Synthetic Long Peptide Derived from \textit{Mycobacterium tuberculosis}
Latency Antigen Rv1733c Protects against Tuberculosis

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Responsible for 9 million new cases of active disease and nearly 2 million deaths each year, tuberculosis (TB) remains a global health threat of overwhelming dimensions. \textit{Mycobacterium bovis} BCG, the only licensed vaccine available, fails to confer lifelong protection and to prevent reactivation of latent infection. Although 15 new vaccine candidates are now in clinical trials, an effective vaccine against TB remains elusive, and new strategies for vaccination are vital. BCG vaccination fails to induce immunity against \textit{Mycobacterium tuberculosis} latency antigens. Synthetic long peptides (SLPs) combined with adjuvants have been studied mostly for therapeutic cancer vaccines, yet not for TB, and proved to induce efficient antitumor immunity. This study investigated an SLP derived from Rv1733c, a major \textit{M. tuberculosis} latency antigen which is highly expressed by “dormant” \textit{M. tuberculosis} and well recognized by T cells from latently \textit{M. tuberculosis}-infected individuals. In order to assess its \textit{in vivo} immunogenicity and protective capacity, Rv1733c SLP in CpG was administered to HLA-DR3 transgenic mice. Immunization with Rv1733c SLP elicited gamma interferon-positive/tumor necrosis factor-positive (IFN-γ+/TNF-α+) and IFN-γ+/CD4+ T cells and Rv1733c-specific antibodies and led to a significant reduction in the bacterial load in the lungs of \textit{M. tuberculosis}-challenged mice. This was observed both in a pre- and in a post-	extit{M. tuberculosis} challenge setting. Moreover, Rv1733c SLP immunization significantly boosted the protective efficacy of BCG, demonstrating the potential of \textit{M. tuberculosis} latency antigens to improve BCG efficacy. These data suggest a promising role for \textit{M. tuberculosis} latency antigen Rv1733c-derived SLPs as a novel TB vaccine approach, both in a prophylactic and in a postinfection setting.

Despite the availability of a vaccine and chemotherapeutic agents, tuberculosis (TB) remains the second leading cause of death from an infectious disease worldwide, annually causing around 9 million new cases and almost 2 million deaths (1). The highest burdens are found in low-income regions: Africa harbors about 25% of the world’s TB cases and more than half have been counted in Asia (1). Furthermore, surveys with tuberculin skin tests (TST) suggest that one-third of the world’s population is latently infected with \textit{Mycobacterium tuberculosis}, which constitutes a huge reservoir with a 3 to 10% lifetime risk of developing TB (1, 2).

It has long been recognized that the efficacy of vaccination with \textit{Mycobacterium bovis} BCG, still the only registered TB vaccine, can be enhanced by a booster or replacement vaccine protecting against reactivating TB from latency (3). \textit{M. tuberculosis} latency antigens (AgS), encoded by the DosR regulon, are upregulated \textit{in vitro} under conditions that tubercle bacilli are thought to encounter \textit{in vivo} during persistence in immunocompetent hosts (4) and in mouse models for latent \textit{M. tuberculosis} infection (LTBI) (5). This discovery caused a change in focus in the search for a novel antigen(s) able to enhance long-term vaccine efficacy. Various human cohort studies showed preferential recognition of DosR-encoded proteins, in particular Rv1733c, Rv2029c, Rv2627c, and Rv2628, by T cells from individuals with LTBI and to a lesser extent by TB patients (6–9). Also, latency antigens can induce CD4+ and CD8+ T cells in TST-converted individuals who were infected with \textit{M. tuberculosis} decades ago in the preantibiotic era and yet never developed TB (10, 11).

In mouse models using chronic, low-dose \textit{M. tuberculosis} infection mimicking human LTBI, the preferential recognition of the DosR regulon in latent infection was further established (5) and provided evidence that BCG fails to induce a significant response to latency antigens despite their presence in the BCG genome. Also, a triple fusion protein, designated H56, including the latency/starvation antigen Rv2660 coupled to the early-stage proteins Ag85B and ESAT-6, provided protection by both pre- and postinfection vaccination in nonhuman primates (12, 13). A similar improvement in long-term protection against \textit{M. tuberculosis} was observed in mice after intradermal inoculation of recombinant BCG expressing the latency-associated antigens Rv2659c, Rv3407, and Rv1733c (14).

