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Applications of bacillus Calmette-Guerin and recombinant bacillus Calmette-Guerin in vaccine development and tumor immunotherapy

Yuan-qiang Zheng^{1,3,¶}, Youssef W. Naguib^{2,¶}, Yixuan Dong², Yan-chun Shi^{1,*}, Shorgan Bou³, and Zhengrong Cui^{1,2,*}

¹Inner Mongolia Key Laboratory of Molecular Biology, Inner Mongolia Medical University, Hohhot 010059, China

²Pharmaceutics Division, College of Pharmacy, the University of Texas at Austin, Austin, TX 78712, USA

³National Research Center for Animal Transgenic Biotechnology, Inner Mongolia University, Hohhot, China

Summary

Bacillus Calmette-Guerin (BCG) vaccines are attenuated live strains of *Mycobacterium bovis* and are among the most widely used vaccines in the world. BCG is proven effective in preventing severe infant meningitis and miliary tuberculosis. Intravesical instillation of BCG is also a standard treatment for non-muscle invasive bladder cancer. In the past few decades, recombinant BCG (rBCG) technology had been extensively applied to develop vaccine candidates against a variety of infectious diseases, including bacterial, viral, and parasite infections, and to improve the efficacy of BCG in bladder cancer therapy. This review is intended to show the vast applications of BCG and rBCG in prevention of infectious diseases and in cancer immunotherapy, with a special emphasis on recent approaches and trends on both pre-clinical and clinical levels.

Keywords

BCG; clinical applications; bacterial vector; recombinant BCG; vaccine development; infectious disease; tumor; bladder cancer

1. Introduction

BCG is a vaccine obtained by attenuating *Mycobacterium bovis*. Being the only human tuberculosis (TB) vaccine available, it is widely used as a part of the World Health Organization (WHO) Global Expanded Immunization Program [1]. In addition, intravesical installation of BCG bacteria following transurethral resection (TUR) is a standard treatment

*Correspondence should be addressed to Yanchun Shi (yeshi5388@163.com) and Zhengrong Cui (zhengrong.cui@austin.utexas.edu).

¶Contributed equally

Conflict of interest

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for the non-muscle invasive form of bladder cancer (NMIBC) to reduce the risks of recurrence of NMIBC and the progression of NMIBC into muscle invasive bladder cancer (MIBC) [2]. BCG has numerous favorable properties such as its extensive safety record in humans (i.e. BCG has been used in humans for about 100 years), thermal stability, low production costs (i.e. a few cents in US dollars), adjuvant activity (i.e. a strong inducer of long-lasting CD4⁺ type 1 helper T cell (Th1) and humoral immunity), and ability to induce mucosal immunity when administered by a proper mucosal route (e.g. oral, intrarectal, or intranasal) [3–6]. BCG is also an attractive bacterial vector in developing vaccine candidates using rBCG technology [7–10], which is based on the use of BCG bacteria as a vector to express foreign genes. In particular, rBCG has been used to develop vaccine candidates against a wide variety of pathogens and diseases, including bacterial, viral, parasite infections, and tumors [11–15]. Recombinant BCG technology has also been applied to improve the effectiveness of BCG as a TB vaccine and in NMIBC immunotherapy [16,17]. This article is intended to briefly i) introduce the current clinical applications of BCG (i.e. prevention of TB in infants and treatment of NMIBC), ii) provide an overview of rBCG technology and its applications, and iii) summarize recent developments in the applications of rBCG technology, with the goal of pointing out recent trends in the applications of rBCG technology. Potential challenges and opportunities in using rBCG technology in vaccine development and tumor therapy are also discussed.

2. BCG as a tuberculosis (TB) vaccine

BCG is one of the most widely used human vaccines, being administered over 4 billion times [18]. It was developed by Albert Calmette and Camille Guérin in France between 1908 and 1921 from a virulent *M. bovis* strain, with more than 230 serial passages *in vitro* [19]. The resultant BCG strain has genomic deletions in multiple regions [20]. Since 1921, BCG has been the only approved human TB vaccine available. Tuberculosis is caused by *M. tuberculosis* (Mtb), although other species can also cause the disease, e.g. *M. africanum* is one of the major TB causative agents in West Africa, and along with *M. tuberculosis* forms what is called mycobacterium tuberculosis complex (MTBC) [21,22]. Mycobacterium tuberculosis complex also includes other species like *M. bovis*, which can be transmitted among humans although being zoonotic, in addition to *M. caprae*, *M. canettii*, and others [23]. It is estimated that about 2 billion people worldwide have latent Mtb [24]. Tuberculosis develops in About 8.6 million people worldwide every year, with about 15% of them die [24,25]. Meanwhile, co-infection with Mtb and human immunodeficiency virus (HIV) represents a terminally severe health condition [26,27]. In this regard, it has been reported by Hesselning et al. that the risk of disseminated BCG infection is several hundreds fold greater in HIV-infected infants compared to HIV-uninfected infant [28,29] following BCG vaccination. The grimness of this particular risk can be better understood based on the fact that, according to the WHO recommendation, all infants in areas with high Mtb risk, are to be vaccinated with BCG at the time of birth unless symptomatic HIV infection is prevalent [30]. However, while HIV infection symptoms can only prevail after 3 months of birth, in most areas where HIV is endemic, diagnostic tools that can point out HIV-infected infants at the time of birth (e.g. PCR) are not generally available [31]. In addition, and aside from the increased disseminated BCG disease risk, it was later found that HIV infection may

dramatically limit the BCG immune response in infants < 1 year old [32]. BCG vaccination guidelines were later amended to include that BCG vaccine is not to be given to any infant known to have HIV infection [33].

For infant meningitis and miliary (disseminated) TB, BCG has proven effective [34]. Nevertheless, due to variable responses, BCG does not provide a reliable protection against pulmonary TB in adults [24:35]. Therefore, new TB vaccines that are generally safe in some high risk populations (e.g. HIV-infected infants) whilst still being consistently effective in adults are strongly needed, and recent clinical trial data showed that some of the TB vaccine candidates engineered using creative rBCG technology are very promising.

As early as it was first developed in the 1920s, BCG has been distributed to many countries worldwide, and since lyophilization techniques were not available to store lots of BCG, the vaccine had to be grown and passaged continuously along the years, leading to the development of more than 14 sub-strains with various genetic differences due to accumulative mutations [36–41]. This practice was mainly carried out until the production of lyophilized seed-lots in the 1960s took place, and since then, it was recommended that BCG vaccine preparation should not go through more than 12 passages from each seed-lot [35:42]. Different sub-strains are mainly divided into early group (group 1), including BCG Moscow (also known as BCG Russia), BCG Moreau, and BCG Tokyo (also known as BCG Japan), which share a lot of the features of the original BCG [35:37:43], group 2 and group 3 BCG vaccines, including BCG Sweden, BCG Birkhaug, BCG Jena, BCG Glaxo and BCG Danish, and late group (group 4) vaccines, including BCG Tice (Chicago) BCG Connaught (Toronto), and BCG Pasteur [43]. Elaborate description of the historical development of these sub-strains can be found elsewhere [41:44]. Many of these strains are now not available commercially, and some of them are only used in bladder cancer therapy (e.g. Tice and Connaught strains in USA and Europe) [36:45]. In order to investigate whether, and subsequently why, they differ from one another, various BCG sub-strains have been studied and compared in terms of proteomic analyses [38:46], biochemical characteristics [39], genomics [35:47], mycolic acid composition [48], immuno-stimulatory effects as Mtb vaccines [37:40], and as immunotherapeutic agents for bladder cancer [49:50].

