rBCG30-Induced Immunity and Cross-Protection against *Mycobacterium leprae* Challenge Are Enhanced by Boosting with the *Mycobacterium tuberculosis* 30-Kilodalton Antigen 85B

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Leprosy remains a major global health problem and typically occurs in regions in which tuberculosis is endemic. Vaccines are needed that protect against both infections and do so better than the suboptimal *Mycobacterium bovis* BCG vaccine. Here, we evaluated rBCG30, a vaccine previously demonstrated to induce protection superior to that of BCG against *Mycobacterium tuberculosis* and *Mycobacterium bovis* challenge in animal models, for efficacy against *Mycobacterium leprae* challenge in a murine model of leprosy. rBCG30 overexpresses the *M. tuberculosis* 30-kDa major secretory protein antigen 85B, which is 85% homologous with the *M. leprae* homolog (r30ML). Mice were sham immunized or immunized intradermally with BCG or rBCG30 and challenged 2.5 months later by injection of viable *M. leprae* into each hind footpad. After 7 months, vaccine efficacy was assessed by enumerating the *M. leprae* bacteria per footpad. Both BCG and rBCG30 induced significant protection against *M. leprae* challenge. In the one experiment in which a comparison between BCG and rBCG30 was feasible, rBCG30 induced significantly greater protection than did BCG. Immunization of mice with purified *M. tuberculosis* or *M. leprae* antigen 85B also induced protection against *M. leprae* challenge but less so than BCG or rBCG30. Notably, boosting rBCG30 with *M. tuberculosis* antigen 85B significantly enhanced r30ML-specific immune responses, substantially more so than boosting BCG, and significantly augmented protection against *M. leprae* challenge. Thus, rBCG30, a vaccine that induces improved protection against *M. tuberculosis*, induces cross-protection against *M. leprae* that is comparable or potentially superior to that induced by BCG, and boosting rBCG30 with antigen 85B further enhances immune responses and protective efficacy.

Leprosy, caused by the bacterium *Mycobacterium leprae*, remains a major global health problem. Although the reported incidence has decreased over the past few decades, the annual decline has leveled off. The World Health Organization reported 232,875 new cases in 2012 with 71% of the cases occurring in the southeast Asian region of the world (1). In many parts of the world, including Southeast Asia, the number of new cases reported increased over the previous year.

Leprosy presents across a clinical and immunopathological disease spectrum ranging from multibacillary lepromatous leprosy, characterized by many skin lesions with numerous bacilli and an absence of cell-mediated immunity to *M. leprae*, to paucibacillary tuberculoid leprosy, characterized by few lesions and a paucity of bacilli but the presence of specific cell-mediated immunity. Infection of peripheral nerves and nerve damage are a frequent and often debilitating manifestation of leprosy. Leprosy is treated with a prolonged course of antibiotics used in combination. As in the case of tuberculosis (TB), drug resistance has emerged, although worldwide levels of resistance to the major drugs dapsone and rifampin have stabilized in recent years. Nevertheless, because of the extremely long treatment regimens for the disease, new multidrug strategies remain at risk of failing due to emergence of resistance.

No specific vaccine against leprosy is currently available. However, the *Mycobacterium bovis* BCG vaccine developed against tuberculosis has modest albeit highly variable efficacy against leprosy: two meta-analyses of clinical trials estimated the protective effect of BCG in preventing leprosy to be 26% (95% confidence interval [CI], 14 to 37%) (2) and 41% (95% CI, 16 to 66%) (3),

respectively. Thus, an improved vaccine is needed as part of a multicomponent strategy to combat leprosy (4).

rBCG30, a recombinant BCG vaccine overexpressing the *Mycobacterium tuberculosis* 30-kDa major secretory protein antigen 85B, a mycolyl transferase, has previously been demonstrated to be significantly more potent than BCG in inducing immunoprotection against *M. tuberculosis* aerosol challenge in the stringent guinea pig model of pulmonary tuberculosis and subsequently in the mouse model of pulmonary tuberculosis (5–7); similarly, a replication-limited version of rBCG30 designed for use in HIV-positive persons for whom BCG is contraindicated was also demonstrated to be more potent than BCG in protecting against *M. tuberculosis* aerosol challenge in the guinea pig model (8). rBCG30 was also significantly more potent than BCG in inducing immunoprotection against *Mycobacterium bovis* challenge in the guinea pig model (9), and a different recombinant BCG vaccine overexpressing *M. tuberculosis* antigen 85B was recently shown to induce protection against *M. bovis* challenge in cattle (10). In a phase I human clinical trial, rBCG30 was shown to be safe and immunogenic; rBCG30, but not BCG, induced signifi-

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cantly enhanced immunologic responses to antigen 85B over baseline values for each of eight different immunologic assays, including antibody responses, lymphoproliferation, gamma interferon (IFN- γ) secretion, IFN- γ enzyme-linked immunospot responses, direct *ex vivo* CD4⁺ and CD8⁺ T cell IFN- γ responses, CD4⁺ and CD8⁺ memory T cells capable of expansion, and the capacity of antigen-specific T cells to inhibit the growth of intracellular mycobacteria (11).

