Review Article

Exploiting the mycobacterial cell wall to design improved vaccines against tuberculosis

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Abstract

The only vaccine available against tuberculosis (TB), the Bacille Calmette-Guerin (BCG), does not provide effective protection against the most common forms of adult TB and in recent years efforts have been made to develop a new and improved vaccine. Among the strategies implemented, the generation of a new live attenuated mycobacterial strain is seen as one of the most promising and feasible, for scientific, ethical and practical reasons. The new understanding of the biology of the tubercle bacilli and of host-pathogen interaction processes, coupled with the possibility to engineer BCG or *M. tuberculosis*, opened new avenues to design "intelligent" vaccines, capable of eliciting the immune response associated with protection while avoiding the induction of the host immune response associated with immunopathology. The complex and highly immunogenic mycobacterial cell wall can shape the general and antigen specific immune response elicited following immunization, and the possibility to exploit this knowledge may lead to the development of new vaccines that could help conquer this ancient human disease.

Key words: tuberculosis; vaccine; BCG; cell wall; HBHA

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Introduction

Among the infectious diseases that afflict mankind, tuberculosis (TB) has the almost unique feature of being one of the most ancient and deadly [1]. The 2011 WHO report quotes estimates of 8.8 million incidence cases worldwide, 1.1 million deaths in the HIV-negative population, and an additional 35 million deaths of HIV-positive subjects [2]. About one million cases involve children, with 130,000 deaths per year in the pediatric population, making TB among the top 10 causes of deaths in childhood. The highest prevalence rates (> 300/100k) are found in developing countries, particularly in sub-Saharan Africa, including South Africa, Zimbabwe and Congo, and in Southeast Asia, including Cambodia, Vietnam and the Philippines [2]. However, TB rates have been declining worldwide since 2006, and the target of the "Stop TB partnership" of halving TB mortality by 2015 compared to 1990 may be met. The impact of the introduction of new diagnostic tools in high-burden countries, strongly supported by the WHO and other international institutions, on TB epidemics remains to be determined. The emergence of multi-drug resistant strains (MDR-TB), extensively resistant strains (XDR-TB) [3] and totally resistant strains (TDR-TB) [4] is raising concerns at the global level and prompted health authorities to strengthen and reinforce control strategies to limit their spread. Even when these ambitious targets are met, the burden of TB at the global level will remain substantial, posing a major threat primarily to the young adult population, and as a result TB will continue to be a major obstacle toward sound economic, social, and political development of the countries where TB is endemic.

Need for a new vaccine

The Bacille Calmette-Guerin (BCG) vaccine, introduced almost one hundred years ago, is still the only vaccine available and used against TB. BCG has a widely proven safety record and it is certainly one of the most frequently administered vaccines in the world [5]. In countries where TB is endemic, newborns are immunized with BCG immediately after birth since it protects infants from the most insidious forms of TB, such as disseminated infection and meningeal TB. Nevertheless, the efficacy of BCG in protecting against the most common forms of adult TB has been challenged by several clinical studies [6,7] and there is

a common consensus that BCG is unable to provide significant protection against pulmonary TB, which is the only form of TB that causes transmission of the bacilli [8].

In the last two decades, thanks to a renaissance in TB research, many efforts have been made to develop a new and improved vaccine. Subunit, viral-vectored, DNA-based vaccines have been developed using many different *M. tuberculosis* antigens and tested in preclinical animal models of TB infection. The results obtained varied depending on the vaccine used, though very few experimental vaccines were capable of inducing a protective immune response superior to that induced by BCG [9-12]. Attempts have also been made to obtain attenuated *M. tuberculosis* strains that, while being equally or even less virulent than BCG, could induce improved protection against *M. tuberculosis* infection [13]. In any case, none of these new vaccines was completely protective and capable of preventing infection. These results, coupled with the fact that BCG is effective in preventing the most severe forms of TB disease in infants, make the substitution of BCG with a new vaccine not feasible for ethical concerns [14]. For these reasons, an option that is being pursued aims to develop an improved version of BCG which could be administered in substitution of the currently available strain. The most common strategy has been that of engineering BCG to express selected *M. tuberculosis* antigens [15] or other heterologous proteins that would improve immunogenicity and enhance the protective activity against TB. Some of these recombinant strains have successfully passed preclinical testing and evaluation in human clinical trials has been started.

