Title: NOVEL LIVE RECOMBINANT BOOSTER VACCINE AGAINST TUBERCULOSIS

Abstract: Embodiments of the invention comprise an improved vaccine for generating an immune response and preventing or treating mycobacterial diseases such as tuberculosis in humans and animals. Embodiments of the invention also comprise a method for using the vaccine against such mycobacterial diseases.
NOVEL LIVE RECOMBINANT BOOSTER VACCINE AGAINST TUBERCULOSIS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of United States provisional patent application number 61/355,052, filed June 15, 2010, the entire contents of which are incorporated herein by reference.

STATEMENT OF GOVERNMENT SUPPORT

This invention was made with Government support of Grant No. AI031338 awarded by the National Institutes of Health. The U.S. government has certain rights in this invention.

1. Field of the Invention.

The present invention relates to methods and compositions of matter that are useful for preventing or reducing the possibility of infection caused by Mycobacterium tuberculosis, the agent of tuberculosis, and infection by other pathogenic strains of mycobacteria in humans and/or animals including Mycobacterium bovis and Mycobacterium leprae.

2. Background.

Around the world, intracellular bacteria are responsible for millions of deaths each year and untold suffering. Tuberculosis, caused by Mycobacterium tuberculosis, is a leading worldwide cause of death from an infectious disease, with millions of new cases and deaths reported each year. Initial infections of M. tuberculosis almost always occur through the inhalation of aerosolized particles as the pathogen can remain viable for weeks or months in moist or dry sputum. Although the primary site of the infection is in the lungs, the organism can also cause infection of the bones, spleen, meninges, and skin. Depending on the virulence of the particular strain and
the resistance of the host, the infection and corresponding damage to the tissue may be minor or extensive.

While *M. tuberculosis* is a significant pathogen, other species of the genus Mycobacterium also cause disease in humans and animals and are clearly within the scope of the present invention. For example, *M. bovis* is closely related to *M. tuberculosis* and is responsible for tubercular infections in domestic animals such as cattle, pigs, sheep, horses, dogs and cats. Further, *M. bovis* may infect humans via the intestinal tract, typically from the ingestion of raw milk. The localized intestinal infection eventually spreads to the respiratory tract and is followed shortly by the classic symptoms of tuberculosis. Another important pathogenic vector of the genus Mycobacterium is *M. leprae* which causes millions of cases of the ancient disease leprosy. Currently, there is no effective vaccine to prevent it. A vaccine to prevent leprosy would potentially have widespread use in endemic areas such as India and Brazil. Other species of this genus which cause disease in animals and humans include *M. kansasii, M. avium intracellulare, M. fortuitum, M. marinum, M. chelonei, M. africanum, M. ulcerans, M. microti,* and *M. scrofulaceum.* The pathogenic mycobacterial species frequently exhibit a high degree of homology in their respective DNA and corresponding protein sequences and some species, such as *M. tuberculosis* and *M. bovis* are highly related.

With regard to alveolar or pulmonary infections by *M. tuberculosis,* the guinea pig model closely resembles the human pathology of the disease in many respects. Accordingly, it is well understood by those skilled in the art that it is appropriate to extrapolate the guinea pig model of this disease to humans and other mammals. As with humans, guinea pigs are susceptible to tubercular infection with low doses of the aerosolized human pathogen *M. tuberculosis.* Unlike humans where the initial infection is usually controlled, guinea pigs consistently develop disseminated disease upon exposure to the aerosolized pathogen, facilitating subsequent analysis. Further, both guinea pigs and humans display cutaneous delayed-type hypersensitivity reactions characterized by the development of a dense mononuclear cell induration or
rigid area at the skin test site. Finally, the characteristic tuberculor lesions of humans and guinea pigs exhibit similar morphology including the presence of Langhans giant cells. As guinea pigs are more susceptible to initial infection and progression of the disease than humans, any protection conferred in experiments using this animal model provides a strong indication that the same protective immunity may be generated in man or other less susceptible mammals. Accordingly, for purposes of explanation only and not for purposes of limitation, the present invention will be primarily demonstrated in the exemplary context of guinea pigs as the mammalian host. Those skilled in the art will appreciate that the present invention may be practiced with other mammalian hosts including humans, mice and domesticated animals.

The only currently available vaccine, Mycobacterium bovis strain Bacille Calmette-Guerin (BCG), is of variable efficacy. Many studies have failed to demonstrate significant protection [see, e.g. Fine (1989). "The BCG story: lessons from the past and implications for the future." Rev Infect Dis 11 Suppl 2: S353-9]. A large carefully conducted meta-analysis has estimated the potency of BCG to be approximately 50% [see, e.g. Colditz, et al. (1994). "Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature." JAMA 271(9): 698-702]. Despite its variable efficacy, several hundred million doses of the BCG vaccine are still administered to humans each year. Hence, a better vaccine or a vaccine that improves the potency of BCG by even a small amount could have a tremendous impact on the disease incidence.

A previous study examined the use of M. tuberculosis major extracellular proteins for immunizing against tuberculosis [see, e.g. Horwitz, et al. (1995). "Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of Mycobacterium tuberculosis." Proc Natl Acad Sci USA 92(5): 1530-4]. Another study examined a prime-boost vaccination strategy for boosting the level of protective immunity conferred by a prime vaccine such as BCG or recombinant BCG [see, e.g. Horwitz, et al. (2005). "Enhancing the protective efficacy of Mycobacterium bovis BCG vaccination against tuberculosis by boosting with the
Mycobacterium tuberculosis major secretory protein." Infect Immun 73(8): 4676-83. In this study, the prime vaccine consisted of BCG or a recombinant BCG and the booster vaccine consisted of an M. tuberculosis major extracellular protein in adjuvant. Boosting BCG with such a vaccine enhanced the level of protective immunity conferred by BCG alone.


PLoS One 4(6): e5856). However, such vectors are difficult to produce because the viruses have to be grown in cell culture. Moreover, the efficacy of these virus-vectored vaccines as booster vaccines for BCG has not been high, especially with routes of administration other than intranasal. These vaccines have given little or no protection in the more challenging guinea pig model.

Therefore, a safe and effective vaccine against M. tuberculosis or other species of the genus Mycobacterium that is more potent than the currently available vaccines is sorely needed. There is also a need for a booster vaccine or a vaccine that can improve the potency of the currently available vaccines by even a small amount. The disclosure provided herein meets this need.
SUMMARY OF THE INVENTION

Current commercially available vaccines are of limited efficacy against pulmonary tuberculosis. The present disclosure provides a vaccine and method for preventing, reducing the possibility of or treating tuberculosis in humans and animals that is more potent than the current commercially available vaccines and methods in protecting against pulmonary tuberculosis and dissemination of bacteria to the spleen and other organs. The present disclosure also provides a vaccine and method for preventing, reducing the possibility of or treating leprosy and other mycobacterial diseases. Moreover, the present invention provides a booster vaccine that is surprisingly and unexpectedly more potent than a protein-in-adjuvant vaccine or an adenovirus-based vaccine. In addition, the present disclosure provides a vaccine that is easier and cheaper to manufacture than both virus-vectored vaccines, which must be grown in tissue culture cells and then purified, and protein-in-adjuvant vaccines, where the protein needs to be purified. The vaccine described in the present disclosure can simply be grown in broth culture - no purification is necessary.

The invention disclosed herein has a number of embodiments. A typical embodiment comprises a composition of matter that includes attenuated *Listeria monocytogenes* that expresses a *Mycobacterium tuberculosis* polypeptide, for example the 30kDa antigen 85B protein (SEQ ID NO: 4). In such compositions, the *Listeria monocytogenes* is attenuated by inactivation (e.g. via deletion) of one or more genes so that it does not express a functional protein such as a ActA protein (SEQ ID NO: 1) and/or a InlB protein (SEQ ID NO: 2). In certain embodiments of the invention, attenuated *Listeria monocytogenes* expresses prfA protein having a G155S substitution mutation (SEQ ID NO: 3). In some embodiments of the invention, the composition comprises one or more agents commonly used in vaccines such as a pharmaceutically acceptable carrier.

While the 30kDa antigen 85B protein (SEQ ID NO: 4) is a commonly discussed embodiment of the invention, other proteins, either alone, or in combination can be expressed in the attenuated *Listeria monocytogenes*. Such proteins include:
Mycobacterium tuberculosis 12kDa fragment of 16 kDa membrane protein (SEQ ID NO:5); Mycobacterium tuberculosis 14 kDa MPT53 protein (SEQ ID NO: 6); Mycobacterium tuberculosis 16 kDa MPT63 protein (SEQ ID NO: 7); Mycobacterium tuberculosis 23 kDa SOD protein (SEQ ID NO: 8); Mycobacterium tuberculosis 23.5 kDa MPT64 protein (SEQ ID NO: 9); Mycobacterium tuberculosis 24 kDa MPT51 protein (SEQ ID NO: 10); Mycobacterium tuberculosis 32 kDa antigen 85A protein (SEQ ID NO: 11); Mycobacterium tuberculosis 32 kDa antigen 85C protein (SEQ ID NO: 12); Mycobacterium tuberculosis 45 kDa MPT32 protein (SEQ ID NO: 13); Mycobacterium tuberculosis 58 kDa glutamine synthetase protein (SEQ ID NO: 14); Mycobacterium tuberculosis 71 kDa HSP 70 protein (SEQ ID NO: 15); Mycobacterium tuberculosis 10.4 kDa EsxH protein (SEQ ID NO: 16); Mycobacterium tuberculosis 14 kDa alpha crystalline homolog protein (SEQ ID NO: 17); Mycobacterium tuberculosis 47 kDa isocitrate lysate protein (SEQ ID NO: 18); Mycobacterium tuberculosis 7.6 kDa hypothetical protein (SEQ ID NO: 19); Mycobacterium tuberculosis 80kDa glcB protein (SEQ ID NO: 20) Mycobacterium tuberculosis 110 kDa can protein (SEQ ID NO: 21); and Mycobacterium tuberculosis 9.9 kDa ESAT-6 protein (SEQ ID NO: 22). A wide variety of combinations of proteins can be expressed in various embodiments of the invention. For example, in certain embodiments of the invention, one or more latency associated proteins (e.g. SEQ NOS: 17-19) are expressed in combination with one or more of the other proteins disclosed herein (e.g. SEQ NOS: 4, 6-16 and 20-22).

In certain embodiments of the invention, the Mycobacterium tuberculosis 30kDa antigen 85B protein is fused in frame with a heterologous protein sequence. Optionally, for example, the Mycobacterium tuberculosis 30kDa antigen 85B protein is coupled to a heterologous protein sequence comprising the N-terminal 100 amino acids of the ActA protein. The protein expression can further be controlled by constructing expression cassettes to include certain regulatory sequences. In one illustrative embodiment of the invention, the expression of the Mycobacterium tuberculosis 30kDa antigen 85B protein is controlled by an ActA promoter.
Another embodiment of the invention is a method of generating an immune response to a specific polypeptide, for example a Mycobacterium tuberculosis polypeptide such as the 30kDa antigen 85B protein (SEQ ID NO: 4). Such methods include immunizing a mammal with a composition of matter disclosed herein, for example one comprising attenuated Listeria monocytogenes constructed to express Mycobacterium tuberculosis 30kDa antigen 85B protein so that an antibody and/or a cellular immune response to Mycobacterium tuberculosis 30kDa antigen 85B protein is generated. In an illustrative embodiment of invention, the Listeria monocytogenes does not express a functional ActA protein (SEQ ID NO: 1); does not express a functional InlB polypeptide (SEQ ID NO: 2); expresses prfA protein having a G155S substitution mutation (SEQ ID NO: 3); and expresses Mycobacterium tuberculosis 30kDa antigen 85B protein (SEQ ID NO: 4). While the 30kDa antigen 85B protein (SEQ ID NO: 4) is a commonly discussed embodiment of the invention, other proteins, either alone, or in combination can be expressed in the attenuated Listeria monocytogenes to generate an immune response.

Those of skill in this art understand that the immunization methods disclosed herein can be combined with other methodological steps. For example, certain embodiments of the invention include the step of further comprising immunizing the mammal with Mycobacterium bovis strain Bacille Calmette-Guerin (BCG). Typically in these embodiments, the BCG is used in a primary immunization and the attenuated Listeria monocytogenes is used in a booster immunization. In such embodiments of the invention, the mammal can be immunized intradermally, intranasally, orally, subcutaneously, percutaneously, intramuscularly, intravenously, or by another conventional route of vaccine delivery.

Other objects, features and advantages of the present invention will become apparent to those skilled in the art from the following detailed description. It is to be understood, however, that the detailed description and specific examples, while indicating some embodiments of the present invention are given by way of illustration and not limitation. Many changes and modifications within the scope of the present
invention may be made without departing from the spirit thereof, and the invention includes all such modifications.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Referring now to the drawings and figures in which like reference numbers represent corresponding parts throughout:

Figure 1 is a graph illustrating measured diameters of induration (mm ± SE) of guinea pigs after being injected intradermally with 10 µg of purified recombinant *M. tuberculosis* 30 kDa major extracellular protein (Antigen 85B; r30). As described herein under Experiment 1, guinea pigs were first immunized with various vaccines and then tested for cutaneous delayed-type hypersensitivity (c-DTH) to purified recombinant *M. tuberculosis* 30 kDa major extracellular protein (r30).

Figure 2 is a graph illustrating splenic lymphocyte proliferation to r30, PPD, and media alone (RPMI). As described herein under Experiment 1, guinea pigs were first immunized with various vaccines and then assayed for splenic lymphocyte proliferation to purified recombinant *M. tuberculosis* 30 kDa major extracellular protein.

Figure 3 is a graph illustrating measured reciprocal antibody titer for r30. As described herein under Experiment 1, guinea pigs were first immunized with various vaccines and then assayed for antibody responses to purified recombinant *M. tuberculosis* 30 kDa major extracellular protein.

Figure 4 is a graph illustrating net weight gains or losses of guinea pigs after being challenged with an aerosol generated from a 7.5 ml single-cell suspension containing 6.3 x 10^4 colony-forming units (CFU) of *M. tuberculosis*. As described herein under Experiment 2, the guinea pigs were first immunized with various vaccines and then subsequently challenged with the aerosol containing *M. tuberculosis*.

Figure 5 is a graph illustrating assay measurements (mean log CFU ± SE) of colony forming units (CFU) of *M. tuberculosis* in the lungs and spleens of guinea
pigs. As described herein under Experiment 2, the guinea pigs were first immunized with various vaccinations and then subsequently challenged with an aerosol generated from a 7.5 ml single-cell suspension containing 6.3 x 10^4 colony-forming units (CFU) of *M. tuberculosis*.

Figure 6 illustrates cellular and humoral immune responses induced by boosting BCG-immunized animals with rLm/Mtb30 vaccines. Mice in groups of 4 were immunized intradermally with PBS (Sham) or BCG at week 0 and groups of BCG-immunized animals were boosted intradermally with one of various rLm/Mtb30 vaccines, with the rAd30 vaccine, or with r30 in adjuvant at weeks 3 and 6. At week 7, mice were anesthetized, bled and euthanized. Splenocytes were assayed for lymphocyte proliferation (A) or intracellular cytokine (INFgamma) expression in response to r30 (C and D) stratified as to CD4+ or CD8+ cells. Serum was assayed for immunoglobulin-G level in response to the r30 protein (B). Values represent mean ± SE.

