Title

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Abstract

Bacille de Calmette et Guérin (BCG) was attenuated from a virulent strain of *Mycobacterium bovis* a century ago and has since been administered as an anti-tuberculosis (TB) vaccine to more than 4 billion people, making it the most widely used vaccine of all time. Although BCG provides significant protection against disease and death due to childhood and disseminated forms of TB, the efficacy of BCG against adult, pulmonary disease is inconsistent. Thus, despite near universal vaccination with BCG in TB endemic areas, TB remains a heavy burden worldwide, especially in developing nations. In recent years, BCG has been utilizied in two major vaccine development strategies. First, BCG has been used as a vector to express foreign antigens in studies aimed at developing new vaccines against a variety of viral, parasitic, and bacterial pathogens, and against cancer and allergic diseases. More recently, in a new vaccine paradigm, BCG has been used as a homologous vector to overexpress native mycobacterial antigens in studies aimed at developing improved vaccines against TB. As a vaccine vector, BCG has several major advantages including a very well established safety profile, high immunogenicity (excellent CD4+ and CD8+ T cell responses and strong TH1 Type immune responses), and low manufacturing cost. As a vector for recombinant TB vaccines, BCG has the additional advantages of providing a broad array of relevant mycobacterial antigens in addition to the recombinant antigens, moderate efficacy to begin with, high acceptability as a replacement vaccine for BCG in TB endemic countries, and the capacity to express *M. tuberculosis* proteins in native form and release them in a way that results in their being processed similarly to *M. tuberculosis* proteins. In addition to the overexpression of native proteins to improve their immunogenicity and protective efficacy against TB, recombinant BCG vaccines have been developed that express immunomodulatory cytokines or have been engineered for enhanced

antigen presentation. Several new recombinant BCG vaccines against TB have demonstrated improved protective efficacy against *M. tuberculosis*, *M. bovis*, and *M. leprae* in small animal models. Against non-TB targets, results have been variable, but several recombinant BCG vaccines have demonstrated excellent immunogenicity and protective efficacy; stable and high level expression of foreign antigens in recombinant BCG, in a way that will make them available for proper processing and presentation, have been recurrent challenges.

I. Introduction

Bacille de Calmette et Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, was developed as a vaccine against human tuberculosis (TB), which is primarily caused by the closely related species *Mycobacterium tuberculosis*. Although its efficacy against TB is suboptimal, BCG is the most widely used vaccine of all time, having been administered to more than 4 billion people since 1921. In addition to its use as a prophylactic agent against TB, BCG has also been used as a therapeutic agent in the treatment of bladder cancer.

BCG is an intracellular bacterial parasite that survives and multiplies in mononuclear phagocytes. Akin to *M. tuberculosis*, inside the human macrophage, BCG resides in an endosomal compartment that does not fuse with lysosomes [1, 2] and is not highly acidified [3].

In the past two decades, BCG has been used as a vaccine vector for a number of purposes. First, it was used as a heterologous vector to express foreign antigens of a variety of pathogens, such as HIV and *Borrelia burgdorferi*, the agent of lyme disease [4-9]. Subsequently, in studies initiated in this laboratory, it was used in a new vaccine paradigm as a homologous vector overexpressing native antigens, so as to improve its protective efficacy against TB [10]. Most recently, a variety of additional modifications of BCG have been explored in an effort to improve its protective efficacy against TB. These include endowing it with immunomodulatory cytokines capable of directing the immune response; altering its life cycle in antigen presenting cells by engineering it to escape its phagosome and enter the cytoplasm, thereby enhancing class I MHC antigen presentation; and arming it with a protease to enhance its ability to present antigens on MHC molecules. This chapter will focus on these new approaches to improving the immunogenicity

and protective efficacy of BCG against TB and on the use of BCG as a vector for new recombinant vaccines against non-tuberculous pathogens.

II. A brief history of BCG

Albert Calmette and Camille Guérin developed BCG as a TB vaccine by passaging *M. bovis* 230 times between 1906 and 1919 on medium consisting of pieces of potato cooked and soaked in ox bile containing 5% glycerine, after which the organism lost its pathogenicity for animals. Their strategy was essentially an amalgam of Jenner's smallpox vaccine strategy centered on using a less virulent species (at least for humans) closely related to the target pathogen and Pasteur's anthrax and rabies vaccine strategy centered on the attenuation of pathogens by culture under non-natural conditions. The vaccine was first administered to children in 1921. Although controlled studies were not conducted at that time, the vaccine was believed effective in reducing the mortality of TB in children below estimated levels prior to its use, and the vaccine gained wide acceptance in Europe and subsequently elsewhere. [11].

Modern molecular analyses have elucidated genetic differences between BCG and *M. tuberculosis*. Many of these differences, involving about 3% of the ~4000 genes of these organisms and clustered in gene segments or Regions of Difference, reflect genomic differences between *M. bovis* and *M. tuberculosis* [12, 13]. In addition, during its original attenuation from *M. bovis*, the original BCG strain lost a gene segment containing 9 open reading frames known as Region of Difference 1 or RD1 [13]. After BCG was distributed to different countries and strains propagated and maintained separately, genetic differences among BCG strains evolved including gene deletions and duplications [12, 13]. Genealogically, strains with relatively few subsequent gene deletions are characterized as "early" strains, and include BCG Russia, Moreau, and Japan, and strains that have acquired additional deletions are characterized as late strains, and include BCG Tice, Connaught, Pasteur, Glaxo, and Danish.

Controlled studies conducted in 1937 and afterwards demonstrated that BCG was efficacious in reducing the incidence and mortality of TB in children and in reducing the incidence of disseminated forms of TB such as meningitis and miliary TB [14, 15]. However, the efficacy of BCG against adult pulmonary TB was inconsistent, ranging from -35% to +80% [16]. A large meta-analysis calculated the overall efficacy of BCG against adult TB at 50% [14], but this figure disguises the fact that trials tended to divide into those demonstrating either high or low efficacy, rather than conform to a normal distribution. Trials in non-tropical regions of the world have tended to have high efficacy and trials in tropical regions of the world have tended to have low efficacy [14].

In 2007, the most recent year for which data are available, there were an estimated 9.27 million cases of tuberculosis worldwide [17]. Most of these cases occurred in populations where BCG vaccination is near universal and hence these cases can be viewed as vaccine failures. Thus, whatever the efficacy of BCG, there is considerable room for improvement of this century old vaccine.

III. Why is the efficacy of BCG so inconsistent?

A number of hypotheses have been advanced as to why BCG protects well against TB in some trials and not in others. These include:

Prior exposure to atypical environmental mycobacteria that mask or interfere with the immune response to BCG

In support of a direct antagonistic effect of environmental mycobacteria, Brandt et al. [18] reported that mice presensitized with a mixture of three environmental bacteria (*Mycobacterium avium*, *Mycobacterium scrofulaceum*, and *Mycobacterium vaccae*) clear BCG more rapidly and are less well protected against *M. tuberculosis* challenge. On the other hand, in a different experimental murine model, presensitization with *M. vaccae* and *M. scrofulaceum* before BCG vaccination enhanced protection against *M. tuberculosis* challenge, and presensitization with *M. avium* had no influence on the protective efficacy of BCG [19].

Malnutrition that interferes with the development of a protective immune response

In support of this idea, McMurray et al. have demonstrated that, in contrast to well-nourished guinea pigs, protein deficient guinea pigs fail to develop mature well-organized granulomas; moreover, when vaccinated with BCG, the protein-deficient guinea pigs are less well-protected against *M. tuberculosis* aerosol challenge than well-nourished guinea pigs [20].

Use of different BCG strains

Some have postulated that some strains of BCG are more attenuated than others and that they consequently induce an inferior protective immune response [12, 21]; Brosch et al.

postulated that evolutionarily early strains, with fewer gene deletions, may be more potent than evolutionarily late strains. Against this idea, a study in this laboratory showed that an evolutionarily early strain (BCG Japan) and several evolutionarily late strains of disparate genealogy (BCG Tice, Connaught, and Pasteur, and Danish) were comparably efficacious in protecting against *M. tuberculosis* aerosol challenge in the demanding guinea pig model of pulmonary tuberculosis [22]. Moreover, a large meta-analysis failed to find differences in efficacy among strains of BCG or for that matter between BCG and *M. microti* (vole bacillus) [14, 23].

Exposure to helminthes

Helminthe infection is highest in tropical regions of the world. Helminthes induce TH2 type immune responses, which may interfere with the capacity of BCG to induce a protective TH1 type of immune response against *M. tuberculosis*. In support of this, Elias et al. found that worm-infected volunteers have significantly diminished TH1 type responses compared with dewormed controls [24].

High levels of IL-4

Rook et al. have proposed that high levels of IL-4 in tropical regions, in part as a result of helminthe infection, interferes with the protective immune response to BCG [25].

IV. Recombinant BCG overexpressing native proteins as vaccines against tuberculosis

A. Rationale for the choice of BCG as vector

In addition to recombinant BCG vaccines, many different types of vaccines have been evaluated for efficacy against tuberculosis in animal models including subunit vaccines, DNA vaccines, and attenuated *M. tuberculosis*. With the exception of attenuated *M. tuberculosis*, none of these vaccines matches, let alone surpasses, the efficacy of BCG in the most challenging animal models. Because of the risk of reversion to virulence, attenuated *M. tuberculosis* vaccines are burdened with a significant safety concern; multiple gene deletions will almost certainly be required to obtain regulatory approval of these vaccines, and with these additional deletions, the vaccines are likely to exhibit reduced efficacy. Thus, at present, improving BCG offers the greatest potential for a vaccine that is more efficacious and at least as safe as BCG.

Aside from its moderate efficacy to begin with, BCG has several major advantages as a vector for new recombinant BCG vaccines against tuberculosis (Table 1). First, it has a very well established safety profile, having been administered to over four billion persons. Serious adverse effects are exceedingly rare except in immunocompromised individuals, for whom the vaccine is not recommended. Second, as a live vaccine, it has high immunogenicity. Third, and of great importance, modified versions of BCG that are superior to BCG have very high acceptability as a replacement vaccine for BCG in regions of the world where the burden of tuberculosis is greatest; for all of its shortcomings, BCG is life-preserving in such parts of the world, especially in infants, and health care workers are reluctant to accept an alternative vaccine in a vaccine trial that is not clearly at least as efficacious as BCG. In such parts of the world, recombinant BCG is considered “BCG+” and is therefore readily acceptable as an alternative to BCG in vaccine trials,

provided of course that it has demonstrated sufficient safety and efficacy in preclinical studies. Fourth, BCG occupies the same intraphagosomal compartment as *M. tuberculosis* in host cells, and consequently processes and presents antigens similarly. In human mononuclear phagocytes, both BCG and *M. tuberculosis* multiply extensively if not exclusively within a phagosome; a small minority of *M. tuberculosis* may exit the phagosome at very late stages of infection (Clemens and Horwitz, unpublished data) as has been reported in myeloid cells [26], possibly as a prelude to lysis of the host cell, but the majority of mycobacterial multiplication takes place in an endosome-like compartment that favors antigen presentation via class II MHC molecules. Fifth, BCG expresses *M. tuberculosis* proteins in native form. Non-mycobacterial vectors may express highly conserved *M. tuberculosis* proteins in native form, but a mycobacterial host is frequently required to express proteins unique to mycobacteria, such as the mycolyl transferases, in native form [27]. Finally, BCG can be manufactured cheaply; cost is of course a significant consideration in the developing regions of the world where tuberculosis has the highest prevalence.

B. rBCG30

rBCG30, the first vaccine demonstrated more potent than BCG against tuberculosis, is also the first vaccine of any kind incorporating the strategy of utilizing a homologous vector to over-express native antigens [10, 28]. This is a powerful vaccine strategy that combines the approach of using a live attenuated homologous vector with the approach of immunizing with key immunoprotective antigens of the target pathogen. In the case of rBCG30, the live attenuated homologous vector is BCG and the key native antigen is the *M. tubeculosis* 30 kDa major secretory protein, a mycolyl transfersase also known as the alpha antigen or Antigen 85B.

