Promiscuous Peptide of 16 kDa Antigen Linked to Pam2Cys Protects Against *Mycobacterium tuberculosis* by Evoking Enduring Memory T-Cell Response

Uthaman Gowthaman,1 Vijender Singh,1 Weiguang Zeng,2 Shweta Jain,1 Kaneez F. Siddiqui,1 Sathi Babu Chodisetti,1 Rama Krishna Gurram,1 Pankaj Parihar,1 Pushpa Gupta,2 Umesh D. Gupta,3 David C. Jackson,2 and Javed N. Agrewala1

1Immunology and Cell Biology Division, Institute of Microbial Technology, Chandigarh, India; 2Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria, Australia; and 3Central JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India

One of the main reasons considered for BCG failure in tuberculosis-endemic areas is impediment by environmental mycobacteria in its processing and generation of memory T-cell response. To overcome this problem, we developed a unique lipopeptide (L91) by linking the promiscuous peptide (sequence 91-110) of 16 kDa antigen of *Mycobacterium tuberculosis* to Pam2Cys. L91 does not require extensive antigen processing and generates enduring Th1 memory response. This is evidenced by the fact that L91 significantly improved the activation, proliferation, and generation of protective T cells. Furthermore, L91 surmounts the barrier of major histocompatibility complex polymorphism and induces better protection than BCG. This peptide has self-adjuvanting properties and activates dendritic cells. Importantly, L91 activates T cells isolated from purified protein derivative–positive healthy volunteers that responded weakly to free peptide (F91). In essence, L91 can be a potent future vaccine candidate against tuberculosis.

*Mycobacterium tuberculosis* affects more than one-third of the world’s population [1]. Although the only available tuberculosis vaccine, BCG, has been administered to >3 billion people worldwide, the disease continues to be a major threat to public health [2, 3]. The maximum number of deaths and ~80% of the global tuberculosis burden is reported in tuberculosis-endemic areas [2, 4]. Furthermore, meta-analysis of BCG efficacy in different populations shows extreme variability, including a total failure in tuberculosis-endemic regions, raising concerns over the efficacy of BCG in these areas [5–7]. This apparent lack of efficacy may possibly be explained by the masking and blocking effects of environmental mycobacteria that ultimately affect BCG efficiency and also by their interference in the antigen-presenting abilities of antigen-presenting cells (APCs) [3, 4, 8–14]. Consequently, BCG antigens may not persist for sufficient time to generate long-lasting memory T cells [7, 15–18]. Hence, in tuberculosis-endemic areas, a vaccine that requires extensive processing may not be quite successful. Peptide vaccines may not require extensive antigen processing and can directly bind to major histocompatibility complex (MHC) molecules to activate T cells [19]. Therefore, peptide vaccines offer an attractive alternative to BCG. Unfortunately, the majority of peptides are poor immunogens, because they fail to initiate a productive T-cell response. Furthermore, peptides work unsuccessfully in genetically diverse populations because of human leukocyte antigen (HLA) polymorphism [20].

To overcome these problems, we identified a promiscuous peptide F91 (sequence 91-110) from 16 kDa antigen of *M. tuberculosis*. This peptide results in robust T helper 1 (Th1) response in > 85% tested donors with diverse HLA-alleles [21–23]. To make this peptide
immunogenic, we adjuvantized it by linking to S-[2, 3-bis (palmitoylxyloxy) propyl] cysteine (Pam2Cys), a ligand for Toll-like receptor 2 (TLR2). We termed this construct L91. The immunogenicity of Pam2Cys is due to its ability to target TLR2, which is extensively expressed on dendritic cells (DCs), leading to their activation and generation of productive immunity [24–27]. Moreover, Pam2Cys induces Th1 immunity by evoking interleukin 12 (IL-12) secretion in DCs [26, 28–30]. In the present study, we reveal that the lipopeptide L91 activates DCs and induces Th1 responses. Importantly, immunization with L91 provides long-term protection by eliciting enduring memory T cell response against M. tuberculosis and renders significantly better protection than does BCG. Hence, L91 may be an attractive vaccine candidate, especially in tuberculosis-endemic areas.