Figure 7 illustrates assay measurements of colony forming units (CFU) of *M. tuberculosis* in the lungs and spleens of mice. As described herein under Experiment 4, the mice were first immunized with various vaccinations and then subsequently challenged with an aerosol generated from a 7.5 ml single-cell suspension containing 6.3 x 10^4 colony-forming units (CFU) of *M. tuberculosis*.

Figure 8 is a graph illustrating interferon-γ production in mice in response to *M. tuberculosis* antigens. As described herein under Experiment 5, mice were immunized with various vaccines and the splenocytes were stimulated with medium alone, the recombinant 30 kD protein (r30) or *M. tuberculosis* Purified Protein Derivative (PPD) for three days. The splenocyte supernatant fluid was collected and assayed for the level of IFNγ by ELISA.

Figure 9 illustrates the calculated the integrated MFI (iMFI) for cytokine-secreting CD4+ T-cells. As described herein under Experiment 5, mice were immunized with various vaccines and the splenocytes were stimulated with the mature recombinant 30 kD protein (r30) or a pool of three peptides of r30 (30p). The
cells were then stained for the cytokines IFNy, IL-2, and TNFa, and analyzed by multi-parameter flow cytometry.

**DETAILED DESCRIPTION OF THE INVENTION**

Unless otherwise defined, all terms of art, notations and other scientific terms or terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

In the following description of the typical embodiment, reference is made to the accompanying drawings which form a part hereof, and in which is shown by way of illustration a specific embodiment in which the invention may be practiced. It is to be understood that other embodiments may be utilized and structural changes may be made without departing from the scope of the present invention.

In one aspect of the present disclosure, a novel vaccine vector comprising attenuated *Listeria monocytogenes* is described. In one embodiment, a vector comprising attenuated *Listeria monocytogenes* is used by itself as a primary vaccine or vaccinating agent. In another embodiment, a vector comprising attenuated *Listeria monocytogenes* is used for delivering *M. tuberculosis* proteins as a booster vaccine for another tuberculosis (TB) vaccine, such as BCG or recombinant BCG. In a further embodiment, a vector comprising attenuated *Listeria monocytogenes* is used for delivering mycobacteria proteins, such as proteins from *Mycobacterium bovis* and *Mycobacterium leprae*, as a primary or booster vaccine or vaccinating agent. Unexpectedly, utilizing a vector comprising attenuated *Listeria monocytogenes* for delivering mycobacteria proteins as a booster vaccine induces greater protective
immunity than boosting with just the purified proteins in adjuvant or boosting with a recombinant adenovirus encoding the same proteins.

Attenuated *Listeria monocytogenes* can be used as vectors to deliver *M. tuberculosis* major extracellular proteins, which include but are not limited to the 30 kDa major secretory protein (Antigen 85B), 32A major secretory protein (Antigen 85A), 32B major secretory protein (Antigen 85C), the 23.5 kDa major secretory protein (a.k.a. MPT64), the 16 kDa major secretory protein, the 23 kDa subunit mass superoxide dismutase, the 58 kDa subunit mass glutamine synthetase, the 71 kDa subunit mass heat shock protein, the 12 kDa subunit mass exported fragment of the 16 kDa alpha-crystallin protein, and the 14 kDa secreted protein, etc. Such extracellular proteins have been shown to be immunoprotective against *M. tuberculosis* [see, e.g. Horwitz, *et al.* (1995). "Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis.*" *Proc Natl Acad Sci USA* 92(5): 1530-4).

In one exemplary implementation of the present disclosure, BCG is utilized as a first vaccine with a booster vaccine being a recombinant attenuated *Listeria monocytogenes* expressing the *M. tuberculosis* 30 kDa major secretory protein, a.k.a. Antigen 85B or r30 (rLm/Mtb30). In the Examples described herein, boosting with rLm/Mtb30 has been compared with boosting with purified *M. tuberculosis* 30 kDa major secretory protein in adjuvant or boosting with a recombinant Adenovirus expressing the *M. tuberculosis* 30 kDa major secretory protein. Surprisingly, this method of immunization is found to induce significantly greater protective immunity than boosting with just the purified *M. tuberculosis* protein in adjuvant or boosting with a recombinant adenovirus encoding the same protein.

In another aspect of the present disclosure, a composition of matter is described that is useful for preventing or reducing the possibility of infection caused by *Mycobacterium tuberculosis*, the agent of tuberculosis, or infection by other pathogenic strains of mycobacteria in humans and/or animals including *Mycobacterium bovis* and *Mycobacterium leprae*. Administration of the composition
of matter comprising attenuated *Listeria monocytogenes* expressing a major extracellular protein induces a cell-mediated immune response to the recombinant major extracellular protein. This subsequently protects against infection by *M. tuberculosis* or other mycobacterial diseases. In yet another aspect of the present disclosure, a vaccination strategy is described wherein an attenuated *Listeria monocytogenes* expressing *M. tuberculosis* proteins is administered by itself. In one embodiment, rLm/Mtb30 is administered by itself.

In a further aspect of the present disclosure, a vaccination strategy is described wherein an attenuated *Listeria monocytogenes* expressing *M. tuberculosis* proteins is administered as a booster vaccine following immunization with another TB vaccine. In one embodiment, rLm/Mtb30 is administered as a heterologous booster vaccine following immunization with another TB vaccine, such as BCG or a recombinant BCG expressing the same protein. For example, BCG or recombinant BCG is administered first, and after a period of time, the rLm/Mtb30 vaccine is administered one or more times. The initial vaccination may be with BCG or any recombinant strain of BCG overexpressing and secreting one or more *M. tuberculosis* major extracellular proteins, including but not limited to the 30 kDa major secretory protein (Antigen 85B), 32A major secretory protein (Antigen 85A), 32B major secretory protein (Antigen 85C), the 23.5 kDa major secretory protein (a.k.a. MPT64), the 16 kDa major secretory protein, the 23 kDa subunit mass superoxide dismutase, the 58 kDa subunit mass glutamine synthetase, the 71 kDa subunit mass heat shock protein, the 12 kDa subunit mass exported fragment of the 16 kDa alpha-crystallin protein, the 14 kDa secreted protein, etc. The subsequent vaccination would be with recombinant attenuated *Listeria monocytogenes* expressing the same *M. tuberculosis* protein.

The attenuated *Listeria monocytogenes* can be administered intradermally or by another route, e.g. intranasally, subcutaneously, percutaneously, intramuscularly, or even orally to a mammalian host. The vaccines or immunotherapeutic agents and methods for their use disclosed herein may be used to impart acquired immunity in a mammalian host against various intracellular pathogens, including but not limited to...

As illustrated above, the invention disclosed herein has a number of embodiments. A typical embodiment comprises a composition of matter that includes live attenuated Listeria monocytogenes that expresses a Mycobacterium tuberculosis polypeptide, for example the 30kDa antigen 85B protein (SEQ ID NO: 4). In such compositions, the Listeria monocytogenes is attenuated by inactivation (e.g. via deletion) of one or more genes so that it does not express a functional protein such as a ActA protein (SEQ ID NO: 1) and/or a InIB protein (SEQ ID NO: 2). In certain embodiments of the invention, attenuated Listeria monocytogenes expresses prfA protein having a G155S substitution mutation (SEQ ID NO: 3).

While the 30kDa antigen 85B protein (also called herein the M. tuberculosis 30 kDa major secretory protein r30) is a commonly discussed embodiment of the invention, other proteins, either alone, or in combination can be expressed in the attenuated Listeria monocytogenes (e.g. an attenuated Listeria monocytogenes of strain 10403S). Such proteins include: Mycobacterium tuberculosis 12kDa fragment of 16 kDa membrane protein (SEQ ID NO:5); Mycobacterium tuberculosis 14 kDa MPT53 protein (SEQ ID NO: 6); Mycobacterium tuberculosis 16 kDa MPT63 protein (SEQ ID NO: 7); Mycobacterium tuberculosis 23 kDa SOD protein (SEQ ID NO: 8); Mycobacterium tuberculosis 23.5 kDa MPT64 protein (SEQ ID NO: 9); Mycobacterium tuberculosis 24 kDa MPT51 protein (SEQ ID NO: 10); Mycobacterium tuberculosis 32 kDa antigen 85A protein (SEQ ID NO: 11); Mycobacterium tuberculosis 32 kDa antigen 85C protein (SEQ ID NO: 12); Mycobacterium tuberculosis 45 kDa MPT32 protein (SEQ ID NO: 13); Mycobacterium tuberculosis 58 kDa glutamine synthetase protein (SEQ ID NO: 14); Mycobacterium tuberculosis 71 kDa HSP 70 protein (SEQ ID NO: 15); Mycobacterium tuberculosis 10.4 kDa EsxH protein (SEQ ID NO: 16); Mycobacterium tuberculosis 14 kDa alpha crystalline homolog protein (SEQ ID NO: 17); Mycobacterium tuberculosis 18.5 kDa glutamine synthetase protein (SEQ ID NO: 18).
17); Mycobacterium tuberculosis 47 kDa isocitrate lysate protein (SEQ ID NO: 18); Mycobacterium tuberculosis 7.6 kDa hypothetical protein (SEQ ID NO: 19); Mycobacterium tuberculosis 80kDa glcB protein (SEQ ID NO: 20) Mycobacterium tuberculosis 110 kDa can protein (SEQ ID NO: 21); and Mycobacterium tuberculosis 9.9 kDa ESAT-6 protein (SEQ ID NO: 22). A wide variety of combinations of proteins can be expressed in various embodiments of the invention. For example, in certain embodiments of the invention, one or more latency associated proteins (e.g. SEQ NOS: 17-19) are expressed in combination with one or more of the other proteins disclosed herein (e.g. SEQ NOS: 4, 6-16 and 20-22).

In some embodiments of the invention, the composition comprises one or more agents used in vaccines such as a pharmaceutically acceptable carrier. Methods for formulating compositions of the invention for pharmaceutical administration are known to those of skill in the art. See, for example, Remington: The Science and Practice of Pharmacy, 19th Edition, Gennaro (ed.) 1995, Mack Publishing Company, Easton, PA. Typically the immunogenic agents used in the methods of the invention combined with at pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" is used according to its art accepted meaning and is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated into the compositions. A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Certain compositions of the invention can include an adjuvant. Immunologic adjuvants are commonly added to vaccines to stimulate the immune system's response to the target antigen, but do not in themselves confer immunity. Adjuvants can act in various ways in presenting an antigen to the immune system. A wide variety of
adjuvants are known in the art, see e.g. Handbook of Pharmaceutical Additives: An International Guide to More Than 6000 Products by Trade Name, Chemical, Function, and Manufacturer by Michael Ash and Irene Ash (1996). In addition, certain embodiments of the invention further comprising a buffer system, for example phosphate buffered saline.

In certain embodiments of the invention, a protein such as the *Mycobacterium tuberculosis* 30kDa antigen 85B protein is fused in frame with a heterologous protein sequence. Optionally, for example, the *Mycobacterium tuberculosis* 30kDa antigen 85B protein is coupled to a heterologous protein sequence comprising the N-terminal 100 amino acids of the ActA protein. The protein expression can further be controlled by constructing expression cassettes to include certain regulatory sequences. In one illustrative embodiment of the invention, the expression of the *Mycobacterium tuberculosis* 30kDa antigen 85B protein is controlled by an ActA promoter.

Another embodiment of the invention is a method of generating an antibody to a *Mycobacterium tuberculosis* polypeptide such as the 30kDa antigen 85B protein (SEQ ID NO: 4) by immunizing a mammal with a composition of matter disclosed herein, for example one comprising attenuated *Listeria monocytogenes* constructed to express this protein so that an antibody to a *Mycobacterium tuberculosis* 30kDa antigen 85B protein is generated. The term "mammal" as used herein refers to any organism classified as a mammal, including mice, rats, rabbits, dogs, cats, cows, horses and humans. In one embodiment of the invention, the mammal is a mouse. In another embodiment of the invention, the mammal is a human. In an illustrative embodiment of invention, the *Listeria monocytogenes* does not express a functional ActA protein (SEQ ID NO: 1); does not express a functional InlB polypeptide (SEQ ID NO: 2); expresses prfA protein having a G155S substitution mutation (SEQ ID NO: 3); and expresses *Mycobacterium tuberculosis* 30kDa antigen 85B protein (SEQ ID NO: 4). While the 30kDa antigen 85B protein (SEQ ID NO: 4) is a commonly discussed embodiment of the invention, other proteins, either alone, or in combination
can be expressed in the attenuated *Listeria monocytogenes* to generate an immune response.

Those of skill in this art understand that the immunization methods disclosed herein can be combined with other methodological steps. For example, certain embodiments of the invention include the step of further comprising immunizing the mammal with *Mycobacterium bovis* strain Bacille Calmette-Guerin (BCG). Typically in these embodiments, the BCG is used in a primary immunization and the attenuated *Listeria monocytogenes* is used in a booster immunization. In such embodiments of the invention, the mammal can be immunized intranasally, orally, subcutaneously, percutaneously, intramuscularly, or by another conventional route of vaccine delivery.

Antibodies generated by methodological embodiments of the invention have a number of uses. In one exemplary use in vivo, antibodies to an antigen such as a *Mycobacterium tuberculosis* protein are useful to prevent and/or diminish the severity of a disease such as tuberculosis. In yet another exemplary use, antibodies to a *Mycobacterium tuberculosis* protein such as 30kDa antigen 85B protein (SEQ ID NO: 4) can function as an essential element in a diagnostic assay. In particular, those of skill in this art understand that a wide variety of diagnostic assays use antigen specific antibodies to provide substantial beneficial information to medical personnel including ELISA assay, Western Assays, radioimmunoassays and the like. Such assays use antibodies specific for an antigen such as a protein expressed by a pathogenic organism to, for example, identify the presence of that organism in a sample. Examples of tuberculosis diagnosis using antibodies in ELISA systems are described in publications such as Radhakrishnan et al, *J Infect Dis*, 1991 163(3): 650-652 and Kashyap et al, *Clin DiagLab Immunol*. 2005 12(6): 752-758, the contents of which are incorporated by reference herein. In addition, a number of commercially available kits are known in the art such as the Clearview TB ELISA system. As is known in the art, antibodies useful in such assays can be obtained, for example, from the supernatants of hybridomas generated by conventional methods and/or affinity purified from human sera (see, e.g., Groen et al, *J Virol Methods*. 1989
Feb;23(2): 195-203, the contents of which are incorporated by reference). In this context, antibodies generated by the methods of the invention can be readily adapted for use in such assays.

Those skilled in the art will appreciate that the exemplary discussions of *M. tuberculosis* that are provided herein are in no way intended to limit the scope of the present invention to the treatment of *M. tuberculosis*. Similarly, the teachings herein are not limited in any way to the treatment of tubercular infections. On the contrary, this invention may be used to advantageously provide safe and effective vaccines and immunotherapeutic agents against the immunogenic determinants of any pathogenic agent expressing extracellular products and thereby inhibit the infectious transmission of those organisms.