Regions of the world with large numbers of leprosy cases, including India, Brazil, Indonesia, Bangladesh, Democratic Republic of the Congo, Nepal, and Myanmar, are also regions with large numbers of cases of tuberculosis. BCG is routinely administered to infants in these countries to protect against tuberculosis. A positive by-product of BCG vaccination is some protective efficacy against leprosy, as noted above. It is likely that an improved tuberculosis vaccine that ultimately replaces BCG will be a recombinant BCG vaccine, as these vaccines are the most promising among the few vaccines that provide protective immunity greater than that provided by BCG in rigorous animal studies (12). Given the overlapping nature of the tuberculosis and leprosy pandemics, it would be highly desirable for replacement vaccines against tuberculosis (TB), such as rBCG30, also to provide enhanced protection against leprosy. With this in mind, we sought here to determine the efficacy of rBCG30 in a murine model of leprosy.

MATERIALS AND METHODS

Bacteria. The BCG Tice parental strain and rBCG30 Tice were prepared as described previously (6). *M. leprae* strain Thai-53 was isolated, maintained, and purified as described previously (13). For the immunology experiments (at University of California—Los Angeles [UCLA]), an improved version of rBCG30 (rBCG30-ARMF-II Tice) that is antibiotic resistance free and that expresses the 30-kDa antigen from the chromosome (immediately downstream of *glnA1*) for enhanced stability was used. This strain expresses the 30-kDa antigen in 15.5-fold-greater amounts than BCG and 2.6-fold-greater amounts than the original rBCG30 (M. A. Horwitz and M. V. Tullius, 9 April 2009, World Intellectual Property Organization, patent application WO 2009/045575).

Mice. (i) Protection studies at NHDP. Six- to 8-week-old female BALB/c mice were purchased from The Jackson Laboratory, Bar Harbor, ME; held 5 per cage; and provided food and water *ad libitum* for 2 weeks prior to vaccination and throughout each 10-month study. All studies with animals were approved by the National Hansen's Disease Programs (NHDP) Institutional Animal Care and Use Committee (IACUC) and conducted within the ethical guidelines outlined under the U.S. Public Health Service policy for the care and use of laboratory animals.

(ii) Immunology studies at UCLA. Six- to 8-week-old female BALB/c mice were purchased from Charles River Laboratories, held 4 per cage, and provided food and water *ad libitum*. Mice were acclimated for 1 week prior to initiating the experiments. Animal research was conducted within the ethical guidelines outlined under the U.S. Public Health Service policy for the care and use of laboratory animals according to protocols approved by the animal research committee of UCLA.

Proteins. The *Mycobacterium tuberculosis* 30-kDa major secretory protein antigen 85B (r30Mtb) was purified as described from culture filtrates of recombinant *Mycobacterium smegmatis* 1-2c containing plasmid pMTB30 (pSMT3 containing full-length gene for 30-kDa *M. tuberculosis* antigen 85B) and demonstrated to be indistinguishable from the native protein (14, 15). The homologous *Mycobacterium leprae* antigen 85B (r30ML) was purified similarly from culture filtrates of recombinant *Mycobacterium smegmatis* 1-2c containing plasmid pNBV1-PLCDS ML30 (pNBV1 containing full-length gene for 30-kDa *M. leprae* antigen 85B).

Other antigens. A peptide encompassing an immunodominant epitope from *M. leprae* antigen 85B (SWEYWG AQLNAMKPD LQ, amino

acids 301 to 317 of full-length protein with signal peptide and amino acids 263 to 279 of the mature protein) was obtained from Synthetic Biomolecules (San Diego, CA). The *M. leprae* antigen 85B epitope differs from the *M. tuberculosis* and *M. bovis* BCG antigen 85B sequences by one amino acid (shown in bold in the abovementioned sequence, **P**→**G**) and is identical to a sequence found in the *M. tuberculosis* and *M. bovis* BCG antigen 85A proteins. *Mycobacterium leprae* SDS-soluble cell wall fraction (MLCwA), NR-19333, was obtained through BEI Resources, NIAID, NIH. Heat-killed *M. leprae* (HKML) was prepared by heating the bacteria in phosphate-buffered saline (PBS) at a concentration of 1×10^9 bacteria/ml at 60°C for 30 min; the bacterial suspension was dispersed prior to and following heating by drawing the suspension through a 25-gauge needle on a tuberculin syringe 20 times.

Vaccination. (i) Protection studies. Mice were sham immunized with PBS or immunized intradermally with 1×10^7 viable BCG or rBCG30 bacteria at week 0 in two experiments (experiments I and II). In experiment II, additional mice were immunized subcutaneously with incomplete Freund's adjuvant (IFA), 20 μ g purified *M. tuberculosis* 30-kDa antigen 85B (r30Mtb) in IFA, or 20 μ g purified *M. leprae* 30-kDa antigen 85B (r30ML) in IFA at week 7, and still other mice were primed with BCG or rBCG30 at week 0 and boosted with 20 μ g r30Mtb or r30ML at week 7. Intradermal injections were given on the backs of mice near the tail following hair removal by shaving, raising a "blister" at the injection site.

(ii) Immunology studies. Mice were immunized intradermally at the base of the tail with 1×10^6 viable BCG or rBCG30-ARMF-II Tice bacteria (diluted from a frozen stock in sterile phosphate-buffered saline [PBS]) or sham immunized with PBS at week 0. At week 7, half of the BCG and rBCG30 mice were boosted intradermally with 20 μ g purified *M. tuberculosis* 30-kDa antigen 85B (r30Mtb) in Syntex adjuvant formulation containing alanyl muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) (MDP) (50 μ g/mouse) (15). Immune responses were assessed 1 week following the booster vaccination.

