdominant epitopes)	[95]

(*continued on next page*)

Table 1 (*continued*)

Vaccine candidates (VC)	Subunit VCs developed from Mtb CEC							Comments	Ref
	Model	Route	Adjuvant	Prime	Boost	Challenge	Protec Y/N		
EsxR	Mouse	SC	MPL-TDM	BCG	VC	Mbo	Y-CFU	C-IR	[139]
HBHA	Mouse	IN	CT	VC	VC	BCG-**IN**	Y-CFU	H&C-IR.	[140]
				BCG/VC	VCx4	Mtb-**IT**	Y-CFU	H&C-IR. Protec > BCG (as BCG-booster)	[141]
Mtb10.4-HspX	Mouse	SC	TDM + DDA	BCG	VCx2	Mtb-**IV**	Y-CFU	Protec = BCG. H&C-IR	[91]
Mtb72F	Mouse	IM	ASO2A/ASO1B	VC	VCx2	Mtb-**AER**	Y-CFU	H&C-IR	[142]
	Mouse/Guinea pig	SC	ASO2A	VC/BCG	VCx2	Mtb-**AER**	Y-S	H&C-IR	[143]
	Rabbit	IM	ASO1A/AS01B	BCG/VC	VCx3	Mtb-**intratecal**	Y-CFU	H&C-IR.	[144]
	NHP	IM	ASO2A	BCG/VC	VCx2/3	Mtb-**IT**	Y-CFU & S	C-IR. Protec > BCG	[145]
	HHA-TST-	IM	ASO2A	VC	VCx2	No	–	Phase I. H&C-IR. AT	[146]
	HHA-BCG-Vac/Mtb infected	IM	ASO2A	VC	VCx2	No	–	Phase I/II. H&C-IR. AT	[147]
	HHA-TST-No-BCG-Vac	IM	ASO2A	VC	VCx3	No	–	Phase I. H&C-IR. AT	[148]
	HHA-TST-	IM	ASO1/AS02	VC (M72/ M72F)	VC	No	–	Phase I/II. H&C-IR. Protec > BCG (M72-ASO1). AT	[149]
PE20	Guinea pig	IM	DDA/TDB	VC	VCx2	Mtb-**AER**	Y-CFU	H&C-IR	[150]
Culture Filtrate Proteins (CFPs)	Cattle	SC	DDA + MLP or synthetic PIM_2 or Pam_3CSK_4 with BCG	VC	VCx2	Mbo-**IT**	Y-CFU	Better results with Pam_3CSK_4	[102]
Carbohydrates									
AM (Arabinomannan)	Mouse	SC/IN	TT/Eurocine^{TM}L3	SC	IN	Mtb-**IN**	Y-S	C-IR	[97]
	Guinea pig	SC/IN	TT/Eurocine^{TM}L3	SC	IN	No	–	H&C-IR	
	Mouse	IN	TT/Eurocine^{TM}L3	BCG	VC	Mtb-**IV**	Y-CFU	C-IR	[98]
AM-Ag85B	Mouse	SC	Alum	VC	VC	Mtb-**IV**	Y-S	–	[97]
	Guinea pig	SC	No	SC	IN	Mtb-**AER**	Y-CFU & S	–	
	Mouse	SC	Alum	VC	VCx2	Mtb-**AER**	Y-CFU & S	H-IR Inhibit Mtb dissemination	[99]
Lipids									
Ac_2SGL + PIM_2 (LipVac1) SL37 + PIM_2 (LipVac2)	Guinea pig	IM	DDA & TDB (liposomes)	VC	VCx2	Mtb-**AER**	Y-CFU		[101]
PIMs	Mouse		liposomes & lipid A	VC		Mtb	Y-S	H&C-IR	[151]
TDM (cord factor)	Mouse	IV/IP		VC	VC	Mtb-**IV**	Y-S	–	[152]
TDM-MBSA	Mouse	SC	IFA	VC	VC	Mtb-**IV**	Y-CFU & S	–	[153]

