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Identification of Mycobacterial Ribosomal Proteins as Targets for CD4$^+$ T Cells That Enhance Protective Immunity in Tuberculosis

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ABSTRACT *Mycobacterium tuberculosis* remains a threat to global health, and a more efficacious vaccine is needed to prevent disease caused by *M. tuberculosis*. We previously reported that the mycobacterial ribosome is a major target of CD4$^+$ T cells in mice immunized with a genetically modified *Mycobacterium smegmatis* strain (IKEPLUS) but not in mice immunized with *Mycobacterium bovis* BCG. Two specific ribosomal proteins, RplJ and RpsA, were identified as cross-reactive targets of *M. tuberculosis*, but the breadth of the CD4$^+$ T cell response to *M. tuberculosis* ribosomes was not determined. In the present study, a library of *M. tuberculosis* ribosomal proteins and *in silico*-predicted peptide libraries were used to screen CD4$^+$ T cell responses in IKEPLUS-immunized mice. This identified 24 out of 57 *M. tuberculosis* ribosomal proteins distributed over both large and small ribosome subunits as specific CD4$^+$ T cell targets. Although BCG did not induce detectable responses against ribosomal proteins or peptide epitopes, the *M. tuberculosis* ribosomal protein RplJ produced a robust and multifunctional Th1-like CD4$^+$ T cell population when administered as a booster vaccine to previously BCG-primed mice. Boosting of BCG-primed immunity with the *M. tuberculosis* RplJ protein led to significantly reduced lung pathology compared to that in BCG-immunized animals and reductions in the bacterial burdens in the mediastinal lymph node compared to those in naive and standard BCG-vaccinated mice. These results identify the mycobacterial ribosome as a potential source of cryptic or subdominant antigenic targets of protective CD4$^+$ T cell responses and suggest that supplementing BCG with ribosomal antigens may enhance protective vaccination against *M. tuberculosis*.

KEYWORDS antigen, cryptic, mycobacteriology, ribosome, T cells, tuberculosis, tuberculosis vaccines

Tuberculosis is an enormous burden on global health systems. The World Health Organization has concluded that nearly one-fourth of the global population is latently infected with *Mycobacterium tuberculosis* (http://www.who.int/news-room/fact-sheets/detail/tuberculosis). With 10.4 million new cases and 1.5 million deaths annually, *M. tuberculosis* remains one of the most serious threats to global public health, and new research is desperately needed to combat its spread (http://www.who.int/tb/publications/global_report/en/). The only currently available vaccine for the prevention and control of *M. tuberculosis* infection, the attenuated live *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) strain, has limited and variable efficacy in children and generally

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fails to prevent pulmonary tuberculosis in adults (1, 2). Lengthy antibiotic treatments that are required for the cure of *M. tuberculosis* infection are costly and plagued by low compliance, which leads to the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *M. tuberculosis* strains (3–5). The HIV epidemic has also led to unforeseen treatment complications for those coinfected with *M. tuberculosis* (6–8). These issues highlight the necessity of identifying new candidates for vaccination against *M. tuberculosis*.

M. tuberculosis vaccine candidates that have shown potential for protection greater than that provided by BCG in animal models are currently in every stage of the vaccine development pipeline (9–12). Candidates in clinical trials can be divided into three broad categories, as live mycobacterium vaccines, subunit recombinant protein vaccines, and subunit vaccines delivered by viral vectors (9). The majority of vaccine candidates have focused on immunodominant secreted antigens of *M. tuberculosis*, such as members of the antigen 85 complex (Ag85a and Ag85b) and proteins secreted by the virulence-related type VII secretion systems of the bacteria (13–22). However, it remains unclear whether any of these antigens will provide useful targets for protective vaccines, and in at least one case, the results from extensive phase II clinical testing have been disappointing (23, 24). Thus, the discovery and analysis of additional antigens for incorporation into new *M. tuberculosis* vaccines remain areas of high priority in the ongoing effort to develop better strategies for the control and eradication of *M. tuberculosis* (11, 12, 25).

We previously reported on a genetically modified strain of *Mycobacterium smegmatis*, which contains the ESX-3 type VII secretion system of *M. tuberculosis*, designated IKEPLUS, that stimulates strong bactericidal $CD4^+$ T cell responses against subsequent *M. tuberculosis* challenge in mice (26). Our detailed analysis of the specificity of the $CD4^+$ T cells evoked by IKEPLUS and cross-reactive with *M. tuberculosis* showed that a majority of this response is specific for structural proteins of the mycobacterial ribosome (27). Using $CD4^+$ T cells from IKEPLUS-immunized mice and epitope mapping with synthetic peptide libraries, we identified conserved epitopes within the ribosomal RplJ/L10 and RpsA/S1 proteins as targets of the immune response. Responses to these antigens were not detected following BCG immunization or aerosol infection with *M. tuberculosis*, suggesting that they are cryptic antigens during virulent mycobacterial infection and may be a previously untapped source of immunogens for the development of new vaccine candidates.