Live attenuated mycobacterial strains

Since it is widely accepted that, for ethical reasons, BCG immunization of infants cannot be discontinued, many efforts have been made to develop improved live and attenuated mycobacterial strains capable of inducing enhanced and long-lasting immunity against *M. tuberculosis*. Several groups have attempted to develop new attenuated strains by inactivating one or more selected genes in *M. tuberculosis* to obtain a safe vaccine capable of expressing all *M. tuberculosis* antigens. Many strains have been obtained and tested and the results of preclinical studies have been promising in some cases, with enhanced protective activity of some of these vaccines compared to BCG [16]. Some of the recently developed live-attenuated *M. tuberculosis* strains are reported in Table 1. Among the most advanced *M. tuberculosis* attenuated vaccine is the phoP *M. tuberculosis* mutant (SO2) developed by Carlos Martin's group, which demonstrated better protection and safety than BCG [17] and was further attenuated by deleting the gene encoding virulence factor fadD26 [18], obtaining the attenuated *M. tuberculosis* strain MTBVAC01. However, even if promising in terms of protection against *M. tuberculosis* infection, live-attenuated *M. tuberculosis* strains must fulfill stricter safety issues than other vaccines and obtaining approval for them could take a long time. On these premises, immunization with BCG remains the path of choice.

Recombinant BCG

Another option to develop an improved live attenuated vaccine is to engineer the current vaccine BCG to improve its protective activity. In the last two decades many attempts have been made and human clinical trials have been initiated for three of the recombinant BCG strains developed: rBCG30, VPM1002 and AERAS-422. rBCG30 is a recombinant BCG expressing one of the most actively secreted antigens of *M. tuberculosis*, Ag85B, in a five-fold amount compared to that expressed by the wild type strain [15], and results of the phase I clinical trial have already been reported [19]. The rBCG30 was shown to induce enhanced protection in animal models compared to the parental strain while maintaining a good safety record [20]. Another rBCG strain in an advanced stage of development is the VPM1002, which is a BCG recombinant strain that has been engineered to express listeriolysin O (LLO) from *Listeria monocytogenes*. In VPM1002, LLO is expressed in fusion with the N-terminal region of Ag85B, under the control of the hsp60 promoter, and this cassette was inserted so to replace the ureC gene of *M. tuberculosis* [21,22]. VPM1002 induced better protection over parental BCG, and this effect was related to its ability to stimulate broader and different T cell populations involved in protective immunity [23]. Now that its safety has been proven (www.clinicaltrials.gov, ID# NCT01113281 and NCT00749034), VPM1002 is currently undergoing phase IIa trials to assess its immunogenicity and safety in the target population [24]. Lastly, the AERAS-422 vaccine, which basically combines concepts underlying VPM1002 and rBCG30 [25], was shown to induce effective protection in preclinical animal models; however, though the phase I clinical trial was stopped because of an adverse effect observed in two participants (www.clinicaltrials.gov ID# NCT01340820).

Table. List of *M. tuberculosis* components that have been used to exploit the mycobacterial cell wall to modulate the host immune response elicited in a vaccine setting