Ac2SGL: Diacylated sulfoglycolipids; **AER**: aerosol**; AM:** Arabinomannan; **AT:** Acceptable tolerability; **AS&T:** Acceptable Safety & tolerability; **BCG:** Bacille Calmete-Guerin.
BCG-Vac: BCG-vaccinated**; C** cellular; **CFU:** colony forming unit; **HHA:** human healthy adults; **HHAd:** human healthy adolescents; **H:** humoral; **IM:** intramuscular.
IV: intravenous; **IT:** intratracheal; **IP**: intraperitoneal; **IR:** immune response; **Inf-adult:** infected adults; **LT:** long term; **Mtb:** *Mycobacterium tuberculosis*; **M72**: point mutation of Mtb72F; **O:** oral; **Pam3CSK4:** synthetic lipopeptide; **PIMs:** Mannophosphoinositides; **PLG:** Poly-α-L-glutamine; **Protec:** Protection; **SC:** subcutaneous; **S:** survival.
TDB: Trehalose-6,6-dibehenate; **TDM:** Trehalose-6,6-dimycolate (cord factor); **TDM-MBSA:** TDM methylated BSA; **TT:** tetanus toxoid; **VC:** Vaccine candidate; **Y/N:** yes/no.

5.2.2. DNA

DNA vaccines, with their capacity to elicit humoral and cellular Th1 IRs, including $CD8^+$ cytotoxic responses have been considered obvious candidates for new generation TB vaccine development [154,155].

DNA VCs, containing CE Ags genes, with different adjuvants, delivery systems and prime-boost schemes demonstrated good immunogenicity and protective capacity in different animal models (Table 2).

Table 2
DNA based vaccines.

