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Journal

Microbes and Infection, 7(5-6)

ISSN

1286-4579

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Publication Date

2005-05-01

Peer reviewed

Recombinant BCG expressing *M. tuberculosis* major extracellular proteins

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Key Words:

Tuberculosis

Vaccine

BCG Vaccine

Microbes and Infection 7 (2005) 947-954

Forum in Immunology: Focus on rational vaccine development against tuberculosis.

Abstract

rBCG30, the first vaccine against tuberculosis demonstrated more potent than BCG in preclinical studies, is the prototype of a class of vaccines that utilize BCG as a host organism for expressing and secreting *M. tuberculosis* major extracellular proteins. The vaccine is based on the concept that extracellular proteins of intracellular pathogens are key immunoprotective molecules. rBCG30, which expresses and secretes large amounts of the *M. tuberculosis* 30 kDa major secretory protein, is currently in human clinical trials.

1. Introduction

rBCG30, the first vaccine against tuberculosis demonstrated more potent than BCG in preclinical studies, is the prototype of a class of recombinant vaccines against tuberculosis that utilize bacillus Calmette-Guérin or BCG as the host organism for expressing *Mycobacterium tuberculosis* major extracellular proteins. rBCG30 is a recombinant BCG Tice strain expressing large amounts of the *M. tuberculosis* 30 kDa major secretory protein, a mycolyl transferase also known as the α Antigen or Antigen 85B [1, 2]. The recombinant protein, designated r30, is expressed from the plasmid pMTB30 (Fig.1) [2, 3]. Derived from the mycobacterium - *E. coli* shuttle vector pSMT3 [4], pMTB30 contains a full-length copy of the *M. tuberculosis* 30 kDa major secretory protein gene and flanking 5' and 3' regions including its own promoter region [2]. rBCG30 expresses 5-6 times more of the 30 kDa major secretory protein than the parental strain, which expresses and secretes a homologous endogenous 30 kDa protein that differs from the *M. tuberculosis* 30 kDa protein by two contiguous amino acids [1, 2].

2. Rationale for rBCG30 and other recombinant BCG vaccines expressing *M. tuberculosis* major extracellular proteins

2.1. Life Style of *M. tuberculosis*

The design of a vaccine must take into account the life style of the pathogen against which the vaccine is targeted. In the case of *M. tuberculosis*, the central immunobiological feature of the organism is that it is an intracellular pathogen of human mononuclear phagocytes. After ingestion by conventional phagocytosis [5], mediated

primarily by complement receptors and mannose receptors [5,6], which recognize fragments of complement component C3 and lipoarabinomannan, respectively, on the bacterial surface [5-7], the organism resides and multiplies within a membrane-bound phagosome. The phagosome, which is impermeable to small molecular weight substances in the cytoplasm [8], interacts throughout its life cycle with early endosomes and to some extent late endosomes [9]. However, it arrests the maturation of its phagosome at an endosomal stage, and it does not proceed along the endolysosomal pathway to fuse with lysosomes [9]. The phagosomal membrane contains both MHC class I and II molecules but whether the MHC molecules on the phagosome are involved in presentation of *M. tuberculosis* antigens is not known. The *M. tuberculosis* phagosome is only modestly acidified [9-11].

2.2. Role of Cell-mediated Immunity

Against intracellular pathogens, cell mediated immunity plays a dominant role in host defense. In cell-mediated immunity, lymphocytes interact with macrophages in two major ways. First, they secrete lymphokines that activate mononuclear phagocytes such that they resist intracellular pathogens. One mechanism by which activated macrophages resist intracellular pathogens is by reducing their uptake, for example by down-regulating complement receptor function [12], as in the case of *Mycobacterium leprae*, thereby denying pathogens their preferred intracellular niche. Another way is to inhibit the multiplication of pathogens that are ingested by denying them access to essential nutrients, such as iron in the case of *Legionella pneumophila* [13, 14], or by assaulting them with toxic oxygen molecules in the case of *Toxoplasma gondii* [15]. Second,

cytotoxic lymphocytes lyse infected macrophages, again denying the pathogens a host cell in which to multiply.

2.3. The Extracellular Protein Hypothesis

How are lymphocytes to recognize a host cell that is infected with an intracellular pathogen so that they can exert an antimicrobial effect against the host cell? How can a vaccine induce a population of lymphocytes in a naive host with the capability of recognizing infected macrophages at some later time when the host is infected?