**MATERIALS AND METHODS**

**Experimental Animals**

Female BALB/c mice aged 6–10 weeks were procured from the National Institute of Pharmacological Education and Research, Mohali, Central Drug Research Institute, Lucknow, and National Institute of Immunology, New Delhi, India. Female Duncan-Hartley guinea pigs (aged 6–8 weeks) were procured from Chaudhary Charan Singh Haryana Agricultural University, Hisar, India. Animals were housed in a Bio Safety Level 3 facility. Use of animals was approved by the Institutional Animal Ethics Committees of the Institute of Microbial Technology, Chandigarh, and the National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India.

**Patients and Healthy Volunteers**

Serum and peripheral blood mononuclear cells (PBMCs) were separated from specimens of blood from sputum-positive pulmonary tuberculosis patients and purified protein derivative–positive (PPD+ ) healthy volunteers. Use of human blood was approved by the Institute’s Biosafety Committee.

**Reagents and Antibodies**

All standard reagents were purchased from Sigma, unless otherwise specified. Middlebrook 7H9, 7H11, ADC, and oleic acid–albumin–dextrose–catalase were procured from Difco Laboratories and Roswell Park Memorial Institute 1640 medium (RPMI-1640) and fetal calf serum from Gibco. All tissue culture plasticware was purchased from BD Falcon. Anti–mouse antibodies CD16/CD32 (2.4G2), CD4 (H129.19) (R35-95), CD127 (SB/199), CD69 (H1.2F3), interferon γ (IFN-γ) (XMG1.2), CD44 (IM7), CD62L (MEL-14), FoxP3 (MF23), CD11c (HL-3), CD40, CD80 (16-10AH), CD86 (GL-1), CD74 (In-1), IAα (AMS 32.1), isotype controls IgG2a (R35-95), IgG2b (R35-95), IgG2c (A23-1), capture and detection antibodies for IFN-γ, interleukin 6 (IL-6), IL-12 (p40/p70) enzyme-linked immunosorbent assay (ELISA), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 4 (IL-4), and CD4 T-cell enrichment cocktail were purchased from BD Biosciences; ³H-thymidine was from Amersham Pharmacia Biotech; and Pam2Cys from Invivogen.

**Synthesis of Lipidated Peptides**

The synthesis, purification, and characterization of peptides SEFAYGSFVRTVSLPVGADE (from the 16 kDa antigen of M. tuberculosis) and KYVKQNTLKL (from influenza hemagglutinin) and lipopeptides were performed as described elsewhere [24, 25].

**Mycobacterial Strains**

M. tuberculosis H37Rv and M. bovis BCG (Danish strain) were cultured in 7H9 medium containing 0.05% Tween-80 supplemented with 10% ADC enrichment.

**Immunization**

Mice were immunized intraperitoneally and subcutaneously with either L91 or F91 (20 nmol per animal). Forty-five days later, a booster dose (10 nmol) was given. Animals were killed 45 days after booster immunization. For protection studies, animals were immunized with constructs (20 nmol per mouse or 100 nmol per guinea pig) and 21 days later a booster (10 nmol per mouse and 50 nmol per guinea pig) or immunized with BCG (1 x 10⁵ colony-forming units [CFU]/animal). Animals were challenged with M. tuberculosis 75 days after booster and killed 30 days later.

**Protection Studies, Aerosol Infection, and Mycobacterial Burden in Lungs**

Immunized animals were rested for 75 days and then aerosol challenged with M. tuberculosis to deposit ~100 (mice) or 30 (guinea pigs) live bacteria in the lungs. Thirty days after infection, the bacterial burden in the lungs was determined by inoculating homogenates on plates to count CFUs. For histopathological analysis, fixed tissues were processed and stained with hematoxylin and eosin.

**Cultures of Spleen and Lung Leukocytes**

Splenocytes/lung cells were prepared as described elsewhere [15]. Lymphocytes (2 x 10⁵/well) isolated from spleens or lungs were cultured in 96-well U bottom plates for 48–72 hours. Different concentrations of peptides (F91/L91) were added to the cultures. For flow cytometry experiments, cells were stimulated (1 nmol) with peptide for 48 hours. A pretitrated dose of Pam2Cys (50 or 100 ng/mL) was used as control. Activity of Pam2Cys was assayed on bone marrow–derived cell (BMDC) cultures by activation markers and cytokine ELISA.