Vaccine candidates (VC)	DNA based VC							Comments	Ref
	Model	Route	Adjuvant	Prime	Boost	Challenge	Protec (Y/N)		
19 kDa/Rv3763	Mouse	IM	No	VC	VCx2	Mtb-**IV**	N	H&C-IR	[156]
27 kDa	Mouse	IM	No	BCG	VCx2	Mtb-**IV**/BCG	N -CFU	Abrogates BCG Protec	[110]
Ag85A	Mouse	IM	PLG	VC	VCx2	Mtb-**AER**	Y-CFU	C-IR	[157]
	Mouse	SC	No	VC	VCx2	Mtb-**AER**	Y-CFU	C-IR	[158]
	Guinea pig	GG	IFA	VC	VC/Ag85A	Mtb-**AER**	Y-CFU	–	[159]
Ag85A (S. thyphimurium as delivery system)	Mouse	O/IN	No	VC	VCx2	Mtb-**IV**	Y-CFU	H&C-IR	[160]
Ag85B	Mouse	IM	Cardiotoxin	VC	VCx3	Mbo	Y-partial	H&C-IR	[161]
	Mouse	IM	–	VC	VC/BCG	Mtb-**AER**	Y-CFU	–	[162]
Ag85B-CFP10-CFP21	Mouse	IM	No	VC	VCx2	Mtb-**IV**	Y-CFU	H&C-IR.	[163]
Ag85B-MTP64-MTP83	Cattle c.	IM	No	VC	BCG	Mbo	Y-CFU	C-IR	[164]
	Mouse	IM	IL2 gene	VC	VCx2	Mtb-**IV**	Y-CFU	H&C-IR	[165]
	Cattle c.	IM	DDA	VC	VCx2	Mbo-**IT**	Y-CFU)	H&C-IR	[166]
Ag85B-ESAT6-KatG-Rv1818c-MTB8.4-MTB12-MTB39A-MPT63-MPT64-MPT83	Mouse	IM	Fused to TPA/ ubiquitin-Ub	VC	VCx2	Mtb-**AER**	Y-CFU & S	C-IR	[167]
Ag85A-ESAT6	Mouse	IM	No	VC	VC/BCG/ attenuated Mb	Mbo-**AER**	Y-CFU	H&C-IR. Protec = BCG (with booster)	[168]
Ag85B-ESAT6	Mouse	IM	No	VC/BCG	VC	Mtb-**AER**	Y-CFU & S	C-IR-IR. Protec > BCG (as booster)	[169]
	Mouse	IM	No	VC	VC	Mtb	Y-CFU	H&C-IR. Protec = BCG	[170]
Ag85A-Ag85B-CFP10 ESAT6 (Tcell epitopes fused to HSP65)	Mouse	IM	No	VC	VCx3	BCG-**IN**	Y-CFU	H&C-IR	[171]
Ag85B-ESAT6-MPT83	Mouse	IM	DDA	VC	VCx2	Mtb-**IV**	Y-CFU	H&C-IR. Protec > BCG	[172]
Apa	Mouse	SC/IM	TDM + PLGA	BCG-SC	VC-IM	Mtb-**IT**	Y-CFU	Protec > BCG	[90]
Apa/Pro	Mouse	SC/IM	CMV-IE/ubiquitin	VC	VC	BCG-**IV**	Y-CFU	H&C-IR	[173]
	Guinea pig	ID		VC	–	No	–	H&C-IR	
ESAT6	Mouse	IM	No	VC/rBCG	VC/VCx2	No	–	C-IR	[174]
	Guinea pig	IM	No	VC/rBCG	VC/VCx2	Mtb-**AER**	N-CFU	No protec: VC. Abrogates protec (as booster of rBCG)	
ESAT6/16kDa/SodA	Mouse	IM	No	VC	VCx2	No	–	H&C-IR	[175]
	Guinea pig	IM	No	VC	VCx2	Mtb-**SC**	Y-CFU		
ESAT6-CFP10	Bull calves	IM	IFA + DNA:GM-CSF + DNA:CD80/CD86	VC	VC	No	–	H&C-IR. Best results with: VC + DNA:GM-CSF + DNA:CD80/CD86	[176]
	Calves	IM		VC/BCG VC + BCG	VC	Mbo-**AER**	Y-CFU	Best results with: BCG + VC + DNA:GM-CSF + DNA:CD80-CD86)	
ESAT6/KatG/MPT64/HBHA	Mouse	IM	No	VC	VCx2	Mtb-**AER**	Y-CFU	H&C-IR	[177]
HSP65	Mouse	IM/GG	No	VC	VCx2	Mtb-**IT**	Y-CFU	H&C-IR. Protec:Y **(IM)**: Protec: N **(GG)**	[178]
	Mouse/ Guinea pig	IM	TDM + PLGA	VC	–	Mtb-**IT**	Y-CFU	H&C-IR. Protec = BCG	[89]
	Mouse	IN/IM	No	BCG-**IN**/**SC**	VCx2	Mtb-**IT**	Y-CFU	C-IR. Protec > BCG (BCG-IN + VC)	[179]
Hsp65-Hsp70-Apa	Mouse	IM	No	VC	VCx3/BCG	Mbo-**IV**	Y-CFU	C-IR. Protec: (with BCG boost)	[180]
LppX (22 kDa)	Mouse	IM	No	VC	VCx2	Mtb-**IV**	N-CFU	H&C-IR	[181]
MPT64 fused to ubiquitin	Mouse	IM	No	VC	VCx2	Mtb-**AER**	Y-CFU	H&C-IR	[182]
MPB83	Mouse	IM	No	VC	VCx3	Mbo-**IV**	Y-CFU	H&C-IR	[183]
	Cattle c.	IM	No	VC	VCx2	No	–	C-IR	
MPB70/MPB83	Cattle	IM	No	VC	VCx2/ Proteinx2	Mbo-**IT**	N-CFU	H&C-IR (after priming with protein)	[184]
Mtb72F	Mouse	IM	No	VC	VCx2	Mtb-**AER**	Y-CFU	H&C-IR	[142]
	Guinea pig	IM	No	VC	VCx2	Mtb-**AER**	Y-S	–	
PstS1/PstS2/PstS3	Mouse	IM	No	VC	VCx2	Mtb-**IV**	Y-CFU	H&C-IR. Protec (PstS3)	[185]

AER: aerosol; **BCG:** Bacille Calmete-Guerin; **Cattle c.:** Cattle calves **C** cellular; **CFU:** colony forming unit; **GG:** Gene-gun; **H:** humoral; **ID:** intradermal; **IFA:** incomplete Freund adjuvant; **IM:** intramuscular; **IN:** intranasal; **IR:** immune response; **IT:** intratracheal; **IV**: intravenous; **Mbo:** *Mycobacterium bovis* **Mtb:** *Mycobacterium tuberculosis*; **O:** oral; **Protec:** Protection.
S: survival; **SC:** subcutaneous; **SodA:** superoxide dismutase A; **VC:** Vaccine candidate; **Y/N:** yes/no.