Hayashi *et al.* compared the *in vitro* immunostimulating activities of various BCG sub-strains and found that the earlier strains (e.g. BCG Russia, Moreau, Japan, and Sweden) are more immunostimulatory than the late strains (e.g. BCG Danish, Glaxo, Tice, and Connaught) [37]. An earlier report by Behr *et al.* revealed that a single nucleotide polymorphism in the *mma-3* gene is found in sub-strains produced after the period of 1927–1931 and is responsible for the inability of such sub-strains to produce methoxymycolates [41:48], which is one of three major classes of mycolic acids in Mtb and BCG strains, along with alpha- and ketomycolates [51]. It is thought that methoxymycolate production may be correlated with the virulence and immunogenicity of BCG sub-strains and may explain the stronger immunostimulatory activity of the earlier sub-strains relative to the late ones [37]. However, as outlined by Behr *et al.* the exact influence of the *mma-3* mutation on the selection of a BCG sub-strain for vaccination is yet to be determined [48]. Other researchers studied the effect of the mycolic acid composition on both Mtb and BCG growth, sensitivity, and infectivity. For example, Yuan *et al.* found that mycobacteria modified to overexpress

mma-3 gene have increased methoxymycolate production, but are deficient in ketomycolate [51]. *Mtb* bacteria and BCG strains that lack ketomycolate have impaired glucose uptake and increased sensitivity to ampicillin and rifampicin [51]. On the other hand, the *in vivo* growth of ketomycolate-deficient BCG strains (i.e. modified Connaught and Pasteur) within THP-1 cells was seriously retarded, as compared to the unmodified parent strains, which may have an impact on the future design of new BCG-based vaccines. It is worth noting that the infectivity of BCG Connaught in THP-1 cells was much higher than Pasteur strain, either in the parent strains or in strains modified to overexpress *mma-3* gene [51].

Castillo-Rodal *et al.* compared 10 BCG sub-strains in terms of protection against tuberculosis infection and *Mtb* progression in a mouse model of progressive pulmonary tuberculosis [40]. Interestingly, none of the tested sub-strains provided protection against lung infection, although they all provided variable degrees of protection against disease progression (represented by development of pneumonia and the number of colony forming unit (CFU) in lungs), with the BCG Connaught and Phipps sub-strains showing relatively better and more persistent reduction in the area of pneumonia and a significant decline in the number of CFU [40]. Davids *et al.* studied the immune responses induced in 10-week old infants after immunization with either BCG Danish intradermally, BCG Tokyo intradermally, or BCG Tokyo percutaneously at birth, and found that percutaneous BCG Tokyo induced stronger specific Th1 immune responses than intradermal BCG Danish or BCG Tokyo, which highlighted the significance of the route of administration [52]. Finally, besides the different sub-strains themselves, it is known that the effectiveness of BCG vaccine is also influenced by factors such as the age, history of pre-exposure to certain diseases, and nutritional status of the patients [53-54].

3. BCG in cancer therapy

3.1. BCG in bladder cancer immunotherapy

About 70–85 % of patients with bladder cancer are initially diagnosed with NMIBC [2-55], which includes carcinoma in situ (CIS), Ta (low grade noninvasive), and T1 (invasive into lamina propria) stages [55-56]. The standard and most successful treatment of NMIBC following TUR is the intravesical instillation of BCG as an immunotherapeutic agent to reduce the risks of recurrence and progression into MIBC [57-59]. This approach has shown superiority to TUR alone, and to TUR followed by intravesical instillation of chemotherapeutic agents such as mitomycin C [60-64]. Although intravesical instillation of BCG following TUR was first reported by Morales *et al.* (1976) when they observed a significant reduction of recurrence in 9 patients [65], intravesical BCG instillation was used earlier to eradicate metastatic melanoma lesion in the urinary bladder of a patient (Table 1) [66]. In one of the studies that paved the way towards the application of BCG intravesical instillation in bladder cancer, Bloomberg *et al.* studied the immunostimulatory effects of BCG inoculated into the bladders of sensitized (with purified protein derivative of tuberculin, PPD) or non-sensitized mongrel dogs, either by intramucosal injection into the exposed bladder or by urethral inoculation through a catheter [67], and found that in general intravesical instillation of BCG elicited an inflammatory reaction, especially in sensitized dogs, that seemed to be temporary and well-tolerated by the study subjects. Consequently,

the authors suggested that this strategy may have a potential for the treatment of bladder cancer [67].

For BCG therapy, the typical induction regimen consists of a 6-week course, 2–3 weeks after the TUR. Each dose consists of lyophilized BCG ($2\text{--}19 \times 10^8$ colony forming units (CFUs), depending on the BCG strains) in normal saline to be instilled through a catheter into an empty bladder [62;68–71]. Many randomized clinical trials highlighted the superiority of BCG instillation therapy, relative to intravesical chemotherapy. For example, in a randomized clinical trial in 837 patients with intermediate to high risk Ta and T1 bladder cancer, Sylvester *et al.* reported that intravesical BCG (with or without isoniazid) for 6 weeks following TUR was more efficacious than intravesical epirubicin in terms of first incidence of recurrence, distant metastasis, and overall survival [72].

In order to achieve the maximum effect, the induction BCG therapy needs to be followed by maintenance schedule of BCG instillations that should continue for 1–3 years [43;73;74]. For example, in a recently published randomized study, Oddens *et al.* applied a therapy regimen consisting of once-a-week instillations of either a full dose (5×10^8 CFU) or 1/3 dose for 6 weeks, followed by a three weekly instillations in months 3, 6, or 12 (i.e. 1-year maintenance, total of 15 instillations) or in months 3, 6, 12, 18, 24, 30, and 36 (i.e. 3-year maintenance, 27 instillations) [75]. It was found, among other findings, that the 3-year full dose reduces recurrence, but not disease progression or mortality rate, in high-risk patients compared to the 1-year full dose [75]. Other schemes for maintenance BCG therapy can be found in literature as well [70;76;77].

Meta-analyses of several clinical studies comparing the efficacy of intravesical BCG, with or without maintenance schedule, to chemotherapy revealed a noticeable divergence in terms of clinical outcomes. Malmstrom *et al.* established a meta-analysis based on individual patient data (IPD) to compare the efficacy of BCG and mitomycin C in randomized trials. The meta-analysis, which included 9 randomized trials and 2820 patients, revealed that studies with maintenance BCG therapy showed an overall 32% reduction in recurrence risk compared to mitomycin C therapy ($p < 0.0001$), whereas BCG without maintenance showed a 28% higher recurrence risk compared to mitomycin C therapy ($p = 0.006$) [63]. However, the meta-analysis did not reveal any significant benefit from the BCG maintenance therapy in terms of disease progression to muscle invasive disease and survival, as compared to mitomycin C therapy [63]. Bohle *et al.* completed a meta-analysis that included 11 clinical studies on intermediate-to-high risk patients with stage Ta or T1 bladder cancer (1421 patients treated with BCG and 1328 treated with mitomycin C) and found similar superiority of BCG intravesical therapy with maintenance scheme in reducing disease recurrence as compared to mitomycin C [61]. Overall, it appears that BCG therapy with maintenance schedule shows better long-term clinical outcomes, as compared to TUR alone, TUR followed by standard 6-weeks BCG, or TUR followed by chemotherapy.

The mechanism of action of BCG as an immunotherapeutic agent against bladder cancer involves both innate and adaptive immune responses [78], even though the immunotherapeutic responses induced by BCG are usually not memory responses, which may explain the need of maintenance schedules for better immunotherapeutic outcome. In

an early attempt to unveil the mechanism of action, Kavoussi *et al.* found that the intravesically instilled BCG bacteria adhere to the bladder mucosa in areas where urothelial damage had been induced, and that this binding can be inhibited by the use of anti-fibronectin antibodies [79]. The authors found that inhibition of the fibronectin-mediated luminal binding of BCG inhibited the efficacy of BCG intravesical immunization [79], highlighting the dependence of BCG internalization on fibronectin binding. BCG is internalized into cancerous urothelial cells following their binding, which was later found to be mediated by $\alpha_5\beta_1$ integrin receptors [80:81]. The negative charges on the mycobacterial cell surface inhibit the cellular internalization of the bacteria, which may in part explain the need for large doses of BCG for bladder cancer immunotherapy [43]. Following cellular internalization, primary innate immune response involves the release of several cytokines, including TNF- α , IFN- γ , IL-2, IL-6, IL-8, and IL-12 [82–86]. The release of cytokines activates neutrophils, T lymphocytes, BCG-activated killer cells, and natural killer cells, which eliminate the urothelial cancerous cells that internalized the mycobacteria [56:81]. T lymphocytes are probably the most effective cells in the antitumor activity of BCG [86–88]. Neutrophils contribute in cytokine secretion and were found recently to express the TNF-related apoptosis-inducing ligand (TRAIL), which induces apoptosis in tumorigenic cells, but not normal cells [89:90]. After their exposure to BCG, TRAIL expression was also found up-regulated on macrophages [87:91], and in general, high urinary levels of TRAIL-expressing neutrophils following BCG adjuvant immunotherapy is associated with better BCG responsiveness [92]. It was also found that priming neutrophils with IFN- α is associated with high levels of intracellular TRAIL, and these IFN- α -primed neutrophils are able to secrete more TRAIL after BCG therapy has started, as compared to unprimed neutrophils [93]. This observation emphasizes the importance of the addition of IFN in combination therapy or the use of IFN-secreting recombinant bacilli, as will be discussed later.