Isolation of splenocytes. At 8 weeks after the initial prime vaccination (1 week after booster vaccination), mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (80 mg/kg of body weight) and xylazine (10 mg/kg) and then euthanized. Spleens were removed, and single-cell suspensions of splenocytes were prepared by gently pressing the cells out of the spleen sac, lysing red blood cells with PharmLyse (BD Pharmingen), washing the cells, and filtering the cells through a 70- μ m nylon cell strainer (Falcon). Advanced RPMI 1640 (Invitrogen) supplemented with 2% heat-inactivated fetal bovine serum, 2 mM L-alanyl-L-glutamine (Glutamax; Invitrogen), 10 mM HEPES buffer, 50 μ M β -mercaptoethanol, and penicillin (100 IU/ml)-streptomycin (100 μ g/ml) was used as the medium.

IFN- γ secretion assay. Single-cell suspensions of splenocytes (5×10^5 viable cells per well) were stimulated with individual antigens or left unstimulated for 3 days in V-bottom, 96-well tissue culture plates in 200 μ l medium at 37°C in a humidified incubator (95% air, 5% CO₂). For antigen stimulation, r30ML protein, r30ML peptide, purified protein derivative (PPD), or MLCwA was each used at 10 μ g/ml and HKML was used at 1×10^6 bacteria/well. After 3 days of stimulation, culture supernatant fluid was collected following centrifugation and stored at -80°C until assayed. Mouse gamma interferon (IFN- γ) in the culture supernatant fluid was assayed using a commercial enzyme-linked immunosorbent assay (ELISA) kit (BD OptEIA; BD Biosciences) according to the manufacturer's instructions.

Intracellular cytokine staining and flow cytometry analysis. Single-cell suspensions of splenocytes (5×10^5 viable cells per well) were stimulated with individual antigens or left without antigen for 6 h or 24 h in V-bottom, 96-well tissue culture plates in 200 μ l medium at 37°C in a humidified incubator (95% air, 5% CO₂). For antigen stimulation, r30ML protein, r30ML peptide, PPD, or MLCwA was each used at 10 μ g/ml and HKML was used at 1×10^6 bacteria/well. Anti-CD28 antibody (clone 37.51) was included in all wells as a costimulant at 2 μ g/ml. BD GolgiPlug (protein transport inhibitor containing brefeldin A) was added to all wells

for the final 3 h of incubation. Cells were washed with PBS and stained with Live/Dead fixable near-infrared (near-IR) dead cell stain (Invitrogen) for 30 min on ice. After washing and blocking of Fc receptor molecules with anti-mouse CD16/32 (TruStain fcX; BioLegend), surface CD4 and CD8 were labeled with V500 rat anti-mouse CD4 (L3T4) (clone RM4-5) and Alexa Fluor 647 rat anti-mouse CD8a (clone 53-6.7) for 30 min on ice. The cells were then washed, fixed, permeabilized with Cytotfix/Cytoperm (BD Biosciences), and then stained for CD3, IFN- γ , interleukin-2 (IL-2), and tumor necrosis factor (TNF) (V450 rat anti-mouse CD3 molecular complex [clone 17A2], Alexa Fluor 488 rat anti-mouse IFN- γ [clone XMG1.2], phycoerythrin [PE] anti-mouse IL-2 antibody [clone JES6-5H4], and peridinin chlorophyll protein [PerCP]/Cy5.5 anti-mouse TNF- α antibody [clone MP6-XT22]) for 30 min at room temperature. Antibodies were purchased from BD Biosciences and BioLegend. A minimum of 10,000 live CD3⁺ T cells per sample (median, ~22,000) were acquired with a BD LSRII flow cytometer with a high-throughput sampler. The frequencies of live CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T cells expressing each of the seven different possible combinations of IFN- γ , IL-2, and TNF were determined using FACSDiva software (BD). Background numbers of cells producing cytokines without antigen stimulation were subtracted.

Protection against *M. leprae* challenge. At 2.5 months (week 10) after the start of the experiment, all mice (7 sham-immunized and 10 to 11 vaccinated mice/group in experiment I; 9 to 12 sham-immunized or vaccinated mice/group in experiment II) were challenged with 5,000 live *M. leprae* bacteria in each hind footpad. At 210 days after challenge, the mice were euthanized, and the footpads were removed and processed for counting of acid-fast bacilli (AFB) (16). Counting was based on the Shepard protocol (17), counting the number of AFB in 60 oil immersion fields and calculating AFB per footpad.

Statistics. For intracellular cytokine staining, one-way analysis of variance (ANOVA) with the Tukey-Kramer *post hoc* test was used. Protective efficacy in the mouse footpad model was analyzed as follows. In experiment I, means (log₁₀ AFB/footpad; all log figures are log₁₀ throughout the paper) were compared across groups by a parametric ANOVA and non-parametric Kruskal-Wallis rank sum test; for statistical purposes, mice with 0 AFB detected were assigned a value at the limit of detection (log 4). In experiment II, where many mice were scored as having no AFB detected, AFB counts in 60 oil immersion fields were compared across groups using a parametric analysis of deviance under a negative binomial model and using a nonparametric analysis of variance (regression) model with bootstrapping.