1. Rationale for selecting the *M. tuberculosis* 30 kDa protein for a recombinant BCG vaccine

The rationale for using BCG as a homologous vector is discussed above. The rationale for overexpressing the major secretory protein of *M. tuberculosis* is derived from the Extracellular Protein Hypothesis for vaccines against intracellular pathogens [29-34]. This hypothesis holds that proteins secreted or otherwise released by intracellular pathogens are key immunoprotective antigens because they are available for proteolytic processing by the host cell and presentation on the host cell surface as MHC-peptide complexes, thus allowing the immune system to generate a population of T cells capable of recognizing the MHC-peptide complexes and exerting an antimicrobial effect against the host cell. The hypothesis further holds that appropriate immunization of a naïve host with such proteins incorporated into a vaccine allows the immune system to generate a functionally equivalent population of T cells later capable of recognizing and exerting an antimicrobial effect against host cells infected with the target intracellular pathogen. Finally, the hypothesis holds that among the extracellular proteins released by intracellular parasites, the most abundant ones will figure most prominently because they would provide the richest display of MHC-peptide complexes on the host cell surface.

The *M. tuberculosis* 30 kDa mycolyl transferase is the most abundant protein released by *M. tuberculosis*, making up almost one-quarter of the total extracellular protein released [33]. The 30 kDa protein (Antigen 85B) is highly homologous with two other mycolyl transferases of ~32 kDa mass - Antigen 85A and Antigen 85C [35]. The 30 kDa protein is not only the major protein secreted into broth culture, it is also among the major proteins of all types expressed by *M. tuberculosis* in infected human macrophages [36]. The 30 kDa protein is highly

immunogenic and, when administered as a purified protein with adjuvant, it induces strong cell-mediated and protective immunity in the guinea pig model of pulmonary tuberculosis [33].

2. Construction of rBCG30

rBCG30 is a recombinant BCG Tice strain overexpressing the 30 kDa protein from plasmid pMTB30 [10], derived from the *Mycobacterium-Escherichia coli* shuttle vector pSMT3 [37]. The plasmid pMTB30 contains the full-length *M. tuberculosis* 30 kDa protein gene and flanking 5'and 3' regions including the promoter region. rBCG30 expresses approximately 5.5-fold the amount of 30 kDa protein that the parental BCG strain expresses. The *M. tuberculosis* and BCG 30 kDa proteins are nearly identical, differing from each other by 2 contiguous amino acids. Other commonly used BCG strains including Connaught, Glaxo, Japanese, Copenhagen, and Pasteur produce amounts of 30 kDa protein comparable to that produced by BCG Tice [28].

3. Preclinical Studies

rBCG30 was tested in the guinea pig model of pulmonary tuberculosis, a model noteworthy for its resemblance to human disease clinically, immunologically, and pathologically, and the gold standard among small animal models of tuberculosis. BCG protects well in this model, in which animals are immunized and then challenged with *M. tuberculosis* by aerosol. Compared with sham-immunized animals, BCG-immunized animals are protected against weight loss, a hallmark of TB, and death, and they have significantly less lung pathology and a lower burden of *M. tuberculosis* in the lung and spleen (~1.5 - 2 logs fewer 10 weeks after challenge).

Despite the fact that the 30 kDa protein is a relatively abundant secreted protein of BCG, parental BCG induces negligible immune responses to the protein in guinea pigs. In contrast,

rBCG30 induces strong cell-mediated immunity, manifest by cutaneous delayed-type hypersensitivity to the 30 kDa protein, and humoral immunity, manifest by high serum antibody titer to the 30 kDa protein.

Paralleling these immune responses, in guinea pigs immunized with BCG or rBCG30 and challenged 10 weeks later by aerosol with virulent *M. tuberculosis* Erdman strain, rBCG30 induces greater protective immunity than BCG. Ten weeks after challenge, rBCG30-immunized guinea pigs had fewer CFU in the lung (0.8 ± 0.1 log fewer) and spleen (1.1 ± 0.1 log fewer) than BCG-immunized animals [34]. In a survival study, rBCG30-immunized guinea pigs survived significantly longer than BCG-immunized animals (Figure 1) [28]. Remarkably few rBCG30 organisms are required to induce strong cell-mediated and protective immunity [38].

In addition to enhanced protective efficacy against *M. tuberculosis*, rBCG30 induces greater protective immunity than BCG against *Mycobacterium bovis*, the primary agent of tuberculosis in domesticated animals, in the guinea pig model and against *Mycobacterium leprae*, the agent of leprosy, in a murine model (see below) [39, 40].

In preclinical safety studies, rBCG30 was well-tolerated. rBCG30 is cleared at the same rate as BCG in guinea pigs, i.e. rBCG30 and BCG are comparably avirulent [28]. No adverse effects were observed in guinea pigs or mice in safety studies conducted by the Aeras Global TB Vaccine Foundation.

4. Clinical Studies

rBCG30 was tested in a Phase 1 human study, the first live recombinant BCG vaccine against tuberculosis to enter clinical trials [41]. The trial was double-blinded with volunteers randomized to rBCG30 or parental BCG Tice. There was no significant difference between the two vaccines in clinical reactogenicity. rBCG30, but not BCG, induced significantly increased Antigen 85B-specific immune responses including significantly increased lymphocyte proliferation, interferon-γ (IFNγ) secretion, IFNγ enzyme-linked immunospot responses, direct *ex vivo* CD4+ and CD8+ T cell IFNγ responses, and CD4+ and CD8+ memory T cells capable of expansion. Moreover, in a novel assay of effector cell function, rBCG30 but not BCG significantly increased the number of antigen-specific T cells capable of inhibiting the growth of intracellular mycobacteria (Figure 2). Thus, rBCG30 was well-tolerated and more immunogenic than BCG.

C. rBCG expressing other native *M. tuberculosis* proteins

Recombinant BCG expressing other native *M. tuberculosis* proteins and *M. tuberculosis* fusion proteins have been constructed and evaluated. Noteworthy studies in which protective efficacy has been investigated are discussed below.

1. rBCG/Antigen 85A

Sugawara and colleagues studied the protective efficacy of a recombinant BCG over-expressing Antigen 85A in guinea pigs, cynomolgous monkeys, and rhesus monkeys [42-44]. Cynomolgus and rhesus monkeys exhibit different susceptibility to *M. tuberculosis* [45]. The rhesus monkey is highly susceptible to progressive infection culminating in death whereas the cynomolgus monkey is relatively resistant and able to contain low challenge doses [45].

Compared with guinea pigs vaccinated with parental BCG before aerosol challenge with *M. tuberculosis*, guinea pigs vaccinated with rBCG/Antigen 85A showed a trend toward fewer CFU in the lung and spleen [42].

In cynomolgus monkeys vaccinated before intrathecal challenge with *M. tuberculosis* H37Rv, both the parental and recombinant vaccines protected relative to sham-immunized controls, reducing CFU in the lung and spleen by ~ 2 logs. Compared with cynomolgus monkeys vaccinated with the parental BCG vaccine, monkeys vaccinated with rBCG/Antigen 85A had fewer CFU in lung sections, but not in spleen sections [43].

In rhesus monkeys vaccinated before intrathecal challenge with *M. tuberculosis* H37Rv, monkeys vaccinated with rBCG/Antigen 85A had significantly fewer CFU of *M. tuberculosis* in the lung and spleen than monkeys vaccinated with parental BCG [44].

2. rBCG/Antigen 85C

Jain et al. [46] investigated a recombinant BCG vaccine over-expressing Antigen 85C, a member of the 30-32 kDa Antigen 85A, B, C family of mycolyl transferases. Compared with BCG, the vaccine gave enhanced protection in the guinea pig model against high-dose aerosol challenge with *M. tuberculosis* H37Rv, including significantly reduced CFU in the lung and spleen, reduced pathology in the lung, liver, and spleen, and reduced pulmonary fibrosis.

3. rBCG/ESAT-6 (± CFP10)

Pym et al. evaluated a recombinant BCG vaccine complemented with the RD1 region that is missing in BCG, having been deleted from all BCG strains during its attenuation from *M. bovis* [47]. The vaccine secretes both ESAT-6 and CFP10, two proteins encoded by the RD1

region. The vaccine was tested in both the mouse and guinea pig models. Compared with mice immunized with BCG before intravenous or aerosol challenge with *M. tuberculosis*, mice immunized with the recombinant vaccine had comparable numbers of *M. tuberculosis* in the lung but fewer CFU in the spleen; differences in the spleen were significant in two of four experiments. In a single guinea pig study, animals immunized with the recombinant vaccine before aerosol challenge with *M. tuberculosis* had comparable numbers of *M. tuberculosis* in the lung but fewer CFU in the spleen than animals immunized with control BCG. The recombinant vaccine however was more virulent than BCG in severely immunocompromised SCID mice [48], and clinical development of the vaccine has not proceeded.

Brodin et al. studied a potentially safer version of the vaccine constructed in *Mycobacterium microti* [49]. In SCID mice, the recombinant *M. microti* vaccine complemented with the RD1 region was less virulent than the recombinant BCG vaccine complemented with the RD1 region but still much more virulent than a BCG control. In a mouse model, in which immunized mice were aerosol challenged with *M. tuberculosis*, mice vaccinated with the recombinant *M. microti* strain had significantly fewer CFU in the spleen than mice immunized with the control BCG vaccine at two of three time points. In the guinea pig model, the recombinant *M. microti* vaccine and control BCG vaccine were comparably protective.

Bao et al. studied two recombinant BCG vaccines expressing ESAT-6 in a murine model [50]. One recombinant BCG secreted ESAT-6 and one expressed ESAT-6 as part of a nonsecreted fusion protein. There was no significant difference in protective efficacy between either of the two recombinant BCG vaccines and BCG.

4. rBCG/38 kDa protein

Castanon-Arreola et al. investigated a recombinant BCG vaccine over-expressing a secreted *M. tuberculosis* 38 kDa glycoprotein and reported that mice immunized with the recombinant vaccine before challenge with either *M. tuberculosis* H37Rv or *M. tuberculosis* Beijing strain survived longer than mice immunized with the parental BCG Tice vaccine [51].

5. rBCG/19 kDa protein

Rao et al. investigated a recombinant BCG vaccine over-expressing a 19 kDa lipoprotein of *M. tuberculosis* and found that it abrogated the protective effect of BCG [52]. Compared with splenocytes of mice immunized with BCG, splenocytes of mice immunized with the recombinant vaccine exhibited an enhanced TH2-type immune response to BCG sonicate (increased IL-10 and decreased IFNγ and IgG2a:IgG1 ratio). In guinea pigs, BCG but not the recombinant vaccine induced immunoprotection against subcutaneous *M. tuberculosis* challenge.

D. rBCG expressing *M. tuberculosis* fusion proteins

1. rBCG/72f

Kita et al. investigated a recombinant BCG secreting a hybrid of two proteins (Mtb39 + Mtb32) named 72f in the cynomolgus monkey model [53]. The recombinant vaccine induced immune and protective responses but they were not significantly different from BCG controls.

2. rBCG/Antigen 85B-ESAT-6

Shi et al. tested recombinant BCG vaccines secreting fusion proteins of Antigen 85B and ESAT-6 in a mouse model [54]. The amount of the fusion protein secreted was not quantitated but appeared to be small on the Western blots on which it was detected. Splenocytes from mice immunized with the recombinant vaccines produced significantly more IFNγ in response to *M.*

tuberculosis culture filtrate proteins than splenocytes from mice immunized with control BCG. However, there was no significant difference between the recombinant vaccine and BCG in protective efficacy.

Xu et al. evaluated recombinant vaccines expressing Antigen 85B, an Antigen 85B-ESAT-6 fusion protein and an Antigen 85B-ESAT-6-mouse IFNγ fusion protein [55]. The biological activity of the mouse IFNγ was not evaluated and was not likely active. In the mouse model, the recombinant vaccines appeared to give slightly better protection than BCG in the lung but not the spleen at late time points.

3. rBCGAntigen 85B-Mpt64$_{190\text{-}198}$-Mtb8.4

Qie et al. investigated a recombinat BCG vaccine expressing a fusion protein of Antigen 85B, an immunodominant peptide of Mpt64, and Mtb8.4 [56]. In a murine model, the recombinant vaccine had comparble or slightly better efficacy than BCG.

V. Recombinant BCG overexpressing native proteins as vaccines against leprosy

BCG has shown efficacy against leprosy in addition to TB, but as with TB, protection is inconsistent. Two recombinant vaccines have been compared with BCG for efficacy against leprosy in murine models of leprosy.

A. rBCG30

Gillis et al. immunized BALB/c mice with BCG, rBCG30, or a recombinant BCG vaccine carrying plasmid pNBV1 encoding the *M. leprae* 30 kDa Antigen 85B (rBCG30ML), and then challenged the animals 2.5 months later by administering viable *M. leprae* into each hind foot pad [40]. Seven months later, the number of *M. leprae* per foot pad was enumerated. In addition, splenocytes and lymph node cells from immunized animals were evaluated for lymphocyte transformation to *M. tuberculosis* Purified Protein Derivative (PPD). All vaccinated groups showed sensitization to PPD; splenocytes from mice immunized with rBCG30 and rBCG30ML showed the highest responses. In the one experiment in which an efficacy comparison was feasible, rBCG30 and rBCG30ML gave protection superior to BCG and the difference between rBCG30 and BCG was statistically significant, as was the difference between the two rBCG30 groups combined and BCG.