5.2.3. Mtb CE-antigens expressed in attenuated vectors

The use of bacterial and viral vectors expressing heterologous Ags as new generation vaccines is one of the most important technological platforms due to their multiple advantages [186,187]. Expression of CE Ags and in various antigen combinations, either by themselves or combined with other VCs in prime-boost schedules, exemplify some of the candidates at the advanced stages of clinical evaluation (Table 3).

5.2.4. CE extracts

The presence in the mycobacterial CE of multiple components associated with the interaction with host receptors, virulence and important processes of relevant importance for the survival of the bacteria, in addition to their accessibility to the host IRs, has influenced the use of different CE extracts as experimental VCs (Table 4).

These candidates represent multi-antigenic formulations with

Table 3
TB VCs based on Mtb CE antigens expressed in attenuated vectors.

Vaccine candidate (VC)	Mtb CE antigens expressed in attenuated vectors						Comments	Ref
	Model	Route	Prime	Boost	Challenge	Protec (Y/N)		
Adenovirus (Ad)								
Ag85A	Mouse	IM/IN	VC-IM/IN	VCx2-IM/IN	Mtb-**IN**	Y-CFU	C-IR. Protec: IN	[188]
			VC-IM/IN	VCx3 = 2:IM; 1:IN/IM	Mtb-**IN**	Y-CFU	Protec: IN + IM better results	
	Mouse	IN/ID	BCG-	ChAd-MVA	Mtb-**AER**	Y-CFU	Protec: ChAd (IN) followed by MVA (ID/IN)	[189]
	Mouse	SC/IN	BCG	VCx2-IM/IN	Mtb-**IN**	Y-CFU	C-IR. Protec > BCG: IN	[190]
	Mouse	IM/IN	DNA-IM	VC-IN	Mtb-**IN/AER**	Y-CFU	Protec > BCG: IN	[188]
	Mouse/	IM/IN	VC	–	Mtb-**IN**	Y-CFU	C-IR. Protec: IN	[191]
	Guinea pig	IN/IM	BCG/VC	VC-IN/IM	Mtb-**AER**	Y-CFU & S	Protec > BCG: IN	[192]
	Calves	ID	BCG	VC	Mbo-**IT**	Y-CFU, pathology	C-IR. Protec > BCG	[193]
Ag85A-TB10.4	Mouse	IN/IM	VC	–	Mtb-**IN**	Y-CFU	C-IR. Protec > BCG	[194]
Ag85A-Ag85B-TB10.4	Mouse	IN/IM	VC	–	Mtb-**IN**	Y-CFU	C-IR	[195]
Ag85B	Mouse	ID/IN	DNA-ID	VC-IN	Mtb-**AER**	Y-CFU	C-IR	[196]
BCG								
16 kDa	Mouse	SC	VC	–	Mtb-**IV**	Y-CFU	H&C-IR	[197]
72f	C. monkey	ID	VC	VCx2	Mtb-**IT**	Y S	C-IR	[198]
Ag85A	C. monkey	ID	VC	–	Mtb-**IT**	Y-CFU	H&C-IR	[199]
Ag85B-16kDa	Mouse	SC	VC	–	Mtb-**IN**	Y-CFU	H&C-IR	[200]
Ag85B	Mouse	SC	VC	–	Mtb-**IV**	Y-CFU	H&C-IR	[197]
	Guinea pig	ID	VC	–	Mtb-**AER**	Y-CFU & S	C-IR	[201]
	HHA PPD-	ID	VC	–	No	–	C-IR. AT	[202]
Ag85B-ESAT6	Mouse	SC	VC	–	Mtb-**IV**	Y-CFU	H&C-IR	[203]
Ag85B-ESAT6-INFγ	Mouse	SC	VC	–	Mtb-**IV**	Y-CFU	H&C-IR. Protec > BCG	[204]
Ag85B-CFP10-ESAT6- Mtb8.4-MTP40	Mouse	IP	VC	VCx2	No	–	H&C-IR	[205]