Over fifteen years ago, we proposed a hypothesis to address these issues - the Extracellular Protein Hypothesis [16-22]. The Extracellular Protein Hypothesis has three major components. First, it holds that during natural infection, extracellular proteins of intracellular pathogens, i.e. proteins secreted or otherwise released by the organisms, play a key role in inducing cell-mediated immune responses and immunoprotection against this group of pathogens. Such proteins, by virtue of their release by live organisms into the intracellular compartment of the host cell, are available for proteolytic processing and subsequently presentation on the surface of the infected host cell as MHC-bound peptide fragments. These surface-exposed fragments serve as flags for the host immune system and allow the immune system to recognize live pathogens sequestered within a host cell and to exert an antimicrobial effect against them.

Second, the hypothesis holds that appropriate immunization of a naive host with extracellular proteins of intracellular pathogens can induce a population of lymphocytes capable of later recognizing and exerting an immune response against infected host cells. These lymphocytes would recognize infected host cells by identifying MHC-bound

fragments of extracellular proteins displayed on the host cell surface consequent to the release of the proteins by the intracellular pathogen.

Finally, the hypothesis holds that among the extracellular proteins of intracellular pathogens, the major extracellular proteins, i.e. those released in greatest abundance, would figure prominently in inducing immunoprotection. We reasoned that such proteins, by virtue of their abundance in the phagosome, would provide the most plentiful display of MHC-peptide complexes on the surface of host cells, and therefore induce a particularly strong cell-mediated immune response.

Initial evidence in support of the Extracellular Protein Hypothesis derived from our studies of *L. pneumophila*, the agent of Legionnaires' Disease. Like *M. tuberculosis*, *L. pneumophila* is an intracellular respiratory pathogen transmitted by the airborne route and a facultative intracellular pathogen that resides throughout its intracellular life cycle within a phagosome [23, 24]. Like the *M. tuberculosis* phagosome, the *L. pneumophila* phagosome does not fuse with lysosomes and is not highly acidified [25, 26]. However, in contrast to the *M. tuberculosis* phagosome, the *L. pneumophila* phagosome does not interact with the endolysosomal pathway [9, 27].

L. pneumophila secretes a single major protein, a 39 kDa metalloproteinase, designated Major Secretory Protein or MSP [16]. Interestingly, MSP is not a virulence determinant for *L. pneumophila* in human mononuclear phagocytes or in guinea pigs, an excellent small animal model of Legionnaires' disease [17]. Immunization of guinea pigs with MSP in adjuvant protects the animals from a lethal aerosol challenge with virulent *L. pneumophila* [16,18]. In addition to MSP, *L. pneumophila* releases another major protein into its extracellular milieu, the major cytoplasmic membrane protein (MCMP), a

60 kDa heat shock protein [19]. This protein, which lacks a leader peptide, is probably released as a result of membrane blebbing. Immunization of guinea pigs with MCMP also protects the animals from lethal aerosol challenge with *L. pneumophila* [19].

Immunization with MSP does not prevent infection with *L. pneumophila*. In guinea pigs immunized with MSP and challenged by aerosol with *L. pneumophila*, the bacterium multiplies several logs in the guinea pig lung during the first 24 hours after infection at a rate comparable to that of *L. pneumophila* in the lung of unimmunized animals [16]. However, by 24 hours, the infection is contained and the concentration of bacteria in the lung plateaus, generally at a level below that at which the animal exhibits signs of illness. In contrast, *L. pneumophila* continues to multiply in unimmunized animals until these animals succumb to the infection. The difference between immunized and control animals in the level to which *L. pneumophila* multiplies in the lungs is approximately 1.5 logs. This result indicates that for a vaccine to protect against disease and death, it must induce in the immunized animal a capacity to control infection but not necessarily to prevent it.

3. *M. tuberculosis* major extracellular proteins as immunoprotective antigens

M. tuberculosis secretes numerous proteins into its extracellular milieu of which 12 are particularly abundant [21]. The *M. tuberculosis* 30 kDa major secretory protein is the most abundant. It makes up almost one-quarter of the total extracellular protein released by *M. tuberculosis* into broth culture. This protein, as already noted, is a mycolyl transferase [28]. It is highly homologous to two other mycolyl transferases of mass 32 kDa, designated 32A (Antigen 85A) and 32B (Antigen 85C), that are also among the 12

major extracellular proteins [21, 29]. The *M. tuberculosis* 30 kDa protein is not only the major protein secreted into broth culture, but it is also among the major *M. tuberculosis* proteins of all types expressed in human macrophages infected with *M. tuberculosis*. [30].