B. rBCG/Antigen 85A, Antigen 85B, and MPB51

Ohara et al. examined the protective efficacy of a recombinant BCG vaccine over-expressing Antigen 85A, Antigen 85B, and MPB51 [57]. C57Bl/6 mice vaccinated with recombinant vaccine but not with the control BCG vaccine had significantly reduced *Mycobacterium leprae* in footpads 30 weeks after challenge with *M. leprae*. Compared with unimmunized controls,

BALB/c mice vaccinated with either the control BCG or recombinant BCG had reduced numbers of *M. leprae* in footpads. While there was no significant difference in the number of footpad *M. leprae* between mice immunized with the recombinant BCG or control BCG, there was a trend toward fewer *M. leprae* in the footpads of recombinant BCG-immunized mice.

VI. Recombinant BCG overexpressing native proteins and attenuated *M. bovis* as vaccines against bovine tuberculosis

Mycobacterium bovis is the principal etiologic agent of tuberculosis in domesticated animals. *M. bovis* infection of domesticated animals exacts a significant economic toll; for example, in cattle it results in reduced fertility, milk production, and meat value [58]. Control measures, where they can be afforded, center on testing animals for a cell-mediated immune response to *M. bovis* antigens (indicative of exposure) and culling herds of animals testing positive. One approach to reducing the incidence of *M. bovis* infection in domesticated animals is vaccinating the domesticated animals and/or the wild animals that serve as reservoirs of infection. BCG has been tested as a vaccine in cattle, but as in humans, its efficacy is suboptimal [59] (<50%), prompting a search for vaccines that are better. Standard tests for TB in domesticated animals frequently rely on assessment of a cell-mediated immune response to PPD. Since BCG vaccination can interfere with tests involving PPD, the use of a BCG vaccine in domesticated animals may need to be coupled with the use of a diagnostic test employing antigens absent in BCG but present in *M. bovis*, such as members of the ESAT-6 family.

Two new generation vaccines have been compared with BCG for efficacy against *M. bovis* infection.

A. rBCG30

rBCG30, described above, was tested in a guinea pig model of *M. bovis* infection in which vaccinated animals were challenged with *M. bovis* by aerosol. Compared with BCG, rBCG30-immunized animals had a lower burden of *M. bovis* in the lung and spleen [39].

B. WAg533

WAg533 is a newly attenuated strain of *M. bovis*. It was tested for efficacy in brushtail possums, an important reservoir of *M. bovis* infection in New Zealand; vaccinated animals were challenged with *M. bovis* by aerosol. Compared with animals immunized with BCG, animals immunized with WAg533 by three different routes (conjunctival/intranasal, oral, and subcutaneous) had reduced severity of illness and lower CFU burdens in the lung and spleen [60].

VII. Recombinant BCG overexpressing native proteins and additionally attenuated for safety in HIV-positive persons

The tuberculosis and AIDS pandemics are closely intertwined. Approximately 12 million people throughout the world are infected with both *Mycobacterium tuberculosis* and HIV, or about a third of all persons infected with HIV. These co-infected people have the greatest susceptibility to developing active tuberculosis, the major opportunistic infection in AIDS patients.

BCG is an extremely safe vaccine in immunocompetent people, but it can cause serious and even fatal disseminated disease in immunocompromised individuals, including AIDS patients. The World Health Organization has advised against administering BCG to HIV-positive infants because of their increased risk of disseminated BCG infection [61]. This has created a need for a vaccine that is safe and effective in HIV-positive persons, especially infants. Since the HIV status of infants in high-risk regions of the world is often unknown, such a vaccine may be a prudent choice for all infants of unknown HIV status in regions of the world where HIV prevalence is high.

Tullius et al. approached this problem by developing versions of rBCG30 that are readily grown in the laboratory but are replication limited in the host [62]. Although their replication is limited, it is nevertheless sufficient to induce a strong immunoprotective response.

A. rBCG(*mbtB*)30

rBCG*(mbtB*)30 is the first vaccine that is safer than BCG in the SCID mouse and yet more potent than BCG in the guinea pig model of pulmonary tuberculosis [62]. rBCG*(mbtB*)30 was rendered

siderophore-dependent by deletion of the gene *mbtB*, which encodes an enzyme necessary to produce the iron siderophores mycobactin and exochelin. The vaccine grows normally *in vitro* in broth culture and in human macrophages provided the iron-loaded siderophore mycobactin is provided, and during *in vitro* growth, it is able to store iron-mycobactin. This stored iron-mycobactin allows the vaccine to multiply for several divisions *in vivo*, sufficient to induce cell-mediated and protective immunity. In the SCID mouse, rBCG*(mbtB*)30 is much safer than BCG. In the guinea pig, rBCG*(mbtB*)30 is cleared much faster than BCG; nevertheless, in contrast to BCG, it induces a strong cell-mediated immune response to the 30 kDa protein. Most importantly, rBCG*(mbtB*)30 induces protective immunity that is significantly greater than that induced by BCG (Figure 3).

B. rBCG(*panCD*)30

rBCG(*panCD*)30 was rendered pantothenate dependent by deletion of the *panCD* genes [62]. This vaccine can multiply *in vitro* in broth culture or human macrophages in the presence of high concentrations of pantothenate, but multiplication is highly limited *in vivo*. In the SCID mouse, rBCG(*panCD*)30 is much safer than BCG. In the guinea pig, rBCG(*panCD*)30 is cleared very rapidly, allowing high doses to be administered safely. In guinea pigs administered high doses of rBCG(*panCD*)30, protection is comparable to BCG.

VIII. Recombinant BCG expressing immunomodulatory cytokines

Cytokines play a central role in the workings of the immune system, potentiating some responses and dampening others. Recognizing this, investigators have attempted to beneficially modulate immune responses to vaccines by incorporating cytokines into them. Early studies by O'Donnell et al. and Murray et al. explored the effect of recombinant BCG secreting cytokines on immune responses in mice [63, 64]. O'Donnell et al. constructed a recombinant BCG secreting IL-2 and showed that, compared with wild-type BCG, it induced enhanced splenocyte secretion of interferon-γ (IFNγ) [64]. Murray et al. constructed BCG secreting a number of different cytokines and studied a variety of immune responses in mice immunized with the constructs. Compared with splenocytes from mice immunized with parental BCG, splenocytes from mice immunized with BCG secreting interleulin-2 (IL-2), granulocyte macrophage colony stimulating factor (GM-CSF), or IFNγ, but not BCG secreting interleukin-4 (IL-4) or interleukin-6 (IL-6), had increased lymphocyte proliferation and increased production of cytokines, especially IFNγ, IL-2, and IL-10, upon stimulation with PPD [63].

Later studies have focused on the efficacy of cytokine-secreting BCG vaccines in protection against tuberculosis, and in the therapy of bladder cancer and allergy.

A. Cytokine-secreting Vaccines for Tuberculosis

1. rBCG/GM-CSF

Ryan et al. studied mice immunized with BCG secreting murine GM-CSF [65]. Compared with mice immunized with control BCG, mice immunized with BCG secreting GM-CSF had greater numbers of antigen presenting cells ($CD11c^+$ $MHCII^+$ cells) in draining lymph

nodes; increased numbers of IFNγ-secreting spleen cells stimulated *ex vivo* with BCG lysate 5 and 17 weeks after immunization; and ~1 log fewer CFU in the spleen after aerosol challenge with *M. tuberculosis*.

2. rBCG/IFNγ and rBCG30/IFNγ

Tullius et al. have constructed rBCG and rBCG30 expressing various forms of human IFNγ, including strains encoding monomeric and covalently linked dimeric forms, and studied their effect on antigen presentation in human monocytes. Infection of human monocytes with these constructs results in upregulation of class I and II MHC molecules and enhanced presentation on an MHC class II molecule of a peptide of the 30 kDa Antigen 85B major secretory protein (Tullius and Horwitz, unpublished studies).

3. rBCG/IL-2

Young et al. studied the capacity of recombinant BCG secreting murine IL-2 to counter a Type 2 immune response in mice and to alter the immune profile of immunosuppressed mice [66]. As noted above, one theory as to why BCG is sometimes poorly effective is that it is administered in a setting in which a TH2 type of immune response predominates, e.g. as a result of helminthe infection. The investigators showed that rBCG/mIL-2 but not control BCG can induce a TH1 profile in mice immunosuppressed with dexamethasone and in IL-4 transgenic mice. Compared with BCG-immunized mice, the rBCG/mIL-2-immunized mice exhibit greater splenocyte proliferation and IFNγ production in response to PPD and a higher IgG2a:IgG1 antibody ratio (consistent with a TH1 type immune response) in both types of mice.

In a separate study, Young et al. investigated the protective efficacy of rBCG/mIL-2 in mice [67]. Although the IL-2 secreting BCG vaccine induced a longer-lasting splenic lymphocyte

proliferative response to PPD, a higher IFNγ level in response to PPD, and a higher IgG2a:IgG1 antibody ratio than control BCG, the recombinant vaccine was not more protective against *M. bovis* aerosol challenge than control BCG.

Slobbe et al. studied a BCG vaccine secreting cervine IL-2 in outbred red deer [68]. rBCG/cIL-2 induced a smaller delayed-type hypersensitivity (DTH) response to PPD than parental BCG. RT-PCR studies of lymphocytes from the deer revealed that IL-2 and IFNγ levels were similar in deer vaccinated with rBCG/cIL-2 or parental BCG but that IL-4 levels were reduced in the deer vaccinated with rBCG/cIL-2.

4. rBCG/IL-18

Young et al. evaluated a recombinant BCG secreting murine IL-18 [67]. Disappointingly, this vaccine induced significantly less IFNγ in splenocytes of immunized mice that control BCG, and paralleling this finding, it was significantly less protective than control BCG.

5. rBCG/IL-15

Tang et al. studied a recombinant BCG vaccine secreting a fusion protein of Antigen 85B and murine IL-15 [69]. Whether the IL-15 portion of the fusion protein was biologically active was not investigated. In any case, the investigators report that the vaccine was cleared more rapidly than a control recombinant vaccine secreting only Antigen 85B and that mice immunized with the recombinant vaccine secreting the fusion protein, compared with mice immunized with the control vaccine, had greater absolute numbers of CD4+ and CD8+ T cells in lung, spleen, and peritoneal exudate cells, greater numbers of IFNγ-secreting CD4+ cells to two *M.*

tuberculosis antigens, and a lower bacterial burden in the lung, but not the spleen, after intratracheal challenge with *M. tuberculosis*.

B. Cytokine-secreting Vaccines for Therapy of Bladder Cancer

In addition to its use as a vaccine against TB, BCG plays an important immunotherapeutic role in the treatment of superficial bladder cancer. Such treatment is associated with induction of TH1 cytokines [70]. Approximately 30% of patients do not respond to current BCG therapy and 50% of patients suffer a recurrence [71]. This has prompted investigators to explore new generation BCG vaccines secreting TH1-inducing cytokines for the treatment of bladder cancer.

1. rBCG/IFNγ

Arnold et al. tested the immunotherapeutic efficacy of rBCG expressing murine IFNγ in a mouse model [70]. They found that rBCG/mIFNγ upregulated class I MHC expression in murine bladder cancer cells to a greater extent than a BCG control strain transfected with an empty vector. Intravesicular instillation of rBCG/mIFNγ resulted in greater recruitment of CD4+ T cells into the bladder and increased expression of IL-2 and IL-4 compared with intravesicular instillation of control BCG. Finally, rBCG/mIFNγ but not BCG treatment of orthotopic bladder cancer significantly prolonged survival compared with untreated animals; however, while survival of rBCG/mIFNγ - treated animals was greater than that of BCG – treated animals, the difference did not reach statistical significance.

2. rBCG/IFNα

IFNα has been shown to improve the response of patients to BCG therapy [71], prompting investigators to evaluate the immunogenicity of rBCG secreting IFNα 2B *in vitro*.

Luo et al. found that rBCG/IFNα induced more IFNγ and IL-2 from human PBMC *in vitro* than BCG [72] and Liu et al. found that rBCG/IFNα induces more potent PBMC cytotoxicity than BCG against human bladder cancer cell lines and the effect was dose-dependent [71]. The addition of neutralizing antibodies against IFNα, IFNγ, or IL-2 to PBMC cultures stimulated with rBCG/IFNα reduced PBMC cytotoxicity against the cancer cells.