COMPONENT	FUNCTION	EMPLOYMENT
p19	Apoptosis induction [38] Downregulation of IFN-γ induced MHCII expression [36] MΦ apoptosis [37]	Expression in saprophytic mycobacteria [40] Deletion in BCG [41] Deletion in H37Rv [42]
HBHA	Bacterial agglutination [45; 46] Dissemination from the primary site of infection [45]	Protection in the mouse model of TB [55] Diagnostic marker in QFT-test [59]
ESX secretion systems	**ESX-1:** Secretion of ESAT-6 and CFP-10 which are employed in IGRAs to diagnose TB infection [71] Encoded by the RD1 region which loss is the main molecular mechanism of attenuation of BCG [69] **ESX-3:** Essential for growth and viability in MTb [79] Growth in iron-deprived environment [78] **ESX-5:** Modulates MΦ response (*M. marinum*) [82]	**ESX-1:** H37RvΔRD1 protects mice against MTb infection similarly to BCG [72] Its deletion is one of the main causes of BCG attenuation [70] **ESX-3:** IKEPLUS *M. smegmatis* is susceptible to immune killing and stimulates the clearance of *M. tuberculosis* in infected mice [81] **ESX-5:** its disruption in Mtb causes loss of PPE protein secretion, changes in cell wall integrity, strong attenuation and protection similar to BCG [66]
PhoP	H37RvΔphoP shows reduced growth *in vitro*, is impaired in replication in BMMΦ and in mice [74] Involved in the biosynthesis of lipids [75] Functionally linked with ESX-1 system [73]	SO2 mutant is more attenuated than BCG, eliciting high protection in mice and superior protection in guinea pigs [17] SO2 mutant is safe once used as live-attenuated vaccine against TB [77]
SigE	As ECF, is involved in response to surface stress such as heat-shock, SDS [85] Its depletion in H37Rv results in attenuation in HMΦ and MMΦ, and is more sensitive to killing in the latter[85]	S*igE* null mutant is unable to grow in THP1-MΦ and severely attenuated in mice [85, 86] *SigE* mutant elicits stronger immune response (IFN-γ and TNF-α) and improved protection than BCG in mouse model of TB once administered as live-recombinant vaccine [87]
PE delivery system	PE domain of PE_PGRS33 delivers proteins to the mycobacterial surface [95, 96]	Fusion of antigens with the PE domain results in the expression of the latter on the surface [96] which can result in higher immunogenicity and protective activity when employed for rBCG engineering [99]

Exploiting the mycobacterial surface

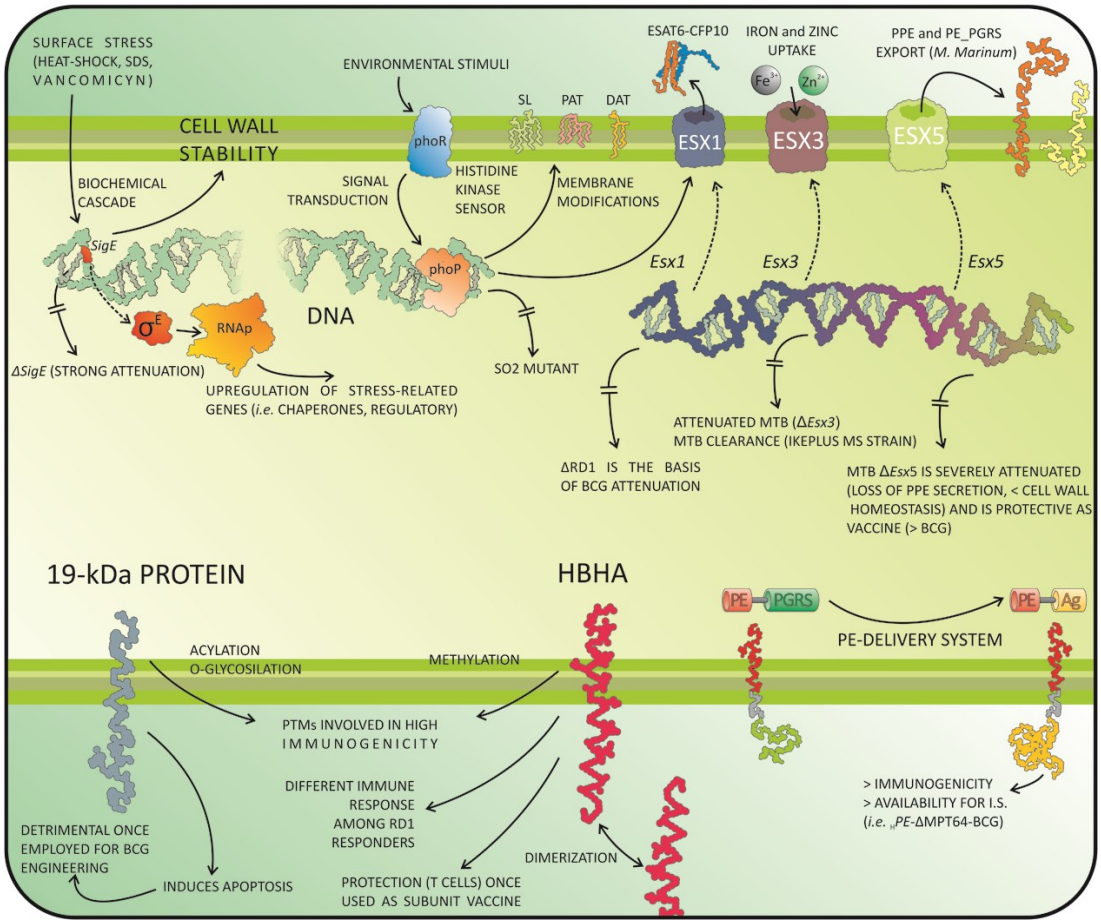
M. tuberculosis is an elusive pathogen and as such is an active manipulator of the host immune system, exploiting a large arsenal of biomolecules which allows the bacilli to interact with a wide array of immune ligands [26,27]. Most of these biomolecules are localized in the complex mycobacterial cell wall, which has a very peculiar composition known to play a pivotal role in the pathogenesis of mycobacterial infections. The main components of the mycobacterial cell wall are lipoarabinomann, arabinogalactan and other sugars; mycolic acids; glycolipids and phenolic lipids; and peptidoglycan. It is well-established that the mycobacterial cell wall is a very immunogenic component with strong immunostimulatory properties as classically highlighted by the use of the Freund's adjuvant, which is made of oleic acid and heat-killed *M. tuberculosis*. The adjuvant properties are linked to the pro-inflammatory activity of these molecules that induce TNF, IL-6, IL-1, IL-12, and trigger upregulation of MHC-II and CD1d1 on macrophages [28]. Not surprisingly, some of the most immunogenic antigens of *M. tuberculosis* are localized on the cell wall or are actively secreted, using specialized secretion systems apparatus such as the type seven secretion system (T7SS) [29,30]. The elucidation at the molecular level of the mycobacterial cell wall components is shedding some light on the mechanisms implemented by the tubercle bacilli to subvert and manipulate the host immune response. This information could be instrumental in designing new and innovative strategies aimed at engineering BCG or *M. tuberculosis* wild type strains to develop a new vaccine with enhanced protective activity against TB. The main cell wall components of *M. tuberculosis* that can be exploited to design new live attenuated vaccines against TB are shown in the Figure.