Initial studies in our laboratory examined the capacity of purified *M. tuberculosis* extracellular proteins in adjuvant to induce protective immunity against tuberculosis. For these studies, we utilized the outbred guinea pig model of pulmonary tuberculosis because of its high relevance to human tuberculosis. In contrast to the mouse and rat, the guinea pig is highly susceptible to *M. tuberculosis*, and it develops disease that closely resembles human tuberculosis clinically, immunologically, and pathologically [1, 2]. In these studies, we immunized the animals several times with protein in adjuvant and then challenged them by aerosol with the highly virulent Erdman strain of *M. tuberculosis*.

In further support of the Extracellular Protein Hypothesis, immunization of guinea pigs with a crude extract of *M. tuberculosis* extracellular proteins or with purified *M. tuberculosis* extracellular proteins induced strong cell-mediated and protective immunity against aerosol challenge with *M. tuberculosis* [20, 21]. The *M. tuberculosis* 30 kDa major secretory protein as a single protein was a particularly potent vaccine [21]. Combinations of the 30 kDa major secretory protein and other major extracellular proteins induced somewhat greater protection than the 30 kDa protein alone. The use of strong adjuvants and powerful immunostimulators, such as IL-12, also enhanced protective immunity. However, even with the most potent combination of proteins and adjuvants, protection was modest in comparison with BCG, the currently

available vaccine. This result was unsatisfactory to us from a practical viewpoint since the ultimate goal of our studies was a vaccine more potent than BCG.

4. rBCG30

To obtain a more potent immune response to *M. tuberculosis* extracellular proteins, we turned to a live vector to express them. We reasoned that the most efficacious vector would be one that was avirulent on the one hand, but able to survive and replicate within the host for some period of time on the other. In addition, we considered that it may be important that the vector be capable of expressing and secreting *M. tuberculosis* major extracellular proteins in native form. If protein processing were significantly influenced by the conformation of the secreted protein, then a vaccine vector that released the proteins in native form would induce a population of T cells better equipped to recognize the display of MHC-peptide complexes on the surface of host cells infected with *M. tuberculosis*. Our previous studies had informed us that a mycobacterial host was required to secrete *M. tuberculosis* major extracellular proteins in native form [3]. These considerations lead us to select BCG as the vector.

Aside from the aforementioned considerations, BCG offered several additional advantages. First, as an attenuated strain of *Mycobacterium bovis*, a member of the *M. tuberculosis* complex, BCG is highly homologous to *M. tuberculosis* at the DNA and protein level; hence it shares many antigens with *M. tuberculosis*. Second, over 4 billion doses of BCG have been administered to humans; hence its safety record is well-established. Third, from a practical standpoint, BCG provides a high baseline level of

protection against tuberculosis. Hence, any enhancement in its potency would meet our goal of a vaccine more potent than BCG.

To test the concept that a live BCG vector expressing and secreting *M. tuberculosis* major extracellular proteins would induce a more potent protective immune response, we generated recombinant BCG strains expressing the *M. tuberculosis* 30 kDa major secretory protein (Fig. 2) [1, 2]. We initially constructed two recombinant strains -- one with BCG Connaught as the parent and one with BCG Tice as the parent. rBCG30 Connaught produced 2-fold and rBCG30 Tice 5.5 fold the amount of 30 kDa protein produced by their respective parental hosts, which express a homologous endogenous protein, as noted above [1, 2]. The parental strains produced very nearly the same amount of endogenous 30 kDa protein as each other and four other commonly used BCG strains – Glaxo, Japanese, Copenhagen, and Pasteur [2]. Production of r30 was highly stable. Even in the absence of selective antibiotic pressure, the rBCG30 strains maintained high production of r30 when subcultured monthly in broth culture for at least one year [1].

4.1 Efficacy of rBCG30

4.1.1. Cell-mediated Immunity

Intradermal immunization of guinea pigs with rBCG30 induced strong cell-mediated immune responses to r30 as evidenced by a vigorous cutaneous delayed-type hypersensitivity (DTH) response to r30 9 weeks after immunization (Fig. 3). The cutaneous DTH response to r30 in guinea pigs immunized with rBCG30 Connaught or

Tice was significantly greater than the negligible cutaneous DTH response of guinea pigs immunized with the parental strains. rBCG30 Tice, which produced the greatest amount of r30 -- about 3-fold the amount produced by rBCG30 Connaught -- consistently induced the strongest cutaneous DTH response and one significantly greater than that of rBCG30 Connaught (Fig. 3) [1, 2].