MPT64-PE_PGRS33	Mouse	SC	VC	–	Mtb-**AER**	Y-CFU & S	H&C-IR. Protec > BCG	[206]
Human Parainfluenza type2 virus (rhPIV2)								
Ag85B Influenza virus	Mouse	IN	VC/DNA	VCx3	Mtb-**AER**	Y-CFU	C-IR. Protec > BCG	[207]
ESAT-6	Mouse/	IN	VC	VCx2	Mbo-**IV**	Y-CFU	C-IR. Protec = BCG	[208]
	Guinea pig	SC/IN	VC	VC	Mtb-**SC**	Y-CFU	Protec = BCG	
Mycobacterium smegmatis								
19 kDa/Rv3763	Mouse	SC	VC	–	Mtb-**IV**	N-CFU & S	Deleterious effect	[156]
Ag85B epitope	Mouse	SC	VC	VC	No	–	H&C-IR	[209]
Mycobacterium vacae								
19 kDa/Rv3763	Mouse	SC	VC	–	Mtb-**IV**	N-CFU & S	Deleterious effect	[156]
Salmonella Thyphimurium								
Ag85B	Mouse	O/IV	VC	VCx2	Mtb-**IV**	Y-CFU	C-IR	[210]
ESAT6	Mouse	IV	VC/DNA	DNA/VC	Mtb-**IV**	Y-CFU	C-IR	[211]
Ag85B-ESAT6	Mouse	O/IN	VC-O/IN	Protein-IN	No	–	H&C-IR	[212]
	Guinea pig	O/SC	VC-O	Protein-SC	Mtb-**AER**	Y-CFU & S	–	
Modified Vaccinia Virus Ankara (MVA)								
Ag85A	Mouse	IN/	BCG-IN	VC-IN/parenteral	Mtb-**AER**	Y-CFU	C-IR. Protec > BCG (VC-IN)	[213]
	Guinea pig	SC	BCG	VC/Fowlpox-85A	Mtb-**AER**	Y-S	Protec > BCG	[214]
	Calves	ID	BCG	VC	Mbo-**IT**	Y-CFU & pathology	C-IR. Protec > BCG (VC booster)	[193]
	R. macaques	ID/AER	BCG	VC	No	–	IR (AER). AS.	[215]
	HHA	ID/AER	BCG	VC	No	–	Specific-IR. Both were safe	[216]
	HHI	ID	BCG	VC	No	–	C-IR. AS&T. Efficacy = BCG	[217]
Ag85B-ESAT6-	Mouse	SC	VC	–	Mtb-**IV**	Y-CFU	H&C-IR. Protec > BCG	[218]
Ag85A-Ag85B-ESAT6-HSP60-MTB39-IL15	Mouse	SC	VC/ESAT& Ag85Bx3	VCx2/ESAT& -Ag85Bx3	Mtb-**IV**	Y-CFU	C-IR	[219]
Vesicular stomatitis virus (VSV)								
Ag85A	Mouse	IN/IM	VC/Ad	VC-IN	Mtb-**AER**	Y-CFU	C-IR. Protec: (with prime-boost)	[220]
VSV-846 (Rv3615c-Mtb10.4-Rv2660c)	Mouse	IN	VC/BCG	VC	BCG-**IN**	Y-CFU	C-IR. Protec > BCG (with prime-boost)	[221]
VSV-846	Mouse	IN	VC	–	BCG-**IN**	Y-CFU	C-IR. LT-Protec > BCG	[222]

AER: aerosol; **AS:** Acceptable safety; **AT:** Acceptable tolerability; **BCG:** Bacille Calmete-Guerin; **C:** cellular; **CFU:** colony forming unit; **ChAd:** Chimpanzee Ad; **C.monkey:** Cynomolgus monkey; **H:** humoral; **HHA:** human healthy adults; **HHI:** human healthy infants; **ID** intradermal; **IM:** intramuscular; **IN:** intranasal; **IP:** intraperitoneal; **IR:** immune response; **IT:** intratracheal; **IV**: intravenous; **LT:** long term; **Mbo:** *Mycobacterium bovis* **Mtb:** *Mycobacterium tuberculosis*; **Protec:** Protection; **R. macaques**: Rhesus macaques; **S:** survival; **SC:** subcutaneous, **T:** tolerability;**VC:** Vaccine candidate; **Y/N:** yes/no.

