Eur J Clin Microbiol Infect Dis (2016) 35:535–543
DOI 10.1007/s10096-016-2579-y

REVIEW

Manipulation of BCG vaccine: a double-edged sword

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Received: 25 November 2015 / Accepted: 7 January 2016 / Published online: 25 January 2016

Abstract *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG), an attenuated vaccine derived from *M. bovis*, is the only licensed vaccine against tuberculosis (TB). Despite its protection against TB in children, the protective efficacy in pulmonary TB is variable in adolescents and adults. In spite of the current knowledge of molecular biology, immunology and cell biology, infectious diseases such as TB and HIV/AIDS are still challenges for the scientific community. Genetic manipulation facilitates the construction of recombinant BCG (rBCG) vaccine that can be used as a highly immunogenic vaccine against TB with an improved safety profile, but, still, the manipulation of BCG vaccine to improve efficacy should be carefully considered, as it can bring in both favourable and unfavourable effects. The purpose of this review is not to comprehensively review the interaction between microorganisms and host cells in order to use rBCG expressing *M. tuberculosis* (*Mtb*) immunodominant antigens that are available in the public domain, but, rather, to also discuss the limitations of rBCG vaccine, expressing heterologous antigens, during manipulation that pave the way for a promising new vaccine approach.

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Introduction

Mycobacterium tuberculosis (*Mtb*) have attained global importance as a human pathogen [1]. In the early part of the 20th century, Bacillus Calmette–Guérin (BCG) vaccine was prepared at the Pasteur Institute, Lille, France in 1921 by Calmette and Guérin, consisting of a live attenuated strain of *M. bovis*, a closely related subspecies of *Mtb*. By repeating sub-culturing (231 passages) of the virulent strain on ox bile glycerine–potato media every 3 weeks over 13 years, they produced an attenuated strain, which, by the year 1919, was shown to be avirulent in guinea pigs, cows, horses, hamsters, mice and rabbits [2]. This attenuation promoted genetic deletions from *M. bovis* and resulted in 16 genomic regions of differentiation (RD1–RD16) as compared to the *Mtb* genome [3]. With reference to the regions of differentiation, RD1 is a DNA segment comprising a 10-Kb region, is deleted from all BCG strains but present in *Mtb* and *M. bovis*, encodes T-lymphocytes epitopes, viz. ESAT-6, CFP-10 and PPE proteins [4]; RD2 consists of a 10.7-Kb DNA segment and encodes proteins Mpt64 and CFP-21 [5]; RD4 corresponds to a 12.7-Kb region, deleted from *M. bovis* and all *M. bovis* BCG strains [3]. RD12 and RD13 are each about 2.5 Kb in size, and encode genes for a methyltransferase cytochrome P450 (RD12), a transcriptional regulator, a cytochrome P450 and a dehydrogenase (RD13). Both genomic regions are deleted in *M. bovis* and *M. bovis* BCG [3]. RD14 is a 9.1-Kb region of DNA encoding proteins of PE-PGRS and Rv1771 families [6]. The original BCG strain was maintained at the Pasteur Institute and it has also been distributed throughout laboratories in many countries before the original strain was lost. Most of the laboratories produced their own BCG strain and maintained it by sub-culturing [7], culminating in the evolution of more than 14 daughter strains, viz. BCG Russia, BCG Sweden, BCG Prague, BCG Moreau, BCG Phipps, BCG Pasteur

1173, BCG Tokyo, BCG Glaxo, BCG Tice, BCG Birkhaug, BCG Denmark, BCG China, BCG Frappier and BCG Connaught [8].

Following the establishment of *Mtb* infection within the lung, the acquired immune response is slow to identify the infection site [9] and by the time specific T cells arrive at the infection site, they play a critical role in determining the outcome of the immune response [10]. But, when the acquired immune response is absent, bacteria grow and the host dies rapidly [11]. The immunity to *Mtb* mainly recognises the participation of macrophages and cells of the adaptive immune system $CD4^+$ and $CD8^+$ T lymphocytes, as well as the cytokines TNF, IL-12 and IFN-γ, which are critical in the control of mycobacteria [12], but the improvement of current tuberculosis (TB) vaccines is limited by a lack of knowledge regarding the protective T cells capable of limiting the development of active TB [13].