### EXAMPLES

#### Materials and Methods

**A. BCG Strain (Wild-type *M. bovis* BCG Tice)**

This strain was maintained in 7H9 medium pH 6.7 (Difco) at 37°C in a 5% C0₂-95% air atmosphere as unshaken cultures. Cultures were sonicated once or twice weekly for 5 min in a sonicating water bath to reduce bacterial clumping, as described *(see, e.g. Horwitz, et al. (2000). "Recombinant bacillus calmette-guerin (BCG) vaccines expressing the Mycobacterium tuberculosis 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model." Proc Natl Acad Sci USA 97(25): 13853-8).*

**B. Recombinant Attenuated Listeria monocytogenes Vaccines**

1. rLm/Mtb30(01)

   a. Construction of rLm/Mtb30(01)

   rLm/Mtb30(01), an attenuated recombinant *Listeria monocytogenes* expressing the *M. tuberculosis* 30kDa major secretory protein (Mtb30), was
constructed using the attenuated *L. monocytogenes* host strain, LmAactA, an *L. monocytogenes* strain 10403S (serotype 1/2a) with a deletion of actA encoding one of virulence factors, ActA. The coding sequence for the mature peptide of the 30kDa major secretory protein was PCR amplified from the genomic DNA of the *M. tuberculosis* 37HRv strain. The DNA fragments were cloned into pZErO (Invitrogen) vector. The identity of the inserted 30kDa coding sequences was confirmed by nucleotide sequencing, and subcloned into the BamHI and Pad sites of the cloning vector pKB199, in such a way that the 30kDa coding sequence was fused to the listeriolysin O signal sequence (LLO) downstream of the hemolysin (hly) promoter of *L. monocytogenes*. The expression cassette of the LLO-30kDa fusion protein driven by the hly promoter was excised from the resultant vector and subsequently cloned into a site-specific integration vector pDP4189. The integration vector was transformed into SM10, the *E. coli* conjugation donor strain. Through conjugation, the plasmid was mobilized and transferred from its *E. coli* SM10 host into the recipient LmAactA strain. The conjugation mixture was selected on plates containing streptomycin and chloramphenicol. The LmAactA strain is insensitive to streptomycin since it is derived from the *L. monocytogenes* strain 10403S, a spontaneous mutant resistant to streptomycin. *E. coli* SM10 is sensitive to streptomycin, therefore was not able to grow on the selective plate. The plasmid pDP4189 carries the chloramphenicol resistance gene and is unable to replicate in Listeria. Under the selection pressure from chloramphenicol, the plasmid pDP4189/Mtb30 integrated in the 3' end of an arginine tRNA gene on the chromosome of LmAactA strain. The resultant recombinant LmAactA strain, rLm/Mtb30(01), carries a single copy of the 30kDa expression cassette and is stable in the absence of antibiotic selection.

b. Protein expression of Mtb30 in broth.

The expression of 30kDa major secretory protein by rLm/Mtb30 was tested in broth. A single colony of rLm/Mtb30(01) was inoculated into 3 ml Brain Heart
Infusion (BHI) medium containing streptomycin and chloramphenicol and the bacteria were grown overnight at 37°C with shaking. The overnight culture was inoculated into 40 ml fresh MOPS-buffered BHI medium containing streptomycin and chloramphenicol at an initial optical density (OD) of 0.05 at 540 nm. The culture was grown until late logarithmic phase at 37°C with vigorous shaking before being harvested by centrifugation. The supernate was passed through a 0.2 µm filter membrane, and proteins in the culture filtrate were precipitated by trichloroacetic acid. The expression of the 30kDa major secretory protein was analyzed by Western blotting using rabbit polyclonal antibody against the 30kDa major secretory protein. It was found that the antibody reacted specifically with a protein band of 30 kDa, which was absent from the LmAactA parental strain. This confirmed that the recombinant rLm/Mtb30 expresses the 30kDa protein.

c. Protein expression of Mtb 30 by rLm/Mtb30(01) in human macrophages

THP-1 cells were differentiated into a monolayer on a 24-well plate (2 x 10^5 cells/well) in the presence of 100 nM PMA (phorbol 12-myristate 13-acetate) and in the absence of antibiotics. The rLm/Mtb30(01) culture was grown to late logarithmic phase (OD of 1.0 at 540 nm) in BHI broth and used to infect the THP-1 cell monolayer at a multiplicity of infection (MOI) of 50:1. After infection for 1 h at 37°C, the monolayer was washed twice with RPMI to remove extracellular bacteria and then treated with 1 ml medium containing gentamicin at a final concentration of 10 µg ml\(^{-1}\) to kill any remaining extracellular bacteria. At 24 h post-infection, cells were harvested, washed once with PBS, and boiled for 7 min in Laemmli buffer before analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. It shows that bands of ~30kDa in size were detected in samples of macrophages infected by two clones of rLm/Mtb30(01), but not by the parental rLmAactA strain. These two clones (#8 and #10) were amplified in BHI broth and used for animal experiments.
2. rLm/Mtb30(02) (Codon Optimized)
   rLm/Mtb30(02), rLmAactA expressing the 30kDa protein codon-optimized for higher level of protein expression in L. monocytogenes (30kDa(C-O)), was constructed using a method similar to that described above for rLm/Mtb30(01). The DNA sequence for a codon-optimized version of the 30kDa major secretory protein (30kDa(C-O)) was synthesized by DNA2.0 (Menlo Park, CA).

3. rLmAactAAinlB/Mtb30 (rLm/Mtb30(l 1))
   To further reduce the toxicity of the LmAactA vector, a second generation of rLm/Mtb30 vaccines were constructed using a L. monocytogenes with deletions in actA and inlB (LmAactAAinlB) as a vector. Deletions in actA and inlB retain the potency and diminish the toxicity of the Lm vector in vivo [see, e.g. Brockstedt, et al. (2004). "Listeria-based cancer vaccines that segregate immunogenicity from toxicity." Proc Natl Acad Sci USA 101(38): 13832-7]. It was shown that the 30kDa protein was readily detected by Coumassie blue staining in broth cultures of the vaccine and in extracts of macrophages infected with rLm/Mtb30(l 1).

4. rLmAactAAinlB/ActAN100-Mtb30 (rLm/Mtb30(03)(04))
   It has been reported that the actA promoter has a higher activity than hly promoter in vivo and proteins fused with 100 amino acids of the N-terminus of ActA are highly immunogenic. Therefore to enhance the in vivo expression of 30kDa protein by the rLm/Mtb30 vaccines, the 30kDa major secretory protein was fused with the N-terminal 100 amino acids of ActA (ActAN100) under the control of the actA promoter and introduced the fusion protein expression cassette into the second generation of the rLmAactAAinlB vector. The following 2 additional second generation of rLm/Mtb30 vaccines have been constructed:
   a. rLmAactAAinlB/ActAN100-Mtb30 (03)
   b. rLmAactAAinlB/ActAN100-Mtb30-SL8 (04)
It has been shown that these second generation rLm/Mtb30 vaccines express the 30kDa major secretory protein in broth culture and in human macrophages at levels similar to that of the first generation rLm/Mtb30 vaccines. The safety of these vaccines in animals are further being tested.

5. \textit{rLmAactAAinlBAuvrABprfA*/Mtb30 (12)(07)(08)}

To further enhance the immunogenicity of the rLm/Mtb30 vaccines, the following third generation rLm/Mtb30 vaccines expressing high amounts of the 30 kDa protein have been constructed and characterized.

- \textit{rLmactAAinlBAuvrABprfA*/Mtb30 (12)}
- \textit{rLmactAAinlBAuvrABprfA*/ActAN100-Mtb30 (07)}
- \textit{LmactAAinlBAuvrABprfA*/ActAN100-Mtb30-SL8 (08)}

The third generation rLm/Mtb30 vaccines were constructed using as a vector an LmAactAAinlB with an additional deletion in uvrAB and a single mutation (G155S) in prfA (LmAactAAinlBAuvrABprfA*, or Lm/prfA*) provided by Aduro BioTech. The deletion in uvrAB was designed to facilitate inactivation of the rLm/prfA* vaccines by UV light (Brockstedt et al. 2005) and the prfA* mutation resulted in constitutive expression of the downstream genes, including actA and hly (see, e.g., Lauer, et al. (2008). "Constitutive Activation of the PrfA regulon enhances the potency of vaccines based on live-attenuated and killed but metabolically active Listeria monocytogenes strains." \textit{Infect Immun} 76(8): 3742-53; Yan, et al. (2008). "Selected prfA* mutations in recombinant attenuated Listeria monocytogenes strains augment expression of foreign immunogens and enhance vaccine-elicited humoral and cellular immune responses." \textit{Infect Immun} 76(8): 3439-50). It was shown that all three rLm/prfA*/MtbSO vaccines expressed the 30kDa fusion proteins at significantly higher levels in broth than the corresponding vaccines derived from the first and second generation Lm vectors (rLmAactA and rLmAactAAinlB).

C. **Purified \textit{M. tuberculosis} 30 kDa Major Secretory Protein**

D. Replication-deficient adenovirus expressing the 30 kDa major secretory protein: generation of strains and recombinant protein expression

The replication-deficient recombinant adenovirus strain that expresses the 30 kDa major secretory protein of M. tuberculosis (Mtb30) was constructed using an AdenoVator system (Q Biogen). The Mtb30 coding sequence was first cloned into a transfer vector of pAdenoVator-CMV5 downstream of a modified immediate-early promoter of cytomegalovirus (CMV5), which allows for the production of high levels of heterologous proteins in mammalian cells. The transfer vector contains a kanamycin-resistance gene that allows for selection of recombinant adenoviral DNAs. The transfer vector containing the Mtb30 coding sequence was then co-transformed into E. coli together with an adenoviral plasmid DNA with deletions in viral early genes E1 and E3, pAdenoVatorAEI/E3. The E1 but not the E3 gene is essential for adenovirus growth in mammalian cells. Thus the recombinant adenoviruses with E1 and E3 deletions are replication-deficient and can grow only on cells that express the E1 gene. Through homologous recombination between the transfer vector and the adenoviral plasmid DNA in E. coli, the Mtb30 gene driven by the CMV5 promoter was introduced into the deleted E1 region of the adenoviral plasmid DNA. Recombinant adenoviral DNAs were selected with kanamycin and confirmed by restriction enzyme analysis. A positive recombinant adenoviral DNA that was confirmed to contain the appropriate insert, pAdvAEIE3/Mtb30, was linearized with
restriction enzyme (Pad) and transfected into mammalian cells (293A) that express the E1 protein. The resultant replication-deficient recombinant adenoviruses were plaque purified and amplified in 293A cells for up to 4 passages. The expression of Mtb30 by the recombinant adenovirus, AdvAEIE3/Mtb30, was confirmed by Western blotting using a rabbit polyclonal antibody to Mtb30. The replication-deficient recombinant adenovirus stock was prepared from 6 x 10^8 293A cells and purified by two rounds of ultracentrifugation on CsCl gradients. The amount of virus particles in the virus stock was measured by assessing the DNA content of lysed virus in solution and utilizing the extinction coefficient of 1.1 x 10^{12} virus particles per OD_{260} unit. The amount of infectious virus in the virus stock was measured by determining the tissue culture infectious dose 50 (TCID_{50}) in 293A cells. The purified viral particles were stored at -80°C in 20 mM Tris, pH 8.0, 25 mM NaCl, 2.5% glycerol.

E. Animal Models

The studies of the efficacy of the vaccines utilized guinea pigs because the guinea pig model is especially relevant to human tuberculosis clinically, immunologically, and pathologically. In contrast to the mouse and rat, but like the human, the guinea pig a) is susceptible to low doses of aerosolized M. tuberculosis; b) exhibits strong cutaneous delayed-type hypersensitivity (DTH) to tuberculin; and c) displays Langhans giant cells and caseation in pulmonary lesions. However, whereas only about 10% of immunocompetent humans who are infected with M. tuberculosis develop active disease over their lifetime (half early after exposure and half after a period of latency), infected guinea pigs always develop early active disease. While guinea pigs differ from humans in this respect, the consistency with which they develop active disease after infection with M. tuberculosis is an advantage in trials of vaccine efficacy.

Additional immunology studies including studies requiring special immunology reagents were conducted in C57BL/6 mice.
F. Preparation of Primary Vaccination

Aliquots were removed from logarithmically growing wild-type or recombinant BCG cultures, and the bacteria were pelleted by centrifugation at 3,500 x g for 15 min. The bacteria were then washed with 1 x phosphate buffered saline (1 x PBS, 50 mM sodium phosphate pH 7, 150 mM sodium chloride) and resuspended at a final concentration of 1 x 10^4 colony forming units per ml in 1 x PBS. The immunization inoculum contained 1,000 viable wild-type or recombinant BCG bacteria in a total volume of 100 µl.

G. Preparation of Booster Vaccination

1. rLm/Mtb30.

The various rLm/Mtb30 vaccines were grown in broth. Aliquots were removed from late logarithmically growing rLm/Mtb30 cultures and the bacteria were pelleted by centrifugation at 3,500 x g for 15 min. The bacteria were then washed once with 1 x phosphate buffered saline (1 x PBS, 50 mM sodium phosphate pH 7, 150 mM sodium chloride) and re-suspended at a final concentration of 2x10^9 colony forming units per ml in 1x sterile PBS. The stock was stored at -80°C, and the titer was checked periodically by plating serial dilutions of the stock on BHI plates. No significant titer loss was found over the period of storage. Before each use in animals, one vial of rLm/Mtb30 was thawed immediately at 37°C, diluted in sterile saline to a final concentration of 2 x 10^7 colony forming units per ml in 1x sterile PBS and kept on ice until use. The rLm/Mtb30 was administered intradermally at a dose of 1 x 10^6 bacteria in a total of 50 µL per animal.

2. r30 in adjuvant

100 µg of the r30 protein was mixed with Syntex Adjuvant Formulation (SAF) as described in Horwitz et al. 1995 (see, e.g. Horwitz, et al. (1995). "Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of Mycobacterium tuberculosis." Proc Natl Acad Sci USA 92(5): 1530-4).
3. rAd/Mtb30

The rAd/Mtb30 stock was thawed on ice, diluted in sterile PBS to a final concentration of $1 \times 10^{11}$ viral particles per mL and administered intradermally at a dose of $1 \times 10^{11}$ viral particles in a total 50 µL per animal.

