Review Article

The quest for a new vaccine against tuberculosis

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Abstract

Tuberculosis (TB) is still one of the deadliest human diseases, killing 1,6 million people each year, mostly in developing countries. The vaccine currently used, Bacille Calmette and Guerin (BCG), is effective in preventing the most severe disseminated forms of disease in children and newborns, but its efficacy against active TB in adults has been challenged by several clinical studies. It is a common opinion that only the development of a new and more effective vaccine against TB would significantly ease the pandemic. In the last few years, the search for a new vaccine has gained a new momentum. New live and attenuated strains of *M. tuberculosis*, improved recombinant BCG strains and subunit vaccines have been tested in preclinical animal models, and some of them showed promising results. Unfortunately, the lack of immunological correlates of protection makes very difficult to anticipate or foresee the efficacy of any of these new vaccines and only phase III clinical trials, that are expected to start in 2009 and last few years, will tell us the real value of these new prophylactic tools. This review highlights the different strategies that are being implemented for the development of an improved vaccine against TB, the rationale behind them and the potential and feasible vaccination schedules that could be implemented.

Key Words: tuberculosis; DNA vaccines; *Mycobacterium*

J Infect Developing Countries 2009; 3(1):5-15.

Received 20 July 2008 - Accepted 25 August 2008

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Introduction

Tuberculosis (TB) is an ancient scourge that has afflicted mankind for millennia and *Mycobacterium tuberculosis*, the etiologic agent, is one of the oldest and most successful of human pathogens [1]. The World Health Organization (WHO) report on Global Tuberculosis Control 2008 [2] indicates that in 2006 more than one and a half million people died of TB, new cases were estimated at 9.1 million, and the number of total TB cases worldwide is about 14 million. As a total number, most of the TB cases occur in Southeast Asia's most populous countries, with the highest number in India (1.9 million new cases and 3,4 million total number), followed by China and Indonesia. The heaviest TB burden is observed in Sub-Saharan Africa, with South Africa, Uganda and Mozambique showing prevalence rates higher than 500 cases/100.000 population. Conversely, the TB prevalence in Western countries is dramatically lower, ranging from 10 to 50 cases per 100,000 population, and it has been estimated that in Western European countries half of the TB cases occur in foreign-born citizens who migrated from countries with high TB prevalence. These statisitics indicate that TB is one of the deadliest infectious diseases in the world, included together with AIDS and malaria (known collectively as The Big Three) in the poverty-related diseases that correlate with the tremendous imbalance between rich and poor countries.

The last few years have also seen the emergence of multi-drug resistant *M. tuberculosis* (MDR-TB). These *M. tuberculosis* strains are resistant to isoniazid and rifampicin, the two antibiotics used most often, and are therefore associated with a very high mortality rate as witnessed in an outbreak that ravaged New York City almost two decades ago [3]. The emergence of MDR-TB has been dramatic in the former Soviet Union countries, where the breakdown of the health system led to improper treatments of TB patients resulting in the emergence of MDR-TB strains. More recently, *M. tuberculosis* strains that show an extended spectrum of antibiotic resistance (Extensively Drug Resistant TB strains, XDR-TB) have been isolated. These are MDR-TB strains that are also resistant to a fluoroquinolone and at least one second-line injectable drug such as kanamycin, capreomycin or amikacin [4]. The emergence of XDR-TB is posing a major threat in many parts of the world [5], primarily in countries such as South Africa, where health authorities are testing new

antibiotic regimens to treat TB and more restricted containment measures to prevent the spread of XDR-TB strains [6].

Overall, these numbers depict a dramatic scenario that prompted the WHO to declare TB a global emergency. There is a consensus among health authorities and the scientific community that a new and more effective vaccine against TB might provide the tool to stop or at least control the epidemic. In this review, we will discuss the current vaccination strategies against TB and provide an overview of the new vaccines that are being developed, tested in preclinical animal models, and undergoing clinical trials in many parts of the world.