19-kDa protein

The relevance of lipoproteins in bacterial physiology and their potential for serving as virulence factors led many researchers to focus on such bacterial components. Many of the lipoproteins have been found to be targets of both the innate and acquired immune responses, and they represent a significant class of cell envelope proteins involved in interactions between the organism and the host [31]. Among such proteins, one of the most extensively studied is the 19-kDa antigen encoded by the *Rv3763* gene in *M. tuberculosis* H37Rv [32]. The 19-kDa glycolipoprotein is abundantly expressed in *M. tuberculosis*, where it is secreted or found in association with the cell wall. As seen in other mycobacterial antigens such as HBHA, post translational modifications have a great impact on the immune response elicited by the 19-kDa protein, since a considerable role for acylation and *O*-glycosilation on the cellular localization and the immune response associated with this antigen have been established [33]. Originally, this glycolipoprotein raised the interest of the scientific community because it was demonstrated that murine antibodies recognized it as a major antigen on crude *M. tuberculosis* extracts; furthermore, other homologues proteins were found in other pathogenic mycobacteria including *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium leprae* [34], suggesting the putative role of 19-kDa as a virulence factor. Many insights were offered for the pleiotropic role of the 19-kDa protein in modulating the innate immune response, such as the induction of cytokine genes [35] and the downregulation of IFN-γ induced MHC Class II expression [36]. However, it is now considered an active player in the mechanisms of subversion of the immune system by *M. tuberculosis*, since it induces apoptosis in macrophages through a TLR-2 pathway [37,38]. The finding that the 19-kDa protein is heavily involved in the induction of apoptosis led to idea that it could be possible to enhance vaccine-induced immunity by using pro-apoptotic vaccines, which may be a way to generate a more effective immune response without employing subunit or killed vaccines [39]. However, the expression of the 19-kDa protein in saprophytic mycobacteria employed as vaccines against tuberculosis showed a detrimental effect on the protection against the tubercle bacilli [40]. Inactivation of the Rv3763 gene in BCG resulted in a vaccine strain with a similar protective activity compared to the wild type BCG against *M. tuberculosis* infection [41]. On the other hand, inactivation of Rv3763 in *M. tuberculosis* H37Rv resulted in an attenuated strain capable of inducing some protective activity which was, unfortunately, no different compared to that induced by BCG [42].