4.1.2. Protective Immunity

To assess the capacity of rBCG30 to induce protective immunity, we immunized guinea pigs with either a parental BCG strain or a recombinant BCG strain[1]. Control animals were sham-immunized or immunized with r30 in adjuvant. As expected, BCG-immunized animals were highly protected against *M. tuberculosis* aerosol challenge compared with sham-immunized animals. In the first three experiments, compared with sham-immunized animals, BCG-immunized animals had significantly less lung pathology, significantly fewer tubercles, and significantly fewer colony-forming units (CFU) of *M. tuberculosis* in the lung and spleen (Fig. 4) [1]. On average, BCG-immunized animals had 1.5 logs fewer CFU in the lung and 1.7 logs fewer CFU in the spleen than sham-immunized animals. BCG-immunized animals were also completely protected against weight loss, a hallmark of tuberculosis in the human and the guinea pig. In contrast, sham-immunized animals lost 19% of their total body weight compared with uninfected control animals. Animals immunized with r30 in adjuvant were protected somewhat, but not as much as BCG-immunized animals [1].

Most importantly, guinea pigs immunized with the two rBCG30 strains were significantly better protected 10 weeks later from *M. tuberculosis* aerosol challenge than

guinea pigs immunized with the parental strains of BCG (Fig. 4). Compared with BCG-immunized animals, rBCG30-immunized animals had significantly fewer lung lesions, significantly less lung pathology, and significantly fewer *M. tuberculosis* organisms in the lung and spleen. In the first three experiments, rBCG30-immunized animals averaged 0.5 log fewer CFU in the lung and 1 log fewer CFU in the spleen than BCG-immunized animals, differences which were highly significant on statistical analysis. rBCG30 Connaught and rBCG30 Tice were comparably effective in lowering the burden of *M. tuberculosis* in guinea pig lung and spleen.

The results of the first three experiments have been confirmed in 10 consecutive experiments, including 8 consecutive experiments comparing rBCG30 Tice with its parent. When all eight experiments with rBCG30 Tice are combined, animals immunized with rBCG30 Tice averaged 0.8 ± 0.1 log fewer CFU in the lungs and 1.1 ± 0.1 log fewer CFU in the spleen than animals immunized with parental BCG Tice (n = 178 for BCG Tice and n = 179 for rBCG30 Tice).

The capacity of rBCG30 to protect against death after challenge was assessed in a subsequent study in which animals were sham-immunized, or immunized with either BCG or rBCG30, and 10 weeks later challenged with *M. tuberculosis* by aerosol (Fig. 5) [2]. As expected, sham-immunized animals died most rapidly. BCG-immunized animals survived significantly longer. Most importantly, rBCG30-immunized animals survived significantly longer than BCG-immunized animals. Thirty-five percent of the rBCG30-immunized animals survived to the point that uninfected control animals began to die.

4.2. Safety of rBCG30

To assess the virulence of rBCG30, we immunized guinea pigs with rBCG30 Tice or BCG Tice and investigated the impact of immunization on the general health status of the animals [2]. No adverse health effects were noted for either group of animals. Both groups gained weight at the normal rate, including during the first 10 weeks after challenge when the animals harbored the highest numbers of bacteria in their organs.

We also assessed the clearance of the organisms by euthanizing animals at various intervals after immunization and assessing CFU in the regional lymph nodes, lung, and spleen. The parental BCG and rBCG30 exhibited an identical clearance profile (Fig. 6) [2]. CFU rose rapidly after immunization in all three sites, peaked at 3 weeks, and then declined such that by 10 weeks few organisms could be detected. Interestingly, low numbers of organisms (≤ 12) persisted in animal organs for at least 26 weeks after immunization [2].

These studies demonstrated that rBCG30 and BCG are comparably avirulent.

The Aeras Global TB Vaccine Foundation contracted for two toxicology studies of rBCG30. In one study, mice (males and females) were administered one human dose of rBCG30 or BCG intraperitoneally and observed for 14 days. No adverse effects of rBCG30 or BCG intraperitoneally were observed. In the second study, guinea pigs (males and females) were administered 0.25, 1, or 2 human doses of rBCG30 or BCG intradermally, observed for 3 or 28 days, euthanized, and necropsied. The assessment included mortality, injection site pathology, body weight, hematology and clinical chemistry analyses, gross pathology, and histopathology of animal organs. No toxicity of rBCG30 was observed.