Table 4
TB VCs based on Mtb CE extracts.

Vaccine candidate (VC)	VC based on Mtb CE extracts							Comments	Ref
	Model	Route	Adjuvant	Prime	Boost	Challenge	Protec (Y/N)		
TSP-Aq	Mouse	SC	IFA	VC	VCx2	Mtb-**IV**	Y-CFU	C-IR. Protec = BCG	[223]
CE-PPC	Mouse	SC/IM	No	VC-SC	VCx2- SC/IM	Mtb-**IV**	Y-CFU & S	H&C-IR	[224]
LMs	Mouse	SC	With/without Alum	VC	VC	Mtb-**IT**	Y-CFU	Protec = BCG (VC with/without Alum)	[225]
Myco-CE-O	Mouse	IV	No	VC	–	Mtb-**AER**	Y-CFU	Protec: CW from BCG, Mbo, Mtb & non-tuberculous mycobacteria	[78,79]
Mtb-WLE	Guinea pig	SC	QS-21/DDA or both	VC	VCx2	Mtb-**AER**	Y-CFU	Protec (VC + QS-21 + DDA and QS-21)	[85]
RUTI	Mouse	SC	No	VC	VC	Mtb-**AER**	Y-CFU	Immunotherapeutic effect on reactivation after ST chemotherapy. C-IR	[226]
	Mouse	SC	No	VC BCG VC	VC/ VCx2 VCx3	Mtb-**AER**	Y-CFU	ST-Protec = BCG (lung) Protec < BCG (spleen) LT-Protec = BCG (lung)	[227]
				VC	VC	Mtb-**AER**	–	Therapeutic effect (decrease of CFU)	[227]
	Guinea pig	SC	No	VC	VC	Mtb-**AER**	Y-CFU	Immunotherapeutic effect on reactivation after ST-chemotherapy.	[226]
	Guinea pig	SC	No	VC	VC	Mtb-**AER**	N-S	–	[227]
	HHA-no BCG vac Non Mtb infected	SC	No	VC	VC	No	–	Phase I. C-IR. AT.	[228]
	Latently infected HIV (±)	SC	No	VC	VC	No	–	Phase II. C-IR. Reasonable tolerability.	[229]
Myco-CEO	Rhesus Monkey	IV	No	VC	–	Mtb-**AER**	Y-Chest X-rays & gross & microscopic pathology	Protec = BCG (CWs from BCG & Mbo)	[80]

AER: aerosol; **AT**: Acceptable tolerability; **BCG**: Bacille Calmete-Guerin; **C**: cellular; **CFU**: colony forming unit; **CE-PPC**: Mtb CE protein peptoglican complex in liposomes.
H: humoral; **HHA**: human healthy adults; **IFA**: Incomplete Freund adjuvant; **IM**: intramuscular; **IR**: Immune response; **IT**: intratracheal; **IV**: intravenous; **LMs**: lipid extract from *M smegmatis*; **LT**: long term; **Mbo**: *Mycobacterium bovis* **Mtb**: *Mycobacterium tuberculosis*; Mtb-**WLE**: Mtb H37Rv-whole lipid extract in liposomes; **Myco-CE-O**: Mycobacterial-CE in oil; **Protec**: Protection; RUTI Fragmented Mtb cells in liposomes; **S**: survival; **SC**: subcutaneous; **ST**: short term; **TSP-Aq**: Aqueous fraction of Triton X-1 00-soluble Mtb H37Rv CE proteins; **VC**: Vaccine candidate; **Y/N**: yes/no.

Table 5
Natural and artificial membrane vesicles (MVs).