The BCG vaccine

The Bacille Calmette and Guerin (BCG) is an avirulent strain of *Mycobacterium bovis* that was attenuated by serial passages in potato slices imbibed with glycerol for 13 years [7]. In 1921 the first child was immunized and the clinical trials that followed in France and Belgium showed that the vaccine was very effective in children. Soon thereafter, immunization campaigns were implemented throughout Europe, and after World War II the WHO recommended expansion of the campaigns outside Europe. Today, BCG immunization is mandatory in TB-endemic areas, and BCG is the most widely used vaccine in the world. The success of BCG is primarily due to its efficacy in preventing TB meningitis in children [8], which is frequent in TB-endemic areas, as well as to its safe profile for use in humans. Furthermore, it is economical and could therefore be readily used in many parts of the world. On the other hand, the efficacy of BCG in preventing active TB in adults has been challenged by several clinical studies [9,10], with the lowest level of protection observed in countries with the highest incidence of TB [11].

Several hypotheses have been postulated to explain the failure of BCG, including geographic latitude, climate, host genetic background, or the BCG strain used [9,12]. Prior sensitization with environmental mycobacteria, which are very common in tropical areas and communities with poor hygienic standards, has been proposed to impair the efficacy of BCG vaccination. The following two hypotheses have been proposed [9]: The *masking hypothesis* postulates that sensitization with mycobacteria provides a certain level of immunity against *M. tuberculosis*, and subsequent BCG vaccination does not provide a significant improvement over it. Hence BCG, which is administered immediately after birth, effectively protects neonates and infants because prior sensitization with environmental mycobacteria does not occur. Conversely, BCG vaccination is not effective in adults because it does not provide an additive immune response in a population already exposed to environmental mycobacteria. The *blocking hypothesis* postulates that prior sensitization with environmental mycobacteria induces a level of anti-mycobacterial immunity that does not provide significant protection against *M. tuberculosis* infection but impairs the ability of BCG to persist in host tissues following immunization, and as a consequence the activity of BCG is reduced. In the first scenario, a new and improved vaccine against TB has to be more effective than BCG to provide protection in sensitized populations, while in the second hypothesis a new vaccine comparable to BCG may induce improved protection as long as its activity is not affected by a pre-sensitization with environmental mycobacteria, such as is the case for subunit vaccines [13].

Since its introduction in the 1920s, the high demand for BCG led to the distribution of the original strain maintained at the Institute Pasteur to many parts of the world, before proper standards for culture protocol and seed lots were established. For these reasons, many BCG strains exist in the world today with antigenic and immunological differences that may affect their efficacy against TB. Moreover, comparative genomics studies led to the identification of genetic differences between BCG strains, with early strains (BCG Russia, Japan and Moreau) being very similar to the original BCG, and late strains (BCG Pasteur, Danish, Glaxo) containing additional molecular modifications [14,15]. Currently, we have a good understanding of the genetic differences between these strains through the identification of regions of deletions (RD), SNPs and gene duplications. All the BCG strains lack RD1, a 10.7 kb fragment that is found in virulent *M. tuberculosis* and *M. bovis*, and that contains 9 ORFs. RD1 encodes a protein secretory system named ESX-1, involved in the secretion of two of the most immunogenic proteins of *M. tuberculosis*, Esat-6 and CFP10 [16,17]. It has been shown that lack of RD1 is the major determinant of attenuation in BCG, although reintroduction of the entire RD1 region in BCG does not restore full virulence, indicating that other genetic determinants might be responsible for the attenuation [18]. The functional characterization of the ESX-1 secretory system is providing new insights at a molecular level into the mechanism of pathogenesis of TB and may lead to the development of innovative tools

to control TB. In a recent work by Brosch *et al*. [19], a detailed genealogy of the BCG vaccine strains has been extended and, based on these new findings, it has been proposed that early BCG vaccines have been superior to the later ones that are more widely used today [19].