Synthetic long peptides (SLPs), administered with adjuvants, are proven efficient vaccines for tumor therapy (15–19). Despite the success of this vaccine strategy, SLPs have not yet been applied to design new TB vaccines. Recognition of cognate antigen presented by either T cells or B cells will lead to an abrogative proliferative response and death of the responding T cells.

The immunogenicity of Rv1733c, which was the most commonly recognized latency antigen in \textit{M. tuberculosis}-exposed household contacts from South Africa, Gambia, and Uganda (9), as well as the improved long-term protection against \textit{M. tubercu-
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losis in mice by BCG expressing Rv1733c (14), prompted us to investigate the potential of Rv1733c-derived SLPs as vaccines for TB.

Our previous work has shown that HLA-DRB polymorphism controls human T-cell responsiveness (20). In this respect, HLA-DR3, a major class II allele that is present in 20% of the human population, is associated with strong T-cell activity to mycobacterial Ags, in vitro and in vivo, and with high-responder (tuberculous) leprosy (21). Since HLA transgenic (tg) mice are efficient models to study HLA-restricted T-cell responses to mycobacteria in vivo (22–24), SLP vaccine efficacy was tested in vivo in the context of HLA-DR3. For this purpose, we investigated the potency of an Rv1733c-derived SLP in inducing protection in mice and demonstrated an SLP-based vaccine strategy for TB inducing CD4+ T cells that confer protection against a live M. tuberculosis challenge in mice in a prophylactic as well as a postinfection fashion.

MATERIALS AND METHODS

Recombinant proteins. M. tuberculosis genes were amplified by PCR from genomic DNA of M. tuberculosis and cloned using the Gateway technology platform (Invitrogen, Carlsbad, CA) with pDEST17 expression vector containing an N-terminal histidine tag (Invitrogen) (25). Sequencing was performed on selected clones to confirm the identities of all cloned DNA fragments. Recombinant proteins were overexpressed in Escherichia coli BL21 (DE3) and purified as described previously to remove any traces of endotoxin (25). For production of recombinant Rv1733c, which is composed of a 210-amino-acid (aa) protein, including two predicted transmembrane regions (aa 44 to 66 and aa 164 to 186), the 5' and 3' parts of the gene outside the transmembrane region were amplified by PCR using oligonucleotides overlapping the DNA sequence from aa 67 to 163. These PCR products were subsequently used as oligonucleotides to fuse the N-terminal and C-terminal parts with the internal part, resulting in a PCR product without the predicted transmembrane region which was cloned as described above for other recombinant proteins.

Each purified recombinant protein (Fig. 1) was analyzed by 12% SDS-PAGE followed by Coomassie brilliant blue staining and Western blotting with an anti-His antibody (Ab) (Invitrogen) to confirm size and purity. Endotoxin contents were below 50 EU (endotoxin units) per mg recombinant protein as tested using a Limulus amebocyte lysate (LAL) QCL-1000 assay (Lonza Inc., Basel, Switzerland). Recombinant proteins were tested to exclude protein-nonspecific T-cell stimulation and cellular toxicity in gamma interferon (IFN-γ) release assays using peripheral blood mononuclear cells (PBMCs) of in vitro-purified protein derivative (PPD)-negative, healthy Dutch donors recruited at the Blood Bank Sanquin, Leiden, The Netherlands. None of these controls had experienced any known prior contact with TB patients.

Synthetic peptides. Rv1733c p63-77 (16-mer; AGTAVQDSRSRHVY AH), Rv1733c p61-80 (20-mer; AAAGTAVQDSRSRHVYAHQAQ), the synthetic long peptide (SLP) Rv1733c p57-84 (28-mer; IFPAAAGTAVQDSRSRHVYAHQAQTRHVP57-84), Rv1733c SLP1-SLP13 (Fig. 1), Ag85B p131-162 (AMILAAYHQOFVYAGSLSALLDPSGMGP), and Ag85B p143-152 (FYIAGSLSA) were purchased from Peptide 2.0 Inc. (Chantilly, VA, USA). Homogeneity and purity were confirmed by analytical high-pressure liquid chromatography (HPLC) and by mass spectrometry. Purity of all peptides was ≥80%. All impurities consist of shorter versions of the peptide caused by <100% coupling efficiency in each round of synthesis.