**Proliferation Assays**

T-cell proliferation assays were set to incubate human PBMCs or mouse splenocytes with peptides for 72 and 48 hours, respectively, after which [³H]-thymidine (0.5 µCi/well) was incorporated. After 16 hours, plates were harvested and radioactivity
was measured. Human PBMCs were separated by means of the density gradient method using Histopaque (Sigma) from the peripheral blood obtained from healthy PPD\(^+\) volunteers.

**Dendritic Cell Cultures**

BMDCs (2 \(\times\) 10\(^6\)) were isolated and cultured in a 60-mm petri dish in the presence of GM-CSF (10 ng/mL) and IL-4 (5 ng/mL) in RPMI-1640+FBS-10% for 6 days. Cultures were replenished with fresh medium after 3 days. Cells were harvested on day 7 and transferred to 24-well plates (2 \(\times\) 10\(^5\) cells/well) and incubated with 3 nmol of L91 or F91, lipopolysaccharide (LPS) (5 \(\mu\)g/mL). After 12 hours, cytokines were estimated in supernatants and cells were used for immunophenotyping.

**Carboxyfluorescin Succinimidy l Ester Dilution Assay**

Splenocytes (2 \(\times\) 10\(^7\)) were incubated with carboxyfluorescin succinimidy l ester (CFSE) (2 \(\mu\)M) in phosphate-buffered saline (PBS) for 8 minutes at 37\(^\circ\)C. Excess CFSE was removed by extensive washing. Cells (2 \(\times\) 10\(^5\) cells/well) were cultured with F91 or L91 (1 nmol) for 72 hours. Later, cells were stained for CD4 expression.

**DC–T-Cell Conjugates**

Peptide-specific T cells were expanded from splenocytes of L91-immunized mice by culturing with L91 for 48 hours. Cells were then harvested, and CD4 T cells were separated by means of magnet-assisted cell sorting. Cells were then labeled with CFSE as described. L91/F91-stimulated BMDCs (0.5 \(\times\) 10\(^6\)) were cultured with CFSE-labeled T cells (1 \(\times\) 10\(^6\)) in 35-mm plates for 8 hours. Excess medium was removed, and cells were gently rinsed with PBS. The DC–T-cell conjugates were examined and counted using a microscope (Olympus IX71).

**Estimation of Cytokines and Antipeptide Antibodies**

**Cytokine ELISA**

 Supernatants from cultures were collected, and cytokines were estimated by means of standard sandwich ELISA.

**Antipeptide antibodies**

Peptide ELISA was performed as follows. F91 (10 \(\mu\)g/mL) or 16 kDa protein (10 \(\mu\)g/mL) was coated in 96-well ELISA plates in 0.2 M Na\(_2\)CO\(_3\)/NaHCO\(_3\) buffer (pH, 9.4) for 16 hours at room temperature. Plates were blocked with bovine serum albumin (1%). Serum samples (dilution, 1:100) from PPD\(^+\) healthy volunteers and sputum-positive tuberculosis patients were added. Horseradish peroxidase–labeled anti–human IgA + IgG + IgM were added, and color was developed using o-phenylenediamine/H\(_2\)O\(_2\). Regular iterations of washing and incubation were followed at each step.

**Flow Cytometry Staining**

Immunophenotyping and intracellular staining were performed as described elsewhere [15]. For intracellular cytokine staining, peptide expanded cells were restimulated with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 \(\mu\)g/mL) for 6 hours at 37\(^\circ\)C, and during the last 4 hours brefeldin A (10 \(\mu\)g/mL) was added to the cultures. Samples were acquired using Fluorescence Activated Cell Sorter-Aria-II (FACS-Aria II) or FACS-Calibur and analyzed using CellQuest-pro or FACS-DIVA software (BD Biosciences).

**L91 Presentation by Fixed APCs**

CD4 T cells from L91-immunized animals were separated by means of CD4 T-cell enrichment kit (BD Biosciences) and CFSE-labeled. Synergic paraformaldehyde-fixed splenocytes (1 \(\times\) 10\(^7\) cells/well) were cultured with equal numbers of CD4 T cells and L91 (50 \(\mu\)g/well). Cultures without L91 were used as controls. CFSE-dye dilution was monitored through flow cytometry after 72 hours.