Entry of *Mtb* into the macrophage is mediated by an array of receptors, including complement receptors, scavenger receptors and the mannose receptor [12]. While *Mtb* is established within lung, the bacilli are believed to be phagocytosed by the alveolar macrophages [14], neutrophils [15] and dendritic cells (DCs) [16]. There is evidence that *Mtb* modulates phagocytic function to prevent direct elimination from phagocytes by blocking maturation of the phagolysosome and by inhibiting apoptosis [17–19]. *Mtb* promotes necrotic death by inducing LXA_4 (lipoxin A_4), which inhibits the production of prostaglandin E_2, resulting in mycobacterial spread [20–22]. MMP-1 (matrix metalloproteinases) is a collagenase that is up-regulated in TB patients and is associated with increased lung pathology in transgenic mice [23, 24] and MMP-9 has also been implicated in the pathogenesis of TB [25]. It has been demonstrated that, in humans, MMP-9 is responsible for worse outcomes in TB, suggesting a role in susceptibility to *Mtb* infection. Mice treated with anti-TNF antibodies or mice lacking the 55-kDa TNF receptor gene revealed that TNF is essential for the control of mycobacterial infection [19, 26]. However, lung neutrophils elicited the antigen-specific $CD4^+$ T cells during mycobacterial infection and enhanced adaptive immune response by delivering the bacilli to DCs, which are more effective initiators of $CD4^+$ T cell activation [27].

The pathogenicity of *Mtb* is related to its ability to export and secrete selected proteins that possess the capability to interact with the host cell. Mycobacterial export and secretion pathways play a central part in the survival of mycobacteria in divergent environments and hosts. Mycobacteria-specific ESX-1 and ESX-5 systems secrete/export Esx, Esp and PE/PPE proteins, respectively [28]. The key players of ESX-1 systems are ESAT-6 and CFP-10, which are encoded by genes esxA and esxB situated in the middle of the RD1 region. The PE and PPE genes are representatives of two large families of *Mtb* (around 7 % of the coding capacity) that encode proteins, which are derived from the motifs proline–glutamic acid (PE) and proline–proline–glutamic acid (PPE) N-terminal motifs. Within two families, several sub-families can be differentiated on the basis of middle and c-terminal sequences of its members, several of which carry highly repetitive motifs, polymorphic GC-rich repetitive sequences (PGRS) and major polymorphic tandem repeats (MPTR) [4, 29]. From a phylogenetics point of view, PE and PPE proteins seem to be associated with ESX systems [30], which are associated with protein secretion and export of the concerned domains. It has been shown that *Mtb* PE-LipY and *M. marinum* PPE-LipY were both exported to the bacterial surface associated with ESX-5-mediated secretion [31].

BCG vaccine can prevent miliary and meningeal TB in children, but its protective value in adults against pulmonary TB is questionable. A 15-year follow-up trial of BCG vaccination carried out in Chingleput and enrolling more than 360,000 individuals found that BCG offered no protection against pulmonary TB in adults [32]. The effectiveness of BCG in preventing TB in adults and infants is highly variable, with efficacies ranging between 0 to 80 % (average 50 %) having been reported from multiple clinical trials performed during the 20th century [33]. The reason for such variable protection may include BCG strain variation, the genetic variability amongst and different ages of the vaccinated individuals, routes of administration, geographic location, the dose of vaccine, interference by environmental, mycobacterial and helminthic infection, and patient nutritional status [6, 34, 35]. But, still, due to having unique properties, BCG vaccine could not be replaced by another vaccine because: (i) of the route of delivery of BCG vaccine (vaccine was delivered orally to humans between 1921 until the late 1940s and, since the late 1940s, administration followed the percutaneous or intradermal route); (ii) it is feasible to produce as compared to other vaccines; (iii) it is unaffected by maternal antibodies and, therefore, it can be given at any time after birth; (iv) it is stable and secure; (v) BCG is usually given as single dose, eliciting a long-lasting immunity [36].