3. rBCG/IL-2

Yamada et al. studied the cytotoxic effect of recombinant BCG secreting murine IL-2 on murine bladder cancer cells *in vitro* [73]. They constructed a recombinant BCG secreting murine IL-2 fused to the signal sequence of the 30 kDa Antigen 85B of BCG and reported that the fusion protein was functional. Peritoneal exudate cells (PEC) incubated with rBCG/mIL-2 produced greater amounts of IFNγ, TNFα, and IL-12 and were more cytotoxic than PEC incubated with BCG. The enhanced cytotoxicity was neutralized by the addition of anti-IL-2 antibody.

4. rBCG/IL-18

Luo et al. found that, compared with splenocytes from BCG-immunized mice, splenocytes from mice immunized with rBCG/mIL-18 had increased IFNγ, TNFα, and GM-CSF levels and increased lymphocyte proliferation in response to BCG antigens [74]. Mouse PECs (>90% macrophages) stimulated *in vitro* with rBCG/mIL-18 also had greater cytolytic activity against a mouse bladder cancer cell line than PEC stimulated with BCG.

C. Cytokine-secreting Vaccines for Allergy

Antigen-specific TH2 type cells figure prominently in allergic reactions, and TH1 cells, via the secretion of IFNγ, may counter allergic responses by dampening TH2 cell activity [75].

1. rBCG/IL-18

Biet et al. studied recombinant BCG secreting biologically active (increased NF-κB) mouse IL-18 [75]. IL-18 acts synergistically with IL-12 to induce IFNγ, and since BCG itself induces IL-12, Biet et al. hypothesized that rBCG secreting Il-18 might exert a more potent dampening effect on TH2 type immune responses than BCG [76]. rBCG/mIL-18 enhanced TH1 and diminished TH2 type immune responses in mice. Compared with splenocytes form BCG-immunized mice, splenocytes from rBCG/mIL-18 – immunized mice had increased IFNγ and GM-CSF production in response to PPD. In contrast, rBCG/mIL-18 – immunized mice had decreased serum IgG to BCG antigens [75].

Biet et al. explored the therapeutic potential of rBCG/mIL-18 in a murine model of pulmonary allergic inflammation, in which mice were sensitized to ovalbumin [76]. The investigators found that lymph node cells from sensitized mice immunized with rBCG/mIL-18 and challenged with ovalbumin produced more IFNγ when stimulated with ovalbumin than lymph node cells from sensitized mice immunized with BCG and challenged with ovalbumin; in contrast, IL-5 production was suppressed. Moreover, ovalbumin - sensitized mice immunized with rBCG/mIL-18 had less bronchoalveolar eosonophilia after ovalbumin challenge than BCG-immunized mice.

IX. Recombinant BCG with Enhanced Antigen Presentation

In addition to engineering BCG that secrete various cytokines, two other approaches have been used to improve antigen presentation. One approach is aimed primarily at enhancing MHC class I antigen presentation and another approach is aimed at enhancing MHC class II antigen presentation.

A. BCG with altered intracellular pathway

Grode et al. engineered a recombinant BCG vaccine secreting listeriolysin, to promote perforation of the phagosomal membrane, and with a deleted urease gene, to reduce the pH in the phagosome to nearer the pH optimum of listeriolysin [77]. The rationale for this vaccine was to increase class I MHC antigen presentation by allowing egress of BCG antigens to the cytosol. As this vaccine also was proapoptotic in macrophages, it additionally promoted presentation of mycobacterial antigens via cross-priming. In a murine model, this vaccine was more potent than BCG against challenge with *M. tuberculosis* Beijing/W, reducing CFU in the lung and spleen by 1-2 logs. The vaccine was tested for safety in SCID mice; mice administered the recombinant vaccine intravenously survived significantly longer than mice administered the parental BCG vaccine. In a study in the guinea pig model, the vaccine was not more potent than BCG [78].

B. rBCG/Cathepsin S

Sendide et al. [79] reported that IFNγ-induced surface expression of mature MHC class II molecules is suppressed in THP-1 macrophages infected with wild-type BCG compared with macrophages incubated with killed BCG, that the suppression is correlated with reduced cathepsin S activity, and that the reduced cathepsin S activity is mediated via BCG-induced IL-

10 secretion. This prompted Soualhine et al. to engineer and evaluate a recombinant BCG secreting the mature form of human cathepsin S [80]. The rBCG/hCathepsin S strain had increased IFNγ-induced surface MHC class II expression and increased expression of an MHC class II-Antigen 85B peptide complex. Whether a recombinant vaccine secreting cathepsin S was capable of inducing improved protective immunity in an animal model was not investigated.

X. Recombinant BCG overexpressing native proteins and escaping the phagosome

Sun et al. [81] engineered a novel recombinant BCG vaccine that combined the approach of overexpressing native antigens, first employed by Horwitz et al. [10], with the approach of phagosome escape, first employed by Grode et al. [77]. To expand the antigenic repertoire of the vaccine, the investigators engineered it to overexpress, in addition to Antigen 85B (the antigen overexpressed in rBCG30, described above), Antigen 85A and TB10.4, a low molecular mass protein in the ESAT-6 family. Instead of using listeriolysin to promote phagosome membrane lysis, these investigators used a mutant form of perfringolysin O. The vaccine, designated AFRO-1, was safer than BCG in SCID mice. Vaccination of mice and guinea pigs with AFRO-1 induced stronger immune responses to the overexpressed antigens than vaccination with the parental BCG vaccine. In mice vaccinated before aerosol challenge with the hypervirulent *M. tuberculosis* strain HN878 (Beijing-type clinical outbreak strain), the group vaccinated with AFRO-1 survived significantly longer than mice vaccinated with the parental BCG vaccine.

XI. Recombinant BCG expressing foreign antigens

With the development of techniques to genetically manipulate mycobacteria [82-85], researchers rapidly looked to exploit recombinant BCG as a multi-component vaccine vector by expressing foreign antigens from various pathogens [4-9, 86-89]. As noted above, BCG possesses a number of potential advantages as a vaccine (Table 1) – it has been extensively used for many decades, it has a very good safety profile, and it is inexpensive to produce [4, 90]. BCG is unaffected by maternal antibodies, so it can be given in a single dose at birth, and it is capable of inducing a long-lasting cellular immune response. Due to its intracellular location in the phagosome of macrophages and dendritic cells, BCG primarily elicits a CD4+ cellular immune response through MHC class II. However, BCG also elicits a humoral immune response, and potent antibody responses to foreign antigens expressed by recombinant BCG have been obtained in some cases (see Tables 2-6). Recombinant BCG expressing foreign antigens can also elicit a CD8+ cytotoxic T-lymphocyte response through MHC class I. This feature of recombinant BCG is particularly important for protection against viral pathogens and those bacterial pathogens that invade the cytoplasm of the host cell. Presentation of antigen through MHC class I may occur via cross-priming.

Recombinant BCG targeting viral (Table 2), parasitic (Table 3), and bacterial (Table 4) diseases have been developed. Several studies have examined recombinant BCG vaccines expressing toxins to enhance the adjuvant effect of BCG (Table 5) and recombinant BCG vaccines against cancer and allergy as well (Table 6). Despite the promise of recombinant BCG vaccines, results have been decidedly mixed. Good to excellent immune responses and/or protection have been achieved in a number of studies, but many studies have also demonstrated weak to moderate

immune responses and no protection or modest protective efficacy at best. Many studies have used high doses (greater than the human dose of ~10^6 CFU administered intradermally) and/or multiple doses of recombinant BCG in an attempt to obtain better immune responses. Heterologous boosting of recombinant BCG has been effective in generating a more potent immune response in several studies [91-93]. While a boosting regimen that yields an effective vaccine has benefit, it does negate one purported advantage of being able to give recombinant BCG in a single dose at birth. The type of immune response that is obtained with any particular rBCG vaccine cannot be predicted, and it is difficult to draw many generalizations from the numerous immunological studies of rBCG vaccines due to the large number of variables among studies (animal model, route of vaccination, dose and timing of vaccine, method of growth and preparation of vaccine, vaccine viability, expression level and cellular location of the foreign antigen, stability of foreign antigen expression, etc.) [94-97]. However, the expression level of the foreign antigen and its cellular location (intracellular, secreted, or membrane-anchored) have had profound effects on immune responses generated by recombinant BCG vaccines in some studies. In general, higher expression levels and targeting of the foreign antigen to the membrane or extracellular space have yielded more potent vaccines [94, 96]. To achieve high expression of a foreign antigen, episomal plasmids with strong promoters have typically been required. Unfortunately, this has been associated with instability of expression of the foreign antigen in more than a few studies. Integrated vectors are more stable but result in less expression and often lower immune responses. High level and stable expression of foreign antigens in recombinant BCG have been rather difficult to achieve in practice.

A. HIV

rBCG expressing HIV and SIV antigens were among the first rBCG multi-component vaccines constructed. rBCG expressing Gag, Nef, Env, Pol, and RT, as complete genes or as smaller fragments, have been developed and tested for their immunological properties primarily in mice and guinea pigs, although several of these vaccines have been tested in non-human primate models as well (Table 2). Developing a vaccine against HIV has proven extremely challenging [98, 99] and rBCG-HIV vaccines are no exception. It is generally agreed that both broadly neutralizing antibodies and anti-viral cytotoxic T-lymphocytes are needed for a highly effective vaccine, although CD4+ T-cells also have a role mediating these effects. Due to the great difficulty in generating broadly neutralizing antibodies, recent HIV vaccine research has focused more on T-cell vaccines. As rBCG HIV vaccines have been reviewed several years ago [94], this section will focus on some of the most recent studies.

1. Recent Studies:

Cayabyab et al. constructed rBCG strains expressing SIV Gag, Pol, and Env localized to the mycobacterial cell wall with the 19 kDa lipoprotein signal under the control of the α-antigen promoter [91]. Rhesus macaques were vaccinated intradermally or intravenously with 10^6 – 10^9 CFU rBCG (given as a cocktail of all three strains) and boosted with an identical dose 23 weeks later. Twenty weeks after the second immunization, all of the monkeys were boosted with 10^{10} virus particles of recombinant adenovirus 5 expressing the same SIV antigens as the rBCG strains. The monkeys developed very weak CD8+ T-cell responses to the SIV antigens even with two doses of rBCG. However, after the heterologous prime boost with adenovirus, the monkeys developed strong responses to all three SIV antigens, as measured by PBMC IFNγ ELISPOT responses to Gag, Pol, and Env peptide pools. The responses to Gag and Pol were greater for the rBCG immunized animals compared with naïve animals, but a similar response

was obtained for Env in rBCG immunized and naïve animals, which was attributed to instability of expression of Env by the rBCG vaccine.

Promkhatkaew et al. constructed an rBCG expressing HIV Gag intracellularly from the strong *hsp60* promoter (0.26-0.45 mg/L of culture) [100]. Expression was reported to be stable. BALB/c mice were vaccinated subcutaneously with 0.1 mg ($2x10^6$ CFU) and cell-mediated immune responses were measured 2 weeks to 2 months later. Gag-specific CTL to multiple epitopes as well as lymphocyte proliferation was induced in response to vaccination, but no anti-Gag antibodies were detected in sera. In a follow up study, mice were boosted one month or six months after subcutaneous or intradermal rBCG vaccination with a replication deficient vaccinia virus strain also expressing full-length Gag and cell-mediated immune responses were measured one month later [101]. The prime-boost regimen resulted in a more persistent CTL response than the single vaccination with rBCG.

Chege et al. tested two rBCG vaccines expressing HIV Gag localized to the mycobacterial cell wall with the 19 kDa lipoprotein signal for immunogenicity in a baboon model using a prime-boost regimen [102]. Both humoral and cellular Gag-specific immune responses to rBCG alone were very weak, but rBCG succeeded in priming the immune system for a Gag VLP boost (assessed by assaying the IFNγ ELISPOT response to Gag peptides and Gag-specific antibody). rBCG was administered at very high doses (10^8 CFU at 0, 14, 24, and 40 weeks) and boosted twice with Gag VLPs at 92 and 104 weeks. The exact expression level of Gag by the rBCG strains was not reported, but the strain with higher expression, which produced better results, was

reported to be less stable. Low expression and/or instability of the rBCG vaccines may have resulted in a poor immune response in this study.