HLA-DR3 tg mice. HLA-DRB1*0301/DR3 transgenic (tg), murine class II-deficient (HLA-DR3,Ab0) mice were generated as detailed previously (23, 26). Briefly, cosmid carrying HLA-DRA and HLA-DRB1*0301 genes were coinjected into (C57BL/6 × DBA/2)F1 × C57BL/6 embryos and backcrossed to C57BL/10 mice. The HLA-DR3 specificity was introduced into murine class II-negative mice by mating the H2.Ab8 strain (27) with HLA-DR3.B10.M mice (28), as described for HLA-DQ8.Ab0 mice (29). The HLA-DR3,Ab0 mice used in this study were backcrossed for 10 generations with C57BL/10 mice and eventually intercrossed. The resulting mice therefore are considered C57BL/10 congenic. Mice were bred under specific-pathogen-free conditions at the Leiden University Medical Center (LUMC) animal facility. During breeding, PBMCs of each mouse were typed for expression and segregation of the transgene by flow cytometry for HLA-DR (fluorescein isothiocyanate [FITC]-labeled mouse IgG2 κ anti-HLA-DR clone L243; BD 555811; BD Biosciences, Franklin Lakes, NJ, USA) and murine CD4 (phycoerythrin [PE]-Cy5-labeled rat IgG2a,κ anti-mouse CD4 clone H129.19; BD 553654; BD Biosciences; and PE-labeled mouse BALB/c IgG2a κ anti-mouse J-Ab, clone AF6-120.1; BD 553552; BD Biosciences). Littersmates lacking HLA-DR expression, designated HLA-DR3,Ab0, were used as negative controls.

HLA-DR3.A2 tg mice were generated by mating the HLA-DR3,Ab0 with HLA-A2 tg mice, B6.Cg-Tg (HLA-A/H2-D)Enge/J stock no. 004191 (The Jackson Laboratory, Bar Harbor, ME, USA (24, 30).

Immunizations. Mice (4 to 6 animals per group; 6 weeks old) were injected subcutaneously (s.c.) three times with 50 μg CpG (oligodeoxynucleotide [ODN] 1826, 5'-TCC ATG ACG TTC CTG ACG TT-3'; InvivoGen, San Diego, CA) in 200 μl phosphate-buffered saline (PBS) in the right flank at 2-week intervals in combination with either 25 μg recombinant Rv1733c protein, Rv1733c p63-77 (40 nmol), or Rv1733c p57-84 (40 nmol). Control mice were injected s.c. with 106 CFU of BCG strain H37Rv 2 weeks after the third antigen immunization or 12 weeks after BCG immunization. Mice were anesthe-
tized with isoflurane [2-chloro-2-(difuoromethoxy)-1,1,1-trifluoroethane; Pharmachemie BV, Haarlem, The Netherlands] and intranasally (i.n.) infected with 10^7 CFU of M. tuberculosis from frozen ampoules. Mice were sacrificed 6 weeks after M. tuberculosis challenge, and spleen and lungs were aseptically removed. The organs were homogenized in sterile PBS, and the number of bacteria was determined by culturing serial dilutions of the homogenates on 7H11 agar plates (BD Biosciences) supplemented with BD BBL Middlebrook oleic acid–albumin–dextrose–catalase (OADC) enrichment (100 ml per bottle; BD Biosciences), PANTA (BD Biosciences; 1 vial per liter containing polymyxin B [6,000 units], amphotericin B [600 µg], nalidixic acid [2,400 µg], thiomethylprin [600 µg], azlocillin [600 µg]), and ampicillin (3.4 mg/ml; Vepidan, Denmark). Colonies were counted after 3 weeks of incubation at 37°C. In the case of animals that received M. tuberculosis infection combined with BCG vaccination, 7H11 agar plates containing 2-thiophene carboxylic acid hydrazide (2 µg/ml; Sigma) were used to distinguish BCG colonies from M. tuberculosis colonies. Protective efficiencies are expressed as log_{10} bacterial counts in immunized mice compared to BCG-immunized mice.