Experiments

Experiment 1: Immunogenicity of rLm/Mtb30 in Prime-Boost Vaccination Regimen

Specific-pathogen free 250-300g outbred male Hartley strain guinea pigs from Charles River Breeding Laboratories, in groups of 6, were immunized intradermally as follows:

- **Group A**: Sham
- **Group B**: BCG Tice Parental Control (10³ CFU) at Week 0
- **Group C**: BCG Tice Parental Control (10³ CFU) at Week 0 and 100 µg of r30 in SAF adjuvant at Week 4
- **Group D**: BCG Tice Parental Control (10³ CFU) at Week 0 and rAd/Mtb30 at Week 4
- **Group E**: BCG Tice Parental Control (10³ CFU) at Week 0 and Lm Empty Vector at Week 4
- **Group F**: BCG Tice Parental Control (10³ CFU) at Week 0 and rLm/Mtb30 at Week 4
- **Group G**: BCG Tice Parental Control (10³ CFU) at Week 0 and rLm/Mtb30 C-0 at Week 4

At week 8, animals were tested for cutaneous delayed-type hypersensitivity (c-DTH) to r30, and after the skin test was assessed, the animals were euthanized for assay of splenic lymphocyte proliferation and antibody responses to r30.
A. Cutaneous Delayed-type Hypersensitivity (DTH) to Purified Recombinant M. tuberculosis 30 kDa Major Extracellular Protein (r30)

Guinea pigs were shaved over the back and injected intradermally with 10 µg of purified recombinant \textit{M. tuberculosis} 30 kDa major extracellular protein (r30) in 100 µl phosphate buffered saline. After 24 h, the diameter of erythema and induration was measured. Induration is most reflective of a c-DTH response. The results are summarized in Table 1 and Figure 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Test Antigen</th>
<th>Erythema (mm ± SE)</th>
<th>Induration (mm ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sham</td>
<td>r30</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>B</td>
<td>BCG</td>
<td>r30</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>C</td>
<td>BCG + r30</td>
<td>r30</td>
<td>19.6 ± 1.9</td>
<td>19.6 ± 1.9</td>
</tr>
<tr>
<td>D</td>
<td>BCG + rAd/Mtb30</td>
<td>r30</td>
<td>19.7 ± 1.4</td>
<td>19.7 ± 1.4</td>
</tr>
<tr>
<td>E</td>
<td>BCG + Lm Vector</td>
<td>r30</td>
<td>1.2 ± 0.7</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>F</td>
<td>BCG + rLm/Mtb30</td>
<td>r30</td>
<td>13.3 ± 1.1</td>
<td>13.3 ± 1.1</td>
</tr>
<tr>
<td>G</td>
<td>BCG + rLm/Mtb30 C-O</td>
<td>r30</td>
<td>12.3 ± 1.0</td>
<td>6.7 ± 3.1</td>
</tr>
</tbody>
</table>

These results showed that the animals immunized with the parental BCG Tice strain (Group F) and the sham-immunized animals (Group J) had no erythema and induration upon testing with the \textit{M. tuberculosis} 30 kDa major secretory protein r30.
In contrast, animals immunized with BCG and boosted with purified r30 in adjuvant or with recombinant vaccines expressing r30, including both recombinant adenovirus and recombinant *L. monocytogenes* all developed significantly increased levels of both erythema and, more importantly, induration in response to skin-testing with r30. Boosting with the empty *L. monocytogenes* vector did not induce a significant c-DTH response (0 induration). Both rLm/Mtb30 and rLm/Mtb30C-O induced a significant c-DTH response.

**B. Splenic Lymphocyte Proliferation to Purified Recombinant M. tuberculosis 30 kDa Major Extracellular Protein (r30)**

Immediately after skin-testing, the animals were euthanized, their spleens removed, and splenic lymphocyte proliferation to r30 (100 µg/well) and PPD (10 µg/well) assayed. The results are shown in Table 2 and Figure 2.

As expected, all but the sham-immunized animals reacted to the positive control antigen PPD. The sham and BCG-immunized animals had low lymphocyte proliferative activity. Animals immunized with BCG and boosted with either rAd/Mtb30 or the Lm vector had modest lymphocyte proliferative activity. Boosting BCG-immunized animals with r30 markedly increased lymphocyte proliferative activity approximately 4-fold vs. BCG-immunized animals. Boosting BCG-immunized animals with rLm/Mtb30 or rLm/Mtb 30 C-0 also markedly increased splenic lymphocyte proliferative activity by ~7-fold and 4-fold, respectively vs. BCG immunized animals.

Thus, boosting with recombinant *L. monocytogenes* expressing the *M. tuberculosis* 30 kDa major secretory protein markedly increased splenic lymphocyte proliferation to r30 in guinea pigs.
Table 2: Splenic Lymphocyte Proliferation - Experiment 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Test Antigen</th>
<th>r30 (Mean CPM±SE)</th>
<th>PPD (Mean CPM±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sham</td>
<td>r30</td>
<td>328 ± 102</td>
<td>259 ± 111</td>
</tr>
<tr>
<td>B</td>
<td>BCG</td>
<td>r30</td>
<td>194 ± 52</td>
<td>3032 ± 682</td>
</tr>
<tr>
<td>C</td>
<td>BCG + r30</td>
<td>r30</td>
<td>774 ± 188</td>
<td>5483 ± 1164</td>
</tr>
<tr>
<td>D</td>
<td>BCG + rAd/Mtb30</td>
<td>r30</td>
<td>527 ± 137</td>
<td>3620 ± 609</td>
</tr>
<tr>
<td>E</td>
<td>BCG + Lm Vector</td>
<td>r30</td>
<td>544 ± 94</td>
<td>2582 ± 454</td>
</tr>
<tr>
<td>F</td>
<td>BCG + rLm/Mtb30</td>
<td>r30</td>
<td>1331 ± 249</td>
<td>2800 ± 505</td>
</tr>
<tr>
<td>G</td>
<td>BCG + rLm/Mtb30 C-O</td>
<td>r30</td>
<td>851 ± 155</td>
<td>1732 ± 251</td>
</tr>
</tbody>
</table>

C. Antibody Responses to Purified Recombinant M. tuberculosis 30 kDa Major Extracellular Protein (r30)

Immediately after skin-testing, the animals were euthanized and their serum obtained. The serum was tested for antibody to r30 using an ELISA assay. The results are shown in Table 3 and Figure 3.
The results showed that sham-immunized animals and animals immunized only with BCG had negligible antibody titers. In contrast, animals immunized first with BCG and later boosted with r30 in adjuvant or rAd/Mtb30 had high antibody titers. Animals immunized first with BCG and later boosted with rLm/Mtb30 or rLm/Mtb30C-O had low antibody titers, perhaps reflecting the fact that the rLm produces r30 in the cytoplasm of host cells where it is likely processed such that the protein is not available for presentation to B cells.

**Experiment 2: Protective Efficacy of rLm/Mtb30 in a Prime-Boost Vaccination Regimen in Guinea Pigs**

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Test Antigen</th>
<th>Reciprocal Titer (Geometric mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sham</td>
<td>r30</td>
<td>157</td>
</tr>
<tr>
<td>B</td>
<td>BCG</td>
<td>r30</td>
<td>(\leq 125)</td>
</tr>
<tr>
<td>C</td>
<td>BCG + r30</td>
<td>r30</td>
<td>35,919</td>
</tr>
<tr>
<td>D</td>
<td>BCG + rAd/Mtb30</td>
<td>r30</td>
<td>14,254</td>
</tr>
<tr>
<td>E</td>
<td>BCG + Lm Vector</td>
<td>r30</td>
<td>140</td>
</tr>
<tr>
<td>F</td>
<td>BCG + rLm/Mtb30</td>
<td>r30</td>
<td>223</td>
</tr>
<tr>
<td>G</td>
<td>BCG + rLm/Mtb30C-O</td>
<td>r30</td>
<td>(\leq 125)</td>
</tr>
</tbody>
</table>
Specific-pathogen free 250-300g outbred male Hartley strain guinea pigs from Charles River Breeding Laboratories, in groups of 15 (except for the sham group, which had 9 animals), were immunized intradermally as follows:

- **Group A:** Sham
- **Group B:** BCG Tice Parental Control (10^5 CFU) at Week 0
- **Group C:** BCG Tice Parental Control (10^5 CFU) at Week 0 and 100 µg of r30 in SAF adjuvant at Week 4
- **Group D:** BCG Tice Parental Control (10^5 CFU) at Week 0 and 100 µg of r30 in SAF adjuvant at Weeks 4 and 8
- **Group E:** BCG Tice Parental Control (10^5 CFU) at Week 0 and rAd/Mtb30 at Week 4
- **Group F:** BCG Tice Parental Control (10^5 CFU) at Week 0 and rAd/Mtb30 at Weeks 4 and 8
- **Group G:** BCG Tice Parental Control (10^5 CFU) at Week 0 and rLm/Mtb30 at Week 4
- **Group H:** BCG Tice Parental Control (10^5 CFU) at Week 0 and rLm/Mtb30 at Weeks 4 and 8

Twenty weeks after immunization, all animals were challenged with an aerosol generated from a 7.5 ml single-cell suspension containing 6.3 x 10^4 colony-forming units (CFU) of *M. tuberculosis*. (Prior to challenge, the challenge strain, *M. tuberculosis* Erdman strain (ATCC 35801), had been passaged through outbred guinea pigs to maintain virulence, cultured on 7H11 agar, subjected to gentle sonication to obtain a single cell suspension, and frozen at -70°F). This relatively high dose aerosol dose delivered ~75 live bacilli to the lungs of each animal. The airborne route of infection was used because this is the natural route of infection for pulmonary tuberculosis. A large dose was used so as to induce measurable clinical illness in 100% of control animals within a relatively short time frame (10 weeks). Afterwards, guinea pigs were individually housed in stainless steel cages contained
within a laminar flow biohazard safety enclosure and allowed free access to standard laboratory chow and water. The animals were observed for illness and weighed weekly for 10 weeks and then euthanized. The right lung and spleen of each animal was removed and cultured for CFU of *M. tuberculosis* on Middlebrook 7H11 agar for two weeks at 37°C, 5% CO₂-95% air atmosphere. The results were as follows:

A. Deaths

Four of the 9 sham-immunized animals died before the end of the experiment between weeks four and nine after challenge. Two of 15 animals in the group immunized first with BCG and then twice boosted with protein in adjuvant died in the last week of the experiment. All of the animals in the remaining groups survived until the end of the experiment.

B. Net Weight Gain (Loss) after Challenge

Animals in the sham-immunized group lost weight over the course of the experiment, with a mean net weight loss by the end of the experiment of 173 grams for the surviving animals (Fig. 4). Animals in the remaining groups maintained their weights but did not gain an appreciable amount of weight.

C. Organ Burden

The results of the assay for CFU in the lungs and spleens are shown in Table 4 and Figure 5.
Table 4: CFU in Lungs and Spleens - Experiment 2

High Dose Challenge; 20 week immunization-Challenge Interval

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Lung (Mean Log CFU ± SE)</th>
<th>Spleen (Mean Log CFU ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sham</td>
<td>7.28 ± .22</td>
<td>5.59± 0.41</td>
</tr>
<tr>
<td>B</td>
<td>BCG</td>
<td>6.13 ± .20</td>
<td>4.13± 0.31</td>
</tr>
<tr>
<td>C</td>
<td>BCG + r30 x1</td>
<td>6.09 ± .22</td>
<td>4.39± 0.37</td>
</tr>
<tr>
<td>D</td>
<td>BCG + r30 x2</td>
<td>6.31 ± .13</td>
<td>4.52± 0.36</td>
</tr>
<tr>
<td>E</td>
<td>BCG + rAd/Mtb30 x 1</td>
<td>5.93 ± .22</td>
<td>4.34± 0.28</td>
</tr>
<tr>
<td>F</td>
<td>BCG + rAd/Mtb30 x 2</td>
<td>6.18 ± .20</td>
<td>4.13± 0.32</td>
</tr>
<tr>
<td>G</td>
<td>BCG + rLm/Mtb30 x 1</td>
<td>5.86 ± .18</td>
<td>3.98± 0.24</td>
</tr>
<tr>
<td>H</td>
<td>BCG + rLm/Mtb30 x 2</td>
<td>5.97 ± .21</td>
<td>3.73± 0.41</td>
</tr>
</tbody>
</table>

These results showed that animals immunized with BCG had much lower CFU in the lungs and spleens than the sham immunized animals. Animals immunized first with BCG and then boosted with r30 in adjuvant or rAd/Mtb30 once or twice had similar CFU to BCG immunized animals in this high dose challenge experiment with a 20-week immunization - challenge interval. Animals immunized first with BCG and then boosted with rLm/Mtb30 once or twice had fewer CFU in the lung and spleen than BCG immunized animals in all cases. Animals immunized with BCG and boosted once with rLm/Mtb30 had 0.3 log fewer CFU in the lungs and 0.15 log fewer CFU in the spleen than animals immunized with only BCG. Animals immunized with BCG and boosted twice with rLm/Mtb30 had 0.2 log fewer CFU in the lungs and 0.4 log fewer CFU in the spleen than animals immunized with only BCG.

Thus, in an experiment in which animals were challenged with a relatively high dose of aerosolized *M. tuberculosis*, such that nearly half of the sham-immunized animals died before the conclusion of the experiment, boosting BCG-immunized...
animals with rLm/Mtb30 improved protection against *M. tuberculosis* aerosol challenge.

**Experiment 3: Immunogenicity of rLm/Mtb30 in the mouse model**

Specific-pathogen free 6-8 week male C57BL/6 mice from Charles River Breeding Laboratories, in groups of 4, were immunized intradermally as follows:

- **Group A:** Sham
- **Group B:** BCG Tice Parental Control (10⁶ CFU) at Week 0
- **Group C:** BCG Tice Parental Control (10⁶ CFU) at Week 0 and 100 µg of r30 in SAF adjuvant at Weeks 3 and 6
- **Group D:** BCG Tice Parental Control (10⁵ CFU) at Week 0 and rLm/30(01) (rLmAactA/LLO-Mtb30) at Weeks 3 and 6
- **Group E:** BCG Tice Parental Control (10⁵ CFU) at Week 0 and rLm/30(03) (rLmAactAainlB/ActA-Mtb30) at Weeks 3 and 6
- **Group F:** BCG Tice Parental Control (10⁵ CFU) at Week 0 and rLm/30(04) (rLmAactAainlB/ActA-Mtb30-SL8) at Weeks 3 and 6
- **Group G:** BCG Tice Parental Control (10⁵ CFU) at Week 0 and rLm/30(07) (rLmAactAainlBAuvrABprfA*/ActA-Mtb30) at Weeks 3 and 6
- **Group H:** BCG Tice Parental Control (10⁵ CFU) at Week 0 and rLm/Mtb30(08) (rLmAactAainlBAuvrABprfA*/ActA-Mtb30-SL8) at Weeks 3 and 6
- **Group I:** BCG Tice Parental Control (10⁵ CFU) at Week 0 and rLm/Mtb30(11) (rLmAactAainlB/Mtb30) at Weeks 3 and 6
- **Group J:** BCG Tice Parental Control (10⁵ CFU) at Week 0 and rLm/Mtb30(12) (rLmAactAainlB AuvrABprfA*/Mtb30) at Weeks 3 and 6
- **Group K:** BCG Tice Parental Control (10⁵ CFU) at Week 0 and rLm/Mtb30(12) (rLmAactAainlB AuvrABprfA*/Mtb30) at Weeks 3 and 6
rAd/Mtb30 at Weeks 3 and 6

One week after the last immunization (Week 7), animals were anesthetized with Ketamine/Xylazine, bleed and euthanized. Serum was isolated and used to assay for levels of antibodies specific to r30 (see below). In addition, a single cell suspension of splenocytes was prepared for assay of lymphocyte proliferation and intracellular interferon-gamma in response to r30. Red cells were lysed with 1 x PharmLyse (BD Biosciences).