Vaccine candidate (VC)	Natural and artificial membrane vesicles (MVs)							Comments	Ref
	Model	Route	Adjuvant	Prime	Boost	Challenge	Protec Y/N		
MV-BCG/MV-Mtb	Mouse	SC	No	VC/BCG	VC	Mtb-**AER**	**N/Y CFU**	Protec = BCG (MV-Mtb). No Protect: (MV-BCG) Protec > BCG (as BCG-booster)	[235]
PLBCG	Mouse	SC	With/without Alum	VC/BCG	VC	Mtb-**IT**	**Y-CFU**	Protec = BCG (less lung lesions). Protec > BCG (VC as BCG-booster)	[236]
PLMs	Mouse	SC	With/without Alum	VC	VC	Mtb-**IT**	**Y-CFU**	Protec (with/without Alum) Protec = BCG (VC with Alum)	[237]

AER: aerosol; **BCG:** Bacille Calmete-Guerin; **CFU:** colony forming unit; **IT:** intratracheal; **MV:** membrane vesicle; **MV-BCG**: Natural MV from BCG; **MV-Mtb**: Natural MV from *Mycobacterium tuberculosis*; **PLBCG**: Artificial MV from BCG; **PLMs:** Artificial MV from *Mycobacterium smegmatis;* **Protec:** protection.
SC: subcutaneous; **VC:** vaccine candidate; **Y/N:** yes/no.

intrinsic adjuvant effect, which has been evaluated as prophylactic and/or therapeutic experimental vaccines in different animal models and clinical trials (Table 4).

5.2.5. *Natural and artificial membrane vesicles (MVs)*

The important role of bacterial MVs in cell–cell communication, immunomodulation, virulence and cell survival, and their intrinsic potential advantages as VCs have focused the interest for their evaluation in animal and human studies, some of them demonstrating suitable efficacy [230,231].

Mtb produce MVs, which contain relevant Ags and are potentially involved in the virulence and in the interaction with immune effectors inducing different types of IRs [232–234].

Considering the results obtained with the evaluation of MVs as vaccines from different microorganisms, natural and artificial MVs from pathogenic and non-pathogenic mycobacteria have been evaluated in animal models demonstrating their immunogenicity and protective capacity, either used alone or as BCG boosters (Table 5).

6. Concluding remarks

Mtb CE is a structure which have been capitalized as an important part of the research related to Mtb due to its peculiar structural organization and its huge impact in the physiology, survival, virulence, interactions with the host cells and the immune system and its potential importance in diagnosis, therapeutic and vaccine development [6–9,12,15,16].

The great diversity and accessibility of "its" components comprising proteins, lipids, carbohydrates, glycolipids and lipoproteins make them potential targets for vaccine development [238–242].

The important contribution of the CEC in the interaction with the host and the elicitation of non-specific IRs, associated with their intrinsic adjuvant properties have attracted attention in the field of vaccine development [6,15,243–246].

Considering the growing evidence of the importance of the Ab responses in the protection against Mtb, the selection of accessible CEC as VCs is an obvious choice with encouraging results [62,97–99].

The presence of immunodominant protein Ags, eliciting protective IRs in the acute phase of the infection highlights the interest in the CEC, as exemplified by the incorporation of this class of Ags in various vaccine platforms evaluated either in animal models or clinical evaluation [111,140,141,157,159,174,178,179,188,190,202,216,217].

The importance of lipid components in the induction of specific IRs with potential impact in protection also present an added element which demonstrate the importance of CEC for the development of new generation vaccines against TB [3,101,104,105,107,246,247].

The great variety of Ags included in the CE related with different stages of the infection is an added advantage for the development of multi-stage VCs such as fusion proteins, antigen cocktails, CE extracts and natural and artificial membrane vesicles, which have been evaluated successfully in animal models, with some under clinical evaluation [69,85,109,113,130–132,134,136,223–228,235–237].

Another aspect that needs to be considered is the complexities involved in advancing some VCs (extracts, synthetic analogues or combinations) from preclinical to clinical proof of concept. The main potential hurdles that face these VCs are associated with the characterization of the active component, complexity & consistency of manufacturing processes and the associated challenges in quality control issues of manufacturing.

It is interesting to note that apart from whole cell-based VCs, almost all the VCs which have been evaluated and those under evaluation in animal models and in clinical trials belong to the Mtb CEC [3,248]. However, it should be highlighted that the CE based VCs studied thus far, only represent a fraction of the great diversity of the CEC (proteins, lipids and carbohydrates) that are available [239,243,249,250]. Many others have the potential to be further explored as new components for the development of vaccines stimulating all the components of the IR and targeting all the phases of the infection.

Conflicts of interest

The authors declare that they have no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.01.003.

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