**RESULTS**

**L91 Successfully Activates DCs**

We examined the ability of L91 to activate DCs, because they are important in initiating a T-cell response. We reasoned that the Pam2Cys component of L91 would elicit DC activation. Treatment of murine bone marrow–derived DCs (BMDCs) with L91 resulted in their activation. L91 treatment augmented the expression of costimulatory molecules CD40, CD80, and CD86 on CD11c\(^+\) DCs (Figure 1A). Furthermore, DCs treated with L91 produced greater amounts of IL-6 and IL-12 (Figure 1B and 1C), indicative of Th1-skewing response. No noticeable change was observed in interleukin 10 (data not shown). Nonlipidated free peptide, F91, did not lead to activation of DCs. LPS or medium served as positive or negative controls. Furthermore, we also observed that L91 enhanced MHC II but reduced the expression of immature MHC II (invariant chain, CD74) on DCs (data not shown). It has been shown earlier that the peptides associated with TLR ligands make stable MHC-peptide complexes on APCs, resulting in a robust T-cell response [31, 32]. Therefore, we examined this ability in L91 by assaying DC–T-cell conjugates. We reasoned that, because L91 would be more stably presented by DCs, they would form better clusters with T cells. CD4 T cells, obtained from L91-immunized mice, were expanded in vitro, purified, and cocultured with DCs pulsed with L91 or free peptide (F91). We observed a greater number of DC–T-cell conjugates in L91-pulsed cultures (Figure 1D). Cumulatively, the data suggest that L91 substantially enhances activation of DCs and promotes better interaction with T cells.

**Immunization of L91 Results in Robust Th1 Immune Response**

We next examined whether immunization with L91 could initiate a Th1 response. Mice immunized with L91/F91 were rested for 45 days and checked for recall responses (Figure 2A). We observed dose-dependent T-cell proliferation. However, cells isolated from L91-immunized mice on in vitro culturing with L91 induced significantly better T-cell response than F91 (\(P = .0087\))
These results were also corroborated with CSFE-dye dilution assay (Figure 2B). F91 immunization did not induce any noticeable immune response (Figure 2A). Furthermore, genetically diverse strains of mice (C57BL/6, C3He) immunized with L91 showed peptide-specific T-cell proliferation, ensuring its promiscuity (data not shown). We also observed that, as compared with F91, L91 substantially upregulated CD69 and CD44 activation markers on CD4 T cells (Figure 2C). Upon restimulation with L91, we observed predominant production of IFN-γ (Figure 2D and 2E) but not IL-4 (data not shown). Furthermore, cells obtained from rested mice (45 days after immunization), on in vitro peptide restimulation, expanded the pool of both central (CM) (CD44hi CD62Lhi) and effector (EM) (CD44hi CD62Llow) memory CD4 T cells (Figure 2F). The data suggest that L91 immunization induces a Th1 memory response. We used pretitrated doses of ultrapure Pam2Cys (50–100 ng/mL) as controls. The absence of any significant response with Pam2Cys alone on CD4 T cells proves that the response is peptide specific (Figure 2B and 2E). Our data concur with earlier findings that TLR ligands alone, in the absence of T-cell receptor (TCR) signals, do not activate T cells [31, 33]. We also observed that immunization with L91 using either intraperitoneal or subcutaneous routes resulted in comparable T-cell responses (Supplementary Figure 1). Hence, in subsequent experiments, only the intraperitoneal route was adopted. Furthermore, we wanted to ensure that stimulation of T cells by L91 occurs nominally, that is, through MHC-TCR interaction. The addition of anti-IAβ antibodies led to the abrogation of L91-induced T-cell activation, which establishes peptide-specific response (Figure 2G). Interestingly, we observed that L91 could also be directly presented to T cells by fixed APCs (Supplementary Figure 2).

Figure 1. L91 induces dendritic cell maturation. A, Bone marrow–derived cells (BMDCs) were treated with L91/F91 (3 nmol) for 12 hours, and the expression of CD40, CD80, and CD86 on CD11c+ gated cells is depicted as flow cytometry histograms. Numbers in the flow cytometry plots indicate mean fluorescence intensity. B and C, Secretion of interleukin 6 (IL-6) and interleukin 12 (IL-12) was assayed in the culture supernatants by enzyme-linked immunosorbent assay. The data are represented as mean ± standard deviation. ***, P < .001. Data are representative of 3–4 independent experiments. D, Peptide-specific CD4 T cells were expanded by in vitro stimulation of splenocytes from L91-immunized animals. CD4 T cells were further enriched by means of magnet-assisted cell separation and labeled with carboxyfluorescein succinimidyl ester. Labeled CD4 T cells were cultured with BMDCs pretreated with L91/F91 (10 nmol). Photomicrographs depicting DC-T-cell conjugates are representative of 2 independent experiments (original magnification, 400×). LPS, lipopolysaccharide.
Immunity With L91 Protects Against *M. tuberculosis*