Additional safety studies demonstrated that a) rBCG30 and parental BCG vaccines are sensitive to the same antimycobacterial antibiotics; however, rBCG30 is slightly less sensitive to INH, and b) rBCG30 belongs to the *M. bovis* complex by *mmpL6* locus analysis (Codon 551 = AAG, characteristic of *M. bovis* strains) [31]. rBCG30 was documented to have had no exposure to animal products from countries where BSE has occurred.

Finally, studies of the pMTB30 plasmid demonstrated that a) the pMTB30 plasmid DNA sequence has no homologies with human DNA sequences of > 10 bp; b) the pMTB30 plasmid is not mobilizable or self-transmissible to other bacteria; c) the pMTB30 plasmid is not expressed in *E. coli* even when forced into it by electroporation; and d) the pMTB30 plasmid expresses no proteins other than the *M. tuberculosis* 30 kDa protein.

4.3. Stability of pMTB30 and Protein Expression in rBCG30

rBCG30 retains the plasmid pMTB30 and maintains high expression and secretion of r30 when subcultured in broth monthly in the absence of selective antibiotic pressure for at least one year. rBCG30 also stably maintains the pMTB30 plasmid *in vivo* in guinea pigs in the absence of antibiotic pressure for at least six weeks, as evidenced by the retention of hygromycin resistance in 100% of bacteria recovered from the lung, spleen, and regional lymph nodes six weeks after immunization.

5. Other Recombinant BCG Expressing *M. tuberculosis* major extracellular proteins

Cole and colleagues reported the second tuberculosis vaccine superior to BCG [32]. Like rBCG30, this vaccine is a recombinant BCG expressing *M. tuberculosis* extracellular proteins. In this case, the vaccine expresses two proteins, ESAT-6 and CFP-10, whose homologs in BCG are absent because they are on a segment of the *M. bovis* genome that was deleted during the generation of BCG.

In our laboratory, we have generated and tested recombinant BCG expressing *M. tuberculosis* major extracellular proteins other than the 30 kDa protein, including the 23.5 kDa protein (MPT64) [3]. This vaccine, rBCG23.5, was also more potent than BCG but not as potent as rBCG30 (G. Harth and M.A. Horwitz, unpublished studies).

Future studies in our laboratory will explore the efficacy of recombinant BCG expressing combinations of major extracellular proteins. To allow greater flexibility in expressing multiple *M. tuberculosis* major extracellular proteins in BCG, we have developed a two-plasmid system involving two compatible plasmids, where one is a high copy number plasmid (pSMT3 or pNBV1) and the other is a low copy number plasmid (pGB9.2) [33]. The system allows expression of different extracellular proteins in different amounts, something that potentially may allow optimization of the protective immune response. The use of a two-plasmid system also has the advantage of reducing stress on the primary plasmid [33].

6. Manufacture of rBCG30

rBCG30 was manufactured by Good Manufacturing Practices at the Korean Institute of Tuberculosis using a protocol based on the procedure used in our laboratory to culture rBCG30. This process involves harvesting the organism at mid to late log phase. The manufactured lots were extensively tested to rule out contamination and to confirm that rBCG30 maintained its viability and capacity to express high amounts of the 30 kDa protein. Manufactured vaccine was also tested for endotoxin, residual moisture, and residual hygromycin to insure that it conformed to acceptable standards. The pMTB30 plasmid was recovered from the manufactured lot and its sequence was demonstrated to be identical to the plasmid isolated from the original seed lot.

7. Human Clinical Trials.

Early in 2004, the first human clinical trial of a recombinant BCG vaccine was initiated with the testing of rBCG30 in a Phase 1 human clinical study in the United States. The study was designed to test the safety and immunogenicity of rBCG30 compared with BCG. The trial is blinded and on-going; thus far, no adverse events have occurred. Subsequent Phase 1 and 3 studies are anticipated in South Africa.