**In vitro cultures.** Splenocytes were isolated from individual animals by homogenizing spleens through a plastic cell strainer (BD Bioscience), and splenocytes (3 × 10^6 cells/ml) were resuspended in Iscove’s modified Dulbecco’s medium (IMDM) (Invitrogen) supplemented with 2 mM l-glutamine (Invitrogen), 100 U/100 µl penicillin-streptomycin solution (Invitrogen), 8% heat-inactivated fetal calf serum (FCS), and 5 × 10^{-5} M β-mercaptoethanol (Sigma). Cell suspensions (100 µl/ml) were added to 96-well round-bottom microtiter plates (Costar; Corning Incorporated). Cells were incubated in quadruplicate with 100 µl of medium, peptide (1 or 10 µg/ml), or recombinant protein (1 or 10 µg/ml). The mitogen concanavalin A (ConA; 2 µg/ml; Sigma) was used in all experiments as a positive control for cell viability. After 6 days, supernatants were taken from each well and quadruplicates were pooled and frozen at −20°C until performance of enzyme-linked immunosorbent assays (ELISAs).

IFN-γ ELISA. Before ELISAs were performed on supernatants from M. tuberculosis-infected murine material, supernatants or sera were transferred into 0.2-µm filter plates (Corning, NY, USA) and centrifuged for 3 min at 1,300 rpm. The filtered material was collected in clean 96-well plates and transferred out of the biosafety level 3 (BSL3) lab for further analyses. Detection of IFN-γ in culture supernatants of *in vitro*-cultured splenocytes was performed by ELISA (BD Biosciences) according to the manufacturer’s instructions. Optical density (OD) values were converted into concentrations using Microplate Manager software, version 5.2.1 (Bio-Rad Laboratories, Veenendaal, The Netherlands). The cutoff value to define positive responses was set beforehand at 100 pg/ml. The assay sensitivity level was 20 pg/ml. Values for unstimulated whole-blood cultures were typically <30 pg/ml.

**Intracellular cytokine staining.** For polychromatic flow cytometry, splenocytes (3 × 10^8 cells/ml) were cultured in *vitro* with peptide (5 µg/ml). After 6 days, cells were incubated with medium or fresh peptide (5 µg/ml). After 1 h, brefeldin A (Sigma; 5 µg/ml) was added. After 5 h, cells were permeabilized and fixed using Cytofix/Cytoperm (BD Bioscience) and stored in −80°C in PBS before the two populations were mixed at a 1:1 ratio, and a total of 15 × 10^6 cells was injected intravenously in the tail of *M. tuberculosis*-infected mice. After 2 days, spleens were removed and the ratio of CFSElow to CFSEhigh target cells was determined by flow cytometry. Specific killing of pulsed CFSEhigh target cells was calculated as follows: \( \frac{1 - (\text{CFSElow}/\text{CFSEhigh})}{100\%} \).

Ethics statement. Handling of mice was conducted in compliance with European Community Directive 86/609 for the care and use of laboratory animals and in accordance with the regulations set forward by the LUMC animal care committee.

Welfare monitoring. Animals were observed daily to fulfill ethics requirements and to monitor any adverse effects possibly related to vaccinations. *M. tuberculosis*-infected mice were weighed once a week.

**Statistical analysis.** GraphPad Prism (version 5) software was used for statistical analysis. Bacterial titers were analyzed by the Mann-Whitney U test. *In vitro* cytokine levels were compared using Student’s t test. P values of ≤0.05 were considered significant.

**RESULTS**

Rv1733c SLIP induces high levels of IFN-γ-producing, Rv1733c-specific CD4+ T cells in vivo. Previously, the *in vivo* immunogenicity of the HLA-DR3-restricted 15-mer Rv1733c p63-77 was investigated in the context of a multistage polypeptide (18). Based on the 15-mer Rv1733c p63-77 as well as the presence of strong HLA-DR3 binding motifs (33), we constructed the 28-mer Rv1733c p57-84 (Fig. 1) for application of SLP vaccination in HLA-DR3 tg mice. First, Rv1733c p57-84/CpG immunization was used to assess the immunogenicity of Rv1733c-derived, shorter peptides that still contained the HLA-DR3 peptide binding motif. Analysis of intracellular IFN-γ production by splenocytes in response to 6 h of stimulation with equimolar amounts of Rv1733c p63-77 (15-mer), Rv1733c p61-80 (20-mer), and Rv1733c p57-84 (28-mer) showed intracellular IFN-γ production for all peptides with an optimum for the 15-mer Rv1733c p63-77 (Fig. 2A). Immunization of 12 other overlapping SLPs in Cpg (28-mers; Fig. 1) covering Rv1733c did not show any significant responses in HLA-DR3 tg mice (data not shown).