It has been shown that the predominance of Th1 cytokines (e.g. IFN- α , IFN- γ , IL-2, IL-12) is associated with beneficial BCG response and tumor destruction [69:89:94:95], while high levels of Th2 cytokines (e.g. IL-10) may be associated with treatment failure [69]. BCG-induced immune response gradually declines after the induction treatment ends, which explain the need for maintenance therapy to boost the cytokines levels and immune cell infiltration [86:96]. Interestingly, Biot *et al.* reported that priming the immune response with parenteral exposure to a subcutaneous dose of BCG and then followed by a single BCG intravesical instillation produced a stronger inflammatory response and recruited more T cells to the bladder in healthy animals, compared to a mere BCG single-dose instillation [96]. This treatment modality also resulted in a stronger response to BCG intravesical therapy in mice with orthotopic bladder tumors [96]. Additionally, pre-existence of a sustained immunity against BCG in patients with high-risk bladder cancer who received BCG intravesical therapy following TUR privileged the patients with significantly longer recurrence-free survival, as compared to patients with no pre-existing immunity [96]. The importance of these findings is that the strong immune response and accelerated T cell recruitment to the bladder following pre-immunization with BCG may eliminate the necessity of multiple intravesical BCG instillation [96]. Finally, there is also evidence that

certain BCG strains (e.g. BCG S4-Jena) induce apoptosis and exert direct cytotoxic activity on bladder cancer cell lines [97].

Despite being the most successful immunotherapeutic agent so far following TUR of the urinary bladder, BCG intravesical instillation strategy is not drawback-free, however. Major drawbacks can be divided into three categories; namely: 1) treatment failure (i.e. recurrence or progression) or emergence of resistance, which is reported in more than 40% of cases, 2) side effects that can be local or systemic, and 3) the short residence of the instilled BCG in the urothelial lumen due to frequent bladder emptying [98–103]. There is not a standard definition of BCG treatment failure, as different international panels and agencies offer different classifications [2]. Since the standard treatment after BCG failure is radical cystectomy [104], which is least preferred by many patients, many other agents had been evaluated for salvage therapy, including intravesical instillation of gemcitabine [104] or mitomycin C [105], with varying levels of success. In addition, intravesical injection of IFN- α 2b has also shown some success as a bladder salvage therapy following BCG failure [100]. To know more about the different definitions of BCG failures, as well as different approaches to treat patients who fail BCG therapy, readers can refer to a review by Ahn and McKiernan [2].

As to the side effects associated with BCG therapy, local side effects include dysuria, cystitis and macroscopic hematuria [101–106], while systemic side effects include fever (in about 15% of cases) and other rare conditions that can be fatal in some cases (e.g. sepsis) [101–107]. Adverse effects can be managed with careful individualized monitoring during the treatment scheme. In contrast to the common belief that the severity of toxic symptoms may be correlated with the length of the maintenance therapy, Van der Meijden *et al.* reported that in a multicenter phase III clinical trial in 487 patients with Ta-T1 bladder carcinoma, the toxicity associated with the induction and early maintenance therapy does not increase with further instillations in the maintenance phase [108]. Furthermore, these symptoms could be successfully minimized, while retaining the efficacy, by the use of lower doses [109–110]. In a randomized trial in 155 patients with high risk superficial bladder cancer, Martinez-Pineiro *et al.* reported that the use of one third of the standard intravesical BCG dose resulted in similar recurrence and progression incidence rates, and disease-specific survival rates, as compared to the standard dose, while the toxicity was significantly decreased with the lower dose [109]. Using a dose lower than one third of the standard dose was also reported [98–111], and there are reports showing that the BCG sub-strains used in the intravesical instillation affect the subsequent adverse effects as well [43].

Unintended BCG infection, either disseminated or localized, may occasionally arise following intravesical instillation [112]. Anti-tuberculosis drugs and glucocorticoids are usually used to treat BCG infections following intravesical instillation, although late localized infection may require surgical intervention [112]. Secanella-Fandos *et al.* found that an irradiated but metabolically active BCG strain was similar to live BCG in inhibiting tumor cells growth and in inducing the production of cytokines *in vitro*, which led the authors to conclude that the killed but metabolically active BCG may represent a safer alternative to live BCG during the maintenance therapy [113]. However, further preclinical studies are warranted before such approach can be evaluated in humans, especially with

many clinicians believing that killed BCG-based vaccines are much less protective than live BCG. An approach that can potentially minimize the risk of BCG infection is the use of BCG cell wall cytoskeleton (BCG-CWS) [114:115], although BCG-CWS appeared to be more effective against other types of cancers, such as colon cancer, than bladder cancer [116:117].

Various modifications have been introduced to conventional BCG therapy in both preclinical and clinical settings to improve the therapeutic outcomes. For example, Hsu *et al.* reported that the co-intravesical instillation of 1- α , 25-dihydroxyvitamin D3 with BCG increased the BCG-induced IL-8 secretion, which in turn promoted the human monocyte cell line THP-1 migration and improved the BCG-induced THP-1 cytotoxicity against bladder cancer [56]. This co-instillation modality significantly prolonged the survival time of mice with model bladder cancer [56]. Pharmaceutical drug delivery technologies were also integrated into BCG-based therapy to improve the therapeutic outcomes. In one example, in order to prolong the residence of BCG in the bladder, Zhang *et al.* designed a BCG-loaded thermosensitive hydrogel to deliver BCG to the bladder [99]. The thermosensitive hydrogel is free-flowing at room temperature, but gels at body temperature, imparting sustained release properties to the carrier [99]. Furthermore, Fe₃O₄ magnetic nanoparticles had also been included into the BCG hydrogel formulation to help increase the bladder residence by applying an external electromagnetic field [99].

As discussed earlier, various BCG sub-strains have been developed following the worldwide dissemination of the vaccine in the 1920s, and various sub-strains exhibited different immunogenicity in bladder cancer as well. For example, most live attenuated BCG sub-strains contain 90–95 % of dead bacteria, with the exception of BCG Tokyo172 (a commercial BCG Tokyo preparation, Immunobladder[®]), which contains more CFUs than any other sub-strains, and about 25% live bacteria [41:49]. The standard dose (80 mg wet weight) of BCG Tokyo172 contains only a dry weight dose of about 18 mg, as compared to 81 mg of the Connaught sub-strain lyophilized powder [118]. On the other hand, Ikeda *et al.* reported that the bacilli from the BCG Tokyo172 sub-strain have better dispersion in suspension, and about 4-times higher binding to bladder tumor cells *in vitro*, when compared to the widely used Connaught sub-strain [118]. Additionally, several studies outlined that a lower dose of BCG Tokyo172 (40 mg) has similar activity as the standard dose of BCG Tokyo172 (80 mg) [119], or the standard dose of Connaught (81 mg) [50] in terms of recurrence-free survival, but with less morbidity and lower incidence of severe adverse events. However, others reported no significant difference in immunogenicity or adverse events between the standard doses of BCG Tokyo172 and Connaught [49].

In a recent study, Rentsch *et al.* reported that the use of BCG Connaught elicited a stronger Th1-biased immune response in mice and prolonged the recurrence-free survival of patients with NMIBC when compared to BCG Tice [36]. A possible reason for this difference in clinical outcome may be due to the exclusive existence of the *SodC* mutation in the Tice sub-strain [36]. This study not only provided clinical evidence concerning the differences in immunotherapeutic outcome of two of the most commonly used strains of BCG, but also correlated this evidence with a genetic difference between the two strains. Additionally, Secanella-Fandos *et al.* found that the BCG Russia and BCG Connaught exhibit the

strongest anti-proliferative activity and the highest cytokine production (IL-6 and IL-8) in bladder tumor cell lines, among a list of 8 different strains, with the Glaxo sub-strain being the least effective [120]. The difference in immunogenicity was not related to the historical evolution of the strains, as BCG Russia is an early strain, while BCG Connaught is a late one [120]. For an extensive overview of the effect of the sub-strains on the clinical outcomes as well as the adverse event profiles of BCG in bladder cancer therapy, the reader can refer to a review by Gan *et al* [43].