Pathogenesis of TB

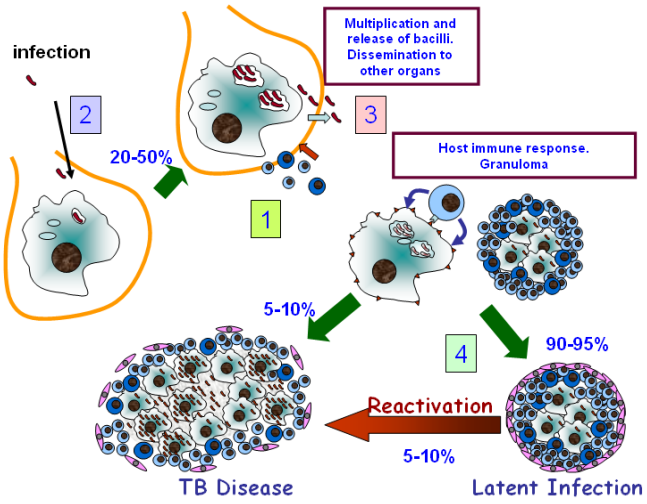
TB is an airborne transmitted disease, with the bacilli released by a patient with active TB inhaled by a healthy person. Once the bacilli reach the alveoli, the bacilli are ingested by alveolar macrophages that usually can effectively kill the bacteria (Figure 1). It has been estimated that in 20-50% of persons exposed to *M. tuberculosis*, the bacilli resist the innate immune response mediated by alveolar macrophages, then actively multiply within the macrophages, infecting nearby cells and activating the immune response [20,21]. The host mounts a cell-mediated immune response that leads to a cellular infiltration in the site of primary infection that organizes to form a granuloma. It is estimated that in 90-95% of cases, this host immune response is capable of inhibiting bacterial multiplication, controlling the growth of the infectious agent and leading to a latent infection (*latent TB*). Latent TB is clinically silent, with no outward signs or symptoms of disease, and is characterized by the presence of a specific cell-mediated immune response specific for *M. tuberculosis*, classically highlighted by the Mantoux test. People with latent TB have a 5-10% chance of developing active TB during their lifetime, indicating that the host immune response cannot completely eradicate the bacteria *in vivo* and that the immune response itself does not provide a lifetime protection against the emergence of active disease. In 5-10% of cases, the host immune response fails to control primary infection and it is the host response itself that is responsible for the extensive tissue damage and necrosis that is the hallmark of active TB in immune-competent patients.

Ideally, a vaccine should induce a host immune response that would rapidly contain bacterial multiplication, limit tissue damage, and block the development of the disease. The lack of correlates of protection in humans, *i.e.*, not knowing which specific immune responses are associated with protection, makes the search for a new and improved vaccine a hard task. Studies conducted in relevant animal models indicate that the cellular immune response, both in the CD4+ and CD8+ T cell subsets, are essential components of an effective immune response, but very little is known about the specific phenotypes of these cells and about the *M. tuberculosis* antigen targets. Moreover, it is thought that the humoral response against *M. tuberculosis* may not be relevant for protection [22].

The new vaccines against TB

Figure 1 illustrates the potential targets of a vaccine-induced immune response that may interfere or block the cycle of *M. tuberculosis* infection. Our current understanding indicates that a vaccine against TB should induce a strong cell-mediated immune response that must warrant a rapid mobilization of lymphocytes to the site of primary infection, to confine and control bacterial replication and to maintain the infection in a latent phase. BCG immunization is thought to induce a strong cell-mediated immune response that lasts few years, probably as a result of the ability of the attenuated strain to persist in host tissues. While many aspects of the immunopathogensis of TB await a better understanding, the search for a new

Figure 1. Natural cycle of tuberculosis and steps of the infectious process that may represent potential targets of the vaccine-induced immune response. Tje numbers in the boxes indicate different strategies that can be pursued: 1) A vaccine should induce a strong cell-mediated immune response, characterized by CD4+ and CD8+ T cells, directed against *M. tuberculosis* antigens actively secreted and abundantly expressed in the early steps of infection. The rapid mobilization of T lymphocytes at the site of infection should contain bacterial replication and reduce the possibility of developing active disease; 2) A vaccine strategy aimed at blocking infection. Such immunization strategy may rely on the induction of antibodies capable of blocking the first steps of infection. While most of the licensed vaccines in humans work in this way, no experimental vaccine has ever been shown to inhibit *M. tuberculosis* infection in animal; 3) A strategy to immunize adult subjects with latent TB infection. A vaccine should boost an anti-mycobacterial immune response in subjects already exposed to *M. tuberculosis*, to warrant control of the infection. This strategy may be used to specifically target the adult population in countries where TB is endemic; 4) A vaccine-induced immune response should target mycobacterial antigens involved in bacilli dissemination. Interference with dissemination will result in fewer bacteria infecting other organs or the upper lobe of the lung, which are the sites where TB disease usually develops. An HBHA-based vaccine may be an example for this immunization strategy.



vaccine against TB is an urgent priority and several approaches, technologies and strategies are being implemented by research groups involved in this challenging quest.