Many factors have forced investigators to look for an alternative to BCG vaccine or to enhance the efficacy of BCG vaccine. In this context, it is required to have a better understanding of the interactions between microorganisms and host cells for a rational recommendation on the use of wild-type BCG and recombinant BCG (rBCG) should be explicated. The interest in BCG vaccine increased due to the development of different genetic systems for expressing foreign antigens in mycobacteria via different shuttle vector systems to express and secrete heterologous antigens and strategies for the transformation of mycobacteria.

Manipulation during rBCG vaccine can have both positive and negative aspects. In one aspect, rBCG expressing and secreting the immunodominant antigen Ag85B of *Mtb* was found to promote levels of protection greater than

conventional BCG [37]. rBCG strains have been constructed which express cytokines such as IFN-γ or IL-2, IL-12 and granulocyte macrophage colony-stimulating factor (GM-CSF) to stimulate more potent immune responses against *Mtb* [38, 39]. In reverse, the protective efficacy of rBCG over-expressing LipY (PE_PGRS63 of *Mtb*) and the profile of host immune response generate an additional concern. During this manipulation, we found that over-expression of LipY in *M. bovis* BCG demolished the efficacy of BCG vaccine to protect against infection of *Mtb*, and the underlying mechanism was found to be down-regulation of the host immune system [40].

This review explains the knowledge available in the public domain with reference to rBCG strains that modulate immune response. In addition, some immunological deficits during the manipulation of rBCG over-expressing *Mtb* antigens are also discussed.

Approaches towards BCG vaccine

The way BCG vaccine has been managed in different countries and manufacturing units for several decades raises serious concern. After the first successful vaccination, BCG strains were distributed throughout the world and generated differently in various laboratories for several decades, resulting in both phenotypic and genotypic differences not only compared to the original BCG parent strain but also between the various BCG daughter strains [41]. Although the effect of these mutations is far from clear, strain variability has been suggested as an explanation for the variable protection found in clinical trials using different strains of BCG [6, 35]. It has been suggested that, over time, BCG vaccine may have lost a number of genes with potential relevance for protective immunity or, in other words, has gradually been attenuated to impotence [42]. Hence, it is important to provide BCG with selected *Mtb*-specific genes in order to enhance its immunogenicity and protective efficacy against TB [6].

Comparative genomics has yielded valuable information on the differences between BCG and virulent *Mtb*, revealing the absence of a number of genes and regions designated RD1–RD16 and encompassing 129 open reading frames in BCG vaccines [6, 43, 44]. Some of these genes which are present in virulent mycobacteria and absent in BCG are even likely associated with virulence and could play an important role for the failure of BCG. Reintroduction of selected genes from RD1–RD16 to BCG has, therefore, been suggested as a way towards enhancing the protective efficacy of the existing BCG vaccine [45]. Reintroduction of the RD1 region encodes *Mtb* ESAT-6 and CFP10 antigens into BCG, enhancing the protection against disseminated *Mtb* infection in mice and guinea pig [46].