We next explored whether L91 immunization could render protection from experimental tuberculosis. Mice were vaccinated with L91 or controls (BCG, F91, LH, and placebo). Later, mice were aerosol challenged with *M. tuberculosis* on day 75 after vaccination and killed 30 days later. We found that L91-immunized mice harbored significantly lower bacterial load in the lungs, compared with mice immunized with BCG (*P* < .05) and other controls. Furthermore, minimal infiltration of cells and few granulomas were noted in L91-immunized mice.

We also used a Pam2Cys conjugated, non–tuberculosis-specific peptide from influenza hemagglutinin (LH) as a control. As expected, LH did not result in any significant protection against *M. tuberculosis*, showed pathology similar to that of the placebo control, and authenticated the tuberculosis-specific response by L91 (Figure 3A and 3B). Furthermore, we sought to determine whether peptide-specific T cells responded to a rechallenge. We observed peptide-specific T-cell recall response evidenced by proliferation and IFN-γ secretion in L91-immunized mice (Figure 3C and 3D). Therefore, L91 immunization induces
long-lasting Th1 cells that can subsequently respond to *M. tuberculosis* infection and engenders better protection than other vaccinations.

**L91 Induces Enduring Memory CD4 T Cell Response**

We next wanted to determine how effectively L91 induced protective recall responses to *M. tuberculosis* challenge. We checked the presence of memory T cells in mice immunized with L91 and challenged with *M. tuberculosis*. Lymphocytes isolated from the spleen were stimulated in vitro with L91, Pam2Cys, and medium. Both central (CD44hi CD62Lhi) and effector (CD44hi CD62Llo) memory CD4 T cells could be expanded on L91 stimulation (Figure 4A). Furthermore, we also found CD4 memory T cells in the lymphocytes isolated from the lungs. L91 stimulation induced a greater percentage of CD4 T cells expressing interleukin 7R (CD127) (19%) than did F91 (6%), Pam2Cys (5%), and medium (3%) (Figure 4B). Furthermore, we also observed a profound increase in the percentage of IFN-γ–producing CD4 T cells isolated from the lungs of L91-immunized mice, compared with controls (Figure 4C). IFN-γ production was further enhanced on in vitro stimulation (Figure 4D). IFN-γ–producing CD4 T cells are known to be very important in mediating protection against *M. tuberculosis* [34]. Cumulatively, these results indicate that L91 augments Th1-memory, which may be responsible for imparting protection. The data reveal that vaccination with L91 results in a favorable immune response that correlated with the improved protection against *M. tuberculosis* (Figure 3A–B).
Figure 4. L91 generates enduring memory CD4 T cells. Mice were immunized with L91 or controls (F91, BCG, LH, and placebo) and challenged after 75 days with *Mycobacterium tuberculosis*. A, Splenocytes from L91-immunized and *M. tuberculosis*-exposed mice were stimulated with peptides for 48 hours. Cells were studied for expression of CD44 and CD62L. Flow cytometry plots depict CD4 central (CD44hi/CD62Lhi) and effector T-cell memory (CD44hi/CD62Llo) populations. B, Lymphocytes isolated from the lungs of L91-immunized and *M. tuberculosis*-exposed mice were cultured with L91, F91, and Pam2Cys for 48 hours. The expression of memory marker CD127 was analyzed. C, Intracellular staining was performed ex vivo for interferon γ (IFN-γ) expression in CD4 T cells obtained from the lungs. D, Intracellular expression of IFN-γ was examined on in vitro peptide stimulation of lymphocytes obtained from the lungs L91-immunized mice. A–D, The numbers in the flow cytometry plots depict the percentage of gated population. All the memory markers and IFN-γ expression were analyzed on CD4 gated T cells. Data shown are representative flow cytometry plots from 2–3 independent experiments.
L91 Renders Significant Protection Against Tuberculosis in Guinea Pigs
After establishing the long-term protective efficacy of L91 in mice, we next ascertained its potency in outbred guinea pigs. Guinea pigs were immunized with L91 or controls and rested for 75 days. Then they were challenged with 
*M. tuberculosis*. Organs were harvested 30 days after immunization. Interestingly, L91 vaccination resulted in significantly better protection than did BCG (P = .0135), F91 (P = .001), LH (P < .001), and placebo (P < .001), as evidenced by reduced mycobacterial load and sparse tubercles and granulomas in the lungs (Figure 5).