RESULTS

rBCG30 and BCG induce significant immune responses to *M. leprae* cell wall antigens (MLCwA) and HKML. To assess the capacity of rBCG30 and BCG to induce a cell-mediated immune response to *M. leprae* antigens in mice, we assayed splenocytes of immunized mice for cytokine-producing CD4⁺ and CD8⁺ T cells in response to *M. leprae* antigens 8 weeks after vaccination in two independent experiments (experiment I is shown in Fig. 1 and 2). After *in vitro* restimulation of splenocytes with antigen for 6 h, mice vaccinated with rBCG30 and BCG, in comparison with sham-vaccinated mice, displayed a significantly higher frequency of cytokine-producing CD4⁺ T cells in response to the broad repertoire of *M. leprae* antigens contained in the MLCwA and HKML antigen preparations (Fig. 1, left); the rBCG30- and BCG-immunized mice also showed a significantly higher frequency of cytokine-producing CD4⁺ T cells in response to *M. tuberculosis* PPD (data not shown; the responses to PPD were qualitatively similar to the responses to MLCwA but somewhat greater in magnitude). The overall magnitudes, as well as the cytokine profiles, of the CD4⁺ T cell responses to MLCwA, HKML, and PPD were similar for the BCG- and rBCG30-vaccinated groups. Increasing the

length of antigen restimulation to 24 h resulted in a greater response to both MLCwA and HKML, but in the case of MLCwA, the sham-vaccinated mice also developed a response not observed at 6 h. In contrast to the responses to MLCwA and HKML, the responses to r30ML protein and peptide were weak. Similar results were seen in the two experiments.

Boosting rBCG30 with *M. tuberculosis* antigen 85B in a heterologous prime-boost vaccination strategy induces significantly more r30ML-specific cytokine-producing CD4⁺ T cells. In the same experiments described above, we also assessed the impact on the cell-mediated immune response of boosting rBCG30 or BCG with r30Mtb. Mice were boosted at 7 weeks after immunization with BCG or rBCG30, and immune responses to *M. leprae* antigens were evaluated 1 week later—the same time as that for mice that were vaccinated with BCG and rBCG30 but not boosted. Data from experiment I are shown in Fig. 1 and 2 (cytokine-producing CD4⁺ T cells and CD8⁺ T cells, respectively), and key data from experiments I and II are summarized in Fig. 3 (r30ML-specific cytokine-producing CD4⁺ T cells and IFN- γ secretion). The most noteworthy effect of boosting with r30Mtb was on the level of response to r30ML protein and r30ML peptide by splenocytes from rBCG30-primed mice. The rBCG30–r30Mtb prime-boosted mice (rBCG30 plus boost) demonstrated a significantly enhanced CD4⁺ T cell response to r30ML protein compared with all other groups (Fig. 1). At 6 h after antigen restimulation, IFN- γ ⁺ TNF⁺ (double-positive), IFN- γ ⁺ TNF⁺ IL-2⁺ (triple-positive), and TNF⁺ (single-positive) cytokine-producing cells were the dominant phenotypes representing 80% of all cytokine-producing CD4⁺ T cells; IFN- γ ⁺ (single-positive) and IL-2⁺ TNF⁺ (double-positive) cytokine-producing cells made up essentially all of the remaining cytokine-producing cells (Fig. 1, left). In the same experiment, when splenocytes were restimulated with r30ML protein for 24 h, splenocytes from rBCG30–r30Mtb prime-boosted mice again demonstrated a significantly enhanced CD4⁺ T cell response compared with all other groups (Fig. 1, right). In the 24-h restimulation, while the magnitude of cytokine-producing CD4⁺ T cells was somewhat increased compared with the 6-h restimulation (1.2% versus 0.9% cytokine-producing CD4⁺ T cells), the profile of cytokine-producing populations was altered more dramatically, now dominated by IFN- γ ⁺ (single-positive) cells and with a much smaller IFN- γ ⁺ TNF⁺ (double-positive) population. These two populations together represented 95% of all cytokine-producing CD4⁺ T cells at 24 h, and IFN- γ ⁺ TNF⁺ IL-2⁺ (triple-positive) cells were essentially absent. The simpler cytokine profile dominated by IFN- γ ⁺ (single-positive) cells at 24 h in response to r30ML protein was also observed for MLCwA, HKML, and PPD (Fig. 1 and data not shown). At both 6 h and 24 h, the response to r30ML peptide was somewhat lower than the response to r30ML protein but otherwise similar. Surprisingly, mice vaccinated with only rBCG30 and mice vaccinated with BCG and boosted with r30Mtb (BCG plus boost) had weak r30ML-specific CD4⁺ T cell responses, similar to the BCG group.

In Fig. 3, data on the frequency of cytokine-producing CD4⁺ T cells in response to r30ML protein and peptide for both experiments are summarized and the results of the assay for IFN- γ secretion are displayed. The splenocytes from rBCG30–r30Mtb prime-boosted mice had a 4- to 5-fold-greater number of CD4⁺ T cells producing one or more cytokines (IFN- γ , TNF, and IL-2) in response to r30ML protein than did the splenocytes from BCG–r30Mtb prime-boosted mice and a 7- to 12-fold greater number

FIG 1 Frequency of cytokine-producing CD4⁺ T cells in response to *M. leprae* antigens. Mice were sham immunized or immunized with BCG or rBCG30-ARMF-II Tice (rBCG30) at week 0. At week 7, half of the mice immunized with BCG or rBCG30 received a booster vaccination with r30Mtb protein in adjuvant. At week 8, splenocytes were isolated from all mice and stimulated *in vitro* with MLCwA, HKML, r30ML protein, or r30ML peptide for 6 h and 24 h. GolgiPlug (containing brefeldin A) was included for the final 3 h of stimulation prior to intracellular cytokine staining, followed by analysis for cytokine-producing T cells by multiparameter flow cytometry. The frequencies of live CD3⁺ CD4⁺ T cells producing each of the seven different possible combinations of IFN- γ , IL-2, and TNF (indicated by the plus and minus signs underneath the graphs), as well as the summation of all cytokine-producing T cells ("All"), are shown. Background numbers of cells producing cytokines without antigen stimulation were subtracted from the data. Values are means and standard errors for 4 mice. The experiment was repeated once with similar results, and the data from both experiments with respect to r30ML-specific cytokine-producing CD4⁺ T cells are summarized in Fig. 3.