There are some vaccination strategies currently in the development, all of them are primarily aimed at delaying disease outbreak and can potentially be optimised to achieve sterile eradication [47]. One of them takes advantage of prime vaccination with conventional BCG to strengthen the immune response by booster with a subunit vaccine. Several subunit vaccines have already entered phase I and phase II clinical trials [48, 49]. Agger and Andersen have shown that a subunit vaccine is not influenced by sensitisation with environmental mycobacteria and stimulates a protective T cell response, whereas BCG is dependent on the initial multiplication for its activity [50]. An alternative vaccination strategy is to replace BCG with a recombinant live vaccine, and two vaccine candidates of that type have now been entered into clinical trials. The first candidate is an rBCG expressing antigen 30-kDa major secretory protein [37]. rBCG expressing membrane-perforating listeriolysin (Hly) of *Listeria monocytogenes* showed better protection against *Mtb* aerosol infection than the parental BCG strain [51]. In a further study, comparison has been made to compare immune responses after vaccination with rBCG:ΔureC:Hly (which expresses Hly of *L. monocytogenes* and is devoid of urease C) and parental BCG with reference to identifying biomarkers that correlate with protection in a murine model of TB infection. The data revealed that rBCG induced type 1 and type 17 cytokine responses, whereas type 1 response was only induced by parental BCG. rBCG:ΔureC:Hly is more efficient than parental BCG against pulmonary TB in pre-clinical studies and has been successfully proven to be immunogenic in phase I clinical trials [52].

Immunological deficit of BCG vaccine

There are a number of issues in developing vaccines with enhanced protective immunity against TB. Several hypotheses have suggested that one reason for the attenuation of immunological characteristics of BCG is the lack of T cell antigens in BCG [45]. $CD8^+$ T cells play a very important role in the host defence against TB infections [19], by using at least three different mechanisms: (a) direct extracellular killing of mycobacteria through antimicrobial activity,, (b) cytolysis of infected cells and (c) release of IFN-γ. Various human studies have demonstrated that $CD8^+$ T cells specifically recognised *Mtb*-infected macrophages, as demonstrated by the production of IFN-γ, and lyse the infected macrophages, resulting in the simultaneous eradication of bacteria by the release of granules containing perforin and granulysin. So, CD8 cytotoxic T lymphocytes reduced the viability of the intracellular *Mtb* and can, hence, contribute to effective immunity against the pathogen [53, 54]. The crucial role of MHC class I-restricted $CD8^+$ T cells was shown by the failure of β2-microglobulin (β2m)-deficient mice to control experimental *Mtb* infection [55].

Second, infection with mycobacteria is not able to induce sterilising immunity against reinfection with the same mycobacterium after clearance of the original infection with antibiotics. So, there is no vaccine against TB that has elicited sterilising immunity [56]. Third, the variability of the BCG vaccine has been attributed to genetic or nutritional differences between populations, as well as several ecological factors such as temperature, sunlight exposure and ultraviolet radiation that correlate with latitude, where the higher prevalence of environmental mycobacteria in tropical regions has been suggested to be the single most important factor for the observed low efficacy of BCG in these regions [35]. Therefore, BCG might attain its full potential only in developed countries where the population is not heavily exposed to environmental mycobacteria, because in the trials performed in the 1940s and 1950s in developed countries like Denmark, UK and North America, the BCG vaccine was found to be highly efficient (70–80 %), whereas more recent trials in developing countries demonstrated less or no protection against pulmonary TB [57].

Animal experiments showing protection provided by environmental mycobacteria partly conceal the effect of a subsequent BCG vaccination [58]. Rook et al. demonstrated that the environmental mycobacteria have a direct antagonistic influence and shift the immune response towards a T helper 2 (Th2) direction [59]. When Th2 cytokines are induced by exposure to high levels of the environmental mycobacteria or by vaccination, they can have a disease exacerbating role and suggested, on the basis of animal studies, that infection with environmental mycobacteria changed the immune reaction towards a detrimental humoral response that could not be abolished by following BCG vaccination [60]. In human TB patients, they correspond with poor clinical outcome [61]. Therefore, it is clear from the evidence that the effects of exposure to environmental mycobacteria on both the level of interfering with the efficacy of BCG and the degree of benefit of protection against *Mtb* are still not clear.