Comparison of IFN-γ production levels after 6-day *in vitro* cultures of splenocytes from mice immunized either with Rv1733c p57-84 (28-mer) or with Rv1733c p63-77 (15-mer) in Cpg showed increased responses after 28-mer (SLP) immunization (Fig. 2B and C), possibly since for SLP vaccines, compared to minimal peptide vaccines, the duration of *in vivo* epitope presentation in the antigen-draining lymph nodes is increased and subsequently enhances IFN-γ production by effector T cells (34). These data could also indicate the requirement for processing and presentation by professional antigen-presenting cells of the 28-mer, whereas the 15-mer can be presented directly by HLA-DR+ cells, including T cells and B cells, with subsequent futile prolifer-
FIG 2 (A) Rv1733c p57-84/CpG immunization of HLA-DR3 tg mice. Splenocytes derived from mice immunized with SLP Rv1733c p57-84 in CpG were stimulated \textit{in vitro} with equimolar amounts of Rv1733c p63-77 (15-mer), p61-80 (20-mer), and p57-84 (28-mer) for 6 h. The percentage of CD4$^+$ IFN-\gamma-producing cells (indicated in each figure) was analyzed by intracellular cytokine staining. (B and C) Rv1733c p57-84/CpG immunization of HLA-DR3 tg mice. Splenocytes derived from mice immunized with Rv1733c p57-84/CpG (B) or with Rv1733c p63-77/CpG (C) were stimulated \textit{in vitro} with Rv1733c p63-77 (15-mer), p61-80 (20-mer), and p57-84 (28-mer) (0.1-\mu g/ml or 1.0-\mu g/ml final concentration). After 6 days, IFN-\gamma production was analyzed by ELISA. ConA was used as a positive control for \textit{in vitro} responsiveness, and recombinant protein HPV16 E6 and hsp65 p1-13 (59) were used as negative protein and peptide controls, respectively. (D) Frequency of polyfunctional CD4$^+$ T cells. Percentages of IFN-\gamma-, IL-2-, and/or TNF-producing CD4$^+$ T cells in splenocytes of HLA-DR3 mice immunized with Rv1733c p57-84/CpG, analyzed without (left panel) or with (right panel) TNF$^+$ cells. Splenocytes were stimulated \textit{in vitro} with stimuli indicated above each graph. After 6 days, cells were incubated with fresh antigen. After 4 h, brefeldin A was added for overnight (20 h) incubation, after which cells were permeabilized, fixed, stained, and analyzed for intracellular cytokine production. Each symbol represents one mouse. Only CD4$^+$ populations of $>5 \times 10^4$ events were analyzed. No significant cytokine production was detected in naive mice or in CD8$^+$ T cells of mice immunized with Rv1733c p57-84/CpG (data not shown). P values were calculated by the Mann-Whitney U test. (E) Frequency of HLA-DR3/p63-77 T$^+$ CD4$^+$ T cells. For determination of Rv1733c p63-77-specific CD4$^+$ T cells, splenocytes of HLA-DR3 mice immunized with CpG alone (--) or Rv1733c p57-84/CpG were stained for 1 h at RT with HLA-DR3/p63-77 TM$^+$ and phycoerythrin-FITC-conjugated anti-CD4. Groups included four mice. All mice were separately analyzed. P values were calculated by the Mann-Whitney U test.
ative response and death of the responding T cells. Alternatively, besides including an HLA-DR3-restricted epitope, the SLP could also harbor a murine class I-restricted epitope and induce a stronger immune response by activating not only CD4$^+$ but also CD8$^+$ T cells to produce IFN-γ.

**Induction of polyfunctional T cells in response to Rv1733c p57-84.** The induction of polyfunctional CD4$^+$ Th1 cells likely correlates with vaccine-induced protection in several models (35, 36), despite contradictory results on their role as biomarkers of protection in *M. tuberculosis* infection either identifying TNF$^+$/CD4$^+$ T cells as specific for active TB (37) or reporting significantly higher levels of IFN-γ$^+$/IL-2$^+$/TNF$^+$ CD4$^+$ T cells in active TB (38, 39). To estimate the contribution of the frequency of cytokine-producing T cells, polyfunctional T-cell analysis was performed in Rv1733c p57–84-immunized HLA-DR3 mice using different *in vitro* stimuli (Fig. 2D). Splenocytes of Rv1733c p57–84-immunized HLA-DR3 showed the predominant presence of Rv17333-specific IFN-γ$^+$/CD4$^+$ T cells (Fig. 2D). Since splenocytes of immunized mice stimulated *in vitro* with medium already produced significant amounts of TNF (data not shown), the data are depicted without TNF$^+$ cells, demonstrating a significantly increased number of Rv17333-specific IFN-γ$^+$/CD4$^+$ T cells (Fig. 2D). Spontaneous TNF production has been described in humans as well (40) and thus requires proper attention during the identification of recall T-cell responses responsible for vaccine-induced protection.