Antibody assay

Serum was analyzed for IgG level by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well high-binding capacity plates (Corning, NY) were coated or not coated with 0.1 ml of the r30 protein diluted in carbonate/bi-carbonate buffer (50 mM NaHCO$_3$, 50 mM Na$_2$CO$_3$) to a final concentration of 10µg/ml. Excess antigen was removed by washing three times with PBS. Sera at a starting dilution of 1:12.5 were diluted further through a two-fold series with PBS. The diluted sera were incubated with r30 coated on 96-well plates at ambient temperature for 3 h. The plates were subsequently incubated for 90 min at ambient temperature with alkaline phosphatase-conjugated goat anti-mouse IgA (Sigma, St. Louis, MO) at a dilution of 1:1000. The plates then were washed three times with PBS and 0.05% Tween-20. One hundred µl of 1/-nitrophenylphosphate substrate in diethanolamine buffer (Phosphatase Substrate kit, BioRad, Hercules, CA) was added to each well. The yellow color that developed was read at 414 nm for absorbance using a multiscan microplate reader (TiterTek, Huntsville, AL). The endpoint antibody titer was calculated as the reciprocal of the highest serum dilution that was 2 fold or above optical density units in r30 coated wells versus non-coated control wells.
Splenic lymphocyte proliferation and intracellular cytokine staining

Splenocytes were incubated with or without r30 antigen and tested for lymphocyte proliferation and intracellular interferon gamma (IFNy) expression. For the lymphocyte proliferation assay, splenocytes were allowed to proliferate for 48 hours. The amount of lymphocyte proliferation was detected by adding radioactive \(^3\)H (tritiated) thymidine for 2 hours, which was incorporated into the newly synthesized DNA of the dividing cells. The amount of radioactivity incorporated into DNA was measured in a scintillation counter and is proportional to the number of proliferating cells, which in turn is a function of the number of lymphocytes that were stimulated by a given antigen to proliferate.

To assay intracellular expression of IFNy, splenocytes were incubated with or without r30 antigen in the presence of interleukin 2 (IL-2) for 24 hours. Intracellular cytokine staining was performed at day 6 after the last vaccination as described previously (see, e.g. Lee, et al. (2006). "Identification, recombinant expression, immunolocalization in macrophages, and T-cell responsiveness of the major extracellular proteins of Francisella tularensis." Infect Immun 74(7): 4002-13; Jia, et al. (2009). "Recombinant attenuated Listeria monocytogenes vaccine expressing Francisella tularensis IgIc induces protection in mice against aerosolized Type A F. tularensis." Vaccine 27(8): 1216-29), using antibodies purchased from BD Biosciences Pharmingen. Briefly, after a 24 h incubation, Golgi-Plug (BD Pharmingen) was added and cells were incubated for an additional 11 h at 37°C in a 5% CO\(_2\) atmosphere. Cells were pelleted at 250 x g for 5 min and resuspended in staining buffer (BD Pharmingen) containing Fc-Block (BD Pharmingen). After incubation for 15 min, cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD4 or PE-Cy5-labeled anti-CD8 antibody at a 1:100 dilution for 30 min, washed twice in staining buffer, fixed with Cytofix solution for 20 min, and washed twice with Perm/Wash solution. Cells were then stained for intracellular interferon gamma (IFNy) with PE-labeled rat anti-mouse IFNy or a PE-labeled isotypic control immunoglobulin G at a dilution of 1:100. All the incubations were performed on ice.
in the dark. Stained cells were washed, resuspended in staining buffer, and analyzed on a FACSCalibur flow cytometer using CellQuest software.

Results of Experiment 3 (Mouse Expt. TB01)

A. Lymphocyte proliferation

As shown in Figure 6A, mice primed with BCG and boosted with rLm/Mtb30 induced higher lymphocyte proliferative response to stimulation by r30 than sham-immunized mice and mice immunized with BCG alone. Among seven rLm/Mtb30 vaccines tested, vaccines derived from the rLmAactAAinlBAuvrABprfA* vector (rLm/Mtb30(12), rLm/Mtb30(07) and rLm/Mtb30(08)) induced stronger immune responses than the corresponding vaccines derived from the rLmAactAAinlB vector (rLm/Mtb30(l1), rLm/Mtb30(03) and rLm/Mtb30(04)). Mice primed with BCG and boosted with r30 or rAd30 also induced strong lymphocyte proliferation to r30.

B. Serum antibody level

As shown in Figure 6B, mice primed with BCG and boosted with r30+SAF had significantly higher antibody levels than sham-immunized mice and mice primed with BCG and boosted with rLm/Mtb30.

C. Intracellular expression of IFNγ

Consistent with the lymphocyte proliferation assay results, mice primed with BCG and boosted with rLm/Mtb30 vaccines derived from rLmAactAAinlBAuvrABprfA* vector (rLm/Mtb30(12), rLm/Mtb30(07) and rLm/Mtb30(08)) had stronger CD4+ mediated immune responses than the corresponding vaccines derived from the rLmAactAAinlB vector (rLm/Mtb30(l1), rLm/Mtb30(03) and rLm/Mtb30(04)), although the difference did not reach statistical significance (Fig. 6C). There was no significant difference in CD8+ mediated immune responses among mice immunized with BCG alone or primed with BCG and boosted with either rLm/Mtb30 or rAd30 vaccines (Fig. 6D).
Experiment 4: Protective Efficacy of recombinant *Listeria monocytogenes* vaccines secreting the *M. tuberculosis* 30 kDa major secretory protein (Antigen 85B) in the mouse model of pulmonary tuberculosis

Specific-pathogen free 6-8 week male C57BL/6 mice from Charles River Breeding Laboratories, in groups of 8, were immunized intradermally as follows:

Group A: Sham

Group B: BCG Tice Parental Control (10^6 CFU) at Week 0

Group C: BCG Tice Parental Control (10^6 CFU) at Week 0 and 100 µg of r30 in SAF adjuvant at Weeks 3 and 6

Group D: BCG Tice Parental Control (10^6 CFU) at Week 0 and LmAactA (Vector control) at Weeks 3 and 6

Group E: BCG Tice Parental Control (10^6 CFU) at Week 0 and rLm/30(01) (rLmAactA/LLO-Mtb30) at Weeks 3 and 6

Group F: BCG Tice Parental Control (10^5 CFU) at Week 0 and rLm/Mtb30(12) (rLmAactAAinlB AuvrABprfA*/Mtb30) at Weeks 3 and 6

Group G: BCG Tice Parental Control (10^5 CFU) at Week 0 and rLm/30(07) (rLmAactAAinlBAuvrABprfA*/ActA-Mtb30) at Weeks 3 and 6

Group H: BCG Tice Parental Control (10^5 CFU) at Week 0 and rAd/Mtb30 at Weeks 3 and 6

At Week 12, all animals were challenged with an aerosol generated from a 7.5 ml single-cell suspension containing 6.3 x 10^4 colony-forming units (CFU) of *M. tuberculosis*. (Prior to challenge, the challenge strain, *M. tuberculosis* Erdman strain (ATCC 35801), had been passaged through outbred guinea pigs to maintain virulence, cultured on 7H11 agar, subjected to gentle sonication to obtain a single cell suspension, and frozen at -70°C). This aerosol dose delivered ~100 live bacilli to the
lungs of each animal. The airborne route of infection was used because this is the natural route of infection for pulmonary tuberculosis.

At 6, 10, and 15 weeks after challenge, animals were euthanized. The lung and spleen of each animal was removed and cultured for CFU of *M. tuberculosis* on Middlebrook 7H11 agar for two weeks at 37°C, 5% CO₂-95% air atmosphere. The results are shown in Figure 7.

These results show that animals immunized with BCG had lower CFU in the spleen and somewhat lower CFU in the lungs than the sham immunized animals. Animals immunized first with BCG and then boosted twice with r30 in adjuvant had slightly lower CFU in the spleen than BCG and significantly lower CFU in the lungs than BCG at all time points. Animals immunized first with BCG and then boosted twice with rAd/Mtb30 had lower CFU than BCG in the spleen only at the 6 week time point; at 10 and 15 weeks, these animals had similar CFU in the spleen. In the lung, these mice had lower CFU than BCG at all time points. Animals immunized first with BCG and then boosted twice with the Listeria vector control had CFU counts similar to BCG in the spleen at the 6 and 10 week timepoints and slightly greater but not significantly greater CFU counts in the spleen at 15 weeks. In the lungs, CFU counts were lower than BCG at 6 and 10 weeks, but similar to BCG at 15 weeks.

Results for the listeria vectored vaccines varied somewhat between early and late timepoints. At 6 weeks after challenge, rLm30(01) had significantly fewer CFU counts than BCG in both the spleen and lung. rLm30(12) and rLm30(07) did not have fewer CFU counts than BCG in the spleen but did have fewer CFU counts in the lung. At 10 weeks, all Listeria vectored vaccines were better than BCG in both the lungs and spleen. rLm30(12) was comparable to rLm30(01) in the spleen but had lower CFU counts in the lungs. rLm30(07) gave the lowest counts of all vaccines in the spleen and CFU counts comparable to rLm30(12) in the lungs. At 15 weeks after challenge, all Listeria vectored vaccines had lower CFU counts than the vector control in the lung and spleen; they also had slightly lower CFU counts than BCG in the spleen and moderately lower CFU counts than BCG in the lungs.
Experiment 5: Mouse immunogenicity of recombinant Listeria vaccines

Mice (4 per group) were sham-immunized, immunized with BCG at Week 0, or primed with BCG at Week 0 and then boosted twice at Week 3 and 6 with a) the recombinant 30 kDa protein in adjuvant; b) the Lm AactA vector, or c) one of five different rLm vaccines expressing the 30 kDa protein - rLm30(01), rLm30(03), rLm30(07), rLm30(11), rLm30(12). At Week 10, the mice were euthanized, the spleen removed, a single cell suspension of lymphocytes prepared and used for studies as follows.

A. Interferon-γ production in response to *M. tuberculosis* antigens

The splenocytes were stimulated with medium alone, the recombinant 30 kDa protein (r30) or *M. tuberculosis* Purified Protein Derivative (PPD) for three days. The splenocyte supernatant fluid was collected and assayed for the level of IFNy by ELISA. The results are shown in Figure 8. Data are the mean ± S.E.

In the absence of antigen, essentially no IFNy was secreted by the splenocytes. In the presence of the purified 30 kDa protein or PPD, splenocytes from sham-immunized mice, mice immunized with only BCG, and mice immunized with BCG and boosted with the Listeria vector control secreted little or no IFNy. In contrast, splenocytes from mice primed with BCG and boosted with the *M. tuberculosis* 30 kDa protein or the recombinant Listeria vaccines expressing the *M. tuberculosis* 30 kDa protein generally produced large amounts of IFNy.

B. Intracellular cytokine secretion

The splenocytes were stimulated with the mature recombinant 30 kDa protein (r30) or a pool of three peptides of r30 (30p) for a total of 6 hours (the last 4 hours in the presence of Golgi-Plug), and stained first for CD4 and CD8 and subsequently for the cytokines IFNy, IL-2, and TNFa, and analyzed by multi-parameter flow cytometry. The total frequency and mean fluorescence intensity (MFI) of each
cytokine-secreting CD4+ T-cell was determined and the integrated MFI (iMFI) was calculated. The results are shown in Figure 9. Data are the mean ± S.E. after background subtraction of the identically gated population of cells from the same sample stimulated without antigen.

In the presence of the purified 30 kDa protein (r30) or the 30 kDa protein peptide pool (30p), splenocytes from sham-immunized mice, mice immunized only with BCG, and mice immunized with BCG and boosted with the Listeria vector control displayed low or negligible iMFI. In contrast, splenocytes from mice primed with BCG and boosted with the *M. tuberculosis* 30 kDa protein or the recombinant Listeria vaccines expressing the *M. tuberculosis* 30 kDa protein generally displayed moderate to large iMFI. Splenocytes from mice immunized with BCG and boosted with rLm30 (01), rLm30 (03), rLm30 (07), and rLm30 (12) had particularly large iMFI for each of the cytokines.

This concludes the description of embodiments of the present invention. The foregoing description of one or more embodiments of the invention has been presented for the purposes of illustration and description. It is not intended to be exhaustive or to limit the invention to the precise form disclosed. Many modifications and variations are possible in light of the above teaching. It is intended that the scope of the invention be limited not by this detailed description, but rather by the claims appended hereto.

It is intended that the scope of the invention be limited not by this detailed description, but rather by the claims appended hereto. The above specification, examples and data provide a complete description of the manufacture and use of the apparatus and method of the invention. Since many embodiments of the invention can be made without departing from the scope of the invention, the invention resides in the claims hereinafter appended and the equivalents thereto.
M. TUBERCULOSIS POLYPEPTIDE AND POLYNUCLEOTIDE SEQUENCES

TABLE 5: Sequences of *M. tuberculosis* Extracellular Protein Genes

<table>
<thead>
<tr>
<th>#</th>
<th>Protein (kDa), (Alternate Names)</th>
<th>Rv numbers /Initial sequences</th>
<th>Lab/Author</th>
<th>Illustrative References</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>12 (fragment of 16 kDa Membrane Protein, alpha crystallian family) MMP</td>
<td>Rv2031c FDTRL…</td>
<td>B.-Y. Lee et al. (Sequenced the 16 kDa Membrane Protein) Verbon et al. (Sequenced the 16 kDa Membrane Protein gene)</td>
<td>Infect. Immun. 60:2066, 1992 J. Bact. 174:1352, 1992</td>
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<td>2</td>
<td>14 (MPT53) (Tuberculist:18kDa)</td>
<td>Rv2878c ADERL…</td>
<td>Horwitz Lab Gen Bank</td>
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<td>---</td>
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<tr>
<td>11</td>
<td>58 (Glutamine Synthetase/GSI/glnA)</td>
<td>Rv2220</td>
<td>Horwitz Lab Harth &amp; Horwitz</td>
<td>J. Biol. Chem. 272:22728, 1997</td>
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<td></td>
<td>Description</td>
<td>Accession Number</td>
<td>Reference</td>
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<tr>
<td>12</td>
<td>71 (hsp/DnaK/Hsp70)</td>
<td>Rv0350</td>
<td>R. Young &amp; D. Young Horwitz Lab</td>
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<tr>
<td>13</td>
<td>10.4 (EsxH/ESAT-6 homolog)</td>
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<tr>
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Note 1: Proteins 14, 15, and 16 are latency-associated proteins

M. tuberculosis  Protein and coding sequences noted in Table 5 above are provided below.

Note: All sequences can be found for example by an online search using the terms:
genolist.pasteur.fr/TubercuI-ist/.