The cloud of doubt surrounding the efficacy of BCG vaccine has inspired investigators to improve BCG by making recombinants of various kinds, including genes of secretory proteins, cytokines, immunomodulators etc. Encouraging but mixed results have been obtained that correlate with both favourable and unfavourable consequences during the construction of rBCG.

rBCG vaccine

Human TB

Recombinant DNA technology enabled the construction of rBCG strains to be used as improved candidate TB vaccines with better immunogenicity. In this line of rBCG constructs with enhanced immunostimulatory properties, BCG was genetically engineered with different immunodominant antigens and cytokines. rBCG expressing and secreting the immunodominant antigen Ag85B of *Mtb* was found to promote levels of protection greater than conventional BCG [37]. Two rBCG strains (Connaught and Tice) over-expressing Ag85B in a guinea pig model of pulmonary TB were more efficient than BCG vaccine. rBCG strains have been constructed which express cytokines such as IFN-γ or IL-2, IL-12 and GM-CSF to stimulate more potent immune responses against *Mtb* [38, 39]. To achieve enhanced immunostimulatory properties, BCG was engineered to secrete r-human IFN-α (rhIFNα) under the control of mycobacterial heat shock protein (Hsp) 60 promoter and the α-antigen signal sequence. When compared with control BCG, rhIFNα-BCG was substantially more active in inducing the production of IFN-γ from human peripheral blood mononuclear cells [62].

It is mostly considered that important T cell antigens are missing in BCG, which is backed by extensive data published on ESAT-6, a low molecular mass protein of the RD1 region. It is considered a strong inducer of T cells [63–66] but deleted in BCG. Immunisation with ESAT-6 and DNA vaccines encoding ESAT-6 evoked protective responses [67–69]. Even a single epitope derived from ESAT-6 in the adjuvant DDA/MPL was found to confer efficient protection comparable to the protection afforded by BCG vaccine [70]. The immunomodulator effect of BCG vaccine has been recorded [39, 71], which indicates that cytokines plays a very significant role in improving this effect. This approach has allowed modulation of the immune system to respond with a specific and desired pattern of cytokines [72]. In another similar study, rBCG producing IL-2 enhanced a strong type 1 immune response in a murine model [73] and rBCG secreting IL-18 increased the type 1 immune response with the production of antigen-specific IFN-γ in vaccinated mice [74]. rBCG expressing IFN-γ resulted in an alteration in the pattern of inflammation and local tissue fibrosis.

In addition, local expression of IFN-γ by rBCG resulted in more efficient bacterial clearance, which is accompanied by a reduction in tissue pathology [38]. Guinea pigs immunized with rBCG30, a BCG over-expressing the 85B antigen, and challenged with *Mtb* by aerosol, had less organ pathology, fewer bacteria in their lungs and spleen, and significantly greater survival than guinea pigs immunized with the parent strain of BCG [37]. This rBCG vaccine was the first vaccine reported to induce greater protective immunity against TB than the parent BCG vaccine in an animal model. Recently, the first double-blind phase I trial of rBCG30 in 35 adult humans showed that rBCG30 induced significantly increased Ag85b-specific T cell lymph proliferation, IFN-γ secretion and increased number of Ag85b-specific T cells capable of inhibiting intracellular mycobacteria [75]. An immense attempt has been dedicated to the assessment of BCG over-

expressing members of the Ag85 complex (Ag85A, Ag85B and Ag85C), either individually [76, 77] or Ag85B associated to ESAT-6 [78] or Ag85B associated to other antigens [79] or IL-15 [80]. Several of these constructs afforded better protection than standard BCG. In another study, BCG was equipped with Hly of *L. monocytogenes* and showed significantly improved protection in a mouse model when compared to the parental BCG strain following aerosol challenge with *Mtb* [51]. Mice immunized with rBCG co-expressing Ag85B, CFP10 and interleukin-12 (rBCG::Ag85B–CFP10–IL-12) elicited strong immunogenicity and attenuation of mycobacterial growth as compared to BCG vaccine [81], and further extension of this study showed that rBCG::Ag85B–CFP10–IL-12 augmented the protection against *Mtb* by increasing the Th1 polarised response [82].