3.2. Other immunotherapeutic applications of BCG in tumors

As mentioned above, BCG is used as a standard treatment in bladder cancer therapy. However, there is pre-clinical and clinical evidence that BCG may be used for the treatment of other types of cancers. The use of BCG to treat stomach cancer was first reported in 1936 [121]. Old *et al.* reported that mice that were intravenously injected with BCG (Phipps' strain) prior to tumor challenge showed slower tumor growth and prolonged survival, as compared to control mice [122]. Weiss *et al.* reported a strong immunological reaction against different types of tumor isografts in mice when the live BCG was intraperitoneally injected prior to tumor implantation, which resulted in tumor growth retardation and also prolonged the survival of tumor-challenged mice compared to control [123]. Zbar and Tanaka reported that the intralesional injection of live BCG (Phipps' strain, 12×10^6 organisms) at the base of the tumors in Guinea pigs with hepatocarcinoma intradermal xenografts not only retarded the growth of the xenografts, but also prevented tumor metastasis to draining lymph nodes, with 24 out of 35 tumor-challenged animals showing complete tumor regression, as compared to 0 out of 23 tumor-challenged animals in the control group [124]. It is worth mentioning that BCG-treated Guinea pigs were pre-immunized with a single intradermal injection of BCG (Phipps' strain, 6×10^6 organisms), which may highlight the importance of pre-existing immunity on the outcomes of BCG treatment.

In 1970, Morton *et al.* injected 36 patients with melanoma intralesionally with BCG, and reported complete lesion regression in 684 out of 754 lesions injected, with some patients showing regression of non-injected lesions that are close to the injected lesions [125,126]. Other researchers reported therapeutic benefit following intralesional injection of BCG in melanoma patients as well, and most of those trials have been documented by Rosenberg and Rapp in the review article that was published in 1976 [126]. In a randomized clinical trial in patients with stomach cancer, colon cancer, and pancreatic cancer, Falk *et al.* reported that when BCG was given intraperitoneally to patients followed by several cycles of oral BCG and chemotherapy, prolonged survival was achieved with gastric cancer patients and, to a less extent, with colonic cancer patients, as compared to oral chemotherapy alone, but the survival of pancreatic cancer patients became surprisingly shorter [127]. Survival benefits were also achieved when BCG-based immunotherapy was used in patients with stage III and stage IV melanoma, compared to interferon therapy [128]. Between 1980 and 1999, some clinical trials that evaluated BCG post-operative treatment of renal cell carcinoma (RCC) were reported [129–132]. Furthermore, direct intratumor injection of BCG in patients with prostate cancer showed some benefits in early clinical trials as well [133,134]. Guinan *et al.* also reported prolonged survival of patients with advanced prostate

cancer treated with BCG immunotherapy in addition to the conventional therapy (e.g. estrogens or observation), as compared to the conventional therapy alone [135]. However, severe adverse effects led to the termination of this approach [94], but other immunotherapy-based approaches are now investigated for prostate cancer [136]. Table 1 summarizes some historical landmarks that shaped the current application of BCG in bladder cancer, in addition to its investigational uses in other types of cancer. The table is not intended to be comprehensive, and various other examples and applications can be found in literature.

4. Recombinant BCG technology

As mentioned earlier, rBCG technology refers to the genetic engineering of BCG bacteria to use them as a vector to express foreign genes of interest [137-138]. Various antigen genes from Mtb (e.g. Ag85) and other pathogens (e.g. HIV-1 env), tumor-associated antigen genes (e.g. MUC1), and genes that encode specific mammalian cell cytokines (e.g. IFN- γ , IL-2) had been introduced into BCG bacteria to construct rBCG strains that express those genes. Both extrachromosomal and integrative expression plasmid vectors that carry the regulatory sequences of major BCG heat-shock proteins (Hsp) had been developed to allow the stable expression of foreign genes in BCG bacteria [139]. Foreign proteins of interest may be expressed in the cytoplasm of the BCG, secreted extracellularly outside of BCG, or cell surface-bound. However, it was shown that only those rBCG strains that secreted those foreign antigen proteins or have cell surface-bound foreign proteins were immunogenic, whereas the ones with cytoplasmic foreign antigens were not [140], likely because those intracellular antigens are confined inside BCG bacteria and do not have access to antigen-presenting cells (APCs). Antigens expressed by rBCG strains can elicit long-lasting humoral and cellular immunity, including CD4⁺ and CD8⁺ T cell responses, to the foreign antigens in animals or humans [141-144]. Moreover, depending on the routes of administration, rBCG strains can induce both systemic and mucosal immunity [144-145]. For example, in 1994, Langermann *et al.* showed that a single intranasal immunization with an rBCG strain that expresses the outer-surface protein A antigen from *Borrelia burgdorferi* resulted in a prolonged protective systemic IgG response and a highly sustained secretory IgA response, which was disseminated throughout the mucosal system [8].

4.1. Recombinant BCG strains engineered to express foreign antigens

The BCG bacteria had been engineered to express antigens specific to a wide variety of pathogens and diseases including antigens expressed by bacteria, viruses, and parasites (see Tables 2, 3 and 4 for representative lists). As aforementioned, BCG is effective in preventing infant TB, but its effectiveness is variable against pulmonary disease in adults [10-35]. Therefore, there continues to be an interest in developing new TB vaccines that are effective in adults. BCG had been engineered to express Mtb protective antigens Ag85A, Ag85B, Ag85C, or ESAT-6, and the resultant rBCG strains were shown to have enhanced protective activity against TB than the original BCG [146-149]. Wang *et al.* and Qie *et al.* developed different rBCG strains that overexpress Ag85B, Mpt64, and Mtb8.4 fusion proteins and showed that the rBCG strains induced strong specific antibody responses in mice, and the immune responses were predominantly Th1-biased [150-151]. Mohamud *et al.* designed two rBCG constructs expressing three T cell epitopes of Ag85B antigen fused to the Mtb8.4

protein or a combination of these antigens fused to B cell epitopes from ESAT-6, CFP-10 and MTP40 proteins, and demonstrated that these two rBCG constructs induced specific immunity against Mtb in BALB/c mice [152]. Rizzi *et al.* created an rBCG strain that overexpresses Ag85B based on an auxotrophic BCG strain for the leucine amino acid and showed that the rBCG-Ag85B was more protective against *M. bovis* in cattle than the original BCG [153]. Other examples have also been described in literature [142:154–156]. Despite the large body of preclinical research towards developing a rBCG vaccine for Mtb, only about a dozen Mtb vaccine candidates, not all of them are recombinant BCG-based, have been or are in clinical trials [157], and to our knowledge, no rBCG construct has been to phase III clinical trials. The earliest example was reported by Horowitz and colleagues, when they developed two rBCG constructs (based on Tice and Connaught strains) that stably express and secrete the 30-kDa major secretory protein of Mtb (Ag85B) and showed that these rBCG30 strains are more effective than the current BCG vaccines against TB in Guinea pigs, in terms of bacterial burden, lung pathology, and overall survival [142], with rBCG30 Tice showing better immunological response (e.g. cutaneous delayed-type hypersensitivity, DTH) and longer overall survival than the rBCG30 Connaught [158]. The Tice rBCG30 construct was later evaluated in healthy adult volunteers in a double blind phase I clinical trial, and its safety and improved immunogenicity (relative to Tice BCG vaccine) were confirmed [159]. In another example, a rBCG ureC:hly strain was engineered to express listeriolysin (Hly) from *Listeria monocytogenes* in the cell membrane, while the Urease C (ureC) gene was deleted from the bacteria [143:160:161]. The pore-forming activity of Hly enables *L. monocytogenes* to escape from the phagosomes of the host cells into the cytoplasm, which is essential for the MHC class I presentation of the *L. monocytogenes* antigens and the subsequent enhancement of CD8⁺ T cell stimulation [160]. Constructing rBCG strains that actively secrete Hly while their UreC activity is absent in order to keep the pH optimal for Hly activity allows the rBCG strains to effectively induce apoptosis in infected host cells, and the mycobacterial antigens in the apoptotic bodies can then be taken up by dendritic cells to induce CD8⁺ T cell response [143:160:162]. This is in contrast to the original BCG bacteria, which are weak apoptosis inducer, and thus only membrane-bound or secreted mycobacterial antigens are presented via the MHC II pathway to elicit CD4⁺ T cell response. Prime-boosting approach with the rBCG ureC:hly vaccine candidate (VPM 1002) showed very promising results in a phase I clinical trial in healthy male volunteers in terms of safety and immunogenicity and was later tested in newborns in a phase II clinical trial in South Africa [163]. Another rBCG construct that secretes a mutant protein from another bacterium did not show similar success in phase I clinical trial however, and administration of which was subsequently suspended because of safety problems. AERAS-422 was engineered from BCG Danish-SSI 1331 to secrete a mutant form of perfringolysin O, a ‘phagolytic’ protein normally secreted by *Clostridium perfringens* to enable endosomal/phagosomal escape, and overexpress three strongly immunogenic mycobacterial antigens, Ag85A, Ag85B, and Rv3407 [164:165]. Although the safety and immunogenicity of this construct were confirmed in mice [166], a phase I clinical trial was terminated following emergence of serious side effects (i.e. shingles) [157:165:167].