Kawahara and colleagues examined the long-term immune response of rBCG expressing full-length SIV Gag in guinea pigs [103, 104]. Gag was expressed intracellularly under the control of a strong promoter (P_{hsp60}) at 0.5 ng Gag/mg of rBCG. Guinea pigs were immunized intradermally (0.1 mg) or orally (80 mg x 2) using typical human doses. A strong immune response was achieved from both routes of immunization for up to three years as evidenced by: Gag-specific serum IgG 10^6-fold greater than control (IgG2 > IgG1), DTH, proliferation of PBMC and splenocytes in response to Gag, and increased IFNγ mRNA in PBMC and splenocytes in response to Gag, mediated largely through CD4+ T-cells. The high levels of antibody produced contrasted with the authors' previous work with a rBCG secreting a 19 amino acid CTL epitope from Env fused to *M. kansasii* α-antigen [105]. Intradermal vaccination of guinea pigs with 0.1 mg of this strain resulted in no antibody production, although other routes of immunization did elicit an antibody response.

Im et al. constructed an rBCG expressing the HIVA immunogen localized to the mycobacterial cell wall with the 19 kDa lipoprotein signal under the control of the α-antigen promoter [92]. HIVA is a synthetic gene containing ~73% of gag fused to a multi-CTL epitope from the Gag, Pol, Nef, and Env proteins [106]. The investigators obtained greater expression with an extrachromosomal plasmid compared with an integrated plasmid and used the higher expressing strain for all of their animal studies. A balanced-lethal system was used to apply selective pressure on the rBCG strain to maintain the plasmid (a BCG Δ*lysA* lysine auxotroph was

complemented with a functional copy of *lysA* on the plasmid). This strategy was quite successful in maintaining the plasmid as 10 of 10 colonies isolated from the spleens of mice 15 weeks after immunization still maintained the kanamycin resistance marker on the plasmid and 2 of 2 were positive for the HIVA gene by PCR. Unfortunately, the authors did not go one step further and check the isolated colonies for continued expression of HIVA. BALB/c mice were vaccinated intraperitoneally with 10^6 CFU of rBCG for most experiments and 10^3-10^7 CFU for a dose-titration study. BCG-HIVA induced little or no CD8+ T-cell responses alone, but enabled enhanced responses when used to prime a subsequent boost with MVA-HIVA (given at 102 d). In a dose response experiment, a high priming dose of BCG-HIVA was determined to be important for eliciting a broader T-cell response. Mice that were primed with a DNA-HIVA vaccine, boosted with BCG-HIVA, and challenged with a surrogate replication-competent vaccinia virus expressing HIVA had significantly increased levels of bifunctional CD4+ T-cells.

Yu et al. constructed rBCG vaccines expressing HIV-1 Env either as a surface, intracellular, or secreted protein [93]. For surface expression, the 19 kDa lipoprotein signal was used and for secretion, the α-antigen signal was used. All constructs were under the control of the α-antigen promoter, a moderately strong mycobacterial promoter. In an attempt to address the very difficult challenge of HIV genetic diversity, the authors used two artificial consensus env genes (CON6 gp120 or CON6 gp140CF). BALB/c mice were vaccinated twice (at 0 and 8 weeks) intraperitoneally with 10^6, 10^7, or 10^8 CFU of the different rBCG strains and antigen specific T-cell responses were measured by IFNγ ELISPOT assays on lymphocytes isolated from spleens. Vaccines secreting Env yielded the strongest response. Interestingly, little or no response was obtained with a single dose of rBCG demonstrating a clear boosting effect of rBCG in this assay.

Elevated Env-specific T-cell responses were also obtained with lymphocytes isolated from the lung and female reproductive tract after two doses of rBCG. The antigen-specific T-cell response was primarily due to CD4+ T-cells. rBCG did not elicit anti-Env antibodies on its own, but did prime an antibody response when followed by a boost of recombinant HIV-1 Env oligomer in RiBi adjuvant.

B. Other Viral Diseases

Many other viruses besides HIV have been targeted by recombinant BCG vaccines (Table 2). Very good protective efficacy has been achieved against respiratory syncytial virus (RSV) and encephalomyocarditis virus (EMCV) [107, 108]. Good protection against cottontail rabbit papillomavirus (CRPV) has been achieved [109, 110] and immune responses to hepatitis C virus (HCV) have provided good protection against a surrogate challenge with recombinant vaccinia virus expressing an HCV antigen [111, 112].

C. Parasitic Diseases

Nearly 20 studies on recombinant BCG vaccines targeting malaria, leishmaniasis, schistosomiasis, and toxoplasmosis are cataloged in Table 3. Several studies that examined protective efficacy of the rBCG vaccines will be highlighted here. Matsumoto et al. obtained very good protection against rodent malaria (*P. yoelli*) with a recombinant BCG secreting the 15 kDa C-terminal region of merozoite surface protein 1 (MSP1) fused to the *M. kansasii* α-antigen (six of seven rBCG vaccinated mice survived versus 0% survival for controls) [113]. Protection was significantly better than that achieved with recombinant MSP1 protein with adjuvant. C3H/He mice were immunized intravenously with 10^6 CFU rBCG, boosted one month later

intraperitoneally with 10^6 CFU rBCG, and challenged one month after the second immunization with *P. yoelli*. As neither route can be used to immunize humans and the route of immunization is known to affect the immune response to rBCG in small animals, the results should be interpreted cautiously. In a follow-up study, the authors found that protective immunity had waned significantly by 4 and 9 months post-immunization (only 4 of 9 and 3 of 9 mice survived at 4 and 9 months, respectively), but still was better than after immunization with recombinant MSP1 protein with adjuvant [114].

Connell et al. constructed a rBCG expressing the *Leishmania major* gp63 surface proteinase intracellularly under the control of a strong promoter (P_{hsp60}) [88]. BALB/c and CBA/J mice were vaccinated intravenously (10^4 or 10^5 CFU) or subcutaneously (10^6 CFU) and challenged 10 weeks later. Good protection from cutaneous leishmaniasis was obtained when the mice were challenged with *L. mexicana* (promastigotes and amastigotes) but not against *L. major* promastigotes. Abdelhak et al. also developed rBCG strains expressing *L. major* gp63 [115]. Mice were vaccinated twice at a one-month interval (10^6 CFU, iv; 10^7 CFU, sc) and challenged with *L. major* amastigotes one month after the boost. Partial protection was observed with the rBCG expressing gp63 fused to the N-terminal portion of β-lactamase (a secreted protein) but not with the rBCG expressing gp63 intracellularly.

rBCG expressing the Sm14 antigen from *Shistosoma mansoni* have delivered partial protection from challenge with *S. mansoni* cercaria in two studies [116, 117]. In the first study, Varaldo et al. expressed Sm14 fused with β-lactamase under the control of a strong promoter (pBlaF*) with the fusion protein localized to the cell wall [116]. A single dose of rBCG (10^6 CFU, sc) was as

effective as a three dose regimen of rSm14 protein in alum – ~50% reduction in worm burden in outbred Swiss mice. Protective efficacy could not be boosted by a second dose of rBCG or by rSm14 protein. In a follow-up study, Varaldo et al. constructed a mycobacterial codon-optimized Sm14 gene and obtained 4-fold greater expression with rBCG expressing the codon-optimized gene compared with their earlier construct [117]. However, this did not translate into an increased immune response (IFNγ secretion by splenocytes stimulated in-vitro with rSm14) or protective efficacy. This contrasts with an earlier report in which an rBCG strain expressing codon-optimized HIV Gag was more immunogenic than an rBCG expressing wild-type Gag (~40-fold increase in expression for the codon-optimized Gag) [118].

D. Bacterial Diseases

Some of the earliest rBCG vaccines targeting bacterial pathogens produced very promising results [5, 119, 120]. Stover et al. developed a rBCG vaccine against Lyme borreliosis by expressing the outer surface protein A (OspA) of *Borrelia burgdorferi* as a membrane anchored lipoprotein [5]. Using this construct, they obtained protective antibody responses in inbred and outbred mouse strains that were 100-1,000 times greater than those obtained with rBCG strains expressing OspA intracellularly or as a secreted protein. In protection experiments, inbred and outbred mouse strains were immunized intraperitoneally with 10^6 CFU rBCG, boosted 17 weeks later with an identical dose, and challenged intraperitoneally or intradermally 5 weeks after the booster dose. Excellent protective efficacy was obtained against both challenge routes. Likewise, a single intranasal dose of this rBCG strain provided complete protection against intradermal challenge 13 weeks post-vaccination [120]. This group also obtained very good humoral immune responses in mice immunized with rBCG strains expressing pneumococcal

surface protein A (PspA) [119]. Interestingly, protective immunity was only induced in mice vaccinated with rBCG secreting PspA or expressing PspA as a membrane anchored lipoprotein. Despite inducing a good humoral immune response, no protective efficacy was obtained with rBCG expressing intracellular PspA. The rBCG vaccine against Lyme disease was eventually tested for safety and immunogenicity in the first phase I clinical trial of a rBCG vaccine (and still the only human trial of a rBCG vaccine expressing a foreign antigen) [121]. Unfortunately, in stark contrast to the results obtained in mice, none of the 24 human volunteers vaccinated intradermally with $2 \times 10^4 - 2 \times 10^7$ CFU rBCG developed a humoral immune response.

Similar to the studies with OspA and PspA above, Grode et al. constructed rBCG vaccines expressing secreted, membrane anchored, and intracellular *Listeria monocytogenes* p60 (a major secreted antigen) under the control of a strong promoter (P_{hsp60}) [122]. BALB/c mice were vaccinated intravenously with 10^6 CFU of rBCG and challenged 120 days later. Excellent protective efficacy (80-100% survival at 10 days post-challenge) was obtained with the rBCG strains expressing membrane anchored or secreted p60, but not with the rBCG strain expressing intracellular p60. Interestingly, only CD4+ T-cells were needed for protective efficacy in mice immunized with rBCG secreting p60, but both CD4+ and CD8+ T-cells were required in mice immunized with rBCG expressing membrane anchored p60. The authors suggest that this could be due to decreased access of membrane anchored p60 to the MHC class II loading compartment leading to less stimulation of CD4+ T-cells.

Recombinant BCG vaccines expressing bacterial toxins have produced good protection in several studies as well [7, 123-125].

Conclusions

As a vaccine vector for a recombinant TB vaccine, BCG is an obvious choice since it shares so many antigens with *M. tuberculosis*, and it has efficacy by itself. The emphases of current efforts aimed at an improved TB vaccine are on expanding the repertoire of overexpressed immunoprotective *M. tuberculosis* antigens and improving the processing and presentation of both vector and recombinant antigens by altering the intracellular lifestyle of the vector, endowing the vector with immunomodulatory cytokines, enhancing apoptosis to promote cross-presentation of antigens, etc. Some of these modifications also show promise for improving BCG as a therapeutic against bladder cancer, the only other approved use of BCG vaccine aside from the prevention of TB.

Increasingly, primarily because of its safety record and high immunogenicity, BCG has been chosen as a vaccine vector to express foreign antigens, particularly where no convenient or safe alternative vector homologous to the target exists, e.g. in the case of parasites, cancer, and allergic disease. Recombinant BCG expressing HIV antigens are being intensely studied including in non-human primates. Some of these vaccines have induced strong immune responses against key HIV antigens, particularly when used as part of a heterologous prime-boost vaccination strategy.

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Figure Legends

Figure 1. rBCG30-immunized animals survive longer than BCG-immunized animals after *M. tuberculosis* aerosol challenge. Animals in groups of 20 or 21 were sham-immunized or immunized with BCG or rBCG30 Tice, and ten weeks later challenged by aerosol with virulent *M. tuberculosis*. A group of uninfected animals served as controls. Sham-immunized animals died most rapidly; BCG-immunized animals survived significantly longer than sham-immunized animals; and rBCG30-immunized animals survived significantly longer than BCG-immunized animals. 35% of rBCG30-immunized animals survived to the point where uninfected control animals began to die off. Reproduced with permission of the American Society for Microbiology from Horwitz et al. [28].

Figure 2. Phase I human trial of rBCG30: rBCG30 but not BCG – immunized recipients show increased Ag85B-specific T cell inhibitory activity against intracellular mycobacteria. In a double-blind Phase 1 human trial in which recipients were vaccinated with BCG Tice or rBCG30 Tice, peripheral blood mononuclear cells were harvested from 10 recipients of each vaccine pre-vaccination and on days 56 and 112 post-vaccination and stimulated with recombinant 30 kDa Antigen 85B (Ag85B) protein for 7 days. These Ag85B-specific expanded T cells were then co-cultured with BCG-infected autologous macrophages for 3 days. The macrophages were lysed; viable CFU of BCG were enumerated on Middlebrook agar plates; and the percent inhibition mediated by Ag85B-specific T cells vs. medium rested T cells was calculated. Shown are the median values (points), mid-50% values (boxes), and non-outlier ranges (whiskers). *, $p<0.05$ comparing pre- and post-vaccination responses by Wilcoxon matched pairs test. **, $p<0.05$ comparing rBCG30 and BCG vaccination groups by Mann-

Whitney U test. In other assays, rBCG30 but not BCG – immunized recipients showed significantly increased Antigen 85B-specific lymphocyte proliferation, interferon-γ (IFNγ) secretion, IFNγ enzyme-linked immunospot responses, direct ex vivo CD4+ and CD8+ T cell IFNγ responses, and CD4+ and CD8+ memory T cells capable of expansion. Reproduced with permission of the University of Chicago Press from Hoft et al. [41].