TB vaccines include viral vectored, mycobacterial whole cell or extract, protein or adjuvant, attenuated *Mtb* and recombinant live. Approximately 15 TB vaccine candidates are in various phases of clinical trials. According to the pipeline for new TB vaccines in August 2014, there is a phase I clinical trial including six vaccines, AdAg85A, TB/FLU-04 L and Crucell Ad35/MVA85A (viral vectored), MTBVAC (attenuated *Mtb* strain), ID93 + GLA-SE (protein/adjuvant) and DAR 901(mycobacterial whole cell or extract), and a phase II clinical trial including six vaccines, VPM 1002 (rBCG), H1/H56/H4 + IC3 (protein/adjuvant), RUTI (mycobacterial whole cell or extract) and Crucell Ad35/AERAS-402 (viral vectored). MVA85A (viral vectored) and M72 + AS01E (protein/adjuvant) are in a phase IIb clinical trial and one vaccine using *M. vaccae* (mycobacterial whole cell or extract) is in a phase III clinical trial [83].

Bovine TB

Mycobacterium bovis, causing bovine TB, is not only a serious animal or zoonotic disease that causes economic loss, but it is also a threat to public health [84]. The only current human vaccine, *M. bovis* BCG, provides protection against bovine TB, but with variable protective efficacy [85]. There have been several improvements in cattle vaccine development, like most of the promising approaches, including BCG-DNA [86], BCG–virus-vectored vaccine [87], BCG–protein vaccine [88, 89] and adjuvant vaccines that induce significantly superior protection compared to BCG alone [90]. Improved vaccines based on rBCG vaccines enhanced the protective efficacy of BCG vaccine, as shown in several studies with reference to human TB in a previous section of this paper, and several studies even demonstrated the enhanced protective immunity of rBCG vaccine against *M. bovis* challenge in cattle. It has been described that a live rBCG vaccine, rBCG30, provided more protection against *Mtb* in a guinea pig model of pulmonary TB [37]. The same rBCG vaccine produced greater protective immunity than BCG alone against *M. bovis* challenge, indicating a lower burden of *M. bovis* in the lung and spleen in rBCG30 immunised guinea pig [91].

When the protective immune response of *M. bovis* deleted mce2A and mce2B genes (double deletion mutant, *M. bovis* Δmce2) as an experimental vaccine, evaluation in cattle showed protection against *M. bovis* challenge, indicating that *M. bovis* Δmce2 is a promising vaccine candidate against *M. bovis* pathogenesis in cattle [92]. Khatri et al. tested the immunogenicity of two rBCG strains, namely, BCG Pasteur Δzmp1::aph and BCG Danish Δzmp1, in cattle and found that both strains induced superior T cell memory response compared to BCG alone [93]. Recently, the evaluation of *M. bovis* double knock-out mce2-phoP tested in mice as a vaccine candidate demonstrated that mice immunized with the double mutant protected against challenge with *M. bovis* [94]. A successive trial with a number of animal species specifies that the oral route of BCG vaccination attenuates the disease extremity after experimental challenge with *M. bovis* [85] and the administration of oral BCG vaccination was shown to prevent infection of wild possums against natural exposure to *M. bovis* [95]. The potential of oral vaccination for controlling TB has also been demonstrated in badgers [96].

Limitations of rBCG vaccine

In contrast to the above findings, one report has indicated that there is no effect on the immunogenicity of BCG vaccine during construction and a few others reported attenuation of immunogenicity of BCG vaccine during the construction of rBCG vaccine. Hereof, over-expression of the 19-kDa antigen (lipoprotein) did not change the capacity of BCG vaccine to protect against *Mtb* in mice [97]. It was noted that the 19-kDa antigen (Rv3763), a lipoprotein of *Mtb*, triggers high levels of IL-12 from macrophages in addition to suppressing the antigen presentation signalling cascade and its immunomodulatory properties. The polarisation of host immune responses towards Th2 subtypes confers the abolition of immunogenicity of rBCG19N (rBCG expressing *Mtb* 19-kDa lipoprotein) when used as a live vaccine against *Mtb* in guinea pigs indicates that over-expression of the 19-kDa antigen attenuates the BCG vaccine efficacy [98]. We assessed the immunogenicity of rBCG over-expressing LipY (PE_PGRS63 of *Mtb*) in mice against *Mtb* and found attenuation of the immunogenicity of rBCG vaccine to protect against *Mtb* infection in a murine model. rBCG over-expressing LipY vaccine offered no protection against challenge of *Mtb* as evident by the parameters, viz. viable counts of tubercle bacilli in the lungs and weight of infected mice, and pathology of the lungs and survival of challenged mice and immune response generated by this rBCG vaccine in murine model suggested down-regulation