Recombinant BCG technology was also used to develop vaccine candidates against other bacteria, but clinical evidence of their safety and efficacy is not established yet. For example, Pertussis is a respiratory tract infection caused by *Bordetella pertussis*, which affects over 40 million children every year, with a mortality rate around 1%. *B. pertussis* expresses dozens of pertussis toxins, including the pertussis toxin (PT). PT is not only the primary toxin, but also a protective antigen. The S1 subunit is the largest subunit of pertussis toxin, and its enzymatic activity is related to the toxicity of conventional pertussis vaccine. Nascimento *et al.* constructed an rBCG-S1PT strain and showed that immunization of mice with the rBCG-S1PT induced a strong specific Th1 immune response and protected mice against a lethal intracerebral challenge with *B. pertussis* [168;169]. In a subsequent study, the rBCG-S1PT vaccine was also shown to induce protective immunity in neonate mice [170]. Other examples bacterial vaccine candidates constructed using rBCG technology can be found in Table 2.

Similarly, rBCG had been used to construct vaccine candidates against many viruses, including HIV-1, hepatitis C virus (HCV), human metapneumovirus (hMPV), and human papillomavirus (HPV). For example, Kawahara *et al.* constructed an rBCG strain that expresses HIV-1 Env V3 peptide (rBCG-pSOV3J1) and showed that oral immunization of Guinea pigs with the freeze-dried rBCG-pSOV3J1 strain induced HIV-1-Env-specific T cell responses [4]. Chege *et al.* constructed an rBCG that expresses HIV-1 subtype C Gag protein (rBCGpan-Gag) and showed that immunization of baboons with the rBCGpan-Gag and boosting with HIV-1 Pr55gag virus-like particles (Gag-VLPs) induced a broad and polyfunctional central memory T cell response [171]. Wei *et al.* engineered an rBCG strain that expresses an HCV multi-epitope antigen CtEm (rBCG-CtEm) and showed that the rBCG-CtEm induced HCV epitope-specific cellular immune responses in transgenic mice [172].

In addition, rBCG vaccine candidates against parasites, such as *Plasmodium falciparum*, *Toxoplasma gondii*, *Echinococcus granulosus*, *Schistosoma mansoni*, and *Eimeria maxima*, have been constructed as well. For example, malaria is a mosquito-borne infectious disease of humans and other animals caused by parasitic protozoans. The *P. falciparum* circumsporozoite (CS) protein is a leading pre-erythrocytic vaccine antigen. Arama *et al.* developed a CS-expressing rBCG construct (rBCG-CS) and showed that immunization of BALB/c mice with the rBCG-CS augmented the number of dendritic cells in draining lymph nodes and spleen, upregulated the activation MHC class II, CD40, CD80 and CD86, and induced CS-specific antibodies and IFN- γ -producing memory cells [13;14]. In addition, *in vitro* stimulation of bone marrow-derived dendritic cells (BMDCs) and macrophages with the rBCG-CS induced IL-12 and INF- γ production and enhanced the phagocytic activity of macrophages [13;14]. In another example that also highlighted the significance of the route of administration, Li *et al.* constructed rBCG strains that express the apical membrane antigen1 (AMA1) of *E. maxima* (i.e. rBCG/pMV261-AMA1, rBCG/pMV361-AMA1) and immunized mice with the rBCG/pMV261-AMA1, rBCG/pMV361-AMA1, or BCG via oral, intranasal, and subcutaneous routes and then orally challenged the mice with homologous sporulated *E. maxima* oocysts [173]. It was shown that immunization with the rBCG strains by subcutaneous and intranasal routes was more effective than the oral route in inducing nasal mucosal immune responses, decreasing intestinal lesions, and reducing fecal oocyst

shedding [173]. Other examples of virus and parasite vaccine candidates constructed using rBCG technology can also be found in Tables 3 and 4, respectively.

Unfortunately, such a huge pool of preclinical studies on developing rBCG vaccine constructs against various bacterial, viral, and parasitic infections are not reflected in a matching spectrum of clinical testing. Although those rBCG constructs that express foreign antigens have been tested in various animal models, including normal mice, transgenic mice, cattle, and even non-human primates (Tables 2, 3, and 4), necessary translational research remains needed. For example, a dose designed for bovine parasitic disease may be 10 times the equivalent of human dose, which is about 50 times of the murine dose; an issue that may raise safety concerns. Moving the field ‘vertically’ towards clinical trials, and ultimately towards the development of marketed products for veterinary or human uses, is likely the only way to answer similar questions.

4.2. Recombinant BCG strains engineered to express mammalian cytokines

Besides expressing antigen proteins of interest, rBCG technology had also been used extensively to express mammalian cytokines to enhance or modify the resultant immune responses and the antitumor activity of the constructs. Different rBCG constructs have been engineered to express various Th1 cytokines, and it was reported that high levels of Th1 cytokines are generally associated with favorable immunogenic responses. For example, O'Donnell *et al.* (1994) reported the development of an rBCG strain that secretes IL-2 (rBCG-IL2) [174]. IFN- γ production by splenocytes isolated from mice immunized with the rBCG-IL2 construct was significantly increased when compared to the original BCG [174]. Unfortunately, despite eliciting a strong Th1 immune response, the IL-2-secreting rBCG failed to provide a better protection against a virulent Mtb challenge than the original BCG [175]. On the other hand, in the field of cancer immunotherapy using BCG bacteria, engineering rBCG to express cytokines enabled the application of BCG in a mouse model of mammary tumor. In one of the earliest preclinical reports in developing a BCG-based therapy against breast cancer, Chung *et al.* developed a rBCG construct that expresses the tumor antigen mucin-1 (MUC-1) and human IL-2 (BCG-hIL2-MUC1), and the construct successfully inhibited the growth of MUC-1-positive tumors in SCID mice reconstructed with human peripheral blood lymphocytes [176]. Other cytokine genes introduced in rBCG bacteria include IL-18 [177], IFN- α 2b [178], and IFN- γ [179]. Using this approach to improve the activity of BCG in bladder cancer therapy following intravesical instillation appears to be promising. For example, intravesical instillation of an IFN- γ -secreting rBCG (rBCG-IFN- γ) in mice with model orthotopic bladder tumor induced the expression of IL-2 and IL-4 mRNA and resulted in an increase in the influx of CD4⁺ cells into the bladder following instillation, as compared to the empty BCG vector [179]. On the other hand, treatment with rBCG-IFN- α 2b was found to improve the cytotoxic activity of peripheral blood mononuclear cells (PBMC) against bladder cancer cells [178]. The observed increase in the production of IFN- γ and IL-2 by PBMC after treatment with rBCG-IFN- α 2b was crucial for the enhanced cytotoxicity, because the cytotoxicity was inhibited, or even abolished, upon blockade of IFN- α , IFN- γ , or IL-2. More examples of rBCG constructs used in cancer therapy will be mentioned in the following section. A comprehensive review on constructs of rBCG that express various cytokines can be found in literature [69].