1. 12 kDa (fragment of 16 kDa membrane protein, alpha crystalline homolog, heat shock protein HSPX, and number 14 in the protein list), 110 aa

FDTRLMRLEDEMKEGRYEVRAELPGVDPDKVDIMVRDGQLTIKAERTEQKDFDGRSEFAYGSFVRTVS
L PVGADDEDDIKATYDKGILTSVSVAVERSGKTEKHIQIRSTN (SEQ ID NO: 5)

12 kDa fragment of 16 kDa membrane protein, MMP : 333 bp

ttcgacaccgcgttgatgcccgtggaagagcgatgaaagaggggcgctacgaggtacgcgcggagctttcccggggtcgacccgacaaggacgtcgacattatggtccgcgatggtcagctgaccatatcaaggcgcagcgcaccgacaagaaggacttcgacggtcgctcggaattcgcgtacggttccttcgttcgcacggtgtcgcctgccggtaggtgctgacgaggacgacattaaggccacctacgacaagggcattcttactgtgtcggtgcgggtttcggaagggaagccaaccgaaaagcacattcagatccggtccaccaactga (SEQ ID NO: 23)

2. M. tuberculosis  H37Rv | Rv2878c | Mpt53 : 173 aa - SOLUBLE SECRETED ANTIGEN MPT53 PRECURSOR. First identified protein sequence starts from ADERL...

M. tuberculosis  H37Rv | Rv2878c | mpt53 : 522 bp - SOLUBLE SECRETED ANTIGEN MPT53 PRECURSOR

Note 3: See also U.S. Patent Nos. 7,622,107; 7,300,660; 7,002,002; 6,924,118; 6,818,223; 6,761,894; 6,752,993; 6,599,510; 6,471,967; 6,054,133; 6,013,660; and 5,108,745; and U.S. patent application Nos. 201010129492; 20100284963; 20100183547; and 20100092518, the contents of which are incorporated by reference.
ctgaatcccaccaacctccaatgacgccgatggtgtgatctgggcccgctacaacgtgccttggcaaccggcattgtgtgtttctatcgcgcggacggcacatcgacgttcgtcaacaaccccaccgcggccatgtctcag

gagcagactgctccgccccggtggtgctgtctgctacgctgccaccgctgaacgagcgccgtgatcggtttggcgttcgccggcgggttggtggctgcgctgaacccatgcgggtttgccatgttgccggcctacctgctgttggtggtgtatgggcaggattcggcgggccggacggggccgcttagcgcagtgggccgagcggcagccgcacggtcgggatggcgctgggcttcttgacgg

(SEQ ID NO: 24)

3. M. tuberculosis H37Rv | Rv1926c | Mpt63 : 159 aa - IMMUNOGENIC PROTEIN MPT63 (ANTIGEN MPT63/MPB63) (16 kDA IMMUNOPROTECTIVE EXTRACELLULAR PROTEIN)

MKLTTMIKTAVAWAMAAIATFAAPVALAAYPITGKLGSELTMTDTVGQWLGKWVSKSTAVIPYGPAVGQWEATAVTNFLGRSVPASQFNPARTADGINYRVLWQAAGPDTSAGTIPOGEOSEQSTKGYFDTVGPSPTIVAMDQMDLLEWEP  (SEQ ID NO: 7)

M. tuberculosis H37Rv | Rv1926c | mpt63 : 480 bp - IMMUNOGENIC PROTEIN MPT63 (ANTIGEN MPT63/MPB63) (16 kDA IMMUNOPROTECTIVE EXTRACELLULAR PROTEIN)

atgaagctcaccacaatgcaagacggcagtagcggtcgtggccatggcggccatcgcgacctttggccagcaggcgtcggttggctgcctatcccatcaccgggaacttggcagtgagctaacgatgaccgacaccgttggccaagtcgtgctcggctggaaggtcagtgatctcaaatccagcacggcagtcatccccggctatccggtggccggccaggtctgggaggccactgccgacgcgtcggcagcgtcacgcccgcggtctcgcagttcaatgcccgcaccgccgacggcatcaactaccgggtgctgtggcaagccgcgggccccgacaccattacgga gccactatcccccaagggggaacaatcgagccggcaaaatctacttcgatgtcaccggcccatcgccaaccatcgtcgcgatgaacaacggcatggaggatctgctgatttgggagccgtag

(SEQ ID NO: 25)

4. M. tuberculosis H37Rv | Rv3846 | SodA : 207 aa - SUPEROXIDE DISMUTASE (FE) SODA

VAEYTLPOLDWGYGAEPIESCQINELHSHHHKHTAYVKGANDAVAKLEEARAKEDHSAILLENEKLNLF NLAVHWNHIIWKNLSPNGDKKPTGELALAAAIAIDAFAFGDFDKFRAQFHAAATTVQQSGWAALGMDTLNKLLLIFQVYDQHTNPFLGIVPLLWLLDMNEHAFYLQYKNVKVDFAKAFWNWADVQSYAATSQTKGLIFG  (SEQ ID NO: 8)

M. tuberculosis H37Rv | Rv3846 | sodA : 624 bp - SUPEROXIDE DISMUTASE (FE) SODA

gtggcgcagataacaccttgccaagaccttgaggctggtggcgaactgggacgcaacggcaccactcgcggtcagatcctcagcacaacggcgaaaagctggcagccacagccacaccctaccaaggggaccatggaagcccgaaatcggcagccacagccacaagccacacagccacaccctaccaaggggaccagccaggggagcctggcggcgaactgggacgcaacggcaccactcgcggtcagatcctcagcacaacggc
ttttggaacgtcgtgaactgggccgatgtgcagtcacggtatgcggccgcgacctcgcag
accaaggggttgatattcggctga (SEQ ID NO: 26)

5. **M. tuberculosis** H37Rv |Rv1980c |Mpt64: 228 aa – IMMUNOGENIC PROTEIN MPT64 (ANTIGEN MPT64/MPB64)

VRKIFMLVTLAWCCSGVATAPKTYCCEILGTKTDGTQAQCQMQSDPAYNINISLPSYYPDQKSLENY
IAQTRDKFLSAATSTTPREAPYENILSATYSQAIAPPRTGTQAWLKVKYNQAGGHFTTTTTYKAFWQAY
RKIPITYDILWQAADTPDFPLFWPFPVQGELSKQTGQQVSIPANAGLPVPYFVNDGVIFFFNPEGEL
PEAAGPTQLVLPRSAIDSMLA (SEQ ID NO: 59)

M. tuberculosis H37Rv |Rv1980c |Mpt64: 687 bp – IMMUNOGENIC PROTEIN

MPT64 (ANTIGEN MPT64/MPB64)

5

6. **M. tuberculosis** H37Rv |Rv3803c |FbpD: 299 aa – SECRETED MPT51/MPB51 ANTIGEN PROTEIN FBPD (MPT51/MPB51 ANTIGEN 85 COMPLEX c) (AG58C)

MKGRSALLRALWIAALSFGGGVAAEAETAKAAPENLYMVPSPSMGDRDIPVAFAGPHHAVYLLDAFN
AGPDVSNWVTAGMATLAKGKISPAGPASSYMTWQDGSQWDTLSAEPLWAARNGILAPGG
HAAVAAGGGYAGMALAAAHFIRDPRFGASMSGFPYSPNNTITNGAIAAGMQQFGDVNDNGWAPQLGRW
KWHDPWHSFULLAQNTRNTRVWWSPTNPGASDAMIGQAAMGNNSRMFYQYRSVGGNHGFDPASG
DNNGWSWAPQLGAMSGDIVGAIR (SEQ ID NO: 10)

M. tuberculosis H37Rv |Rv3803c |FbpD: 900 bp – SECRETED MPT51/MPB51 ANTIGEN PROTEIN FBPD (MPT51/MPB51 ANTIGEN 85 COMPLEX c) (AG58C)

(MYCOLYL TRANSFERASE 85C) (FIBRONECTIN–BINDING PROTEIN c) (85C)

MKGRSALLRALWIAALSFGGGVAAEAETAKAAPENLYMVPSPSMGDRDIPVAFAGPHHAVYLLDAFN
AGPDVSNWVTAGMATLAKGKISPAGPASSYMTWQDGSQWDTLSAEPLWAARNGILAPGG
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KWHDPWHSFULLAQNTRNTRVWWSPTNPGASDAMIGQAAMGNNSRMFYQYRSVGGNHGFDPASG
DNNGWSWAPQLGAMSGDIVGAIR (SEQ ID NO: 10)
7. M. tuberculosis H37Rv | Rvl886c | FbpB: 325 aa - SECRETED ANTIGEN 85-B
(FBPB (85B) (ANTIGEN 85 COMPLEX B) (MYCOLYL TRANSFERASE 85B)
(FIBRONECTIN-BINDING PROTEIN B) (EXTRACELLULAR ALPHA-ANTIGEN)

MTDVSRRKIRAWGRRLMI183 GAAALWPLLGLVLAGGAATAGAFSRPGLPVEYLQVPSPS338 SG
GNNSP457 YLDGLRAQD407 NYWNTIPAFcases</noparse>457 QGGLIVP361 VYMD365 DVS720 PAGACACQ1192 TYKWE
TLTS1PQ457 LWSANRAKVP755 TSAAIGL315 SMAH335 AYHPQ350 YAS356 GSLALDL380 DPSQMGPS515 IGLA
MGDAGGYK457 ADDMGPS365 SSDPAWNRNDP720 TQIKPV457 ANVANNTRL755 YW457 CGN457 GTPNELG457 GANIPA457 EFLENFVR457 SSS
NLK457 FDAYNAAGHHNA457 VFNP457 NGTHSWYEY457 GQAQLNAM457 KGDL457 QSSL457 AG

M. tuberculosis H37Rv | Rvl886c | FbpB: 978 bp - SECRETED ANTIGEN 85-B
(FBPB (85B) (ANTIGEN 85 COMPLEX B) (MYCOLYL TRANSFERASE 85B)
(FIBRONECTIN-BINDING PROTEIN B) (EXTRACELLULAR ALPHA-ANTIGEN)

atgacagacgtagccgaaagattctagcttggagcagccgtattgtagtcagctggacgcagcagcagctgta
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965
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970
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atgctctcgccggtctootgcgggttgcgaaacccggccggggcctctcaggggatggggcctagcctgatcggcctcgcg
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975
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aacctgaagttccaggatgcgtacaacgccgcgggcgggcacaacgccgtgttcaacttcccgcccaac
990
ggcacgcacagctgggagtactggggcgctcagctcaacgccatgaagggtgacctgcagagttcgtta
atgacagacgtagccgaaagattctagcttggagcagccgtattgtagtcagctggacgcagcagcagctgta
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atgggtgacgccggcggttacaaggccgcagacatgtggggtccctcgagtgacccggcatgggagcgcc
aacgaccctacgcagcagatccccaagctggtcgcaaacaacacccggctatgggtttattgcgggaac

8. M. tuberculosis H37Rv | Rv3804c | FbpA: 338 aa - SECRETED ANTIGEN 85-A
(FBPA (MYCOLYL TRANSFERASE 85A) (FIBRONECTIN-BINDING PROTEIN A)
(ANTIGEN 85 COMPLEX A)

MQVDRVQAVTGMSRSLWGAGAALSVGAVGTTAGAFRSRPGLPVEYLQVPSGMSRDILTQF
QSGSAPALILDGLGRQDFDWSWNTAAPFWEYDQSLSWMPVQGQSSFYDSWYQPACGKAGCQTY
KWETFLTELPGWLQANRHVKNKTSAGSWLMASSLATLTALYHPQFYVAGAMSLGDLDSQAGMPTL
GLDMGDAAGYKASDMNGPKEDPAWRNQNLNVGKLIANNTRVWYVCNGKPSDLNQGNNLPAKLEG
RTSNIKFQDAYNAGGGMNGVGDFDPSGTSWYGAQLNAMKPDQLRAGATPTNPQG

9. M. tuberculosis H37Rv | Rv0983 | PepD: 464 aa - PROBABLE SERINE PROTEASE PEPD (SERINE PROTEINASE) (MTB32B)

MAKLRWGLQVEEQPSDMTNHPFSSPPPQGPOTPGYAGQQQTQYDWRYPSSPSPPQPTQYRQPE
ALGTRGCLPGVIPTMTPPPGMVRQPRGMLAIAGTIASAGAASLGAVFNRAPGSGPVP
AASAASPIAPAAMMPPSVEQVAAKWPSSWMLDQLGRQSEQGSI ILSEGLILLTHVIAAAAKPL
GSPPPRTVFTVSDGTAPTFWDAPTSDIAVRWQGVSGLPISLGGSSDLRVQPVLAIGSLPLEG
TVTTGVISALNRPVSTTGEAGNOMTVLDIALDIAIPGNSGGALVNMNQLVGVNSAIATLGADSA
QSGSILGFAIPVQAKRIADELISTGKASASLGAVQVTN喀DGALKIVEWAGGAAANAGVPKGW
TKVDRPPNSADALVAAVRSAKAPGAVALTQDPSGSGSSRTQVTLGKAQ

SEQ ID NO: 11

SEQ ID NO: 30

SEQ ID NO: 12
10. *M. tuberculosis* H37Rv | Rv0983 | pepD : 1395 bp - PROBABLE SERINE PROTEASE
PEPD (SERINE PROTEINASE) (MTB32B)

```
5
atgcgccacgttcgcggccagctagttggccctactacagagagaagcaacctagcgacatgacg
ccgcagcgaacacgtacacaccgctgtccacccaccgctgcccgcgacccgggacacccgc
ccgcgcgcacccctgtagttggtgcagccgcgaggcctgacagttgttgttgggtaacatgctc
ccgcgccgctgcccgcgacccgggacacccgc
```  

(SEQ ID NO: 31)

20


(Immunogenic protein MPT32) (Antigen MPT-32) (45-kDa glycoprotein)

```
25
MHQDVPNLTRRKLALAAAILAMASLLTAVPAPATADPEAPPVTAAASPPSTAAPAPAPPTVAPV
PPPPAAANTPAQPGDNAPAPADNPAPAPVPIAPNAQPQVRINDPVSFGFSFALPGWESDAHFDY
GSALSSKTTPDPPFGPQPPVPANDTVLRDQLKYLAESAATDKSAAARLGSDMEFEPYMPTRINQ
ETVSLDANGVSASASYEYKFLPSKPNQIWTVGVSPPANAFDAGPPQRWFWLFGLTANLNPVEKGA
KALAE SIRFLVAP PPAAAPPAE PAPAPAPAGE VAPT TTPTPQRTLPA (SEQ ID NO: 13)
```  

30


(Immunogenic protein MPT32) (Antigen MPT-32) (45-kDa glycoprotein) (45/47 kDa antigen)

```
35
atgcacaggtggaccccaacttggacacgtccgaagggagattgcccagctcctccgcgggtgctg
ccgcagcgcagcgctgtggtcgcctgccgcgagccaccgccacctggcgcgatgcgcgcccccgc
ctggtgcgcgccgcgccgcgccgccgagctgccgcgcgatcgcgcgatgcgcgcccccgccgc
```  

(SEQ ID NO: 13)
11. *M. tuberculosis* H37Rv |Rv2220 |GlnAl: 478 aa - GLUTAMINE SYNTHETASE