L91 Also Induces Proliferation of Human PBMCs
On the basis of the animal data, it seemed that L91 may be an attractive vaccine candidate for humans. Hence, we also monitored the influence of L91 in stimulating PBMCs of PPD+ healthy volunteers. We selected PPD+ individuals who responded weakly to F91. Interestingly, L91 significantly enhanced T-cell proliferation (P < .001) (Figure 6A). We did not note any change with F91. These results suggest that L91 may be exploited for evoking effective immune response in the human population as well. It has been a concern in tuberculosis-endemic areas that vaccination with BCG is of limited efficacy as a result of the presence of preformed antimycobacterial antibodies. The epitope chosen for this study is a CD4 T-cell epitope, and hence there were remote chances to detect the presence of antipeptide antibodies. However, we sought to rule out any such possibility. We evaluated the presence of antipeptide antibodies in the serum of either tuberculosis patients or PPD+ healthy participants (Figure 6B). However, we could detect the presence of the antibodies against the antigen 16 kDa from which this CD4 T-cell epitope is derived. Importantly, the data suggest that unlike BCG, L91 performance will not be compromised by prior exposure to mycobacteria.

**DISCUSSION**

The poor performance of BCG, especially in tuberculosis-endemic areas, is attributed to the interference of environmental mycobacteria in antigen processing and inadequate generation of T-cell memory [3–18]. Hence, a vaccine that does not require extensive antigen processing and induces enduring T-cell memory may work successfully in endemic areas. Immunodominant subunit vaccines are attractive alternatives; and even a single T-cell epitope can be quite successful [35, 36]. Furthermore, this approach circumvents epitopes that adversely influence immunity, which is a choice not offered by whole-cell vaccines, such as BCG. However, the use of peptide vaccines has 2 major shortcomings: (1) peptides are weak immunogens; and (2) they are not effective in an outbred population because of MHC polymorphism. In the present study, we have overcome both these issues by selecting a promiscuous peptide of sequence 91–110 of the 16 kDa protein of *M. tuberculosis* [22, 23] and conjugating it with Pam2Cys to make a potent immunogenic construct. The novelty of L91 lies in its ability to simultaneously incite both innate and adaptive immunity. In this study, we reveal the efficacy of a lipidated promiscuous peptide that has a self-adjuvanting property. This peptide succeeded in inducing long-lasting Th1 memory, which is the cardinal feature of a successful tuberculosis vaccine. The following major findings have emerged from the present study: (1) L91, but not F91, activated DCs; (2) L91 enhanced Th1 responses, as evidenced by the predominant secretion of IFN-γ; (3) vaccination with L91 bolsters generation of enduring T-cell memory; (4) L91 generated better protection against *M. tuberculosis* than did BCG; (5) L91 induced proliferation of T cells obtained from individuals who weakly responded to free peptide.

It is worth mentioning here that L91 has a self-adjuvanting property; hence, no adjuvant was used for immunization. This may be due to the enhanced ability of Pam2Cys of L91 to activate APCs, especially DCs. Furthermore, the attachment of peptides to Pam2Cys increases the stability of the peptide-MHC complexes on APCs, which may result in a quantitative and qualitative difference in T-cell response [31, 32]. Indeed, L91—but not F91—treated DCs formed conjugates with a greater number of T cells. An additional interesting line of reasoning would be that the Pam2Cys may itself provide a costimulatory signal to the T cells, thus reducing the activation threshold [31, 33, 37]. It is noteworthy that the immune response observed was peptide specific, because Pam2Cys alone incubated with APCs failed to stimulate T cells. Furthermore, it has been reported that endocytosis can occur through TLR2 [24, 38]. This will result in phagosome acidification, initiation of downstream signaling, and enhanced presentation, leading to activation of T cells by APCs [38, 39]. This advantage may not be offered by BCG, because mycobacteria are reported to hamper the processing and presentation of antigens [11–14]. We observed that peptide-pulsed fixed APCs activated T cells, suggesting that L91 could also be directly presented to T cells.