5. Recent trends in rBCG technology

5.1. Heterologous priming and boosting to improve the immune responses induced by BCG and rBCG vaccines

Vaccine candidates developed using rBCG technology naturally induce immune responses against the BCG bacteria, which is desirable on one hand, as any rBCG vaccine engineered to express an antigen from a different pathogen would be naturally multivalent. On the other hand, pre-existing immunity against the rBCG vector may make boost immunization with rBCG less effective. In recent years, there is increasing evidence that better immunogenicity could be achieved following rBCG vaccine priming combined with boosting with the same foreign antigens carried or expressed by adenovirus vector, vaccinia virus vector, VLPs, or plasmid DNA [13;14;171;180;181], making this heterologous priming and boosting approach a preferred trend. Various examples can be found in literature, and only a few of them are described herein. For example, Cayabyab *et al.* showed that high frequency polyfunctional SIV-specific cellular immune responses were induced in monkeys that were primed with rBCG-SIV-Gag-Pol and boosted with recombinant adenovirus 5 (Ad5) expressing those SIV antigens, whereas animals primed and boosted with the same rBCG-SIV-Gag-Pol developed only modest SIV-specific CD8⁺ T cell responses [145]. Chege *et al.* reported that a prime-boost immunization regimen using an rBCG strain that expresses Gag from a South African HIV-1 subtype C isolate and HIV-1 subtype C Pr55 (gag) VLPs successfully elicited Gag-specific CD8⁺ T cell and humoral responses in baboons, whereas the rBCG vaccine alone induced only a weak, or an undetectable, HIV-1 Gag-specific response [180]. Hopkins *et al.* showed that priming of adult BALB/c mice with rBCG strains that expresses the immunogen HIVA and boosting with human Ad5-vectored HIVA (HAdV5.HIVA) or sheep atadenovirus-vectored HIVA (OAdV7. HIVA) induced T cells that were capable of *in vivo* killing of sensitized target cells [182]. Similarly, Raham *et al.* showed that priming with rBCG that expresses Mtb Ag85A, Ag85B, and TB10.4 and boosting with non-replicating Ad35 encoding the same antigens in primates was associated with enhanced MHC-I antigen presentation and activation of CD8⁺ T cell responses at the local site of infection in Mtb-challenged animals [183]. Chapman *et al.* reported that priming with rBCG that expresses HIV-1 Gag and boosting with modified vaccinia virus Ankara (MVA) that express HIV-1 Gag elicited HIV-1 specific CD8⁺ T cells in mice [181]. Arama *et al.* reported that heterologous prime-boost regimen with a Ad35-CS protein vaccine and rBCG expressing the *P. falciparum* CS protein induced stronger long-term memory immunity in BALB/c mice [13]. Recently, Chege *et al.* reported that an rBCG-VLP prime-boosting HIV vaccine regimen in nonhuman primates was highly immunogenic and induced a broad and polyfunctional center memory T cell response [171]. Conclusively, the heterologous priming and boosting approach, although not unique to rBCG vaccines, may turn out to be essential for successful vaccinations with rBCG-based vaccines. However, heterologous priming and boosting has an inherent limitation, as more than one vaccine need to be developed and evaluated clinically for safety and efficacy for heterologous priming and boosting using rBCG vaccines to become clinically feasible.

The clinical significance of priming with rBCG that expresses a particular antigen, as compared to BCG, is appealing; especially considering the limited protection improvement

that was met when regular BCG priming was used followed by boosting. For example, in a double blind phase 2b clinical study in BCG-vaccinated non-HIV healthy infants, evidence that boosting with a modified vaccinia Ankara virus that expresses the antigen 85A (MVA85A) following priming with BCG (n = 1399 for MVA85A vaccine and 1398 for placebo) can provide better protection against Mtb was not found [184], even though the safety of the new MVA85A vaccine was confirmed [184]. The approach of priming with BCG and boosting with MVA85A has proven effective pre-clinically in cattle [185] and primates [186], and even clinically in inducing cellular immune responses (i.e. poly-functional CD4⁺ and CD8⁺ T cell responses [187] or potent poly-functional CD4⁺ T cell response [188]). It was expected that the induction of strong cellular responses following the boosting with the MVA85A would be sufficient to provide further protection against Mtb infection in infants [188], but in fact MVA85A could not provide better protection compared to placebo [184]. Infantile malnutrition and other factors were thought to be related to the limited improved protection against Mtb following MVA85A boosting [189–191]. We think that a reasonable justification of the inability of MVA85A to improve the BCG-induced protection against Mtb is that the BCG-induced protection itself is reasonably effective, which does not leave much room for improvement. Probably more effort needs to be directed towards improving the BCG vaccination outcome in adults, as well as in infants in AIDS-endemic regions. In this regard, Tchakoute et al. reported a stronger T cell response when the BCG vaccination was delayed to the 8th week after birth in HIV-exposed Mtb-free infants [168]. This new strategy may not only make Mtb vaccination in HIV-exposed infants possible, which may usually comprise a potential risk when regular BCG is to be used, but also may provide a better priming option, in case a heterologous boosting step is to follow. In our opinion, optimizing the heterologous priming and boosting approach by priming with a potent rBCG construct followed by boosting with another vaccine expressing the same antigen(s) in clinical trials may provide a successful and highly demanding alternative. We expect that in case rBCG priming followed by boosting step develops into a common clinical practice for newborn vaccination, individuals that have been vaccinated at birth with conventional BCG may still benefit from boosting with innovative Mtb vaccines, although it may still be quite challenging to improve the already high protection efficacy of BCG. For this reason, it can be concluded that the most highly demanding priority in BCG research would be to develop a safe, yet effective, vaccination modality against Mtb for infants in high TB risk areas where HIV is endemic.

5.2. Recent trends in tumor immunotherapy using rBCG technology

Recombinant BCG technology had been extensively investigated to induce stronger and more specific antitumor immune response. For example, Nasciemento *et al.* constructed a rBCG strain that expresses the genetically detoxified S1 subunit of pertussis toxin S1PT (rBCG-S1PT), which was much stronger in providing protection against intracerebral challenge of mice with *B. pertussis* than the conventional DPT vaccine [17]. Later, the rBCG-S1PT was found to prolong the survival of mice challenged with MB49 murine bladder cancer cells, as compared to BCG [17,102]. Moreover, the researchers also reported an increased TNF- α and IL-10 levels in mice treated with rBCG-S1PT, compared to an increased TNF- α only in mice treated with BCG [179]. Other researchers reported promising preclinical results in tumor therapy with other rBCG constructs as well. For

example, Arnold *et al.* generated a rBCG strain that secretes murine IFN- γ (rBCG-IFN- γ), and the rBCG-IFN- γ was shown to prolong the survival of mice with model bladder cancer, as compared to the original BCG [103]. Begnini *et al.* constructed an auxotrophic rBCG strain that overexpresses Mtb Ag85B (BCG- leuD/Ag85B), which was shown to modify the gene expression of apoptotic and cell cycle-related genes favorably (e.g. bcl-2 and p53) [103]. The BCG- leuD/Ag85B was more cytotoxic against human bladder carcinoma cell line 5637 than BCG [178;192]. However, there is no evidence that the BCG- leuD/Ag85B induces stronger immune responses than the original BCG. Furthermore, researchers also reported the development of IFN- α 2b-secreting rBCG constructs that showed enhanced immune response and cytotoxicity against human bladder cancer cells [176].

About three decades after the first report of the application of BCG in bladder cancer, rBCG technology was evaluated in a mouse model of breast cancer. Chung *et al.* constructed the BCG-hIL2-MUC1 strain that expresses a truncated form of MUC-1 and human IL-2 [176]. The BCG-hIL2-MUC1 construct successfully inhibited human breast tumor growth in hu-PBL-SCID mice, and in a few cases, tumor development [193]. The mice were vaccinated prior to tumor challenge, which revealed the immunoprotective capability of this approach. Other researchers constructed rBCG strains that express both MUC-1 and colony stimulation factor (CSF) [194] or MUC-1 and CD80 [194]. The growth of MCF-7 tumors implanted in hu-PBL-SCID mice was significantly inhibited using these constructs [195]. Unfortunately, there is not any recent evidence that those rBCG constructs are evaluated in clinical trials.