Figure 3. rBCG(*mbtB*)30, a replication-limited but highly immunoprotective TB vaccine designed specifically for HIV-positive persons. (A) Siderophore-dependence of rBCG(*mbtB*)30 grown in broth culture. rBCG(*mbtB*)30 was cultured in medium containing 0.01 μg/ml mycobactin J and washed before inoculation into broth containing 0 to 100 ng/ml mycobactin J (as indicated to the right of the graph). Growth of the parental BCG strain (grown without mycobactin) is shown for comparison. (B) Pre-loading of rBCG(*mbtB*)30 with mycobactin-iron results in greater residual growth in human THP-1 macrophages. rBCG(*mbtB*)30 was cultured in medium containing 0.01, 1, or 10 μg/ml mycobactin J (as indicated to the right of the graph) and washed before addition to THP-1 monolayers. CFU were enumerated 0, 3, and 7 days after infection. Data are the mean log CFU ± SE for duplicate wells. The number of bacterial generations over the 7-day course of infection is indicated to the far right of the growth curves (data are means for two independent experiments). (C) Attenuation of rBCG(*mbtB*)30 in SCID mice. SCID mice in groups of 20 were injected with 10^6 CFU of BCG or rBCG(*mbtB*)30, or sham treated with PBS via the tail vein, and survival was monitored over a 40-week period. SCID mice vaccinated with rBCG(*mbtB*)30 survived significantly longer than mice vaccinated with BCG. ($P < 0.0001$). (D) Limited replication of rBCG(*mbtB*)30 in guinea pigs. Guinea pigs in groups of 24 were immunized by intradermal administration of 10^6 CFU of BCG or iron mycobactin-loaded rBCG(*mbtB*)30. At one to 15 weeks after immunization, as indicated, three

animals per group were euthanized, and CFU of BCG and rBCG(*mbtB*)30 in the lungs, spleen, and inguinal lymph nodes were assayed. Data are mean log CFU ± SE. The limit of detection was 1 log CFU. (E) Immunogenicity of rBCG(*mbtB*)30 in guinea pigs. Guinea pigs in groups of six were immunized by intradermal administration of 10^3 CFU BCG or rBCG30 or with 10^6 CFU of iron mycobactin-loaded BCG *mbtB* or rBCG(*mbtB*)30, or were sham immunized with PBS. Ten weeks after immunization, the animals were skin tested by intradermal administration of highly purified *M. tuberculosis* 30-kDa major secretory protein (Antigen 85B), and the degree of induration was assessed 24 h later. Data are mean diameters of induration ± SE. *, $P \leq 0.01$; **, $P \leq 0.001$; (ANOVA; compared with BCG-immunized guinea pigs). (F) Protective efficacy of rBCG(*mbtB*)30 in guinea pigs. Guinea pigs in groups of 15 (except for the sham-immunized group of 9 animals) were immunized by intradermal administration of BCG, rBCG30, BCG *mbtB*, or rBCG(*mbtB*)30 as in (E) above. Ten weeks after immunization, the animals were challenged with a low-dose aerosol of *M. tuberculosis* Erdman strain. Ten weeks after challenge, the animals were euthanized and CFU of *M. tuberculosis* in the lungs and spleen were assayed. Data are mean log CFU ± SE. Open symbols indicate sham-immunized animals and groups immunized with BCG strains not overexpressing the 30-kDa protein. Closed symbols indicate groups immunized with BCG strains overexpressing the 30-kDa protein. For (E) and (F), one representative experiment of three is shown. Reproduced with permission of the American Society for Microbiology from Tullius et al. [62].

Table 1. Advantages of BCG as a Vector for Recombinant Vaccines.

General Advantages
Well established safety profile – administered to >4 billion people
Live vaccine – high immunogenicity
Unaffected by maternal antibodies - can be given in a single dose at birth
Elicits excellent CD4+ and CD8+ T cell responses
Elicits strong TH1 Type immune responses
Relatively inexpensive to manufacture
Additional Advantages for Recombinant TB vaccines
Provides a broad array of shared mycobacterial antigens in addition to recombinant antigens
Moderate efficacy against *M. tuberculosis* to start with
High acceptability as a replacement vaccine for BCG in high incidence countries
Similar intracellular compartment to *M. tuberculosis* and hence similar antigen processing
Expresses *M. tuberculosis* proteins in native form

Table 2. Viral diseases targeted by recombinant BCG vaccines.

Disease (organism) **Antigen**	**Animal Model**	**Immunogenicity**	**Protective Efficacy**	**Reference**
HIV and SIV				
Gag, Pol, Env (HIV)	BALB/c mice	Weak Ab to Gag and Env; IFNγ secretion (SP) and CD8+ CTL (SP) to Gag [only Gag tested]		[8]
Gag, Pol, Env, RT (HIV)	BALB/c mice	Weak Ab; CD8+ CTL (SP) [only Env tested]		[4, 6, 7]
HIVA (HIV) [synthetic construct containing ~73% of Gag fused to a multi-CTL epitope from the Gag, Pol, Nef, and Env proteins]	BALB/c mice	No CD8+ T-cell response alone, but a good response obtained after heterologous boost	protection against surrogate recombinant vaccinia virus challenge	[92]
Gag, Pol, Env, Nef (SIV) (cocktail)	Rhesus macaques	IgA and IgG secreted by PBMC; CTL (PBMC)		[126]
Gag, Env, Nef (SIV) (cocktail)	BALB/c mice	mucosal IgA, serum IgG; CTL (SP)		[127]
Gag, Env, Nef (SIV) (cocktail)	Cynomolgus macaques	IFNγ secretion and CTL (PBMC)	no protection against rectal challenge	[128]
Gag, Nef (SIV) (bivalent strain)	BALB/c mice	serum IgG; IFNγ secretion (CD4+ T-lymphocytes, spleen)		[129]
Gag, Pol, Env (SIV) (cocktail)	Rhesus macaques	weak IFNγ ELISPOT and tetramer binding (PBMC) which could be significantly enhanced with heterologous boosting		[91]
Gag (HIV) [p17gag B-cell epitope (aa 92-110) fused to *M. kansasii* α-antigen]	BALB/c mice	high titer Ab induced in 3 of 7 mice		[130]
Gag (HIV)	Chacma baboons	No A; weak IFNγ ELISPOT (PBMC) which could be		[102]

		significantly enhanced with heterologous boosting		
Gag (HIV)	BALB/c mice	40-fold higher expression using codon optimization resulted in stronger immune response; T-cell proliferation and IFNγ ELISPOT (SP); serum IgG		[118]
Gag (HIV)	BALB/c mice	heterologous prime-boost resulted in prolonged CTL (SP)		[101]
Gag (HIV)	BALB/c mice	CTL and T-cell proliferation (SP); no serum Ab		[100]
Gag (SIV)	Rhesus macaques	CD8+ CTL (PBL)		[87]
Gag (SIV)	Rhesus macaques	Heterologous prime-boost; good CTL (PBL); no Ab	no protection against cell-free intravenous SIV challenge	[131]
Gag (SIV)	Cynomolgus macaques	Heterologous prime-boost; IFNγ ELISPOT (PBMC)	protection against mucosal challenge with SHIV	[132]
Gag (SIV)	Guinea pigs, Hartley strain	Long-lasting, very high titer serum IgG; IFNγ mRNA elevated (PBMC)		[104]
Gag (SIV)	Guinea pigs, Hartley strain	DTH; T-cell proliferation (PBMC); IFNγ mRNA elevated (PBMC, SP); very high titer serum IgG		[103]
Env (HIV) [15 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	BALB/c mice	CD8+ CTL (SP)		[133]
Env (HIV) [19 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	BALB/c mice and Guinea pigs, Hartley strain	DTH; CTL (SP); moderate serum Ab; Neutralizing Ab		[134]
Env (HIV)	C57BL/6J	serum IgG; Neutralizing Ab;		[135]

[19 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	mice	IFNγ and IL-2 secretion by CD4+ T-cells		
Env (HIV) [19 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	Guinea pigs, Hartley strain	DTH; T-cell proliferation (PBMC, SP, I-IEL)		[136]
Env (HIV) [19 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	Guinea pigs, Hartley strain	DTH; serum IgG and IgA; Neutralizing Ab		[105]
Env (HIV) [19 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	Rhesus macaques	serum Ab; Neutralizing Ab; weak IFNγ ELISPOT (PBMC)	no protection against pathogenic SHIV	[137]
Env (HIV) [15 aa V3 CTL epitope (PND) fused to *M. tuberculosis* chaperonin-10]	BALB/c mice	T-cell proliferation (SP); serum IgG		[138]
Env (HIV) [11 or 12 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	Guinea pigs	moderate serum IgG; Neutralizing Ab		[139]
Env (HIV)	BALB/c mice	IFNγ ELISPOT (SP, lung, FRT); No serum Ab although could be elicited with heterologous boost		[93]
Env (SIV)	BALB/c mice and Guinea pigs	CD8+ CTL (LN); serum IgG; fecal IgA; Neutralizing Ab		[140]
Nef (HIV)	BALB/c mice	T-cell proliferation (LN)		[9]
Nef (HIV)	BALB/c mice	T-cell proliferation (LN); No serum Ab		[141]
Nef (SIV)	BALB/c mice	T-cell proliferation and CD8+ CTL (LN)		[142]
Nef (SIV)	BALB/c mice	T-cell proliferation (PGLN, MLN, CLN, SP); IFNγ and TNFα ELISPOT (PP, I-IEL, PGLN, MLN, SP); CD8+ CTL (I-IEL, MLN, SP)		[143]
Measles Virus				
Nucleocapsid (N) protein (measles	C3H/He mice	moderate serum Ab but only	partial protection	[144]

virus)		when rBCG given twice; T-cell proliferation (SP); low levels of Neutralizing Ab after infection		
Nucleocapsid (N) protein (measles virus)	Rhesus macaques	CTL and T-cell proliferation (PBMC); no serum IgG	partial protection	[145]
Human Papillomavirus (HPV)				
HPV type 6b late protein L1, HPV type 16 early protein E7	C57BL/6J or BALB/c mice	DTH; serum Ab (weak but could be boosted); T-cell proliferation and low CTL (SP)	no protection against tumor challenge	[146]
Cottontail Rabbit Papillomavirus (CRPV)				
L1 (major capsid protein)	outbred New Zealand White rabbits	serum Ab; Neutralizing Ab	good protection	[109]
L2, E2, E7 or L2E7E2 fusion	outbred New Zealand White rabbits		therapeutic vaccine: 71% of papilloma sites had complete regression with rBCG expressing L2E7E2 fusion protein	[110]
Porcine reproductive and respiratory syndrome virus (PRRSV)				
GP5, M (cocktail)	BALB/c mice	serum Ab; Neutralizing Ab; IFNγ secretion (SP)		[147]
GP5, M (cocktail)	crossbreed F1 (Landrace × Large White) pigs	serum Ab, Neutralizing Ab in 3 of 5 pigs; negative IFNγ ELISPOT (PBMC)	partial protection	[148]

Hepatitis B Virus (HBV)				
HBsAg-Middle S, HBsAg-Large S	BALB/c mice	serum Ab		[149]
Hepatitis C Virus (HCV)				
NS5a (non-structure protein 5a) [12 aa CTL epitope of HCV-non-structure protein 5a (NS5a) as a chimeric protein with α-antigen of *M. kansasii*]	BALB/c × C3H/HeN (CC3HF1) mice	CD8+ CTL (SP); increased IFNγ, IL-2, and IL-12 mRNA (LN)	good protection against surrogate recombinant vaccinia virus challenge	[111]
CtEm [multi-epitope antigen, including the major epitope domains of the truncated core, mimotopes from E2, and six HLA-A2-restricted CTL epitopes from NS3, NS4 and NS5]	HHD-2 mice (transgenic for HLA-A2.1)	serum Ab; T-cell proliferation, IFNγ and IL-2 secretion, and CTL (SP)	good protection against surrogate recombinant vaccinia virus challenge	[112]
Rotavirus				
VP6	BALB/c mice, Guinea pigs, Rabbits	no serum Ab	partial protection	[150]
Respiratory syncytial virus (RSV)				
N, M2	BALB/c mice	no serum IgG; IFNγ and IL-2 secretion and increased CD69+ T-cells (SP)	very good protection	[107]
Encephalomyocarditis Virus (EMCV) D-variant (Human Type I Diabetes model)				
VP1 (major outer capsid protein)	SJL/J mice, inbred Guinea pigs	serum IgG; Neutralizing Ab; DTH; T-cell proliferation (SP)	long lasting (>10 months) protective immunity, very good protection (required at least 6 weeks for full immunity)	[108]

Rabies Virus				
N (nucleoprotein) [B-cell and T-cell epitopes fused to *M. leprae* 18-kDa protein]	BALB/c mice	serum IgG		[151]

Abbreviations: aa, amino acid; Ab, antibody; CLN, cervical lymph nodes; CTL, cytotoxic T-lymphocytes; DTH, delayed type hypersensitivity; ELISPOT, enzyme-linked immunosorbent spot; I-IEL, intestinal intraepithelial lymphocytes; LN, lymph nodes; MLN, mesenteric lymph nodes; PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cells; PGLN, periglandular lymph nodes; PP, Peyer's patches; SP, splenocytes.