It is a well-established fact that enduring T-cell memory response is a cardinal feature of any successful vaccine [34, 40–43]. One of the reasons for BCG failure is that it fails to generate long-lasting memory T cells [3, 15]. Notably, immunization with L91 significantly improved T-cell memory response. Interestingly, it has been reported that TLR2 agonists induce T-cell memory [37, 44]. Protection against *M. tuberculosis* persisted even after 75 days in both mice and guinea pigs, indicative of enduring memory T-cell response elicited by L91. Furthermore, the role of Th1 cells has been proven to be reasonably decisive in protection against tuberculosis [15, 34, 45–48]. The secretion of IL-12 by DCs when treated with L91 hinted at a Th1-biased
immune response. Furthermore, T cells from L91-immunized animals secreted IFN-γ, indicating a predominant Th1 immunity. Moreover, we observed greater numbers of IFN-γ+ CD4 T cells in the lungs of L91-immunized and *M. tuberculosis*–exposed mice, which may be a possible reason for the enhanced protection.

**Figure 5.** Immunization with L91 protects guinea pigs against *Mycobacterium tuberculosis*. Duncan-Hartley guinea pigs were immunized with L91 or controls (F91, LH, BCG, and placebo). After 75 days, animals were aerosol challenged with *M. tuberculosis*. Thirty days after infection, animals were killed. A, Mycobacterial load in the lungs was quantified by inoculating on plates and counting colony-forming units (CFU). Data are mean ± standard deviation (log₁₀ value). B, Representative histopathology sections of guinea pig lungs stained with hematoxylin and eosin. Gross pathology of guinea pigs lungs is also shown (col. 3). Arrows indicate granulomas (left and middle) or tubercles (right). Photomicrograph original magnification, 100×. Data are representative of 2–3 independent experiments with 5–7 animals in each group. *, *P* < .05; **, *P* < .01; ***, *P* < .001.
We were also able to show the potency of L91 in humans. Interestingly, we observed that the T-cell response in poor responders was rescued by L91. Hence, introducing peptides with different contextual signals, such as Pam2Cys, can induce a robust T-cell response. Furthermore, we also could not detect antipeptide antibodies in the serum of tuberculosis patients and PPD⁺ healthy participants. Therefore, our experiments reveal that prior exposure to mycobacteria and the presence of antibodies against mycobacterial antigens would not compromise the T-cell response induced by L91. Our results testify that immunization with L91 can play a vital role in protection against tuberculosis. L91 notably reduced the bacterial load in the lungs and controlled the pathology due to \textit{M. tuberculosis} infection. Furthermore, the vaccination studies clearly revealed that L91 could effectively protect not only mice but also guinea pigs from \textit{M. tuberculosis}. Importantly, immunization with L91 resulted in the induction of long-lasting memory Th1 cells.

Cumulatively, the data indicate that L91 elicits parameters that result in a favorable immune response crucial for evoking long-term immunity against \textit{M. tuberculosis}. Importantly, L91 has self-adjuvanting properties and works effectively in a genetically diverse population. Hence, it may be an attractive vaccine candidate against \textit{M. tuberculosis}.

\section*{Supplementary Data}

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/).

Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

\section*{Notes}

\textbf{Acknowledgments.} We thank Andrea Cooper, PhD, Trudeau Institute, New York, and Robert J. Wilkinson, PhD, Imperial College, London, for their critical reading of the manuscript. We thank Priya Gowthaman for helping with the manuscript preparation. We thank V. M. Katoch, MD, NJIL&OMD, Agra, India, for providing the Biosafety Level 3 facility and to B. N. Dutta, MD, Medicos Centre, Chandigarh, India, for histopathological analysis. U. G. and K. F. S. are recipients of a DBT fellowship, and V. S., S. J, R. G., and S. C. are receipients of a CSIR fellowship.

\textbf{Financial support.} This work was supported by the Council of Scientific and Industrial Research (CSIR) and Department of Biotechnology (DBT), New Delhi, India.

\textbf{Potential conflicts of interest.} All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

\section*{References}