Expert Commentary

As mentioned above, there had been numerous studies of using rBCG as a vector to develop vaccine candidates against various pathogens (e.g. bacteria, viruses, and parasites) and to improve bladder cancer immunotherapy. However, only the original BCG is used in clinical practice. It is expected that future research will continue to be on engineering BCG bacteria to stably express foreign antigens or cytokines to develop potential rBCG-based vaccine candidates, but it is our opinion that increasing efforts should also be focused on translating existing or new rBCG vaccine candidates from bench to clinics, either for humans or animals, including domestic animals (e.g. cattle) and wildlife animals. The traditional route of intradermal injection may be used to administer the vaccine candidates, but other routes such as intranasal and oral routes may be chosen as well, especially if mucosal immunity is desired. BCG was originally given to humans orally [196]. BCG bacteria are highly susceptible (to killing or inactivation) in the acidic condition in the stomach and by the action of duodenal juices [42]. This can be readily addressed now by region-specific vaccine delivery. A major reason for the discontinuation of oral BCG vaccine is that when concentrated BCG vaccine is not swallowed, but drained into the cervical lymph nodes, a high frequency of lymphadenitis developed in children [18]. This will also not likely be an issue now if the vaccines are given to adults in well-designed coated capsules. Some literature reports have already discussed the potential of improving the survival of BCG cells, and thus the *in vivo* immune response, upon oral administration, once the bacilli are encapsulated in certain carriers (e.g. lipid-based oral formulations) [197;198]. Such approaches can facilitate the induction of mucosal immune response in the GI tract. For wildlife animals, catching and injecting them with rBCG vaccines may be feasible on a

small scale [122], but formulating the vaccines into baits for oral consumption and thus vaccination is likely more preferred.

Heterologous priming and boosting with an rBCG vaccine candidate is promising in inducing strong and specific CD8⁺ T cell responses. However, it is cautioned that due to the inherent need for developing at least two vaccines at the same time, the heterologous priming and boosting regimen should not be the first choice in vaccine development. Instead, it should be reserved only for certain human diseases, against which an efficacious vaccine remains elusive. Examples include HIV infections, adult TB, and malaria (note: approval of GSK's first-in-the-world malaria vaccine, which is not BCG-based, is pending). In fact, as shown above, studies in which heterologous priming and boosting immunizations with rBCG strains were used were mainly for HIV and TB vaccine development.

Finally, there had been significant efforts in improving the efficacy of BCG in bladder cancer therapy and exploiting rBCG's potential in the therapy of other types of cancers. Further research in using rBCG in tumor immunotherapy should be directed towards the development of rBCG constructs that secrete a harmonized set of the 'right' cytokines with a well-designed induction and maintenance scheme, which are expected to significantly improve the outcomes of BCG therapy. The rBCG technology may also be utilized to develop immunotherapeutic agents for other types of cancers, including renal cell carcinoma and prostate cancer, because more specific responses and less severe side effects may be achieved, as compared to the wild type. In addition, creative approaches to prolong the residence time of BCG in tumor tissues and to increase the internalization of BCG bacteria by tumor cells only are needed to improve tumor immunotherapy using BCG.

Five-year view

In the next five years, it is expected that rBCG technology will continue to be used to develop vaccine candidates for various infectious diseases, and there will be more efforts in using rBCG technology to improve immunotherapy of bladder cancer, as well as other types of cancers. In addition, the heterologous priming and boosting vaccination regimen will continue to be tested in the development of HIV and likely new TB vaccines. Importantly, several new TB vaccine candidates based on rBCG technology are currently in clinical trials, and it is expected that safety and some efficacy data from those trials will become available in the next five years.

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Key issues

- BCG vaccine is one of the most widely used vaccines in the world. BCG vaccine is one of the first oral vaccines.
- BCG vaccine is effective against infant meningitis TB and miliary TB, but not against TB in adults.
- Intravesical instillation of BCG after TUR is a standard bladder cancer therapy.
- BCG had been used as a bacterial vector to develop vaccine candidates against numerous pathogens and tumors.
- Heterologous priming and boosting regimen with an rBCG vaccine is proven to be effective in eliciting strong CD8⁺ T cell immune responses, but priming and boosting with different vaccines should not be the first choice in vaccine development.
- Recombinant BCG vaccine candidates can induce both humoral and cellular immunity. They can also induce both systemic and mucosal immunity, if given via a proper mucosal route.
- To improve the effectiveness of BCG in bladder cancer therapy, and to enable the use of BCG in the therapy of other cancers, innovative strategies to increase the delivery and targeting of BCG into tumor cells are needed.

Table 1
Representative examples of BCG applications and key findings in the field of tumor immunotherapy.

BCG strain/ formulation	Publication year	Host	Application	Key finding(s)	Ref.
Phipps	1959	Female <i>Hu/ICR</i> Swiss mice and C57 hybrid mice	Infection of mice by intravenous injection of BCG	It is one of the earliest reports to show that BCG-infected mice are resistant to tumor growth. Tumors were inoculated (either subcutaneously or intraperitoneally) after BCG infection was established.	[124]
Phipps	1971	Male Guinea Pigs	Intradermal injection of BCG into tumors pre-established intradermally in Guinea pigs. Guinea pigs were either BCG-immunized or un-immunized.	This study showed that intratumoral injection of BCG caused complete regression of tumors in most animals and inhibited metastasis to drainage lymph nodes. Effect was noticeable in both BCG-pre-immunized and un-immunized Guinea pigs. Contact between tumor cells and injected BCG seemed to be crucial.	[66]
Tice	1975	One human patient with metastatic malignant melanoma in the urinary bladder.	A whole ampoule of a market available BCG was intratumorally injected at the base of a 2 cm metastatic melanoma lesion in the bladder using a cystoscopic needle.	This study shows that a 2 cm metastatic melanoma tumor lesion inside the bladder completely disappeared 1 week following intratumoral injection of BCG. Metastatic melanoma was spread later to the small intestine and liver, and the patient died later. However, no evidence of bladder metastasis recurrence was found.	[67]
Tice	1975	Mongrel dogs	BCG was applied to bladder either by intramucosal injection into the exposed bladder or inoculation into the bladder using a catheter.	BCG inoculation into the bladder of PPD-sensitized dogs seemed to be well-tolerated, which suggested potential for BCG use in bladder cancer.	[125]
Glaxo	1970	36 human patients with subcutaneous melanoma	Intralesional injection of BCG	About 91% of injected melanoma lesions disappeared or showed regression. About 20% of patients showed regression of un-injected lesions that are located within the same drainage area of injected lesions.	[65]
Montreal	1976	9 human patients with superficial bladder cancer	Intravesical instillations of BCG into the bladder	This is the first clinical study that reports the use of intravesical instillation of BCG in bladder cancer. Recurrence of the disease in the	[79]

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BCG strain/ formulation	Publication year	Host	Application	Key finding(s)	Ref.
Pasteur	1990	C3H/HEN mice.	Intravesical instillation of BCG into the bladder	patients treated was reduced. It was found that fibronectin-mediated attachment of BCG to the bladder mucosa was crucial to elicit the desired antitumor activity. This was an important step towards elucidating the mechanism underlying the BCG immunotherapeutic activity in bladder cancer.	[70]
Connaught	2000	Human patients with resected bladder tumors with high risk of recurrence	Maintenance schedule of BCG instilled intravesically and given percutaneously for up to 36 months.	This is one of the earliest large clinical trials that showed the benefit of a maintenance schedule as compared to the standard induction therapy alone in bladder cancer patients.	[36]
Connaught and Tice	2014	142 human patients with NMIBC.	6 intravesical instillations of BCG into the bladder	By comparing 2 commercially available BCG strains, the researchers reported improved recurrence-free survival by the Connaught strain. The genetic difference between the strains may explain the survival advantage.	[176]

BCG: bacillus Calmette-Guerin; PPD: purified protein derivative; NMIBC: non-muscle invasive bladder cancer.