Acknowledgments

Günter Harth engineered rBCG30, played a central role in the testing of its efficacy and safety, helped design the manufacturing protocol, and designed key tests of vaccine potency including its capacity to express the 30 kDa protein. Barbara Jane Dillon and Saša Masleša-Galić were instrumental to the successful testing of the vaccine for efficacy in animals. Barbara Jane Dillon also helped design the manufacturing protocol and performed key tests including stability and endotoxin levels. Chalermchai Chaloyphian provided excellent technical assistance. Carol Nancy, founder of the predecessor to the Aeras Global TB Vaccine Foundation, arranged for the foundation to undertake the clinical development of the vaccine. Ronald Mayner, with the assistance of Sharon Rowland, organized, oversaw, and compiled the Investigational New Drug application to the U.S. Food and Drug Administration. Jerald Sadoff, Lawrence Geiter, Lewellys Barker, and Amy Kinney played critical roles in the design and management of the human clinical studies, and Daniel Hoft directed the first Phase 1 clinical trial. The Korean Institute of Tuberculosis generously made its facilities available for manufacture of the vaccine, and Dr. Hyun Je Han oversaw the production of the vaccine at the institute. National Institutes of Health Tuberculosis Program Directors John Foulds, Ann Ginsberg, and Christine Sizemore provided critical support and encouragement of these studies. The preclinical studies were supported by grants AI31338 and HL077000 from the National Institutes of Health. The clinical studies were supported by the Aeras Global TB Vaccine Foundation with the financial assistance of the Bill & Melinda Gates Foundation.

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Abbreviations

CFU	Colony-forming units
DTH	Delayed-type hypersensitivity
MCMP	Major Cytoplasmic Membrane Protein of <i>Legionella pneumophila</i>
MSP	Major Secretory Protein of <i>Legionella pneumophila</i>
rBCG30	Recombinant BCG expressing and secreting the <i>M. tuberculosis</i> 30 kDa major secretory protein
r30	Recombinant <i>M. tuberculosis</i> 30 kDa major secretory protein

Figure Legends

Fig. 1. Plasmid pMTB30. This plasmid, which is derived from the mycobacterium-*E. coli* shuttle vector pSMT3, contains a full-length copy of the *M. tuberculosis* 30 kDa major secretory protein gene and its flanking regions including the promoter region. The inset is oriented opposite the direction of the plasmid-encoded promoter. Reproduced with permission from Reference 2.

Fig. 2. Hypothesized conceptual basis for recombinant BCG expressing *M. tuberculosis* major extracellular proteins. When a naive host is vaccinated with recombinant BCG, the organism is ingested by a host antigen presenting cell. Inside the phagosome, the recombinant BCG expresses and secretes a recombinant *M. tuberculosis* protein. The recombinant protein is processed and fragments of the protein are ultimately presented on the surface of the cell as MHC-peptide complexes. This induces a population of T cells able to recognize the MHC-peptide complexes. Later, when the host is infected with *M. tuberculosis*, the bacterium is ingested by host mononuclear phagocytes. Inside its phagosome in the host cell, *M. tuberculosis* expresses and secretes extracellular proteins including the one expressed and secreted by the recombinant BCG vaccine. Processing and presentation of this protein by the host cell results in the display of MHC-peptide complexes on the surface of the host cell identical to those earlier displayed on the antigen presenting cell as a result of the secretion of the same

protein by the recombinant BCG vaccine. The T cells induced by the recombinant BCG vaccine are thus able to recognize the MHC-peptide complexes on the surface of the infected host cell and to exert an antimicrobial effect against the host cell, activating it such that it can inhibit the multiplication of *M. tuberculosis* or lysing it so as to deny *M. tuberculosis* its preferred intracellular niche in which to multiply. Copyright 2000 Marcus A. Horwitz. Reproduced with permission.

Fig. 3. rBCG30 induces strong cutaneous DTH to r30. Guinea pigs were sham-immunized or immunized with r30 in adjuvant, parental BCG (Connaught [Conn] or Tice strain), or rBCG30 (Connaught or Tice strain), as indicated. Ten weeks later, the cutaneous DTH response to r30 was assayed. Data are the mean diameter of induration \pm SE. Animals immunized with rBCG30 developed significantly greater cutaneous DTH to r30 than animals immunized with BCG, which had baseline responses. Animals immunized with rBCG30 Tice, which secretes ~ three times as much r30 as rBCG30 Connaught, had the strongest DTH response to r30. Reproduced with permission from Reference 1.