Taken together, these data suggest that the highly immunogenic 19-kDa protein has a central role in *M. tuberculosis* pathogenicity, functioning to mislead the host immune system. Nevertheless, 19-kDa cannot be considered a potential candidate antigen for the development of a new vaccine against TB, since the immune response triggered is detrimental to the ability of the host to control *M. tuberculosis* infection. The pro-apoptotic activity seen in the 19-kDa protein could fall within the immune subversion mechanisms

Figure 1: Factors heavily involved in the regulation and the composition of the cell wall of *M. tuberculosis*



***SigE* (upper left)** is activated in response to surface stresses through a biochemical pathway, promoting the transcription of stress response genes once associated to the RNAp, and the regulation of the cell wall stability. *SigE* is tightly associated with the immune response, as highlighted by the disruption of the gene in *M. tuberculosis* (*ΔSigE* mutant), which leads to a strong attenuation of the mutant while maintaining the ability to elicit a robust immune host response.

The PhoPR regulation system (upper center) has a central role in *M. tuberculosis* virulence, as highlighted by the SO2 mutant severe attenuation. PhoPR is activated in response to environmental stimuli; PhoP modulates the composition of the mycomembrane through the regulation of the biosynthesis of lipids such as SL, PAT and DAT; PhoP is also involved in the regulation of the ESX1 system.

The T7SS of *M. tuberculosis* (upper right): ESX1 is encoded by the RD1 region of *M. tuberculosis*: the deletion of RD1 is accountable for the attenuation of BCG. ESX1 is responsible for the secretion of highly immunogenic proteins such as ESAT6 and CFP10. **ESX3** is involved in Fe^{3+} and Zn^{2+} uptake and is a central virulence factor in *M. tuberculosis*; recombinant *M. smegmatis* equipped with *esx3* (IKEPLUS strain) stimulates *M. tuberculosis* killing and acts as sterilizing vaccine. **ESX5** is heavily involved in cell wall homeostasis and composition, and its abrogation in the *ΔEsx5* strain causes attenuation in *M. tuberculosis* similar to the one observed with BCG.

Differences between two surface-exposed and highly immunogenic antigens (lower left): The **19-kDa protein** and **HBHA** of *M. tuberculosis*. Even if both the antigens are prone to post-transcriptional modifications, which are highly involved in their immunogenicity, their employment as vaccines leads to opposite results. The 19-kDa glycolipoprotein, even if proapoptotic and immunogenic, shows a detrimental effect once used for engineering recombinant BCGs. On the other hand, HBHA, which is highly immunogenic, evokes a strong T-cell response, giving notable protection once used as protein vaccine in the mouse model of TB.

The PE-delivery system (lower right): The PE domain of PE_PGR33 contains sufficient information to deliver the protein to the surface, and it was shown that a fusion between the PE domain and another antigen (i.e. $_{H}$*PE*-ΔMPT64-BCG) results in the exposition of the latter on the mycobacterial surface, which results in an improved availability for the immune system and, consequently, a major immunogenicity. For further explanations and references, see text.

Abbreviations: DAT, diacyltrehaloses; PAT, polyacyltrehaloses; SL, sulfolipids; RNAp, RNA-polymerase; PTMs, post-transcriptional modifications; IS, immune system

operated by the tubercle bacilli, as it has been shown that early apoptosis tends to favor the parasite survival rather than its killing [43]. While the paradigm given by such a protein could be exploited for the development of recombinant vaccines against pathogenic microorganisms other than *M. tuberculosis*, its use for the development of a vaccine against TB appears not feasible. Overall, the results obtained with the 19-kDa protein underscore the implication that an antigen associated with the mycobacterial cell wall can have in the development of an immune response against *M. tuberculosis*.

HBHA

Heparin-binding haemagglutinin (HBHA) is a 21 kDa surface exposed protein which has been involved in the mechanism of TB pathogenesis [44]. HBHA mediates adhesion of the bacilli to epithelial cells but not macrophages and is involved in the dissemination of *M. tuberculosis* from the site of primary infection [44,45]. The molecular characterization of HBHA sheds light on the structure-function relationship and their impact on the mechanism of pathogenesis [46-49], and the information gathered could open new avenues for the development of innovative prophylactic and therapeutic strategies against TB.