Table. 2

A representative list of rBCG vaccines against bacterial infections.

rBCG vaccine candidates	BCG strain	Antigen gene(s)	Hosts	Key findings	Ref.
TB vaccines	China	Ag85A, Ag85B	C57BL/6 mice	The rBCG::AB strain provided a stronger and longer-lasting protection against a virulent Mtb challenge.	[146]
	Tokyo	Ag85A	Rhesus monkeys	The rBCG-85A prevented an increase in old tuberculin test after challenge with Mtb and reaction of Mtb-derived antigen.	[147]
	Danish	ESAT-6	Guinea pigs	A DNA-E6 booster induced an enhanced antigen specific INF- γ response, but obliterated the protection imparted by rBCG against tuberculosis.	[148]
	Danish	HspX	Mice	The rBCG::X produced a more consistent and enduring protective effect against infection with Mtb than the BCG containing the empty vector pMV261.	[149]
	Danish	Ag85B-Rv3425	C57BL/6 mice	The rBCG::Ag85B-Rv3425 induced a predominant Th1 response.	[150]
	Danish	Ag85A, Mpt64, Mtb8.4	C57BL/6 mice	The rBCG-AMM conferred similar, or better, protective efficacy against Mtb infection, compared to BCG or rBCG-A.	[151]
	Danish	Ag85B, ESAT-6, mouse TNF- α	C57BL/6 mice	The rBCG-Ag85B-ESat6-TNF- α induced a stronger immune response than rBCG-Ag85B-ESat6 or BCG.	[154]
	N/A	Ag85B, ESAT-6	THP-1 cells	The rBCG-Ag85B-ESAT-6 showed an enhanced immunostimulatory activity in human macrophages.	[155]
	N/A	Ag85B (P1,P2,P3), Mtb8.4, ESAT-6, CFP-10, MTP40	BALB/c mice	The rBCG constructs expressing either T or T and B cell epitopes of Mtb induced appropriate immunogenicity against Mtb.	[152]
	China	Ag85A, ESAT-6	BALB/c mice	The rBCG-AE was not more protective against Mtb H37Rv infection than the parent BCG.	[156]
	Pasteur	Ag85B	Cattle	Vaccination with the rBCG-Ag85B protected cattle better than the wild type BCG Pasteur.	[153]

rBCG vaccine candidates	BCG strain	Antigen gene(s)	Hosts	Key findings	Ref.
Diphtheria, Pertussis and Tetanus vaccines	Moreau	S1 subunit of pertussis toxin	Neonate mice	The rBCG induced higher PT-specific IFN- γ production and an increase in both IFN- γ (+) and TNF- α (+), CD4(+) T cells and conferred protection against a lethal intracerebral challenge with <i>B. pertussis</i> .	[169]
	Moreau	S1 subunit of pertussis toxin	Neonate mice	An unmarked complemented rBCG-Delta lysA-S1PT-lysA(+)(kan(-)) was constructed, which induced a high level of cellular immune response and protection against <i>B. pertussis</i> challenge.	[170]
	Moreau	Tetanus toxin FC, CRM ₁₉₇	BALB/c and NIH mice	Reciprocal adjuvant effects of rBCG-Fc and rBCG-CRM ₁₉₇ were detected.	[199]
	Moreau	CRM ₁₉₇	BALB/c mice	The rBCG-CRM ₁₉₇ elicited neutralizing antibody responses.	[200]
Shiga vaccines	Tokyo	Shiga toxin 2B subunit	BALB/c mice	The rBCG-Stx2B vaccine protected mice against a challenge by Shiga-toxin-producing <i>E.coli</i> via intraperitoneal vaccination.	[12]

BCG: bacillus Calmette-Guerin; PPD: purified protein derivative; NMIBC: non-muscle invasive bladder cancer.

Table. 3

A representative list of rBCG vaccines against viral infections.

rBCG vaccine candidates	BCG strain	Antigen gene(s)	Hosts	Key findings	Ref.
HIV vaccines	Pasteur	HIV-1 Gag, RT and Gp120	BALB/c mice	The rBCG vaccines were able to prime the immune system for a boost with rMVA expressing matching antigens, and induced robust, HIV-specific T cell responses to both dominant and subdominant epitopes in the individual proteins, when used as individual vaccines or in a mix.	[11]
	Tokyo	HIV-1 Env V3 peptide	Guinea pigs	Oral vaccination of guinea pigs with freeze-dried rBCG-pSOV3J1 induced high levels of functional T cells specific to HIV-1 antigens in both mucosal and systemic compartments.	[4]
	Pasteur	HIV-1 Env V3-concatamer	BALB/c mice, guinea pigs	The rBCG-mV3 was very efficient in inducing humoral and long-lasting cell-mediated immunity against HIV-1 V3 in immunized mice and guinea pigs.	[201]
	Pasteur	HIV-1 subtype C Gag protein	Chacma baboons	The rBCGpan-Gag priming and Gag VLP boosting regimen induced a broad and polyfunctional central memory T cell response.	[171]
SIV vaccines	Tokyo	SIV Gag	CD1 and C57BL/6 mice	The rBCG-SIV Gag priming-recombinant vaccinia virus boosting protocol elicited long-lasting Gag-specific CD8 ⁺ T cells.	[202]
	Pasteur	SIV Gag, Pol	Rhesus monkeys	The rBCG priming and rAd5 boosting protocol elicited SIV-specific T cell responses.	[145]
	Tokyo	SIV Gag	Guinea pigs	The rBCG-SIVgag induced an antigen-specific humoral immune response concomitant with IFN- γ response, even at three years after immunization.	[203]
	Pasteur	SIVmac251 nef, gag and env	Cynomolgus macaques	Mucosal administration of three rBCG induced rectal IgAs and	[6]

rBCG vaccine candidates	BCG strain	Antigen gene(s)	Hosts	Key findings	Ref.
HCV vaccines				boosted systemic cellular immune responses, which are primed by intradermal vaccination.	
	Pasteur	SIV mac Gag	Rhesus monkeys	The rBCG elicited MHC I-restricted CD8 ⁺ SIVmac gag-specific CTL responses.	[204]
	Tokyo	HCV-non-structure protein 5a (NS5a)	BALB/c × C3H/HeN (CC3HF1) mice	The rBCG elicited MHC I-restricted CD8 ⁺ HCV-NS5a-specific CTLs.	[205]
Human metapneumovirus vaccine	Tokyo	HCV multi-epitope antigen CIE _m	HLA-A2.1 transgenic mice	Immunization with the rBCG-CIE _m conferred protection against infection with the recombinant vaccinia virus (rVV-HCV-CNS) <i>in vivo</i> .	[172]
	Danish	Phosphoprotein from hMPV	BALB/c mice	The rBCG induced protective Th1 immunity and the activation of hMPV-specific T cells that produce IFN- γ and IL-2.	[206]

BCG: bacillus Calmette-Guerin; PPD: purified protein derivative; NMIBC: non-muscle invasive bladder cancer.

Table. 4

A representative list of rBCG vaccines against parasitic infections.

rBCG vaccine candidates	BCG strain	Antigen gene(s)	Hosts	Key findings	Ref.
<i>Plasmodium falciparum</i> vaccines	Glaxo	Circumsporozoite protein (CS)	BALB/c mice	The rBCG-CS was highly efficient in activating long-lived plasma cells (LLPCs) for priming of adaptive immunity.	[13-14]
	Japan (Tokyo)	SE22 from the N terminal domain of serine repeat antigen (SERA)	BALB/c mice	The rBCG-SE22 enhanced both humoral and cellular immune responses in mice.	[207]
	Japan (Tokyo)	19kD merozoite surface protein (MSP-1(19))	BALB/c mice	The rBCG expressing the mutated version of MSP-1(19) of <i>P. falciparum</i> induced enhanced humoral and cellular responses.	[208]
<i>Toxoplasma gondii</i> vaccines	N/A	Cyclophilin	BALB/c mice	Both rBCGpMV261-TgCyp and rBCGpMV361-TgCyp elicited Th1 immune responses after i.v. or oral vaccination, and the rBCGpMV361-TgCyp i.v. inoculation resulted in a better protection.	[209]
<i>Echinococcus granulosus</i> vaccines	N/A	Eg95	BALB/c mice	The rBCG-Eg95 induced Th1 response in mice.	[210]
<i>Schistosoma mansoni</i> vaccines	Pasteur	Sm14	BALB/c or Swiss mice	Splenocytes of immunized mice released increased levels of IFN- γ , and one or two doses of rBCG-Sm14 conferred the equivalent protection compared to that obtained by immunization with three doses of rSm14 protein.	[211]
<i>Eimeria maxima</i> vaccines	N/A	Apical membrane antigen1 (AMA1)	Chickens	Intranasal rBCG immunization induced strong humoral and cellular responses directed against homologous <i>E. maxima</i> infection.	[173]

BCG: bacillus Calmette-Guerin; PPD: purified protein derivative; NMIBC: non-muscle invasive bladder cancer.