Additionally, we assessed the frequency of Rv1733c p63-77-specific, HLA-DR3-restricted CD4$^+$ T cells, using FITC-conjugated tetramers composed of HLA-DRB1*0301 and Rv1733c p63-77 (Fig. 2E). The median of TM$^+$/CD4$^+$ splenocytes induced by adjuvanted Rv17333c p57–84 immunization was 1.1%. No TM$^+$/CD4$^+$ T cells (0.19%) were observed in splenocytes derived from HLA-DR3 mice immunized with CpG alone.

To estimate the effect of multiple SLPs combined in one vaccine, Rv1733c-specific CD4$^+$ T-cell responses were analyzed after immunization of HLA-A2/DR3 double tg mice with a mixture of Rv1733c p57–84 together with the HLA-A2-restricted epitope Ag85B p143–152 (22) in CpG. As observed for single transgenic animals, CD8$^+$ T cells responded only to the class I-restricted Ag85B epitope and not to the class II-restricted Rv1733c p57–84 (Fig. 3), whereas CD4$^+$ T cells recognized only the HLA-DR3-restricted Rv1733c epitope. These data confirm the HLA-DR3 restriction of the Rv1733c SLP-induced IFN-γ response. Since IFN-γ responses to both antigens in the SLP mixture were similar to those induced by immunization with one antigen in single HLA tg mice, these data also show that the distinct SLPs did not inhibit each other’s responses in HLA-A2/DR3 double tg mice.

Besides producing cytokines, CD4$^+$ T cells are also known to contribute to protection by exerting cytolytic functions. Therefore, similarly immunized HLA-DR3 mice were used to determine whether Rv1733c p57–84 could induce cytotoxic T-lymphocyte (CTL) responses using *in vivo* cytotoxicity assays (24, 32). For this purpose, mice were immunized with CpG alone or with Rv1733c SLP combined with CpG. Rv1733c SLP immunization induced intermediate cytotoxicity levels (median, 33%) specific for Rv1733c p63–77 15-mer (Fig. 4A). In contrast, the HLA-A2 Ag85B p143–152 showed high *in vivo* cytotoxicity levels of 95% (Fig. 4B).

**Immunization with Rv1733c SLP induces antibodies specific for Rv1733c protein.** The paradigm that humoral immunity is not involved in the protection against TB is slowly making space for increasing evidence for antibody-mediated immunity against *M. tuberculosis* (41). The role of antibody (Ab) responses was previously confirmed by us in *in vivo* studies using HLA-A2 (24) and HLA-DR3 mice immunized with *M. tuberculosis* antigens (42, 43). Thus, we next investigated the humoral response induced by the Rv1733c 28-mer. Immunization with Rv1733c SLP/CpG induced high antibody titers to the peptide itself and induced antibodies to only a limited extent to the Rv1733c protein but not to the unrelated human papillomavirus 16 (HPV16) E6 recombinant protein. Mock-immunized mice, on the other hand, did not show any antibody reactivity, indicating that the Rv1733c SLP is capable of inducing cellular as well as humoral immunity (Fig. 5).

**Rv1733c SLP induces protection against live *M. tuberculosis* challenge in HLA-DR3 mice.** Previously, we have shown that splenocytes of *M. tuberculosis*-infected HLA-DR3 tg mice induced distinct IFN-γ production in response to Rv1733c p63–77, although the level was reduced compared to that obtained by stimulation with the HLA-DR3-restricted epitopes derived from secreted *M. tuberculosis* antigens such as Ag85B (42). To assess the vaccine potential of the Rv1733c SLP adjuvanted by CpG, its prophylactic protective effect was evaluated in a live *M. tuberculosis*...
challenge model in mice, by enumerating the CFU in the lungs (Fig. 6). As expected from the immunogenicity studies described above, Rv1733c SLP/CpG vaccination did not reduce the number of CFU in HLA-DR3 neg mice (data not shown), confirming the HLA-DR restriction of the T-cell-mediated protection. Reduction of CFU required Rv1733c, since no responses were observed in mice injected with CpG alone (42).