FIG 2 Frequency of cytokine-producing CD8⁺ T cells in response to *M. leprae* antigens. Mice were immunized, and T cells were analyzed by multiparameter flow cytometry as described in the Fig. 1 legend. The frequencies of live CD3⁺ CD8⁺ T cells producing each of the seven different possible combinations of IFN- γ , IL-2, and TNF after 24 h of *in vitro* stimulation (indicated by the plus and minus signs underneath the graphs) as well as the summation of all cytokine-producing T cells (“All”) are shown. Background numbers of cells producing cytokines without antigen stimulation were subtracted from the data. Values are means and standard errors for 4 mice.

than did the splenocytes from mice immunized with only rBCG30 (Fig. 3A and B; 6-h restimulation). With a 24-h restimulation (Fig. 3C and D), a similarly high frequency of CD4⁺ T cells producing one or more cytokines was observed for mice prime-boosted with rBCG30–r30Mtb in experiment I (Fig. 3C) but not in experiment II (Fig. 3D). Paralleling these results, splenocytes from mice prime-boosted with rBCG30–r30Mtb secreted a larger amount of IFN- γ after stimulation with r30ML protein or r30ML peptide than did splenocytes from mice in all other groups (Fig. 3E and F).

Cytokine-producing CD8⁺ T cells were not detected for any of the groups with a 6-h restimulation with antigen (data not shown), but after 24-h restimulation, a cytokine profile similar to that of the CD4⁺ T cells at 24 h was observed, although of lesser magnitude and lacking double-positive IFN- γ ⁺ TNF⁺ cells (Fig. 2). While mice prime-boosted with rBCG30–r30Mtb produced a greater r30ML-specific (both protein and peptide) CD8⁺ T cell response than did the other groups, the difference did not reach statistical significance.

BCG and rBCG30 induce protective immunity against *M. leprae* challenge, and in the one experiment in which an efficacy comparison was feasible, rBCG30 induced significantly greater protection than did BCG. To assess the capacity of rBCG30 to induce immunoprotection against *M. leprae* challenge, we immunized mice with BCG or rBCG30, challenged them 2.5 months later with *M. leprae* administered into the hind footpads, and, 7

months after challenge, assayed the number of *M. leprae* bacteria in the hind footpads by counting acid-fast bacilli (AFB). In experiment I (Fig. 4A), both vaccines induced significant protective immunity, decreasing the mean number of AFB by ~1 log from the level (5.3 logs) in sham-immunized animals ($P < 0.0001$ for both vaccines versus sham by ANOVA; $P = 0.0004$ for rBCG30 versus sham and $P = 0.002$ for BCG versus sham by the nonparametric Kruskal-Wallis rank sum test); for statistical purposes, animals at the level of detection were assigned an AFB value of 4 logs. Moreover, rBCG30-immunized animals had 0.3-log-lower AFB counts than did BCG-immunized animals, a difference that was statistically significant ($P < 0.04$ by ANOVA and $P = 0.04$ by Kruskal-Wallis nonparametric test).

In experiment II (Fig. 4B), both BCG and rBCG30 again induced significant protective immunity, decreasing the mean log number of AFB by 0.9 logs from the level (4.6 logs) in sham-immunized animals ($P < 0.001$ for both vaccines versus sham by ANOVA; $P = 0.002$ for both vaccines versus sham by nonparametric analysis). In this experiment, all but one animal in each of the BCG and rBCG30 groups were at the level of detection of 3.7 logs (i.e., 0 or 1 AFB was observed in 60 oil immersion fields from the footpads of all but one animal per group), and there was not a statistically significant difference.

Thus, in both experiments, rBCG30 and BCG significantly induced protection against *M. leprae* challenge, and in the one ex-

FIG 3 Priming with rBCG30 and boosting with r30Mtb protein in adjuvant significantly enhances r30ML-specific cytokine-producing CD4⁺ T cells and IFN- γ secretion. Mice were immunized as described in Fig. 1. At 8 weeks, splenocytes were isolated and stimulated *in vitro* with r30ML protein and r30ML peptide for 6 h prior to intracellular cytokine staining (A and B), for 24 h prior to intracellular cytokine staining (C and D), or for 3 days prior to analyzing the culture medium for IFN- γ by ELISA (E and F). Two independent experiments were performed: experiment I (A, C, and E) and experiment II (B, D, and F). For intracellular cytokine staining (A to D), the summation of all CD4⁺ T cells producing cytokine (IFN- γ , IL-2, and/or TNF) is shown and background numbers of cells producing cytokines without antigen stimulation were subtracted from the data. Values are means and standard errors for 4 mice. Statistical comparisons are indicated by lines above the bars with *P* values calculated by one-way ANOVA and multiple comparisons performed by a Tukey-Kramer *post hoc* analysis.

periment in which a meaningful comparison between the two vaccines was feasible, rBCG30 induced significantly greater immunoprotection than did BCG.