GLNA1 (GLUTAMINE SYNTHASE) (GS-I)

VTEKTPDDVLAKDEKVEYDVRFCDLPGIMQHFTIPASAFDKSVVVFDDGSGISRGFQSIHESDM
LLPLPETARIDPFRAAATNLNFPVFVFDTPLEPSRDPNRNIAKANYLISTGDAATMDGAEAFFYI
FDVSFDRANGSFYEVDASGWGNNTAATEADGSPNRGKYHKGVFYAPVNDQYVDRLDKMNTNLNSGF
ILEKHKHEVSGGQAENYQFNSLHAADEMQLYKYI IKNTAWQGKTVTFPMKPLFDNGSOMCHQSL
WKGAPLMYDTEGTYAGLSLTARHIFTGLLHLHAPSLLAFTNPNTVNSKRLYPYEAPINLVYSSQR
NRSACBPPIGSNPKARLEFRSPDSSNPNYLAFSAMLMLGIKNIQIEQPVDKDLVLPPEAEASSIP
tQPTQLSDVDRADWLEDHYTEGVITNLHIFWTISFKRENEIPVNIRPHYPFEFALYDV (SEQ
ID NO: 33)

12. *M. tuberculosis* H37Rv |Rv0350 |DnaK: 625 aa - PROBABLE CHAPERONE

PROTEIN DNAK (HEAT SHOCK PROTEIN 70) (HEAT SHOCK 70 KDA PROTEIN)
(HSP70)
M. tuberculosis H37Rv [Rv0350] | dnaK : 1878 bp - PROBABLE CHAPERONE PROTEIN DNAK (HEAT SHOCK PROTEIN 70) (HEAT SHOCK 70 KDA PROTEIN)

(HSP70)

15atggctcgtgcggtcgggtcgggatcgacctcgggaccaccaactccgtcgtctcggttctggaa
gggtggcgacccggtcgtcgtcgccaactccgagggctccaggaccaccccgtcaattgtc
gcgttcgcccgcaacggtgaggtgctggtcggccagcccgccaagaaccaggcagtgacc
aacgtcgctgcacccctgcctctggtcaagcgacacatgggcagcgactggtccatagag
attgacggcaagaaatacaccgcgccggagatcagcgcccgcattctgatgaagctgaag
cgcgacgccgaggcctacctcggtgaggacattaccgacgcggttatcacgacgcccgcc
tacttcaatgacgcccagcgtcaggccaccaaggacgccggccagatcgccggcctcaac
gtgctgcggatcgtcaacgagccgaccgcggccgcgctggcctacggcctcgacaagggc
gagaaggagcagcgaatcctggtcttcgacttgggtggtggcactttcgacgtttccctg
ctggagatcggcgagggtgtggttgaggtccgtgccacttcgggtgacaaccacctcggc
ggcgacgactgggaccagcgggtcgtcgattggctggtggacaagttcaagggcaccagc
ggcatcgatctgaccaaggacaagatggcgatgcagcggctgcgggaagccgccgagaag
gcaaagatcgagctgagttcgagtcagtccacctcgatcaacctgccctacatcaccgtc
gacgccgacaagaaccctttgttcttagacgagcagctgacccgcgcggagttccaacgg
atcactcagacacgcttggacacgccactcgcaacctccacgcgtctatcagggggagcgtgag
atcgccgcgcacaacaacatggctcgggtccttcgagctgaccggcatcccgccggcgccg
cgggggattccgcagatcgaggtcactttcgacatcgacgccaacggcattgtgcacgtc
accgccaaggacaagggcaccggcaaggagaacacgatccgaatccaggaaggctcgggc
cctgtccaaggaagacattgacgcatgatcaaggacgccgaagcgacgccgaggaggat
cgcaagcgtcgcgaggaggccgatgttcgtaatcaagccgagacattggtctaccagacg
gagaagttcgtcaaagaacagcgtgaggccgagggtggttcgaaggtacctgaagacacg
cctgaacaaggttgatgccgcggtggcggaagcgaaggcggcacttggcggatcggatatt
tcggccatcaagtcggcgatggagaagctgggccaggagtcgcaggctctggggcaagcg
atctacgaagcagctcaggctgcgtcacaggccactggcgctgcccaccccggcggcgag
ccgggcggtgcccaccccggctcggctgatgacgttgtggacgcggaggtggtcgacgac
ggccgggaggccaagtga (SEQ ID NO: 15)

13. M. tuberculosis H37Rv | Rv0288 | EsxH : 96 aa - LOW MOLECULAR WEIGHT PROTEIN ANTIGEN 7 ESXH (10 kDa ANTIGEN) (CFP-7) (PROTEIN TB10.4)
M. tuberculosis H37Rv | Rv0288 | esxH : 291 bp - LOW MOLECULAR WEIGHT PROTEIN ANTIGEN 7 ESXH (10 kDa ANTIGEN) (CFP-7) (PROTEIN TB10.4)

14. M. tuberculosis H37Rv | Rv2031c | HspX : 144 aa - HEAT SHOCK PROTEIN HSPX (ALPHA-CRYSTALLIN HOMOLOG) (14 kDa ANTIGEN) (HSP16.3)

15. M. tuberculosis H37Rv | Rv0467 | icl : 428 aa - ISOCITRATE LYASE ICL (ISOCITRASE) (ISOCITRATASE)
16. M. tuberculosis H37Rv | Rv2 660c | Rv2 660c: 75 aa – HYPOTHETICAL PROTEIN

VIAGVDQALAATGQASQRAAGGVTGVGVTGTEQQRNLISWAPSQFTFSRSSPDFVDETAGQSWCAIL
GLNQFH (SEQ ID NO: 19)

17. M. tuberculosis H37Rv | Rv1837c | GlcB: 741 aa – PROBABLE MALATE SYNTHASE G GLCB

MTDRVSVGNLRIARVLYDFVNENLPDQTNQAGNLARDELDQIQIDWHR RRVIEPDMAYQFLEIGYLLPEPDDPTITTTSGVDAEIATTAGPQQLWIFILARFALNAANARWGLS
YDALYGTDVIPETDAEKGPTYKVNRGDKVIAYARKFDLDSVLSSSGFGDATGFTVDQQLWALPDK
STGLANPQFGAGTYGAEAESTPSVLLIHNLHIELIDEPQSTTVTDQVASTEILIMTDIEFV
AADVDAADKVLRWNLKINLKDIALAANVKDTGFARLVRNLNDRNYTAPQGGQFTLPGRSLMVNVN
GHLMTNDAIIVDTSVEFVEMIALFTGLIAHGLKASDVNPLINSRTGISYIKPKHMHPAEVAFTELFS
RVDULPVLPQTNMIKIDMPDERTTNNLKALICAAADRWFINTGFDRTGDEIHTSMEEAGPMVRKMG
TMSQPWIAYEHDNVDAAGLASGATQVKGWMETLMAMEVTQIKAYPRASTAWFVSPATATLHA
LYHQVDVAVAAQQGLAGRRATIEQLTTPPLLAKELAWAPEIREEVDNQNCQILSYRWVQDGVCXSK
VPDHSVAMLREDRLRSSQILANWRLHGVTASDVARSLERMAPLVDQNAGDRVYRMPAPNFDDI
AFLAAQELILSGANNPQNPAGENILHRHRREF KARAAE KPA PDSRGDDAAR (SEQ ID NO: 20)
18. *M. tuberculosis* H37Rv | Rvl1475c | Acn: 943 aa - PROBABLE IRON-REGULATED ACONITATE HYDRATASE ACN (Citrate hydro-lyase) (Aconitase)

VTSKSVNSFGAHDTLKVGEKSYQIYRDLAVPNTAKLPSYLSKVLAEMLRNEDDSNITKHIEAIANWDPKAEPSIEIQYTPARWMQDFTGCPCVIDLSTMREIALDLGCPKQNLAPAVLIDHSVIADLFGRADAFERNVEIEYQNREYQFLRWWGQFDFFWPPGTGIVHQNIEYLASWMTGDAYPTDCGTSHTTMTVNLGQVGVGIEAAALVGQVSMILPWFRFLTGIEQPGVTATDVTLMRLQHGWKVFEPFVGQVAGPLANRALTGNMSEPFGSTAAIFIPIDETEIKLRFQRTPEQVALVEAYAKAQNGMWDKHPHEFSEPSEVYELNSLPWSIAQGRKQDORIALAQAKSTFREQIYHYVNGSCPDSPHPSKLEDWEETPFPASDPQQTALFPANDYDETVPHSAAAHAHGRVPSVPRKVSDEGGEFLDLHQAAMIAITSCNTSNPEVMLGAALLARNAVEKGLTSKPWVKTTIAPSGQWNYDYYDRSGLWPELKGFYLVGYCTTGCSN
M. tuberculosis H37Rv |Rv1475c | acn : 2832 bp - PROBABLE IRON-REGULATED ACNITATE HYDRATASE ACN (Citrate hydro-lyase) (Aconitate)
tcgggttcgtcacgggactgggcggccaaaggcacattgctactgggcgtgcgggcggtg
atcgccgagtcattcgagcggatccaccggtccaacctgatcggcatgggcgtgatcccg
cctgcagttccccgaaggaaagtcagcgtcgtcgttgggactcgacggtaccgaggtcttc
gacatcaccggtatcgcgtgttaacgcagcgaacaccaccaagcggtgtgcgtcaggccaccaagggcgacggcgccacgatcgagttcgacgccgtggtgcgcatcgacaccccc
ggtgaggcggactactaccgcaacggcggcatcctgcagtacgtgctgcgcaacatactg
aagtcaggctga

19. M. tuberculosis H37Rv | Rv3875 | EsxA: 95 aa - 6 KDA EARLY SECRETORY ANTIGENIC TARGET ESXA (ESAT-6)

MTEQQWNFAGIEAAASAIQGNVTSIHSLLDEGKQSLTKLAAAWGGSGSEAYQGVQQKWDATATELNNALQNLARTISEAGQAMASTEGNVTGMFA (SEQ ID NO: 22)

M. tuberculosis H37Rv | Rv3875 | esxA: 288 bp - 6 KDA EARLY SECRETORY ANTIGENIC TARGET ESXA (ESAT-6)

atgacagagcagcagtggaatttcgcgggtatcgaggccgcggcaagcgcaatccaggga
aatgtcacgtccattcattccctccttgacgaggggaagcagtccctgaccaagctcgca
gcggcctggggcggtagcggttcggaggcgtaccagggtgtccagcaaaaatgggacgccacggctaccgagctgaacaagcgctgcagaacctggcgcggacgatcagcgaagccggtcaggcaatggcttcgaccgaaggcaacgtcactgggatgttcgcatag (SEQ ID NO: 41)

POLYNUCLEOTIDE AND POLYPEPTIDE SEQUENCES FROM:
LISTERIA MONOCYTOGENES (EGD STRAIN)

ActA

actin-assembly inducing protein precursor (Listeria monocytogenes EGD-e)

MGLNRFMRAMMWFITANCITINPDI IFAATDSEDSSSLNDEWEENEKTEQQPSEVNTGPR
YETAREVSSRD1KELEKSNVRNKTADIALMEKAEKGPNNNNNSEQTENAINEEA
SGADRPAIQVERRPGLPSDAEAIKRRKAIASSDESELTYPDKPTKNKKVAKES
VADASESDELSMQSDESSQPPLKANQQPFPFKVFKIDAKGKVRDKIDENPEVKKAI
VDKSAAGLIDQQLTKKKSEVNASDFPPPPPDEELRLALPETPMLLGFNAPATSEPSSEF
PPPPDDEELRLALPETPMLLGFNAPATSEPSSEFPPPPTEDELEI IREASSLDSFR
GDLASLRNAIRHSSQNFSDPPPPITEEELNGRGRPTSEEFFSSLNSGDFTDENSEETTE
EIIDRRLDRLORDRGKHSRNGAFPPLNPASPPVSPLPKVSKISAPALISDITKTPFPKN
PSQPLNVPNKNTTTKTIVKPTPVKAPKLAELOPTPKQETVLREKTPFIEKQAEIKNQ
SINMPSLPVIQEATESDKEEMKPQTEEMKVEESESANNANNGKNRSAGIEEGKLIKASAE
DEKAKEEPNHNTTLLMAIGVFSLGAIFIKI IQLRKNN (SEQ ID NO: 1)

gene="actA" (Listeria monocytogenes EGD-e)

GTGGGATTAACACAGATTTATCTGGTGGATGTTTCCTTATTACTGCAAATTGCATT
ACGATAAACCCCGACATAATATTTGCAGCAGATAAGTAGGAAGATCCTAGCTAAACACA
GATGAAATGGAAAGAAGAAAAACAGAGACCATCGAAGAGGGAGGACAGAAAAAGAC
TAGAACAGAAGAAGAACACCAACCGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG

TACGAAAC TGCACTTGGAAG TTATCGCTGATT AATAGG ACCA AAAAAA CGA AAA
GTGAAACAA AAGCAAGCAAC ACCACCAAGAAGAAAAAGAC

57
CCAAATATCAATAATAACACGTGAAACTCGGTATATAAATGAAGAGGCT
TCAGGACCGACCGACCGACCGATTCGCTTTCCGATTCGCTTTCCGAT
AGCCGAGCGATAATAAATAGCAAACTCGGTATATAAATGAAGAGGCT