Attenuated M. tuberculosis *mutants*

Since BCG lacks many genes found in *M. tuberculosis* (>120 genes). it has been proposed that a live attenuated strain that contains a more complete antigen repertoire may be more protective than BCG. The introduction and implementation of genetic engineering in mycobacteria opened the possibility of knocking out specific genes in *M. tuberculosis*, resulting in several highly attenuated strains. Auxotrophic strains for the amino acids leucine [23], tryptophan [24], glutamine [25], lysine [26], or purine [27] have been obtained and these strains have been tested in animal models, where they were able to multiply and/or persist without causing disease. Some of these *M. tuberculosis* mutants showed good immunogenicity and were safer than BCG in immunocompromised animals, but the protective activity was always less than or equivalent to that induced by BCG [28]. To enhance the activity of mutant strains, deletion specifically affecting the bacilli's ability to persist in host tissues without dramatically impairing basic biological processes have been developed. An *M. tuberculosis* mutant for genes involved in the panthotenate biosynthesis has been shown to be safe even in immunocompromised animals, but still capable of persisting in host tissues without causing significant damage [29]. The protective activity of this mutant was remarkable even after 7 months post-immunization, although it was not superior to that elicited by BCG [30]. Along these lines other mutants for genes known to be involved in the mechanism of pathogenesis have been developed and some of these have been tested as vaccine candidates with promising results [28,31]. Among them is the *M. tuberculosis* ΔRD1 mutant, which was able to induce a level of protection similar to those induced by BCG despite the fact that it maintained a residual virulence and was less attenuated than BCG itself [32].

Recently, a significant result was obtained with the Δ*sec*A2 *M. tuberculosis* mutant, which is impaired in its ability to secrete virulence-associated proteins and enhances apoptosis of infected macrophages by diminishing secretion of mycobacterial superoxide dismutase. This mutant was capable of a more effective antigen priming than BCG, and vaccination of mice resulted in superior protective activity compared to that induced by BCG [33]. The results of this study underscore the need to tailor mutations that affect the ability of the bacilli to interact with the host and that harness the immune response to enhance immunogenicity, opening a new rationale for the development of improved live attenuated vaccines against TB.

A major concern for the use of live attenuated *M. tuberculosis* strains is the possibility that reversions may occur. For this reason there is a consensus that at least two non-reverting independent mutations should be present in candidate vaccines. However, since auxotroph mutants did not induce a superior level of protection compared to BCG, the most recent strategies aim at combining one mutation conferring auxotrophy with one affecting immunogenicity, as has been observed for the Δ*sec*A2 mutant [33], or two mutations that disable various other immune evasion- and virulence-promoting functions of *M. tuberculosis*.

Recombinant BCG

The ability to genetically manipulate BCG has allowed the development of recombinant strains that may be more effective than the parental strains, and several strategies for developing new strains have been pursued in the last few years. Horwitz's group produced a BCG strain (rBCG30) that overexpresses the highly immunogenic protein Ag85B, and in mouse and guinea pig models, it showed improved protection over the BCG parental strain [34,35]. Another strategy has been to reintroduce selected genes in BCG that were deleted during attenuation, such as Esat-6 and CFP-10, which are encoded by the RD1 region [18]. While this BCG recombinant strain (BCG::RD1-2F9) showed enhanced activity over the parental BCG, the potential for an increase in virulence resulting from the expression of the ESX-1 region raises concerns for its use in humans.

A major limitation of BCG may result from its inability to induce an effective CD8+ T cell immune response, which is known to play an important role in the immunity against *M. tuberculosis* [36]. It has been suggested that BCG maintains a certain degree of resistance to macrophage killing that prevents an effective antigen processing and presentation, primarily through the MHC-I pathway [37]. Hence a strategy to improve BCG is to make a strain more "digestible" by host dendritic cells. Kaufmann's group produced a recombinant BCG strain (rBCGΔ*ureC*:*hly*$^{+}$) that has two major features: i) it can secrete listeriolysin (Hly), a cytolysin of *Listeria monocytogenes* that forms pores in