The first attempts to exploit this knowledge aimed at inducing antibodies directed against HBHA that could somehow neutralize the binding of the *M. tuberculosis* bacilli to epithelial cells, to prevent bacterial dissemination. Interestingly, opsonization of BCG with a monoclonal antibody against HBHA was capable of reducing bacterial dissemination from the lung to the spleen [45]. Indeed, several studies showed that HBHA is properly exposed on the surface and that antibodies could bind and potentially neutralize its adhesion properties [50]. However, when antibodies were induced in mice following immunization with a DNA vaccine encoding HBHA, no protection could be observed [51], though the protein expressed by DNA vaccines lacked the proper methylation of the lysines present at C-terminus, which are known to play an important role for the immunological properties of HBHA [46,52,53]. Moreover, protective activity of an HBHA-based vaccine correlated with the ability to elicit HBHA-specific T cells secreting IFN-γ but not with the ability to induce antibodies [54,55]. Hence the results so far observed in preclinical models of TB do not support the hypothesis that antibodies against the surface exposed adhesion HBHA could exert a protective effect against *M. tuberculosis* infection or TB disease.

The correlation of the HBHA-induced protective activity with a strong T-cell response in mice correlated with another major feature of HBHA. In fact, PBMCs from subjects with latent TB infection, but not patients with active TB, are capable of secreting IFN-γ when stimulated *ex vivo* with HBHA; conversely, patients with active TB, but not subjects with latent infection, show antibodies in the sera specific for HBHA [52-54]. These results imply that during *M. tuberculosis* infection the host-specific immune response directed against HBHA differs depending on the clinical status. These findings are relevant from a diagnostic point of view, since it would be possible to discriminate patients with active TB from those infected but with no overt sign of the disease [56-61]. At the same time, these differential immune responses in TB patients versus healthy TB-infected subjects may suggest that an effective T cell response against HBHA could help to control *M. tuberculosis* replication and prevent disease development [54,62]. Interestingly, promising results were obtained in preclinical animal models, where immunization with HBHA was capable of eliciting levels of protective activity similar to those induced by BCG [54,55]; other studies provided further support of the role of HBHA as a candidate vaccine against TB [63,64]. Interestingly, it was found that intranasal immunization of mice with HBHA adjuvated with the cholera toxin induced an effective humoral and cellular immune response, and protection was measured as a reduction of the bacilli dissemination from the lung to the spleen [63]. These results highlight the potential relevance of mucosal administration of an HBHA-based vaccine and suggest that relevant animal models specifically investigating the dissemination of the bacilli from the site of primary infection should be used, which is known to be a primary step in human TB pathogenesis [65]. It would be interesting to investigate the activity of an HBHA-based vaccine in the guinea pig or rabbit model of TB, and monitor vaccine efficacy by specifically measuring the number of secondary lesions occurring in later stages of infection.

These observations clearly underscore the importance of the host immune response against a mycobacterial cell surface antigen on the clinical status of TB, and highlight that this host immune response could be affected by yet uncharacterized events whose understanding could lead to improved strategies to control TB.

ESX T7SS

The identification of T7SS in mycobacteria [29] provided a new understanding of the molecular mechanisms associated with protein secretion and their impact on TB pathogenesis. In *M. tuberculosis* five ESX secretion systems have been identified (Esx1-5) and their characterization is opening new avenues of inquiry on the complex host-pathogen interactions between *M. tuberculosis* and its host [66,66-68].

ESX-1

The best characterized T7SS in *M. tuberculosis* is ESX-1, which is encoded in the region of difference 1 (RD1) and its deletion is responsible for the attenuation of the vaccine strain BCG [69,70]. ESX-1 encodes for a complex protein machinery that warrants the secretion, among other proteins, of Esat-6 and CFP-10 proteins (EsxA and EsxB), which are the two highly immunogenic proteins used in the IGRAs assays to diagnose TB latent infection [71]. Complementation of the BCG strain with a cosmid containing the RD1 genomic region restored EsxA/B secretion and resulted in a more virulent BCG strain, clearly showing that the deletion of ESX-1 is the major molecular mechanism of attenuation of BCG [69,72]. The importance of the role of ESX-1 in virulence was also indirectly demonstrated by showing that the main mechanism of attenuation of the avirulent *M. tuberculosis* strain H37Ra consists in a point mutation of the *pho*P gene, which abrogates secretion of Esat-6 [73]. Previous studies have shown that inactivation of *pho*P, which encodes the transcriptional regulator of the two-component system PhoPR, causes attenuation of the virulent *M. tuberculosis* H37Rv strain [74], and when this attenuated strain was used as a vaccine, the levels of protective activity induced were higher compared to those afforded by BCG [17]. PhoP controls the synthesis of complex mycobacterial lipids which exhibit immunomodulatory properties; in addition to abrogating the secretion of Esat-6, inactivation of *phoP* in the *M. tuberculosis* SO2 strain results in significant changes in the mycobacterial cell wall composition [75,76]. As of today, the *M. tuberculosis* SO2 attenuated strain is one of the new TB vaccines in the most advanced stage of development and the results obtained so far are promising [77].