Purified *M. tuberculosis* or *M. leprae* 30-kDa antigen 85B induces protective immunity against *M. leprae* challenge. In experiment II, in addition to evaluating the protective efficacy of BCG and rBCG30, we also evaluated the capacity of purified *M. tuberculosis* 30-kDa protein (r30Mtb) and purified *M. leprae* 30-kDa protein (r30ML) to induce protective immunity (Fig. 4B); the proteins were administered in incomplete Freund's adjuvant (IFA) as described in Materials and Methods. Both proteins induced significant protection, reducing *M. leprae* per footpad by 0.4 logs versus sham-immunized animals vaccinated with only IFA ($P < 0.02$ for both vaccines versus sham [IFA] by ANOVA

and $P = 0.02$ and $P = 0.04$ for r30Mtb and r30ML, respectively, versus sham [IFA] by nonparametric analysis).

Thus, as in numerous previous studies, a purified extracellular protein of an intracellular pathogen (or one closely related to it) was immunoprotective (15, 18, 19).

Boosting rBCG30 with purified *M. tuberculosis* 30-kDa antigen 85B in a heterologous prime-boost vaccination strategy further enhances protective immunity to *M. leprae* challenge. Also in experiment II, a prime-boost strategy was employed where animals were primed with BCG or rBCG30 and boosted with purified r30Mtb or r30ML. Surprisingly, in the case of BCG, boosting did not improve protection; indeed, protection was somewhat diminished, although the differences between each of these prime-boosted groups and sham-immunized animals remained statisti-

FIG 4 Protective efficacy. Mice were sham immunized (Sham) or immunized with BCG or rBCG30 at week 0 and challenged at week 10 with 5,000 live *M. leprae* bacteria in each hind footpad. In experiment II, additional mice were sham immunized with IFA (Sham IFA) or immunized with purified r30Mtb in IFA or r30ML in IFA at week 7 and also challenged at week 10. Also in experiment II, additional mice were immunized with BCG or rBCG30 at week 0 and additionally boosted with r30Mtb or r30ML in IFA as indicated at week 7 and challenged at week 10. Seven months after challenge, the animals were euthanized and the number of AFB was enumerated in their hind footpads. Data are the log AFB/footpad; for graphing purposes, mice with 0 AFB per 60 oil immersion fields were assigned a value of 1 equivalent to log 4 in experiment I and log 3.7 in experiment II. (A) Experiment I. *, $P < 0.0001$ by ANOVA and $P = 0.0004$ by Kruskal-Wallis rank sum test; **, $P < 0.0001$ by ANOVA and $P = 0.002$ by Kruskal-Wallis rank sum test; ***, $P < 0.04$ by ANOVA and $P = 0.04$ by Kruskal-Wallis rank sum test. (B) Experiment II. Data where AFB counts were 0 are shown by open circles. †, $P < 0.001$ versus Sham by ANOVA and $P = 0.002$ versus Sham by nonparametric analysis; ‡, $P < 0.02$ by ANOVA and $P = 0.02$ by nonparametric analysis; §, $P < 0.02$ by ANOVA and $P = 0.04$ by nonparametric analysis; γ, $P < 0.02$ by ANOVA and $P = 0.04$ by nonparametric analysis; γγ, $P < 0.02$ by ANOVA and $P = 0.04$ by nonparametric analysis; §, $P = 0.03$ by nonparametric analysis.

cally significant ($P < 0.001$ by ANOVA and $P = 0.002$ by nonparametric analysis). In the case of rBCG30, boosting with r30Mtb did significantly improve protection in this single experiment despite the data clustering at the limit of detection, as not a single AFB was detected in 60 oil immersion fields from the footpads of the 12 prime-boosted animals whereas 5 AFB (1 AFB in each of 3 animals and 2 AFB in one animal) were detected in the footpads of the 11 animals that were only primed with rBCG30 ($P = 0.03$ by nonparametric analysis).

Thus, the enhanced protective immunity observed after boosting rBCG30 with *M. tuberculosis* antigen 85B mirrored the significantly enhanced r30ML-specific CD4⁺ T cell responses observed with this heterologous prime-boost vaccination regimen.

DISCUSSION

This paper demonstrates that a recombinant BCG vaccine (rBCG30) originally designed to provide enhanced protection against human and bovine tuberculosis additionally has the potential to provide enhanced protection against leprosy. In previous studies, rBCG30 was shown to induce significantly stronger protection than that induced by BCG against *M. tuberculosis* and *M. bovis* challenge in animal models (5, 6, 9). In this study, rBCG30 was shown to provide significant protection against *M. leprae* challenge in a murine model of leprosy in two independent experiments, and in the one experiment in which a comparison with BCG was feasible, rBCG30 provided significantly greater protection than did BCG. Thus, in addition to its potential for providing the general population better protection than that by BCG against tuberculosis, rBCG30 has the potential to provide the bonus of better protection against leprosy. rBCG30 is the first new-generation TB vaccine demonstrated to protect against *M. leprae*.

Other recombinant BCG vaccines not previously tested against

M. tuberculosis or other pathogenic mycobacteria have been tested in murine models of leprosy. Ohara et al. evaluated a recombinant BCG vaccine overexpressing the *M. bovis* BCG 32-kDa antigen 85A in both a short-term (mice challenged 4 weeks after immunization) and a long-term (mice challenged 5 months after vaccination) study and showed a trend toward reduced numbers of *M. leprae* in mouse footpads versus BCG, although the difference from BCG was not statistically significant (20). Ohara et al. also evaluated a recombinant BCG vaccine overexpressing *M. bovis* BCG antigen 85A, antigen 85B, and MPB51 in a short-term study (mice challenged 4 weeks after vaccination) and found that the vaccine showed a trend toward reduced numbers of *M. leprae* in mouse footpads versus BCG in both C57BL/6 and BALB/c mice, although the difference from BCG was not statistically significant (21). Maeda et al. studied a recombinant BCG expressing the *M. leprae* major membrane protein II in a short-term study in which mice were challenged 4 weeks after immunization and found that mice immunized with the recombinant BCG had significantly fewer *M. leprae* bacteria than did BCG-immunized mice in their footpads (22).