the phagosomes membrane; ii) it cannot block early acidification of phagosomes as a result of the urease gene deletion (*ure*C) [38,39]. As a consequence, rBCGΔ*ureC*:*hly*$^+$ can translocate to the cytoplasm of infected dendritic cells, where antigens can be readily presented through the MHC-I pathway. Moreover, translocation in the cytoplasm triggers apoptosis of infected cells, which in turn favour killing of the bacilli and antigen presentation, and is itself a strong signal for the induction of cellular immune response. The rBCGΔ*ureC*:*hly*$^+$ strain showed better efficacy than BCG in a mouse model [37], underscoring the potential for a strategy that aims to disable the mycobacterial immune evasion strategies, as also seen for the *M. tuberculosis* Δ*sec*A2 mutant. In line with these findings, researchers at Aeras are developing a BCG that escapes the phagosomes and overexpresses selected *M. tuberculosis* antigens [40].

The Subunit Vaccines

The identification and immunological characterization of the culture filtrate proteins of *M. tuberculosis* in the early '90s, and the finding that some of these antigens are highly immunogenic, was instrumental to the testing of protein-based vaccines against TB. The preliminary studies by Pal *et al*. [41] have shown that it was possible to induce in mice an antigen-specific immune response that could partially control bacterial growth *in vivo*. These findings have been extended following the completion of the *M. tuberculosis* genome sequence, with the identification of more antigen candidates. The rationale for the development of a subunit-based vaccine against TB is that induction by immunization of a Th-1 type immune response against *M. tuberculosis* antigens that are secreted and abundant in the early steps of infection results in a more rapid and more effective mobilization of T cells at the site of bacteria multiplication, and this would contain the infection and reduce the risk of developing active TB.

Most of the candidate antigens are *M. tuberculosis* secreted or cell-bound proteins that can re-stimulate *ex vivo* T cells from infected animals or patients. In the last decade, several of these antigens have been identified (Table 1). Among the most important are Esat-6 and CFP10, which are encoded by the RD1 region that is missing in BCG, Ag85B, MPT64, and other actively secreted antigens, together with some cell bound antigens, such as Mtb39A, that belong to the PPE family of proteins.

Protein-based vaccines

The most promising antigen candidates have been expressed as recombinant proteins in *Escherichia coli*, purified, and then used to immunize mice and guinea pigs with selected adjuvants. The usefulness of protein subunit vaccines against TB gained a new momentum when it was demonstrated that immunization with the poly-protein Ag85B-Esat-6 elicited a strong cell-mediated response that warranted significant protection in mice [42], and that such immunity was not affected by previous sensitization with environmental mycobacteria [13]. Moreover, it was also shown that immunization with the Ag85B-Esat6 poly-protein could also boost the anti-mycobacterial immune response induced by BCG. These two findings had important implications, because they suggested a new vaccination strategy in humans that is boosting a BCG-immunized population.

Table 1. List of the *M. tuberculosis* candidate antigens for a subunit vaccine.

Antigen Location	Antigen Name	Description
Secreted	ESAT-6 CFP10	ESAT-6, CFP10: highly immunogenic T cell antigens, secreted through the ESX-1 system and encoded by the RD1 region; involved in the evasion from the phagosome.
	TB10.4, MPT63, MPT64, MPT83, MTB12, MTB8,4,	Secreted and highly immunogenic antigens of unknown function
	Ag85A, Ag85B	Mycolyl transferase enzymes involved in the coupling of mycolic acids with arabinogalactan. Highly immunogenic and abundant proteins.
	Rpf-like proteins	Latency-resuscitation promoting factor
Cell bound antigens	KATG	Catalase
	PPE18	Belong to the PPE protein subfamily
	MTB32C	Serine protease
	α-crystallin	heat shock protein
	HSP65	heat shock protein
	PST-S	ABC phosphate binding receptor
Surface exposed antigens	HBHA	Heparin-binding hemagglutinin, adhesion involved in dissemination.