ESX-3

The ESX-3 secretion system is involved in iron uptake in *Mycobacterium smegmatis* [78] and iron and zinc uptake in *M. tuberculosis* [79,80]. While the role of ESX-3 in iron uptake was hypothesized to be related to the import of iron-loaded mycobactin [78], the mechanism by which ESX-3 is involved in zinc uptake is still unknown. Interestingly ESX-3 is essential in *M. tuberculosis*, but not in *M. smegmatis*, where a second siderophore (exochelin) can complement the absence of iron-loaded mycobactin uptake [78,79].

High-dose intravenous infection of mice with *M. smegmatis* results in early death of the animals unless the *esx-3* region is deleted. In this case, the mutant (designed IKE, for Immune Killing Evasion) is controlled and cleared by a MyD88-dependent bactericidal immune response. When this mutant was complemented with a cosmid encoding *M. tuberculosis* ESX-3 (IKEPLUS), it remained susceptible to innate immune killing, but was able to stimulate bactericidal immunity against virulent *M. tuberculosis* dependent on CD4+ memory T cells [81], making this strain a powerful candidate for the development of novel, sterilizing vaccines.

ESX-5

ESX-5 secretion is the most recently evolved T7SS in mycobacteria and it has been identified in MTB complex, *Mycobacterium marinum,* and *Mycobacterium ulcerans* [67]. Its role in virulence has been demonstrated in *M. marinum* [82] and in *M. tuberculosis* by inactivating one or more parts of the genes' the locus [66]. Disruption of ESX-5 in *M. tuberculosis* resulted in the loss of PPE protein secretion, changes in cell wall integrity and strong attenuation [66], clearly indicating the impact of this system on cell wall homeostasis. Interestingly, the *M. tuberculosis* ESX-5 mutant strains showed protective activity superior to that induced by BCG when administered as a vaccine in a mouse model of TB [66] [83].

SigE mutant

Sigma factors are proteins that reversibly associate with RNA polymerase (RNAp) to form the RNAp holoenzyme, providing the promoter recognition function. Bacterial genomes encode a variable number of sigma factors. SigE is one of the 13 sigma factors encoded in the genome of *M. tuberculosis* [84]. Its transcription is activated in response to several environmental stresses; a *sigE* null mutant is unable to grow in THP-1-derived macrophages and is severely attenuated in mice [85,86]. Interestingly, despite low bacterial burden, *sigE* mutant-infected mice produce high levels of protective factors as interferon gamma,

tumor necrosis factor alpha, inducible nitric oxide synthase and beta defensins in the lungs, suggesting that SigE function confers the ability to avoid the induction of the host response. Since SigE is involved in the regulation of functions responsible for controlling surface stability and composition, it is possible to hypothesize that the enhanced immune stimulation evoked by this strain might be due to the lack of some immunomodulatory molecule on its surface. When the *sigE* mutant was used to vaccinate mice, it showed protective activity superior to that induced by BCG, and this was particularly evident when mice were challenged with a hypervirulent Beijing strain [87].