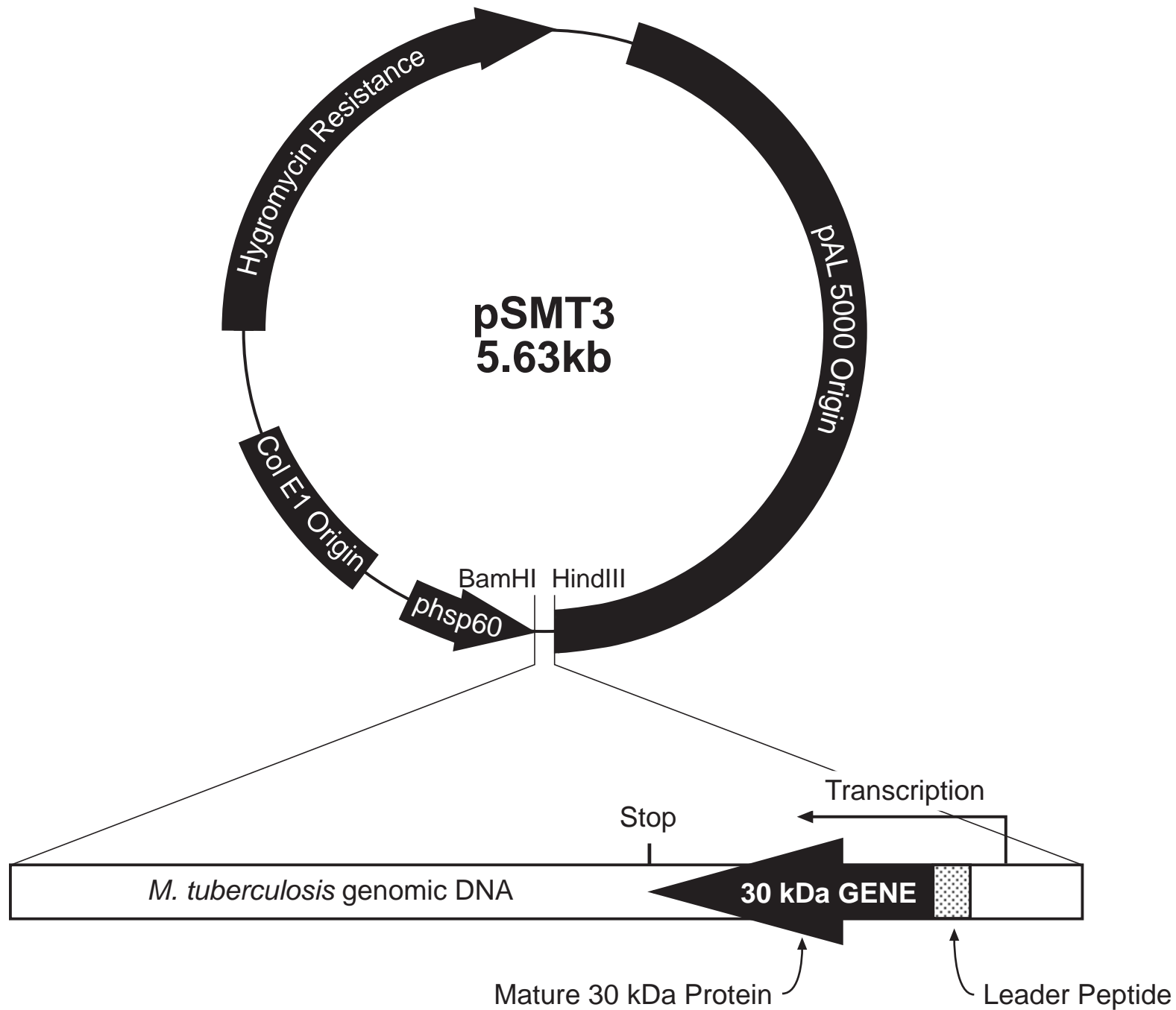
Fig. 4. rBCG30-immunized guinea pigs have a significantly lower burden of *M. tuberculosis* in the lung and spleen than BCG-immunized animals after aerosol challenge with *M. tuberculosis*. Guinea pigs were sham-immunized or immunized with r30 in adjuvant, parental BCG (Connaught [Conn] or Tice