Similarly, a recombinant poly-protein (Mtb72f) based on two antigens, Mtb39A and Mtb32C, was shown to be highly immunogenic and capable of inducing a level of protection equivalent to those provided by BCG, both in the mouse and guinea pig models of infection [43]. The promising results obtained with Mtb72F and Ag85B-Esat6 (Hybrid-1) in the pre-clinical animal models paved the way to human clinical trials [29]. Moreover, attempts are being made to develop new and improved recombinant fusion proteins, such as a fusion between Ag85B and Mtb10.4 (HyVac-4) that would not affect the use of T cell restimulation assays currently used to distinguish

people with *M. tuberculosis* infection from those immunized with BCG, such as the ESAT-6 antigen [44].

The use of recombinant proteins expressed in *Escherichia coli* is not always feasible, since some mycobacterial proteins undergo posttranslational modifications that affect the immunological properties of the protein itself. This is the case for the heparin-binding hemagglutinin (HBHA), a surface-exposed adhesin that mediates interaction with non-phagocytic cells and dissemination of the bacteria from the site of primary infection[45,47]. It has been shown that a Th1-type immune response against HBHA is associated with a latent infection, while a strong humoral response correlates with active disease [48]. Based on these premises, HBHA has been tested as a vaccine candidate and the results obtained in the mouse model indicated that only native HBHA, purified from *M. bovis* BCG and properly methylated, could elicit a protective activity, while the recombinant protein purified from *E. coli* (non-methylated) could not induce a protective effect [49,50]. It was also shown that only modified (methylated) peptides could be recognized by T cells with a significant impact in anti-tuberculosis immunity [50]. It would be of interest to investigate whether the implementation of mucosal immunization strategies relying on HBHA-based vaccines would provide better protection, considering that this is an antigen directly involved in the early steps of infection [51].

DNA Vaccines

Genetic immunization emerged more than ten years ago as a promising strategy to develop new vaccines against several infectious agents. Compared to protein subunit vaccines, DNA vaccine purification and production steps are in general less expensive. DNA vaccines are also simple to develop and for these reasons have been used as models to test the immunogenicity and protective activity of single *M. tuberculosis* antigens. The antigens that have been tested are mainly secreted and immunogenic proteins such as Esat-6, MPT64, Ag85A/B, and MPT83, although other antigens have provided significant results, such as the *hsp*65 [52], KATG [53] and Mtb39a encoding for a PPE protein [54-56]. DNA vaccination in mice elicited significant levels of cell-mediated immune responses with a broader T cell repertoire compared with subunit vaccination [57], characterized by CD4+ and CD8+ T cells. Many studies have dissected the immune response induced by DNA vaccines against TB, but no correlation with protection has been so far identified. The protection afforded by these DNA vaccines varied and accounted for 50-80% of that afforded by BCG in the same experimental settings. The use of DNA vaccine cocktails or immunization with constructs expressing two ore more antigens provided improved levels of protection compared to monovalent DNA vaccines [56,58,59], although no DNA vaccines worked better than BCG in preclinical animal models. Interestingly, when guinea pigs where immunized with a multivalent DNA vaccine and a polyprotein encoding the same antigens (Mtb72F), the levels of protection achieved were similar and equivalent to BCG, suggesting that DNA vaccines are not less effective than protein subunit vaccination, at least in this animal model [43]. Of interest is the fact that DNA vaccination could provide significant protection even in mice lacking $CD4^+$ cells [60], suggesting a possible immunization strategy in populations such as HIV-positive patients who present the highest risk of developing TB.

A major obstacle that DNA vaccines face is their poor immunogenicity in larger mammals [61], probably resulting from the very low transfection of plasmids *in vivo*. To overcome this problem, use of electroporation *in vivo* or plasmid delivery by poly-lactide beads have been introduced. More recently, the possibility of using avirulent bacteria as a DNA vaccine delivery system has been tested in relevant animal models and the results obtained have been promising [58]. In this case, bacteria are cultivated in broth and used to immunize mice by intranasal delivery, eliminating the costly procedures of plasmid purification and providing the possibility of implementing mucosal vaccination strategies against TB.

To enhance the immunogenicty of genetic immunization, other vectors expressing *M. tuberculosis* antigens have been tested, such as alphavirus [62,63], adenovirus [64], or vaccinia virus [65]. The use of these viral-based vectors is under consideration in prime-boosting strategies to enhance an antigen-specific immune response. The most promising results have been obtained using the modified vaccinia virus Ankara (MVA) expressing Ag85A, which gave remarkable immunogenicity and was able to warrant enhanced protection when used in prime-boosting strategies together with BCG [66,67].