Like other pathogenic mycobacteria, *M. leprae* expresses the proteins of the antigen 85 complex (antigens 85A, -B, and -C), which function as mycolyl transferases (23). Mycolyl transferases catalyze the formation of the major mycobacterial cell wall component trehalose 6,6'-dimycolate (TDM) via the transfer of mycolic acid from one trehalose 6-monomycolate to another; crystal structure analysis infers that this transfer takes place via an interfacial mechanism consistent with association of the enzyme with the bacterial surface (24, 25). The 30-kDa antigen 85B protein is the most abundant protein secreted into broth culture by *M. tuberculosis*, and it is among the most abundant proteins of all

types expressed by *M. tuberculosis* residing in infected human macrophages (15, 26). The 30-kDa mature proteins of *M. tuberculosis*, *M. bovis*, and various *M. bovis* BCG strains are identical or virtually so, sometimes differing by 1 amino acid. The *M. leprae* antigen 85B mature protein is highly homologous (85%) with its *M. tuberculosis* and *M. bovis* homologs. Epitope mapping of the *M. tuberculosis* 30-kDa antigen 85B protein in BALB/c mice, the animal strain used here, has revealed seven major Th1 helper T cell epitopes, all of which share high similarity with the corresponding region of the *M. leprae* antigen 85B homolog (27). Hence, it is not surprising that recombinant BCG overexpressing the *M. tuberculosis* antigen 85B confers enhanced protection against *M. bovis* and *M. leprae* in addition to *M. tuberculosis*.

In previous studies, *M. tuberculosis* 30-kDa antigen 85B protein (r30Mtb) was demonstrated to induce significant protective immunity against *M. tuberculosis* challenge in the guinea pig model of pulmonary tuberculosis (15), although protection was always less than that provided by BCG. Similarly, in the present study, both r30Mtb and the *M. leprae* antigen 85B protein (r30ML) were found to induce significant protection against *M. leprae* challenge in the murine model of leprosy. There was no significant difference in the protective efficacies of r30Mtb and r30ML. As in the case of *M. tuberculosis* challenge, protection by these purified proteins was less than that afforded by BCG or rBCG30.

Protective efficacy against tuberculosis can be enhanced significantly by boosting BCG with the *M. tuberculosis* 30-kDa antigen 85B (28). This raised the possibility that boosting BCG with the *M. tuberculosis* or *M. leprae* 30-kDa protein would similarly enhance immunoprotection against *M. leprae*. However, demonstrating this in the murine model of leprosy is challenging in view of the limit of detection for assaying *M. leprae* numbers per footpad in this model. Indeed, in experiment II of the present study, in animals primed with BCG or rBCG30 and boosted with r30Mtb or r30ML, data on *M. leprae* numbers per footpad were clustered at the limit of detection at the end of the experiment. In this single experiment, in the case of BCG, boosting with either r30ML or r30Mtb did not enhance protection and, inexplicably, trended toward diminished protection. This may reflect experimental variation and the limitations of the mouse leprosy model rather than a truly antagonistic effect of boosting, since in numerous prime-boost studies in more sensitive pulmonary tuberculosis animal models, boosting BCG with r30Mtb has consistently enhanced protection, both in guinea pigs and in mice (28) (Q. Jia and M. Horwitz, unpublished studies). However, the failure of the BCG-antigen 85B regimen to enhance protection may have reflected the failure of this prime-boost regimen to induce r30ML-specific CD4⁺ T cell responses (see paragraph below).

In this study, we also evaluated the immunologic and protective impact of boosting rBCG30 with *M. tuberculosis* antigen 85B. One of the most striking and interesting observations was that boosting rBCG30 with r30Mtb protein dramatically enhanced the r30ML-specific CD4⁺ T cell response. Perhaps reflecting this, boosting rBCG30 with r30Mtb also significantly enhanced protection against *M. leprae* challenge, despite the limitations and challenges in demonstrating this in the *M. leprae* mouse model. Interestingly and in contrast, boosting BCG with r30Mtb did not enhance these r30ML-specific CD4⁺ T cell responses, perhaps underlying the failure of the BCG-antigen 85B prime-boost regimen to enhance protection against *M. leprae* challenge, as noted in the

previous paragraph. These results suggest that rBCG30 elicits a greater number of central memory T cells that are capable of expansion than BCG does and/or that these central memory T cells have increased proliferative capacity in the context of reexposure to antigen.