Preinfection immunization with Rv1733c recombinant protein and, to a greater extent, Rv1733c p57-84 significantly reduced the number of CFU from $3.6 \times 10^5$ to $1.4 \times 10^5$ ($P < 0.002; 0.41 \log$) and $0.65 \times 10^5$ ($P = 0.0003; 0.75 \log$), respectively. When protein was administered after infection, CFU reduction caused by the Rv1733c protein and SLP was still notable but significant only for the SLP (2.3 $\times 10^5$ [$P = 0.22$] and 1.96 $\times 10^5$ [$P = 0.018$], respectively). Interestingly, when the SLP was used to boost a prior BCG vaccination, mice boosted with Rv1733c SLP had the highest reduction (0.92 log) in bacterial load in their lungs (from $3.6 \times 10^5$ to $0.44 \times 10^5$; $P = 0.0002$) compared to mice vaccinated only with BCG (from $3.6 \times 10^5$ to $0.76 \times 10^5$ CFU; $P = 0.0004; 0.66 \log$). These data indicate that SLP derived from latency-associated protein Rv1733c may have potential as a booster vaccine for TB.

DISCUSSION

Identification of M. tuberculosis antigens that induce dendritic cell (DC) activation for subsequent priming of protective CD4+ and CD8+ Th1 cell responses is important to the development of new diagnostic tools as well as TB vaccines. Antigen discovery studies have been an essential factor of mycobacterial research for over several decades (44) and were markedly expedited by the availability of the M. tuberculosis genome sequence (45). Besides the secreted antigens in clinical trials (Ag85 and ESAT-6) (3), recent studies show that M. tuberculosis proteins such as PE_PGRS protein Rv0978c and Rv0754, secreted protein Rv0577, and cell wall proteins Rv3812 (48) and Rv3425 (49) recognize TLR2, induce maturation, and activate human DCs, enhancing their ability to stimulate Th1 cells. In addition, we demonstrated previously that the M. tuberculosis latency antigen Rv1733c was well recognized in diverse human populations, including those in several African regions (9). In addition, recombinant BCG expressing latency antigens showed improved long-term protection against TB in mice (14), and latency antigens can also boost the effect of BCG in nonhuman primates (13).

In the context of therapeutic vaccination, SLPs have been shown to induce better in vivo responses and protection against...
tumors than short peptides (16, 50). Since short peptides (8 to 11 amino acids in length) can be loaded directly onto HLA class I molecules, their presentation can take place by nonprofessional antigen-presenting cells (such as T cells or B cells) in the absence of costimulatory signals, which may lead to immunological tolerance rather than immunity. On the other hand, the use of SLP ensures that antigen processing will take place by professional antigen-presenting cells such as DCs and that epitopes can be cross presented to T cells in the context of optimal costimulation (50, 51). Furthermore, the use of SLP containing both T helper and a CTL epitope induces epitope presentation to CD4+ T cells and CD8+ T cells simultaneously. This cross presentation not only causes an increased effective immune response by generating IFN-γ from both CD4+ and CD8+ T cells but also works synergistically, as antigen-specific CD4+ T helper cells provide direct help to CD8+ T cells but also trigger DCs to activate CD8+ T cells to become granule-containing CTL effector cells. Moreover, SLPs also prolong the in vivo epitope presentation in the antigen-draining lymph nodes, which increases clonal expansion and cytokine production by effector T cells (26). Thus, conversion of minimal single epitopes to SLPs may provide an approach to increase immunogenicity. Besides these immunological advantages, however, SLPs have not yet been implemented into new TB vaccination strategies. However, vaccines aimed at treatment of established disease require long-lived presentation of epitopes by HLA on appropriately activated antigen-presenting cells, and thus, SLP would be advantageous for latently infected individuals.