Table 3. Parasitic diseases targeted by recombinant BCG vaccines.

Disease (organism) **Antigen**	**Animal Model**	**Immunogenicity**	**Protective Efficacy**	**Reference**
Malaria (*Plasmodium spp*.)				
CSP (circumsporozoite protein of *Plasmodium falciparum*)	BALB/c and BALB/k mice	no serum Ab; no IFNγ secretion or T-cell proliferation (SP) in response to CSP Th2R peptide (CD4 + T lymphocyte epitope)		[89]
CSP (circumsporozoite protein of *Plasmodium falciparum*)	BALB/c mice	serum IgG; T-cell proliferation and IFNγ and IL-2 secretion (SP)		[152]
CSP (circumsporozoite protein of *Plasmodium yoelii*) [B-cell epitope of CSP fused to *M. kansasii* α-antigen]	BALB/c mice	serum Ab in only one of seven mice		[153]
MSP-1 (merozoite surface protein 1 from *Plasmodium yoelii*) [15 kDa C-terminal region of MSP-1 fused to *M. kansasii* α-antigen]	C3H/He mice	IFNγ secretion (SP); no serum Ab	very good protection against challenge with 10^4 *P. yoelii* 17XL–parasitized erythrocytes	[113]
MSP-1 (merozoite surface protein 1 from *Plasmodium yoelii*) [15 kDa C-terminal region of MSP-1 fused to *M. kansasii* α-antigen]	C3H/He mice		long lasting (9 month) protection against intraperitoneal challenge with 10^4 *P. yoelii* 17XL–parasitized erythrocytes but protection was substantially less than	[114]

			at 1 month post-vaccination	
MSA2 (merozoite surface antigen 2 from *Plasmodium falciparum*)	BALB/c mice	serum IgG; T-cell proliferation and IFNγ and IL-2 secretion (SP)		[154]
F2R(II)EBA (fragment 2 region II of EBA-175), $(NANP)_3$, as well as two T cell epitopes of the *M. tuberculosis* ESAT-6 antigen	BALB/c mice	no expression data presented; serum IgG; T-cell proliferation (SP); increased splenocyte CD4+ IFNγ+, CD4+ IL-2+, CD4+ IL-4+ cells in response to $(NANP)_3$; increased splenocyte CD4+ IL-4+ cells in response to F2R(II)EBA; immune responses also obtained against *M. tuberculosis* ESAT-6 epitopes		[155]
Leishmaniasis (*Leishmania spp.*)				
gp63 (*Leishmania major* surface proteinase)	BALB/c and CBA/J mice		good protection against *L. mexicana* promastigotes and amastigotes, poor protection against *L. major* promastigotes in BALB/c mice	[88]
gp63 (*Leishmania major* surface proteinase)	BALB/c and C57BL/6 mice		partial protection against a challenge with *L. major* amastigotes in BALB/c-mice	[115]
LCR1 (antigen cloned from amastigote *L. chagasi* library)	BALB/c mice		weak protective immunity against *L. chagasi* infection	[156]
Schistosomiasis				

(*Schistosoma spp.*)				
glutathione S-transferase (*Schistosoma mansoni*, Sm28GST)	BALB/c, C57BL/6, and C3H/HeJ mice	T-cell proliferation (LN but not SP) [BALB/c and C57BL/6 mice]		[157]
glutathione S-transferase (*Schistosoma mansoni*, Sm28GST)	BALB/c mice	high titer and long-lasting (1 year) serum IgG; Neutralizing Ab		[158]
glutathione S-transferase (*Schistosoma haematobium*, Sh28GST)	BALB/c mice	high titer serum IgG; serum and BALF IgA; Neutralizing Ab		[159]
glutathione S-transferase (*Schistosoma japonicum*, Sj26GST)	BALB/c mice	T-cell proliferation and IFNγ secretion (SP)		[160]
Sm14 (from *Schistosoma mansoni*)	BALB/c and outbred Swiss mice	no serum Ab; IFNγ secretion (SP)	partial protection against subcutaneous challenge with 100 *S. mansoni* cercaria; not able to boost protection with rSm14 protein or rBCG	[116]
Sm14 (from *Schistosoma mansoni*, codon optimized)	BALB/c and outbred Swiss mice	no serum Ab; IFNγ secretion (SP); codon optimization increased expression level but did not improve immune response	partial protection against subcutaneous challenge with 100 *S. mansoni* cercaria; not able to boost protection with rSm14 protein	[117]
Toxoplasmosis (*Toxoplasma gondii*)				
GRA1 (a major secreted antigen)	OF1 outbred mice, Suffolk crossbred sheep	mice: no serum Ab; no DTH sheep: no serum Ab; T-cell proliferation and IFNγ secretion (PBMC)	mice: very weak protection against oral challenge sheep: shorter duration of pyrexia	[161]

			following oral challenge	
ROP2 (rhoptry protein 2)	BALB/c mice	serum Ab; IFNγ and IL-2 secretion (SP)	slightly increased survival after intraperitoneal challenge	[162]
Coccidiosis (*Eimeria tenella*)				
Rho (rhomboid gene)	Chickens	serum IgG	partial protection	[163]

Abbreviations: Ab, antibody; BALF, bronchial alveolar lavage fluid; DTH, delayed type hypersensitivity; LN, lymph nodes; PBMC, peripheral blood mononuclear cells; SP, splenocytes.

Table 4. Bacterial diseases targeted by recombinant BCG vaccines.

Disease (organism) **Antigen**	**Animal Model**	**Immunogenicity**	**Protective Efficacy**	**Reference**
Lyme Disease (*Borrelia burgdorferi*)				
OspA (outer surface protein A)	BALB/c, C3H/HeJ, and outbred Swiss Webster mice	high titer serum IgG	complete protection	[5]
OspA (outer surface protein A)	BALB/c mice	prolonged systemic IgG and mucosal IgA	complete protection	[120]
OspA (outer surface protein A)	Human phase I clinical trial	None of the 24 volunteers developed anti-OspA Ab		[121]
OspA (outer surface protein A)	White-tailed deer	serum Ab		[164]
Bacterial pneumonia, otitis media, meningitis (*Streptococcus pneumoniae*)				
PspA (Pneumococcal surface protein A)	BALB/c, C3H/HeJ, and CBA/N (Xid) mice	high titer serum Ab	50-100% protection against intraperitoneal challenge	[119]
Leptospirosis (Leptospira spp.)				
LipL32 (Leptospira interrogans external membrane protein)	Golden Syrian hamsters	serum IgG	partial protection against intraperitoneal	[165]

			challenge with *L. interrogans*	
	BALB/c mice	serum Ab		[166]
Listeriosis **(*Listeria monocytogenes*)**				
p60 (major secreted antigen)	BALB/c mice	CD8+ T-cells (SP)	complete protection with rBCG expressing membrane anchored p60	[122]
DPT(Diptheria-Pertusis-Tetanus)				
S1-TTC (hybrid protein of S1 subunit of pertussis toxin fused to fragment C of tetanus toxin)	BALB/c mice	high titer serum anti-TTC IgG but no anti-S1 IgG; toxin neutralizing Ab; IL-2 secretion (SP)		[167]
S1 subunit of pertussis toxin	BALB/c mice	long-term serum IgG (up to 8 months) and memory response (15 months)		[168]
S1 subunit of pertussis toxin (genetically detoxified)	BALB/c and outbred Swiss mice	weak serum Ab; IFNγ secretion and T-cell proliferation (SP)	good protection against intracerebral *Bordetella pertussis* challenge	[123]
S1 subunit of pertussis toxin (genetically detoxified)	outbred Swiss mice (neonates)	no serum Ab; IFNγ secretion (SP)	good protection against intracerebral *Bordetella pertussis* challenge	[124]
CRM197 (mutated nontoxic derivative of diphtheria toxin)	BALB/c mice	weak serum Ab (non-neutralizing); rBCG capable of priming a humoral response to DT vaccine		[169]
FC (tetanus toxin fragment C), CRM197 (mutated nontoxic	BALB/c and outbred NIH	mice: weak serum anti-FC IgG; rBCG capable of	75% protection against a challenge	[125]

derivative of diphtheria toxin) (cocktail)	mice, Guinea pigs	priming a humoral response to DT vaccine guinea pigs: Neutralizing Ab against tetanus toxin and diphtheria toxin	with 100 minimum lethal doses of tetanus toxin	
FC (tetanus toxin fragment C, ToxC)	outbred NIH Swiss mice	serum Ab		[4]
FC (tetanus toxin fragment C, ToxC)	outbred NIH Swiss mice	serum Ab	partial to complete protection against a challenge with 100 minimum lethal doses of tetanus toxin	[7]
Bovine Anaplasmosis **(*Anaplasma marginale*)**				
MSP1a	BALB/c mice	weak serum Ab; IFNγ secretion (SP)		[170]

Abbreviations: Ab, antibody; SP, splenocytes.

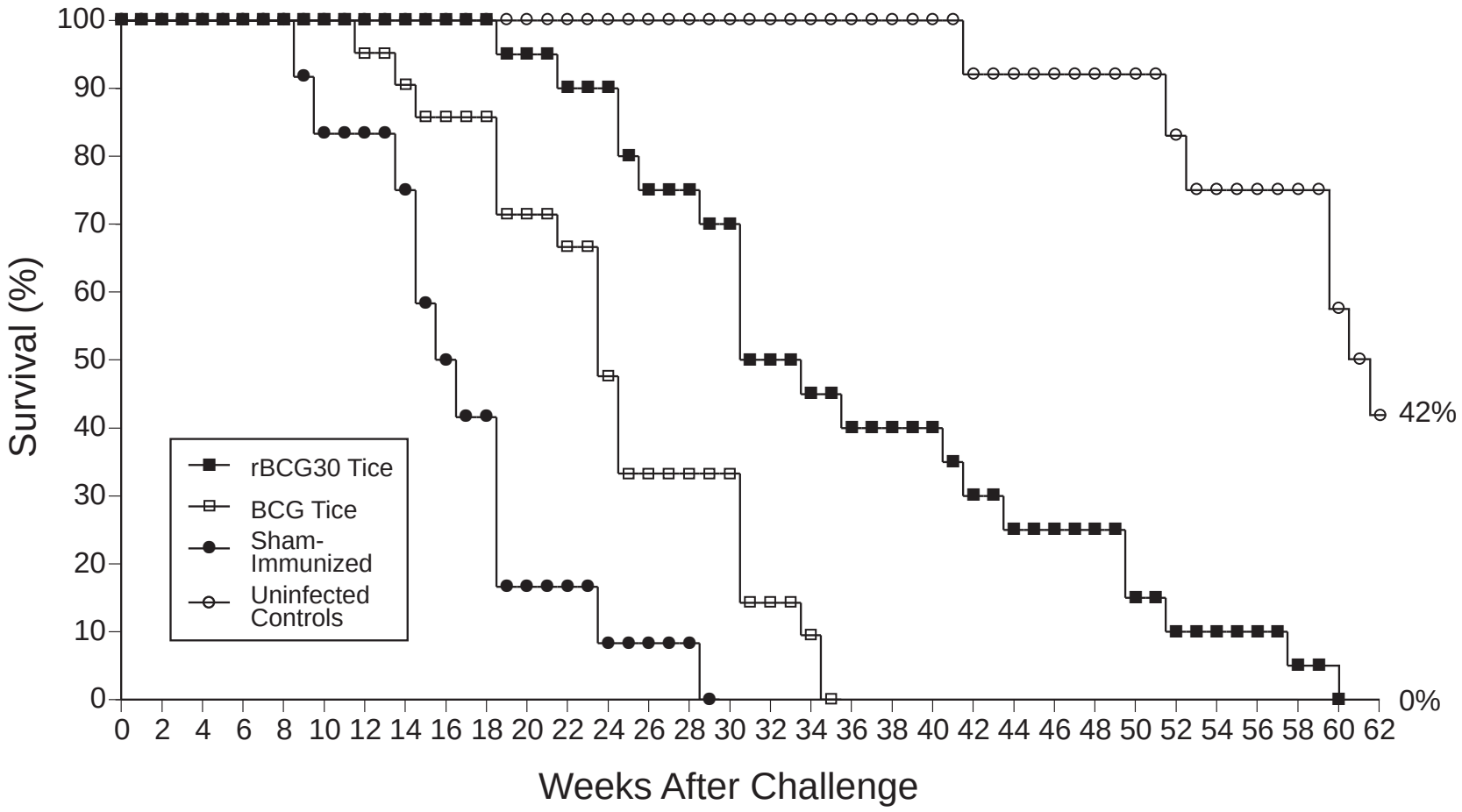
Table 5. Bacterial proteins used to enhance the adjuvant effect of recombinant BCG vaccines.