This study lends further support to the concept first proposed over 25 years ago (18, 29) that extracellular proteins of intracellular pathogens, i.e., proteins secreted or otherwise released from the pathogens, are key immunoprotective proteins (extracellular protein hypothesis). Such proteins, by virtue of their secretion into the phagosome or cytoplasmic milieu of the pathogen, are available for processing and presentation as major histocompatibility complex (MHC)-peptide complexes on infected host cells, allowing recognition of host cells harboring live intracellular pathogens by antigen-specific lymphocytes. Similarly, appropriate vaccination with these extracellular proteins induces a population of antigen-specific lymphocytes capable of later recognizing these MHC-peptide complexes on infected host cells and exerting an antimicrobial effect against them. Consistent with this idea, within hepatic granulomas of mice infected with BCG or rBCG30, lymphocytes specifically recognizing an MHC-peptide complex comprising a major epitope of r30 engaged infected macrophages and increased their CD69 surface expression to a greater extent in granulomas containing rBCG30-infected macrophages than in granulomas containing BCG-infected macrophages, as visualized by intravital imaging (30). Major secreted proteins released by the same or a highly related pathogen have been shown previously to induce and/or enhance protection against *Legionella pneumophila* (18, 19), *M. tuberculosis* (5, 6, 15, 31), *M. bovis* (28), and *Francisella tularensis* (32, 33) and in the present study to enhance protection against *M. leprae* both when administered as purified proteins and when overexpressed by recombinant BCG.

In addition to the 30-kDa antigen 85B, *M. tuberculosis* secretes a large number of other proteins into culture supernatants, approximately a dozen in high quantity. Several of these abundantly secreted proteins have been demonstrated to be immunoprotective, alone or in combination with antigen 85B. These include antigen 85A (r32A; Rv3804c) (15), MPT51 (Rv3803c) (15), superoxide dismutase (SodA; Rv3846) (15), MPT63 (Rv1926c) (15), Hsp70 (DnaK; Rv0350) (15), and ESAT-6 (Rv3875) (34). Administered individually, these proteins are less protective than antigen 85B, but when combined with antigen 85B, they enhance the level of protection conferred by antigen 85B alone, both in terms of reducing organ burden and, in the case of ESAT-6, increasing survival after *M. tuberculosis* challenge (15, 34).

In addition to rBCG30, several recombinant BCG vaccines expressing extracellular proteins have been shown to induce protection against *M. tuberculosis* challenge superior to that by BCG, most notably rBCG expressing antigen 85B plus antigen 85A plus TB10.4 (in combination with perfringolysin O to promote phagosome escape) (35), ESAT-6 plus CFP10 (36), antigen 85A (37, 38), antigen 85C (39), antigen 85B-ESAT-6 fusion protein (40), and surface-expressed MPT64 (41).

A central premise of the extracellular protein hypothesis is that major secreted proteins of intracellular pathogens are protective independently of their function or essentiality as virulence determinants. In support of this idea, vaccination of guinea pigs with the most abundant protein secreted by *L. pneumophila*, the 39-kDa major secretory protein (MSP), a metalloprotease, is highly protective against lethal *L. pneumophila* challenge when adminis-

tered as a single protein in adjuvant (18); yet, this protein is not a virulence determinant in the guinea pig model of Legionnaires' disease, as evidenced by the demonstration that an MSP-negative *L. pneumophila* strain is just as virulent as an isogenic MSP-positive *L. pneumophila* strain in this model (42).

In addition to the provision of an abundant amount of protein, for which multiple T cell epitopes have been identified in mice, guinea pigs, and humans (27, 43–45), overexpression of r30 by rBCG30 could enhance protective immunity in additional ways. Since r30 is a mycolyl transferase involved in the synthesis of the key cell wall component TDM, overexpression of r30 could endow rBCG30 with greater resistance to clearance by host antimicrobial defense mechanisms; persistence of the organism might allow it to induce stronger immunity. Consistent with this idea, rBCG30 is more resistant than BCG to the cell wall active antibiotic isoniazid (46). However, studies in the guinea pig have shown that rBCG30 is cleared at the same rate as is BCG (5).

Another potential mechanism by which rBCG30 may enhance protective immunity is the induction of greater amounts of antibody to r30. Indeed, rBCG30 induces significantly greater titers of anti-r30 antibody than does BCG in guinea pigs (9). Two traditional roles of antibody in host defense against bacterial pathogens—promotion of complement-mediated lysis and promotion of phagocyte killing—evidently do not play a role in control of *M. tuberculosis* infection. With respect to complement, *M. tuberculosis* is highly resistant to complement killing. Indeed, *M. tuberculosis* relies heavily on fixation of fragments of complement component C3 to its surface to induce its uptake into macrophages via CR1, CR3, and CR4 receptors, the dominant receptors involved in its phagocytosis by these host cells (47); similarly, *M. leprae* is ingested via C3 and complement receptors (48). With respect to phagocyte killing, *M. tuberculosis* is highly resistant to killing by professional phagocytes. However, a new role for antibody has emerged in recent studies demonstrating that B cells and antibody can promote Th1 immunity to *M. tuberculosis* and contain infection (49, 50). Although the mechanisms remain to be clarified, one study provided evidence that antibody downmodulates the IL-17-mediated neutrophil response associated with diminished dendritic cell migration to draining lymph nodes, thus potentially enhancing antigen presentation by dendritic cells (50).

In summary, rBCG30, a vaccine developed to protect against human and bovine tuberculosis, offers cross-protective immunity against leprosy. Purified *M. tuberculosis* antigen 85B, the 30-kDa protein overexpressed by rBCG30, administered in adjuvant, also provides cross-protective immunity to *M. leprae* challenge, although to a lesser extent than live BCG or rBCG30. Boosting rBCG30 with *M. tuberculosis* antigen 85B in a heterologous prime-boost vaccination strategy significantly enhances r30ML-specific CD4⁺ T cell responses and, likely reflecting this, further augments protective immunity to *M. leprae* challenge.

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