HLA-DRB polymorphism plays an important regulatory role in controlling human T-cell reactivity against mycobacteria (20, 21). Previously, we demonstrated that the 15-mer Rv1733c p63-77 epitope was immunogenic in vivo either alone or as part of an HLA-DR3-restricted multistage polyepitope. Immunization with the latter adjuvanted with CpG generated high IgG levels as well as polyfunctional CD4+ T cells producing IFN-γ, TNF, and IL-2, specific for these HLA-DR3-restricted epitopes. Also, this multistage-polyepitope immunization reduced the number of bacilli in the lungs after M. tuberculosis challenge when administered as a prophylactic vaccine (18). Here, we have explored the application of SLP Rv1733c p57-84 as a novel approach in the design of new TB vaccines.

Rv1733c p57-84 conferred better protection against live M. tuberculosis challenge in HLA-DR3 transgenic mice than did the whole recombinant Rv1733c protein in a preventive setting. However, HLA-DR3neg mice showed no protection when immunized with SLP, which underscores the specificity of the protection by SLP. Furthermore, SLP booster vaccination significantly improved the protective efficacy of BCG. Since BCG is known not to induce immune responses to M. tuberculosis latency antigens (52, 53), boosting previous BCG vaccination with M. tuberculosis latency antigens is a promising and rational approach. This is supported by the above-mentioned improved long-term protection induced by recombinant BCG expressing latency antigens in mice (14) or by latency antigens as a BCG booster in nonhuman primates (13). However, since BCG does not induce detectable responses to latency antigens (53), it is conceivable that primary SLP...
immunization followed by BCG or simultaneous immunization with antigen and BCG (54) generates similar protection.

Since considerable numbers of individuals in developing countries are latently infected with \textit{M. tuberculosis}, therapeutic vaccines preventing progression from latent to reactivated infection are essential as well. According to mathematical models, the combination of mass preexposure vaccination with postexposure vaccine administered to people with latent TB infection could prevent two-thirds of TB deaths (55). Still, the majority of TB vaccines in current clinical trials are designed for prophylactic use, and only a few studies have reported subunit vaccines in postchallenge animal models (56), including studies on the multistage H56 subunit vaccine in mice and nonhuman primates (12). Furthermore, administration of fragmented \textit{M. tuberculosis} shortly (4 days) after \textit{M. tuberculosis} challenge reduced the bacillary load in lungs of mice compared to BCG-vaccinated animals (57), while in a lethal \textit{M. tuberculosis} challenge mouse model the ID93 vaccine lowered bacterial burden and lung pathology, which allowed shortening of the chemotherapy period (58).

In this study, we show that immunization with \textit{Rv1733c} SLP controls already-established infection. Thus, immunization with \textit{M. tuberculosis} latency antigen SLPs may also offer new tools for therapeutic vaccination against TB. In this respect, it is important to note that SLP production and purification, especially for membrane proteins such as \textit{Rv1733c}, are much less complicated than those of whole proteins, offering another advantage for vaccination.

Since HLA-DR3 is present in 20% of most populations worldwide, application of SLP vaccine approaches would require expansion of the number of SLPs to accommodate epitopes for multiple HLA alleles when targeting different populations (44). Therefore, it is important to note that simultaneous immunization with two SLPs, derived from \textit{Rv1733c} and Ag85B, did not affect the response to either SLP in HLA-A2/DR3 double tg mice. Thus, our data suggest that multiple SLPs, with variable HLA specificity, can be accommodated in one vaccine without loss of T-cell immunogenicity.

In view of the currently emerging evidence for a contribution of humoral immunity to \textit{M. tuberculosis} infection control (41), SLP vaccines may represent a promising strategy since, besides strong T-cell immunity, also robust antigen-specific humoral responses were induced. These data are in line with our previous findings where we observed strong humoral responses against the protective IVE-TB (in vivo-expressed \textit{M. tuberculosis}) antigen \textit{Rv2034} following vaccination (43). Thus, the mechanism by which \textit{Rv1733c} SLP reduces the number of bacteria in murine lungs could be based on a combination of induction of CD4\(^{+}\) T cells (IFN-\(\gamma\)/TNF\(\alpha\) and IFN-\(\gamma\)) and \textit{Rv1733c}-specific antibodies and potentially even a minor population of cytotoxic CD4\(^{+}\) T cells.

In conclusion, our data support the use of \textit{M. tuberculosis} latency antigen-based SLP approaches as novel TB vaccination approaches, both in prophylactic and in postinfection/therapeutic settings as well as in boosting BCG.

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