The results obtained in pre-clinical animal models with the above mentioned new experimental vaccines have been limited, since only a few of them have been shown to be superior to BCG. It has been suggested that the protective activity induced by BCG in animal

models such as mice, guinea pigs and rabbits might be exceptionally high and may not necessarily mimic what happens in humans. These observation are supported by the divergent effect of BCG immunization on *M. tuberculosis* infection in two highly related macaque species [68]. The lack of immunological correlates or biomarkers of protection makes it very difficult to properly assess the reliability of any animal model until data on efficacy studies in humans are available.

The design and management of clinical trials is of pivotal importance and should be carried out in accordance with regulatory authorities [69]. Immunogenicity and safety clinical studies have been performed or are ongoing for several new experimental vaccines (see table 2). At this time it is expected that only phase III efficacy clinical trials will measure the activity of a new vaccine against TB, and will provide the data that may shed light on the relevance of the animal models and on the usefulness, significance, and predictive value of several biomarkers.

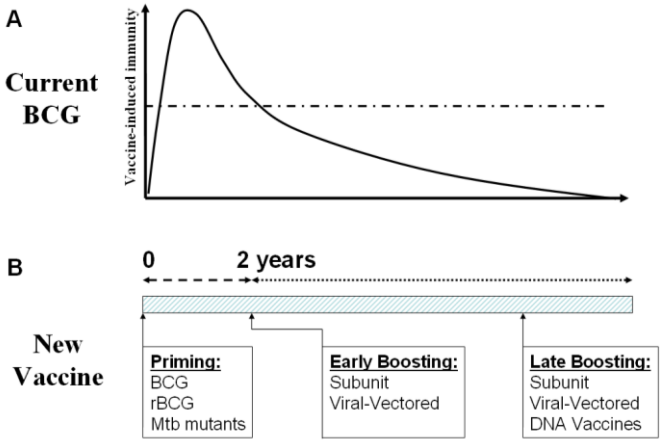
Table 2. Summary of the new TB vaccines under development.

Strain Name	Description	Step	Ref.
Live-, attenuated- *M. tuberculosis*			
M. tuberculosis mc^{2}6020	*Mtb* with deletion of the *lys*A and PanCD locus.	GMP production	27, 29
M. tuberculosis mc^{2}6030	*Mtb* with deletion of the *lys*A and RD-1 locus	GMP production	27, 29
M. tuberculosis PhoP	*Mtb* with deletion of the virulence-associated gene *Pho*P	Preclinical studies	28
M. tuberculosis Δ*sec*A2	*Mtb* with deletion of the virulence-associated protein secretory system	Pre-clinical studies	32
Live-, attenuated modified BCG			
rBCG30	BCG Tice overexpressing Ag85B		33
rBCGΔ*ureC*:*hly*$^+$	BCG Pasteur with deletion in the urease gene and expressing listeriolysin from *L. monocytogenes*.	Phase I	32
BCG::RD1	BCG Pasteur containing the RD1 locus of *Mtb*	Phase I	17
rBCG-AERAS-403	Endosome escape and antigen overexpression		39
Viral Vector			
MVA-Ag85B	Modified Vaccinia Ankara expressing Ag85A	Phase II	64, 65
Advac	Adenovirus vector expressing Ag85B and other antigens	Phase I	62
Subunit Vaccines			
Mtb72F	Recombinant fusion protein composed of PPE18 and Mtb32C, administered in AS02A and AS01B adjuvants.	Phase II	42
Hybrid-1	Recombinant fusion protein composed of Ag85B and Esat-6 administered in IC31 adjuvant.	Phase I	28, 41
HyVac-4	Recombinant fusion protein composed of Ag85B and TB10.4	Phase I	40

Designing Improved Vaccination Strategies

The first vaccination strategy against TB is aimed at substituting the currently used BCG, which does not protect against TB in adults. As it has been already mentioned, BCG vaccination confers protection in neonates and children from developing the most severe forms of disseminated TB and for these reasons its use is recommended and should not be discontinued in TB endemic countries (Figure 2). Hence, it is anticipated that any new vaccination strategy against TB should be based on BCG, or an improved recombinant BCG or another live-attenuated *M. tuberculosis* mutant. In this scenario, newborns in TB endemic populations might be immunized with one of the following: i) normal BCG in tandem with a subunit vaccine, to boost antigen-specific immunity while maintaining the spectrum of immune responses generated by BCG; ii) recombinant, improved BCG vaccine, which should induce a level of immunity superior or at least equal to those induced by normal BCG; iii) live-, attenuated-, *M. tuberculosis* mutants, whose safety has been properly assessed.