from Th1 to Th2 type [40]. These findings suggest that the detrimental effects mask the development of new promising rBCG vaccine approaches.

rBCG vaccine and other diseases

BCG vaccine usually prevents TB, but it is also an effective treatment for some non-muscle-invasive bladder cancers and has been used to treat it for more than 30 years. A putative model of the mechanism of action of BCG in bladder cancer has been shown elsewhere [99]. Raymond Pearl suggested that mycobacteria might be applicable to cancer therapy, observing in an autopsy study that cancer was less common in patients with active TB. Among various cancers including colon cancer, bladder cancer, lung cancer, leukaemia and melanoma, bladder cancer is the only cancer in which BCG is commonly used [71].

Several studies have reported that rBCG induced protection by using parasite, bacterial and viral antigens. Mice immunized subcutaneously with rBCG expressing the LCR1 antigen of *Leishmania chagasi* drove a type 1 immune response with IFN-γ production and, consequently, protection against challenge [100]. Interestingly, this rBCG was unable to cause significant levels of IFN-γ production when mice were immunized intraperitoneally and failed to ensure protection, advising that the route of administration is important for protection against *L. chagasi*. In another supporting study, BCG expressing the Sm14 antigen of *Schistosoma mansoni* induced IFN-γ production and predominantly type 1 cellular immune response in a murine model. Especially, the vaccinated animals were protected against cercarial challenge in this study [101]. Hamsters immunized with BCG expressing the LipL32 antigen of *Leptospira interrogans* were protected against challenge with *L. interrogans*. Autopsy examinations acknowledge, in this study, that rBCG-LipL32 was able to draw sterilising immunity against *L. interrogans* [102]. Rabbits inoculated with BCG expressing the cottontail rabbit papillomavirus L1 antigen developed neutralizing antibodies and showed smaller papilloma than the control group, demonstrating that rBCG could be used as a possible prophylactic against papillomavirus [103]. These studies manifest that rBCG has great potential as a vaccine vector, and rBCG vaccine offering protection against TB and several other diseases is credible.

Concluding remarks

Despite several controversies, Bacillus Calmette–Guérin (BCG) has several advantages, hence it is not easy to replace it with other vaccine candidates. The improvement of BCG remains the best alternative for the rational design of a vaccine against tuberculosis (TB). The post-genomic era could lead to the identification of novel *Mycobacterium tuberculosis* (*Mtb*) antigens that are absent from the BCG proteome and could, consequently, be applied to efficiently enhance the immunogenicity of BCG vaccine. The rationale to give a chance to foreign antigens in BCG is to enhance the efficacy and adjuvanticity of BCG as a recombinant vaccine. Success may play an important role when BCG continues to be applied to neonates, and boost the best subunit vaccine candidate, to stretch out the protection and efficacy of a vaccine. Improvements in recombinant BCG (rBCG) by expressing dominant antigens contained in the subunit vaccine are used for booster vaccinations and further improving the immune stimulatory capacity not merely as a vaccine against TB but also carrying a major role against many infectious diseases and, hence, making it a truly multi-valent vaccine. Manipulation of BCG vaccine should be carefully considered when discussing the potential of substituting BCG with new rBCG vaccines by using heterologous antigen expression in BCG, which also tends to attenuate the immune response in the murine model of TB and shares some disadvantages in rBCG vaccines.

Compliance with ethical standards

Funding None.

Competing interests The authors have no conflicts of interest.

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