PE delivery systems

The PE and PPE protein families of *M. tuberculosis* include more than 169 proteins whose roles and functions remain elusive [32]. The PE proteins are characterized by a highly conserved N-terminal of ≅ 100 aa that includes a conserved motif at position 7-8 of proline (P) and glutamic acid (E) which gives the name to the family (PE) [88]. PE proteins are subdivided into three subfamilies: the PE only, which are usually 100 aa or less long and are often co-expressed with a PPE protein to form a heterodimer [89,90]; the PE unique, where the PE domain is fused at the C-terminus with a unique domain, that at least for some proteins has enzymatic activity, such as for LipY [91,92]; the PE_PGRS proteins, where the PE domain is followed at the C-terminus by the PGRS domain which contains the typical repetitive motifs of gly-gly-ala, gly-gly-N, which varies in size and sequence and that are unique for each protein. The PE and PGRS domains are linked by a highly conserved domain, which functions as a linker between the PE and PGRS domains. PE_PGRS proteins are exposed at the mycobacterial surface [93,94], and it has been shown that the PE domain of PE_PGRS33 contains sufficient information to deliver the protein on the surface [95,96]. Indeed, fusion of a heterologous protein such as MPT64 or GFP to the PE domain results in the exposure of this protein on the surface [96], clearly indicating that the PE domain could function as a surface delivery system in mycobacteria, though the PE itself is not available on the surface. Indeed, recent data confirmed that the PE-delivery system was efficient in different mycobacterial species such as *M. smegmatis*, *M. marinum*, *M. bovis* BCG and *M. tuberculosis*, and that mutations in the PE domain affected the ability of the PE protein to work as a delivery system [96]. These findings suggest that the PE domain may bring the protein to the mycobacterial outer membrane so that the heterologous antigens localize on the outer part and the linker domain remains embedded on the mycomembrane [97,98].

Expression of a heterologous antigen in the context of the highly immunogenic mycobacterial outer membrane would result in enhanced immunogenicity [96]. When the *M. tuberculosis* antigen MPT64, which is naturally secreted by *M. tuberculosis*, was over-expressed as a fusion protein with PE in BCG, significant enhanced protective activity was measured in the mouse model of TB [99]. Interestingly, the enhanced activity of the rBCG was observed only when the MPT64 was over-expressed as a fusion protein with the PE, but not when the same protein was over-expressed to localize in the cytoplasm. Indeed, protein localization was able to affect the quality and intensity of the MPT64-specific immune response induced *in vivo* in the mouse model, and protection correlated with the ability of $_{H}$*PE*-_MPT64-BCG (the rBCG strain overexpressing the PE_MPT64 fusion protein), but not of the other rBCG expressing MPT64, to induce specific T cell clones as determined by spectratyping [99]. These results underscore the relevance that antigen localization has on the type of immune response elicited. It would be interesting to investigate whether similar results will be obtained when other *M. tuberculosis* antigens, which are candidate vaccines against TB, are delivered as a PE fusion. The potential of using the PE delivery system to express antigens of other microorganism to generate a BCG vaccines against other infectious diseases would also be worthy of investigation.

Vaccination strategies based on live attenuated mycobacterial strains

The preliminary results obtained in preclinical studies using these new recombinant BCG and some of the *M. tuberculosis* attenuated strains are overall promising and while their activity in human clinical trials remains to be determined, their potential usefulness for implementing vaccination strategies in humans that could result in enhanced protection is worth mentioning. More specifically, the rBCG strains were engineered to enhance the immunogenicity of one or more specific *M. tuberculosis* antigens selected as valid vaccine candidates against TB, and as such these candidate antigens were shown to induce partial protection when administered as subunit vaccines or as viral-vectored or naked DNA vaccines. Since it has been suggested

that a main limitation of BCG vaccination is its inability to induce long-lasting immunity against TB, prime-boost vaccination strategies based on BCG priming and heterologous boosting with one or more subunit vaccines have been proposed as valid options to induce a sustained immune response capable of controlling *M. tuberculosis* infection [100]. In this regard the possibility of designing specific homologous prime-boosting strategies, where priming consists of immunization with one of the rBCG strains capable of eliciting a potentiated immune response against a specific antigen, such as 19-kDa protein, MPT64 or HBHA, and a boosting with a vaccine specifically targeting the same antigen, may result in anti-mycobacterial immunity maintained at levels sufficiently high to control *M. tuberculosis* replication *in vivo*.

The complex mycobacterial cell wall can affect the host-specific immune response against specific *M. tuberculosis* antigens, which could result in enhanced ability to control infection or cause a detrimental effect for the host. Understanding the immunological mechanism regulating the interaction between mycobacterial cell wall components and host immune responses may lead to the generation of recombinant BCG or *M. tuberculosis* attenuated strains with improved protective activity against TB, specifically designed to be included in prime-boost immunization strategies against TB, which may guide our ability to control this ancient human scourge.

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