Figure 2. New vaccination strategies against TB. A) The anti-mycobacterial immunity against TB is thought to be maximum in children, and is supposed to wane over time. When this immune response, primarily cell-mediated, drops below certain levels, the chances to develop disease, as a result of exogenous infection or endogenous reactivation, increases and this explains the failure of BCG to protect against TB in adults. B) To induce improved levels of immunity at an early age and to warrant a high level of cell-mediated immunity later on, new vaccination strategies based on the combination of live attenuated vaccines and subunit or viral-vectored vaccines have been proposed.



In order to maintain a sufficiently high level of cellular immune responses, a boosting strategy has been proposed (Figure 2). Subunit vaccines based on polyproteins (Mtb72F, Hybrid-1 or HyVac-4) with adjuvants, or viral-vectored vaccines such as MVA-85A or AdVac, may be used to boost a previously BCG-immunized population in the first two years of age, or even at adolescence [40,70]. The antigen-specific immune response, which is known to induce partial protection in animal models, should provide additional immunity that may lower the risk of developing TB. Moreover, priming with an rBCG overexpressing a given antigen, coupled with a boosting immunization

with the same antigen-based subunit or viral-vectored vaccine, might be a strategy to further enhance anti-tuberculous immunity.

Any immunization strategy involving school-age children and young adults in endemic areas means that people already exposed to *M. tuberculosis* (latently infected), might be receiving a post-exposure vaccination. Since very little is known about the components of the immune system that control infection, the effect of a post-exposure vaccine against TB must be carefully evaluated for safety issues before entering clinical trial. A DNA vaccine coding HSP65 administered in a post-exposure regimen showed promising results in the mouse model [71], while a DNA vaccine cocktail that induced protection in a prophylactic regimen did not protect when administered in a therapeutic regimen [72]. Hence, a thorough evaluation in preclinical animal models is required in order to envision post-exposure vaccination strategies against TB.

Concluding remarks

The last twenty years have seen a renaissance in TB research, with scientists coming from different experiences, backgrounds and scientific fields joining forces to dissect the ultrastructure, physiology, mechanisms of pathogenesis, and host-pathogen interaction of one of the most successful human pathogens. A landmark achievement has been the completion of the *M. tuberculosis* genome sequence which shed a new light on the tubercle bacilli [73]. The amount of data that has been accumulating is providing a better understanding of the biology of *M. tuberculosis*, although we are far away from solving the puzzle of TB and some of the basic questions that were posed more than 100 years ago still await proper answers [1]. Some of this knowledge is starting to find practical applications, such as the improved diagnostic tools. On the other hand, the search for new drugs or for a better vaccine has been far less successful. However, in the last ten years, the search for an improved vaccine has gained momentum thanks to the efforts and financial commitments of many governments and philanthropic institutions. The most effective experimental vaccines have been tested in PhaseI/II clinical studies and Phase III trials are expected to start in the near future. Given the complexity of these studies, it is anticipated that the results of the Phase III trials will not be ready until 2014-15. Some prominent scientists argue that there is no scientific evidence to suggest that a fully protective vaccine against TB can be developed [74], fueling scepticism about the quest for a new vaccine. However, it is important to remember that a vaccine that works better than BCG or that shows "only" a 50% efficacy in adults may save millions of lives in the course of few years and will ease the dramatic health, social, and economic burden that TB is posing in low-income and developing countries. Hence the search for new TB vaccines continues and should be properly supported while many scientists in the world are poised to make their contributions.

Acknowledgements

This work has been supported by the Istituto Superiore di Sanità (project 50G.13 awarded to GF), by the EC-VI Framework (contract n. LSHP-CT-2006-036871, awarded to G.D.) and by the Italian MIUR (grant n. 2005060582_002 awarded to G.D).

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Conflict of interest: No conflict of interest is declared.