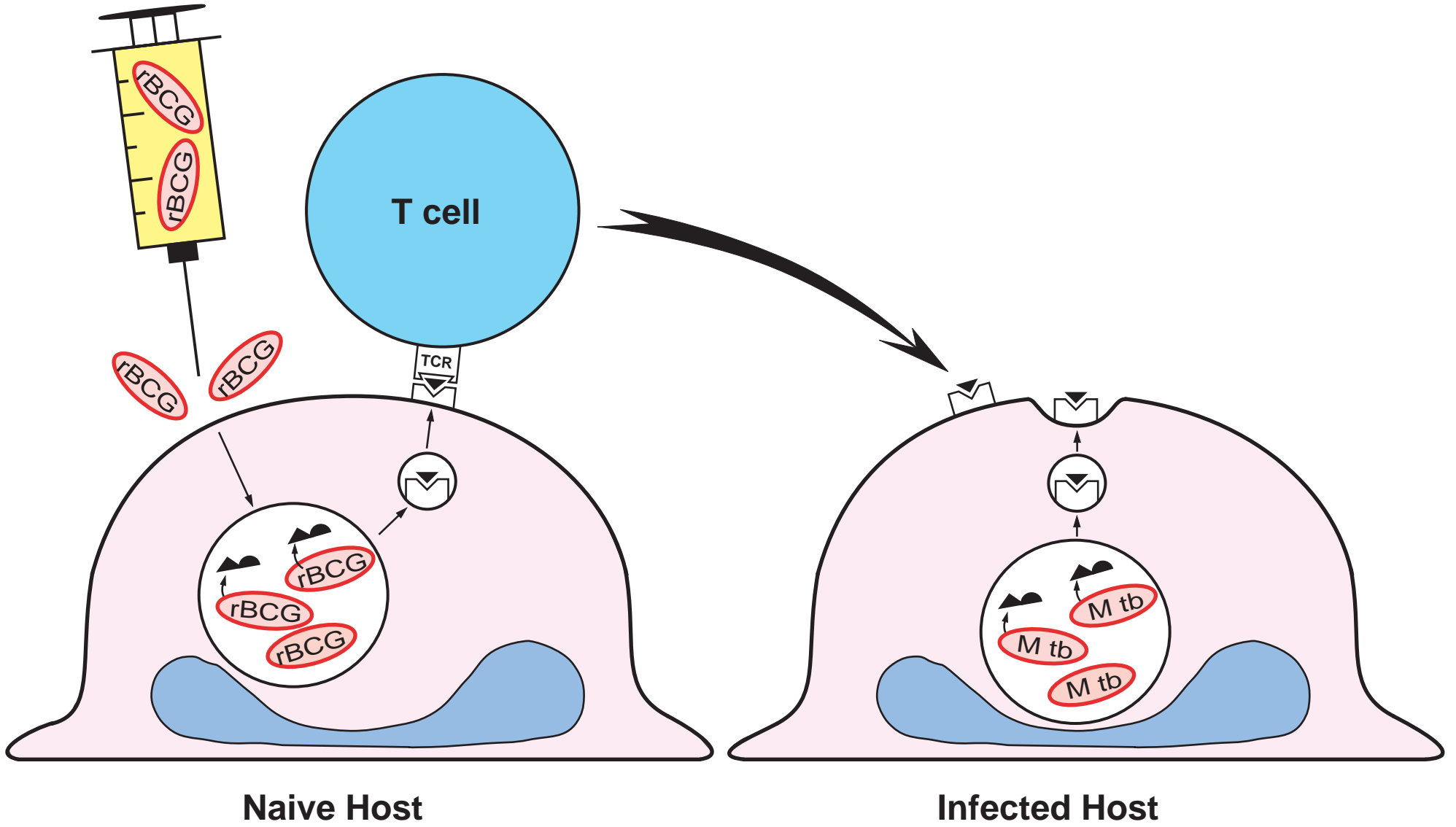
strain), or rBCG30 (Connaught or Tice strain), as indicated. Ten weeks later, the animals were challenged by aerosol with virulent *M. tuberculosis*. Ten weeks after challenge, the animals were euthanized and CFU assayed in the lung and spleen. Data are the mean CFU \pm SE. Animals immunized with BCG had significantly fewer CFU in their lungs and spleens than animals immunized with r30 or sham-immunized animals. Animals immunized with rBCG30 had significantly fewer CFU in their lungs and spleens than BCG-immunized animals. On average, rBCG30-immunized animals had 0.5 log fewer CFU in the lung and 1 log fewer CFU in the spleen than BCG-immunized animals. Reproduced with permission from Reference 1.

Fig. 5. 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. Ten weeks later, the animals were challenged with *M. tuberculosis* by aerosol. A group of uninfected animals served as controls. Sham-immunized animals died most rapidly. BCG-immunized animals survived significantly longer than sham-immunized animals. 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 from Reference 2.

Fig. 6. BCG and rBCG30 are cleared by guinea pigs at the same rate. Guinea pigs were immunized with BCG Tice or rBCG30 Tice. At various time intervals thereafter, animals in groups of three were euthanized and CFU assayed in the regional (inguinal) lymph nodes, lung, and spleen. Data in the large panels are geometric mean CFU \pm SE. Data in the inserts are the mean CFU \pm SE at the indicated time point between 10 and 26 weeks after immunization. Low numbers of bacteria persisted at all sites up to 26 weeks when the experiment was terminated. Reproduced with permission from Reference 2.

pMTB30





Naive Host

Infected Host