GTGCGGATCTGCAATATTGATATCTAGTACATGCTGAGTCGTCATCCAGGATTGCCATCGGAT

CCCAAGCTTCTTGGTTTTAATGCTCCTGCTACATCAGAACCGAGCTCATTCGAATTT

ATGGCGTGTTCTCTTTAGGGGCGTTTATCAAAATTATTCAATTAAGAAAAATAATTAA

AACGGAAAAATCGTTCTGCTGGCATTGAAGAAGGAAAACTAATTGCTAAAAGTGCAGAA

AAC

CCGTTGCGGATGCTTCTGAAAGTGACTTAGATTCTAGCATGCAGTCAGCAGATGAGTCTTCA

GATGCAGTGACAAATGTGCCGCCAAGAAAAGGTTACAAAGATGGAAATGAATATATTGTT

CCACCAGCATCTCCGCCTGCAAGTCCTAAGACGCCAATCGAAAAGAAACACGCGGATGA

CAAACTGAAGCAAAGGATGCATCTGCATTCAATAAAGAAAATTCAATTTCATCCATGGCA

CCACACATCCTCCTCCTCTCCTCTATATGGTTTTAATAATGCTGACCGGCTCTGATAAGTGACATACTAAAAAAACGCCATTTAAGAAT

ATATAGTTTCGCT

AAC

GAT

AAAATATGCTAGTTTTTATTACACTTATATTAGTTAGTCTACCAATTGCGCAA

GAT

AAATACCCCAATGCTTCTTGGTTTTAATGCTCCTGCTACATCAGAACCGAGCTCATTCGAATTT

AGCCTAATTAATATGCC

TCGAAAATAAGCGCACCGGCTCTGATAAGTGACATACTAAAAAAACGCCATTTAAGAAT

ATATAGTTTCGCT
CCTAATGTTAAATGGCATTTACCAGAATTTACAAATGAAGTGAGTTTTATTTTCTATCAG
CCAGTCACTATTGGAAAAGCAAAAGCAAGATTTCATGGGAGAGTAACCCAACCACTGAAA
G
G
T
T
T
A
C
A
C
A
G
T
AAG
TTATGATGTT
G
A
T
G
G
G
AAC
G
G
T
AAT
AAAAAC
AAAAG
TAG
AAGC
A
GGGACGCGGATAACTGCACCTAAACCTCCGACTAAACAAGGCTATGTTTTTAAAGGATGG
TATACTGAAAAAATGGGCGATGAGTGGAATTTTAATACGGATTATATGTCCGGAAAT
GATTTTACTTTGACCGAATTTAAATGGAATAGCAAGAAAGGACTCCTTCTCCTAGCT
AA
GAGATGGAAATGCTGCTTGGGAAAACCCCTCAAACACAGGCAGGCT
AAACACTGTAATAGCTCTCGCTTACCAAGGTTAAAAATATCGGTTCATTTGGCGGAAAGCC
AAAACACCATATCTACATGATGTAATTTGACATTTGCTGGTTAAGTAAATTTGTGGTCTC
G
ATACCGGACATCTAACACAATCTTCAACAAAGCATTGGAAGACCAACGTTTTAC
CGTTATGTCAGCGCCAATAAAGCTGGCGAATCGTACTATAAAGTCCCGGTAGCAGATAAT
CCAGTAAAAAGGGG TACCTTACG CAAG TAAAAAT CAAAG TTAAT TTGTGAT TGTC
GCAACATCGAAGGTCACCTTTGTCAGCCAAATAGGGACGACTTCCACTTCCATGGTGG
ACGAAAGC AGC TAAT TTAAGGGC ACAGAAAT AA (SEQ ID NO: 45)

20

PrfA

_listerialysin positive regulatory protein_ (Listeria monocytogenes EGD-e)

MNAQAEEFKKYLETNGIKPKQHKKELIFQWDPQECFILYDITKLTISENIGHTMLN
QYYKGAFVIMSFDIETSVGYNLEVISEQATAYVIKINELKLSKNTLFHYVFQTL
QKQVSYSLAKFNDFSINGKLSIGCQLLLITYVYGKETPDGKKTIQITLDNLTMLGEYGSSI
AHSSAVSRI ISKLKQEKKVYKNCFYQVDLYLKRYPKLDWYFLAC PATWGTKLN (SEQ ID NO: 3)

gene="prfA" complement (1..714) prfA (Listeria monocytogenes EGD-e)

TTAATTATTTTCCCCAAGTACGACGACATGCTAAAATAAAAACATCCATCTTATTTTGG
GGCA TACTTTTGAGTAAT CAAAG TTTTGTAC TAAAGGC ATGGA TTTTTTACAC TAC
ACTTTTCTGTTTAAATTTGGGAATAATTTTCTGTAACAGCTGACATTGCGGAGTCC
ACTTGAATATCTCACTTCTGCAATGTTAAAAATATCCAGTGTAACTTTGCTGACTTCT
AGTTTTCATACCATACGAGATAGTTAAAAAGTTGACGGCAATAAGAGCACAGACCTT
CCGGTATTTAAGTCGAGATTTTTGACTAGTTAAATATGGAGCTTGGTTTTTTTTAGGT
TTGGAACCAATAAAGAGATCGGTAAGATTTTTGTCCAGATGTTTTTTTTTTGCTGTTTAT
TTTGTACACTCGTACGCTTGTTAGTAAATTAATAGAAGGCGACCCTTTTTCTGATATTGATAATT
CATGAATGCGCCCTGTTCTCCTGAAATACGTCTGAGGCTTTTGTGATACCATCATATAAGAAAAAT
ACAAATATCTTTGGGAGCTCTATTCTTCTTTTTTATGAAAATTTGTGGT
TTTATCCCGGTTAGTTTTCTTAAATATTTTTTGAATTTTCTCCTGTTGACGTC

(SEQ ID NO: 46)
CLAIMS

1. A composition of matter comprising attenuated *Listeria monocytogenes*, wherein the *Listeria monocytogenes*:
   does not express a functional ActA protein (SEQ ID NO: 1); and
   expresses *Mycobacterium tuberculosis* 30kDa antigen 85B protein (SEQ ID NO: 4).

2. The composition of claim 1, wherein the *Listeria monocytogenes* further expresses at least one protein from the following group:
   *Mycobacterium tuberculosis* 12kDa fragment of 16 kDa membrane protein (SEQ ID NO: 5);
   *Mycobacterium tuberculosis* 14 kDa MPT53 protein (SEQ ID NO: 6);
   *Mycobacterium tuberculosis* 16 kDa MPT63 protein (SEQ ID NO: 7);
   *Mycobacterium tuberculosis* 23 kDa SOD protein (SEQ ID NO: 8);
   *Mycobacterium tuberculosis* 23.5 kDa MPT64 protein (SEQ ID NO: 9);
   *Mycobacterium tuberculosis* 24 kDa MPT51 protein (SEQ ID NO: 10);
   *Mycobacterium tuberculosis* 32 kDa antigen 85A protein (SEQ ID NO: 11);
   *Mycobacterium tuberculosis* 32 kDa antigen 85C protein (SEQ ID NO: 12);
   *Mycobacterium tuberculosis* 45 kDa MPT32 protein (SEQ ID NO: 13);
   *Mycobacterium tuberculosis* 58 kDa glutamine synthetase protein (SEQ ID NO: 14);
   *Mycobacterium tuberculosis* 71 kDa HSP 70 protein (SEQ ID NO: 15);
   *Mycobacterium tuberculosis* 10.4 kDa EsxH protein (SEQ ID NO: 16);
   *Mycobacterium tuberculosis* 14 kDa alpha crystalline homolog protein (SEQ ID NO: 17);
   *Mycobacterium tuberculosis* 47 kDa isocitrate lysate protein (SEQ ID NO: 18);
   *Mycobacterium tuberculosis* 7.6 kDa hypothetical protein (SEQ ID NO: 19);
Mycobacterium tuberculosis 80kDa glcB protein (SEQ ID NO: 20);
Mycobacterium tuberculosis 110 kDa can protein (SEQ ID NO: 21); or
Mycobacterium tuberculosis 9.9 kDa ESAT-6 protein (SEQ ID NO: 22).

3. The composition of claim 1, wherein the Listeria monocytogenes further expresses:

at least one protein from group A:

Mycobacterium tuberculosis 12kDa fragment of 16 kDa membrane protein (SEQ ID NO:5);

Mycobacterium tuberculosis 14 kDa MPT53 protein (SEQ ID NO: 6);
Mycobacterium tuberculosis 16 kDa MPT63 protein (SEQ ID NO: 7);
Mycobacterium tuberculosis 23 kDa SOD protein (SEQ ID NO: 8);
Mycobacterium tuberculosis 23.5 kDa MPT64 protein (SEQ ID NO: 9);
Mycobacterium tuberculosis 24 kDa MPT51 protein (SEQ ID NO: 10);
Mycobacterium tuberculosis 32 kDa antigen 85A protein (SEQ ID NO: 11);
Mycobacterium tuberculosis 32 kDa antigen 85C protein (SEQ ID NO: 12);
Mycobacterium tuberculosis 45 kDa MPT32 protein (SEQ ID NO: 13);
Mycobacterium tuberculosis 58 kDa glutamine synthetase protein (SEQ ID NO: 14);

Mycobacterium tuberculosis 71 kDa HSP 70 protein (SEQ ID NO: 15);
Mycobacterium tuberculosis 10.4 kDa EsxH protein (SEQ ID NO: 16);
Mycobacterium tuberculosis 80kDa glcB protein (SEQ ID NO: 20);
Mycobacterium tuberculosis 110 kDa can protein (SEQ ID NO: 21); or
Mycobacterium tuberculosis 9.9 kDa ESAT-6 protein (SEQ ID NO: 22);

at least one protein from group B:

Mycobacterium tuberculosis 14 kDa alpha crystalline homolog protein (SEQ ID NO: 17);

Mycobacterium tuberculosis 47 kDa isocitrte lysate protein (SEQ ID NO: 18); or
Mycobacterium tuberculosis 7.6 kDa hypothetical protein (SEQ ID NO: 19).

4. The composition of claim 1, wherein the Listeria monocytogenes: does not express a functional InlB protein (SEQ ID NO: 2); and/or expresses prfA protein having a G155S substitution mutation (SEQ ID NO: 3).

5. The composition of claim 1, further comprising at least one of a pharmaceutically acceptable carrier or an adjuvant.

6. The composition of claim 1, further comprising a buffer system.

7. The composition of claim 1, wherein the Mycobacterium tuberculosis 30kDa antigen 85B protein is fused in frame with a heterologous protein sequence.

8. The composition of claim 1, wherein the Mycobacterium tuberculosis 30kDa antigen 85B protein is coupled to a heterologous protein sequence comprising the N-terminal 100 amino acids of the ActA protein.

9. The composition of claim 4, wherein the expression of the Mycobacterium tuberculosis 30kDa antigen 85B protein is controlled by an actA promoter.

10. A method of generating an antibody to a Mycobacterium tuberculosis 30kDa antigen 85B protein (SEQ ID NO: 4) comprising the steps of: immunizing a mammal with a composition of matter comprising attenuated Listeria monocytogenes, wherein the Listeria monocytogenes: does not express a functional ActA protein (SEQ ID NO: 1); and expresses Mycobacterium tuberculosis 30kDa antigen 85B protein (SEQ ID NO: 4);
such that an antibody to a *Mycobacterium tuberculosis* 30kDa antigen 85B protein is generated.

11. The method of claim 10, further comprising immunizing the mammal with *Mycobacterium bovis* strain Bacille Calmette-Guerin (BCG).

12. The method of claim 11, wherein the BCG is used in a primary immunization and the attenuated *Listeria monocytogenes* is used in a booster immunization.

13. The method of claim 10, wherein the *Listeria monocytogenes*:
   - does not express a functional InlB protein (SEQ ID NO: 2); and/or
   - expresses prfA protein having a G155S substitution mutation (SEQ ID NO: 3).

14. The method of claim 10, wherein the mammal is immunized intranasally, subcutaneously, intradermally, intramuscularly or orally.

15. The method of claim 10, wherein the mammal is a guinea pig or a mouse.

16. The method of claim 10, wherein the mammal is a human.

17. The method of claim 10, wherein the *Listeria monocytogenes* further expresses at least one protein from the following group:
   - *Mycobacterium tuberculosis* 12kDa fragment of 16 kDa membrane protein (SEQ ID NO: 5);
   - *Mycobacterium tuberculosis* 14 kDa MPT53 protein (SEQ ID NO: 6);
   - *Mycobacterium tuberculosis* 16 kDa MPT63 protein (SEQ ID NO: 7);
   - *Mycobacterium tuberculosis* 23 kDa SOD protein (SEQ ID NO: 8);
   - *Mycobacterium tuberculosis* 23.5 kDa MPT64 protein (SEQ ID NO: 9);
   - *Mycobacterium tuberculosis* 24 kDa MPT51 protein (SEQ ID NO: 10);
Mycobacterium tuberculosis 32 kDa antigen 85A protein (SEQ ID NO: 11);
Mycobacterium tuberculosis 32 kDa antigen 85C protein (SEQ ID NO: 12);
Mycobacterium tuberculosis 45 kDa MPT32 protein (SEQ ID NO: 13);
Mycobacterium tuberculosis 58 kDa glutamine synthetase protein (SEQ ID NO: 14);
Mycobacterium tuberculosis 71 kDa HSP 70 protein (SEQ ID NO: 15);
Mycobacterium tuberculosis 10.4 kDa EsxH protein (SEQ ID NO: 16);
Mycobacterium tuberculosis 14 kDa alpha crystalline homolog protein (SEQ ID NO: 17);
Mycobacterium tuberculosis 47 kDa isocytrate lysate protein (SEQ ID NO: 18);
Mycobacterium tuberculosis 7.6 kDa hypothetical protein (SEQ ID NO: 19);
Mycobacterium tuberculosis 80kDa glcB protein (SEQ ID NO: 20);
Mycobacterium tuberculosis 110 kDa can protein (SEQ ID NO: 21); or
Mycobacterium tuberculosis 9.9 kDa ESAT-6 protein (SEQ ID NO: 22).

18. The method of claim 10, wherein the Listeria monocytogenes further expresses at least one protein from group A:
Mycobacterium tuberculosis 12kDa fragment of 16 kDa membrane protein (SEQ ID NO:5);
Mycobacterium tuberculosis 14 kDa MPT53 protein (SEQ ID NO: 6);
Mycobacterium tuberculosis 16 kDa MPT63 protein (SEQ ID NO: 7);
Mycobacterium tuberculosis 23 kDa SOD protein (SEQ ID NO: 8);
Mycobacterium tuberculosis 23.5 kDa MPT64 protein (SEQ ID NO: 9);
Mycobacterium tuberculosis 24 kDa MPT51 protein (SEQ ID NO: 10);
Mycobacterium tuberculosis 32 kDa antigen 85A protein (SEQ ID NO: 11);
Mycobacterium tuberculosis 32 kDa antigen 85C protein (SEQ ID NO: 12);
Mycobacterium tuberculosis 45 kDa MPT32 protein (SEQ ID NO: 13);
Mycobacterium tuberculosis 58 kDa glutamine synthetase protein (SEQ ID NO: 14);
Mycobacterium tuberculosis 71 kDa HSP 70 protein (SEQ ID NO: 15);
Mycobacterium tuberculosis 10.4 kDa EsxH protein (SEQ ID NO: 16);
Mycobacterium tuberculosis 80kDa glucB protein (SEQ ID NO: 20);
Mycobacterium tuberculosis 110 kDa can protein (SEQ ID NO: 21); or
Mycobacterium tuberculosis 9.9 kDa ESAT-6 protein (SEQ ID NO: 22);
at least one protein from group B:
Mycobacterium tuberculosis 14 kDa alpha crystalline homolog protein (SEQ ID NO: 17);
Mycobacterium tuberculosis 47 kDa isocytrate lysate protein (SEQ ID NO: 18); or
Mycobacterium tuberculosis 7.6 kDa hypothetical protein (SEQ ID NO: 19).

19. The method of claim 10, wherein the Mycobacterium tuberculosis 30kDa antigen 85B protein is coupled to a heterologous protein sequence comprising the N-terminal 100 amino acids of the ActA protein.

20. The method of claim 19, wherein the expression of the Mycobacterium tuberculosis 30kDa antigen 85B protein is controlled by an ActA promoter.
FIG. 5
FIG. 6
FIG. 7
iMFI of cytokine-producing r30 specific CD4+ T cells (TBm010)

![Graph showing cytokine production by r30 specific CD4+ T cells across different treatments.]

iMFI of cytokine-producing 30p specific CD4+ T cells (TBm010)

![Graph showing cytokine production by 30p specific CD4+ T cells across different treatments.]

**FIG. 9**