Antigen	**Animal Model**	**Immunogenicity**	**Reference**
CTB (cholera toxin B subunit)	BALB/c mice	increased IgA and TGF-β1in bronchial alveolar lavage fluid	[171]
LTB (B subunit of *E. coli* heat labile enterotoxin)	BALB/c mice	serum IgG and IgA; oral rBCG also induced mucosal IgA	[172]
LTB (B subunit of *E. coli* heat labile enterotoxin) fused to R1 repeat region of P97 adhesion from *Mycoplasma hyopneumoniae*	BALB/c mice	LTB used for adjuvant effect; serum anti-R1 IgG and IgA (greater response to LTB-R1 fusion than to R1 alone)	[173]

Table 6. Cancer and allergic disease targeted by recombinant BCG vaccines.

Antigen	Animal Model	Immunogenicity	Protective Efficacy	Reference
OVA	C57BL/6 mice	IFNγ-secreting CD8+ T cells and CTL (SP)	significant protection against challenge with OVA-expressing tumor cells (B16-OVA)	[174]
OVA (SIINFEKL epitope)	C57BL/6 and $TAP1^{-/-}$ mice	weak CTL	partial protection against challenge with OVA-expressing tumor cells (B16-OVA)	[175]
MUC1 (22 variable-number tandem repeats) + mIL-2	SCID mice reconstituted with 10^7 human PBL	IFNγ secretion; low-level serum IgG and IgM; rBCG secretes functional IL-2 but effect of cytokine is unclear as rBCG expressing MUC1 alone was not tested		[176]
MUC1 (1, 4, or 8 variable-number tandem repeats) + hGM-CSF	SCID mice reconstituted with 5×10^7 human PBL	increased IFNγ ELISPOTand CTL; no data on whether coexpressed hGM-CSF was functional	partial protection against tumor challenge	[177]
S1 subunit of pertussis toxin (genetically detoxified)	C57BL/6 mice	increased TNFα mRNA (qPCR)	reduction of bladder tumor volume	[178]
Der p I (a major allergen from house dust mites, immunodominant peptide containing T- and B-cell epitopes)	C57BL/6J and BALB/b mice	IFNγ secretion (SP)		[179]

Abbreviations: CTL, cytotoxic T-lymphocytes; ELISPOT, enzyme-linked immunosorbent spot; PBL, peripheral blood lymphocytes; SP, splenocytes.


Survival (%)
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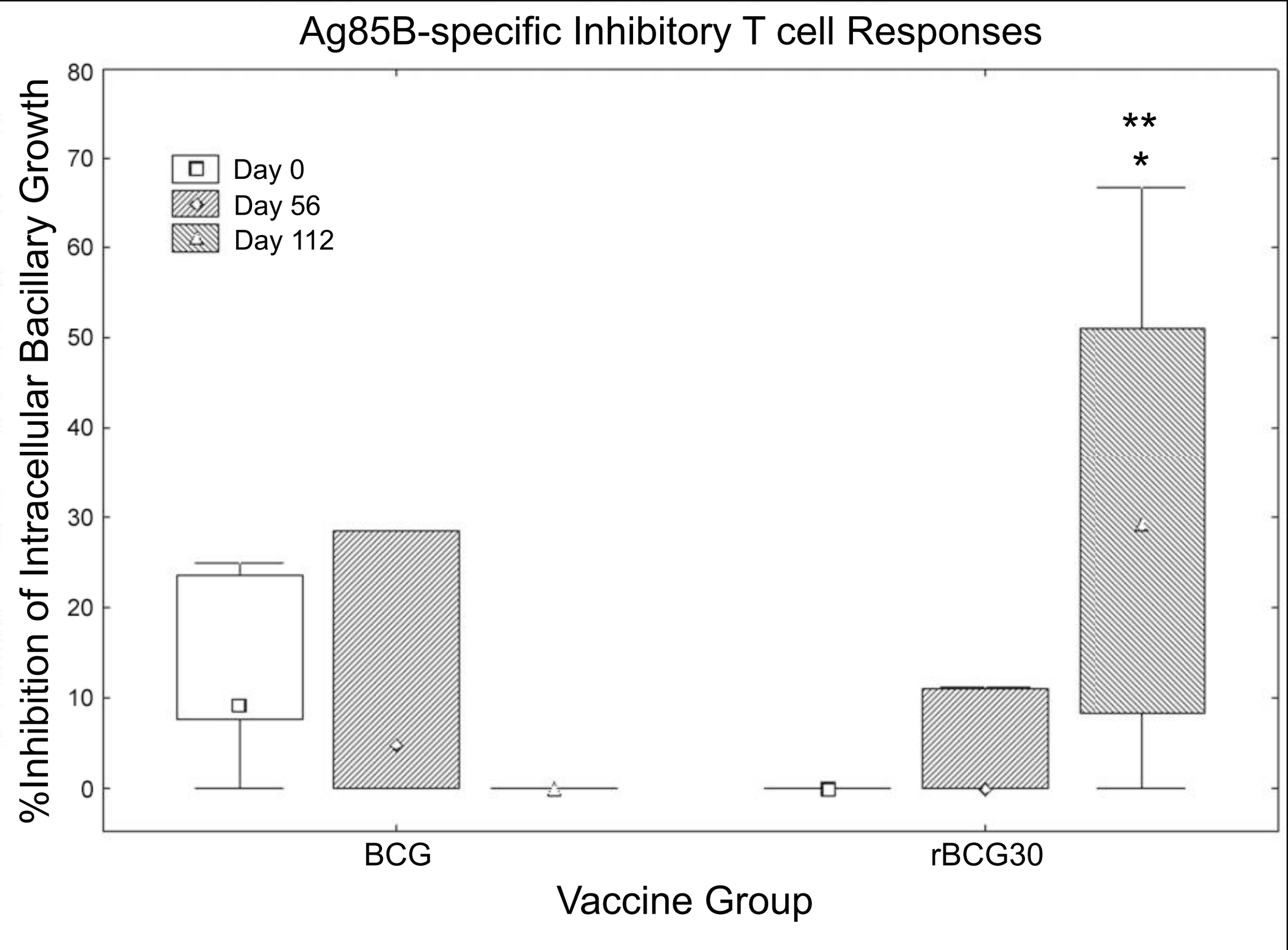
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62
Weeks After Challenge
rBCG30 Tice
BCG Tice
Sham-Immunized
Uninfected Controls
42%
0%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%Inhibition of Intracellular Bacillary Growth
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70
60
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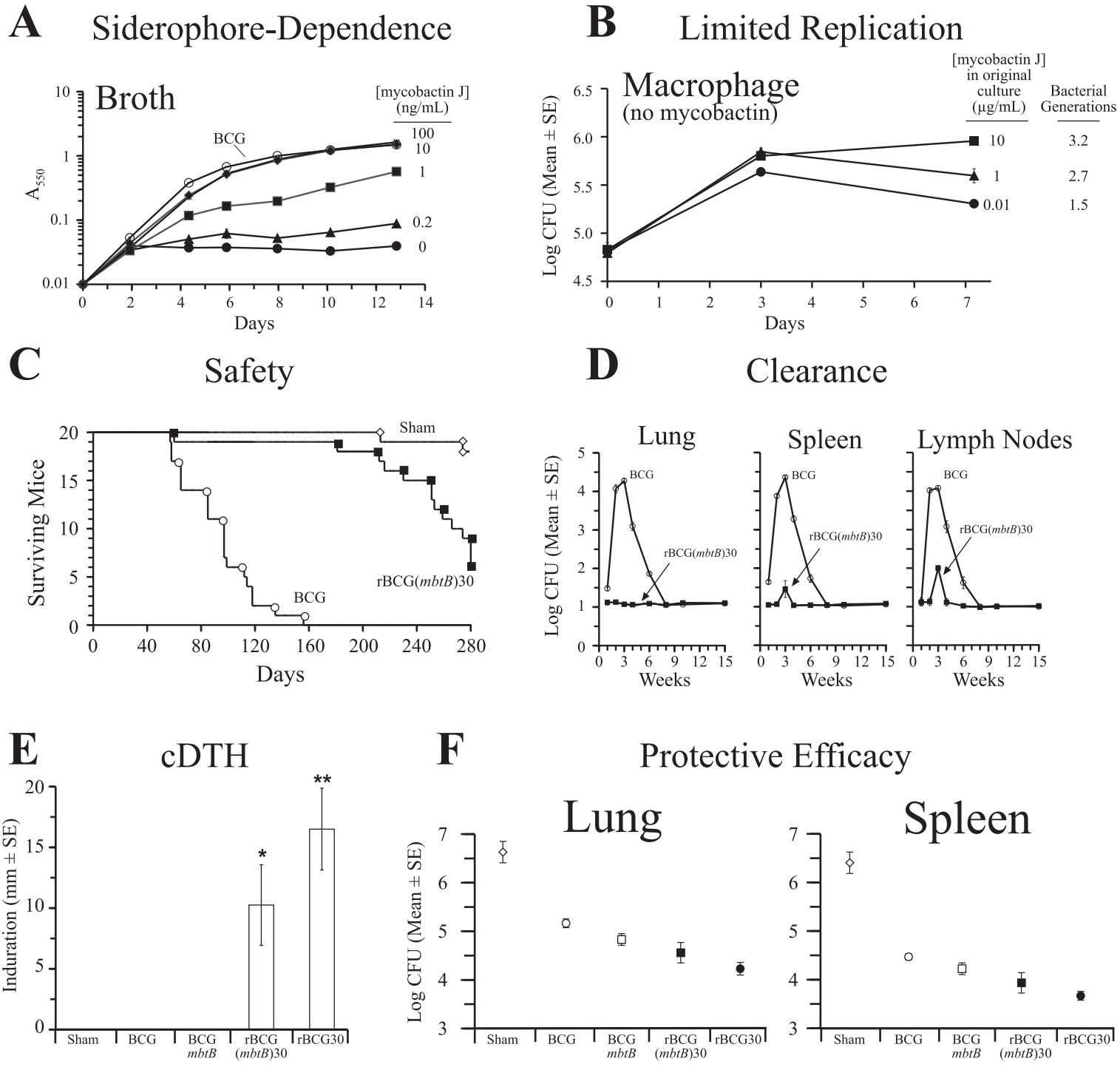
A
Siderophore-Dependence
Broth
[mycobactin J] (ng/mL)
100
10
1
0.2
0
BCG
A_{550}
Days
B
Limited Replication
Macrophage
(no mycobactin)
[mycobactin J] in original culture (µg/mL)
Bacterial Generations
10
3.2
1
2.7
0.01
1.5
Log CFU (Mean ± SE)
Days
C
Safety
Sham
rBCG(mbtB)30
BCG
Surviving Mice
Days
D
Clearance
Lung
Spleen
Lymph Nodes
BCG
rBCG(mbtB)30
Log CFU (Mean ± SE)
Weeks
E
cDTH
Induration (mm ± SE)
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**
Sham
BCG
BCG mbtB
rBCG (mbtB)30
rBCG30
F
Protective Efficacy
Lung
Spleen
Log CFU (Mean ± SE)
Sham
BCG
BCG mbtB
rBCG (mbtB)30
rBCG30