In the present study, we have expanded on those previous results by broadly screening all structural proteins of the *M. tuberculosis* ribosome for their ability to be targeted by the $CD4^+$ T cell responses of appropriately immunized mice. We used IKEPLUS immunization along with a recombinant mycobacterial ribosomal protein library to probe for the immune response to the 57 proteins that make up the mycobacterial ribosome. Synthetic peptide libraries were then used to identify specific epitopes within ribosomal proteins that were immunogenic after IKEPLUS immunization. This study also used recombinant RplJ protein to assess the ability of ribosomal proteins to complement BCG immunization. Our findings showed that the mycobacterial ribosome was highly immunogenic and contained many epitopes for the stimulation of T cell responses. Our results also showed that BCG did not inhibit $CD4^+$ T cell responses to ribosomes and that BCG vaccination could be potentially augmented with mycobacterial ribosomal epitopes to enhance protection against *M. tuberculosis*.

RESULTS

Identification of mycobacterial ribosomal proteins recognized by $CD4^+$ T cells. To broadly survey which proteins of the mycobacterial ribosome could induce an immune response in mice immunized with live mycobacterial vaccine strains, we generated all 57 ribosomal proteins of *M. tuberculosis* by expressing them individually in *Escherichia coli* and isolating them via affinity tag purification (see Fig. S1 and Table S1 in the supplemental material). $CD4^+$ T cell responses from mice immunized with IKEPLUS or BCG were analyzed for responses to the individual recombinant mycobac-

terial ribosomal proteins by a gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay of splenic $CD4^+$ T cells. Among the 57 purified recombinant ribosomal proteins, 24 elicited significant numbers of IFN-γ-producing $CD4^+$ T cells in IKEPLUS-immunized mice (Fig. 1A). In contrast, only one ribosomal protein antigen elicited a response that achieved statistical significance with BCG-immunized $CD4^+$ T cells (Fig. 1B). Based on the reported three-dimensional structure of the *M. smegmatis* ribosome as resolved by cryoelectron microscopy (28, 29), we observed a random distribution in the locations of proteins that stimulated $CD4^+$ T cell responses (Fig. 1C), without obvious clusters in specific areas of either the large or small subunit. Overall, these results indicated that IKEPLUS primed a broad immune response to proteins distributed throughout the structure of the mycobacterial ribosome, while standard BCG vaccination was mostly ineffective at inducing detectable levels of such T cell responses.

Mapping minimal epitopes of immunogenic mycobacterial ribosomal proteins. Ten ribosomal proteins that consistently stimulated significant numbers of IFN-γ-secreting $CD4^+$ T cells in the ribosomal protein library screen (Rv0640, Rv0652, Rv0682, Rv0683, Rv0700, Rv0701, Rv0707, Rv0714, Rv2058c, and Rv2904) (Fig. 1 and additional data not shown) were analyzed *in silico* for candidate major histocompatibility complex (MHC) class II-presented epitopes within all potential 15-amino-acid stretches using the consensus method, as previously described (30). After ranking by predicted binding affinity for MHC class II I-A^b, the results were trimmed by selecting the top 33% strongest predicted binders for each ribosomal protein. Within this group, peptides with overlaps of >3 amino acids were removed, yielding a set of 109 peptides, which were then synthesized (see Table S2 in the supplemental material). To test the recognition of these candidate epitopes, splenic $CD4^+$ T cells were isolated from IKEPLUS-immunized mice and examined for responses to each of these peptides by an IFN-γ ELISPOT assay. Using this approach, we detected 16 stimulatory peptides corresponding to $CD4^+$ T cell epitopes (Fig. 2), 11 of which were nonoverlapping sequences from 7 different ribosomal proteins (Table 1).

In a complementary but broader approach to epitope identification, we synthesized a second peptide library consisting of predicted I-A^b binding peptides among all 15-mers with fewer than three overlapping residues contained within the entire set of 57 mycobacterial ribosomal proteins. This prediction was done as described above, using the consensus method (30). Peptides were ranked based on hypothetical binding affinity for I-A^b, and the top 10% (153 peptides) were synthesized (Table S3). This library was screened by using an IFN-γ ELISPOT assay as described above, which identified six additional stimulatory peptides from five ribosomal proteins. Two of these overlapped peptides from the proteins RplL and RpsJ identified in the initial, more directed screen. Peptides from the previously reported RplJ and RpsA proteins were also identified, as was one peptide derived from a protein that was not found to be immunogenic in the initial protein screen (RpsD) (Fig. 3 and Table 1). In all, our combined peptide screening identified at least 13 epitopes derived from 10 different *M. tuberculosis* ribosomal proteins.

Lack of inhibitory effects of BCG on responses to mycobacterial ribosomal proteins. To determine if BCG immunization could inhibit T cell priming against mycobacterial ribosomal proteins or suppress previously primed responses to these antigens, mice were immunized with either IKEPLUS alone, IKEPLUS followed by BCG 2 weeks later, or BCG followed by IKEPLUS 4 weeks later. Splenic $CD4^+$ T cells were isolated and incubated with recombinant RplJ protein or intact 70S mycobacterial ribosomes and naive antigen-presenting cells (APCs). Analysis by an IFN-γ ELISPOT assay showed that mice responded to intact mycobacterial ribosomes when immunized with IKEPLUS, or with IKEPLUS and BCG in either combination, and also responded to recombinant RplJ protein when immunized with IKEPLUS or IKEPLUS followed by BCG (Fig. 4A). Although mice immunized with BCG followed by IKEPLUS did not show responses that reached significance when stimulated with RplJ protein (Fig. 4A), the overall trend revealed that IKEPLUS immunization in any combination elicited

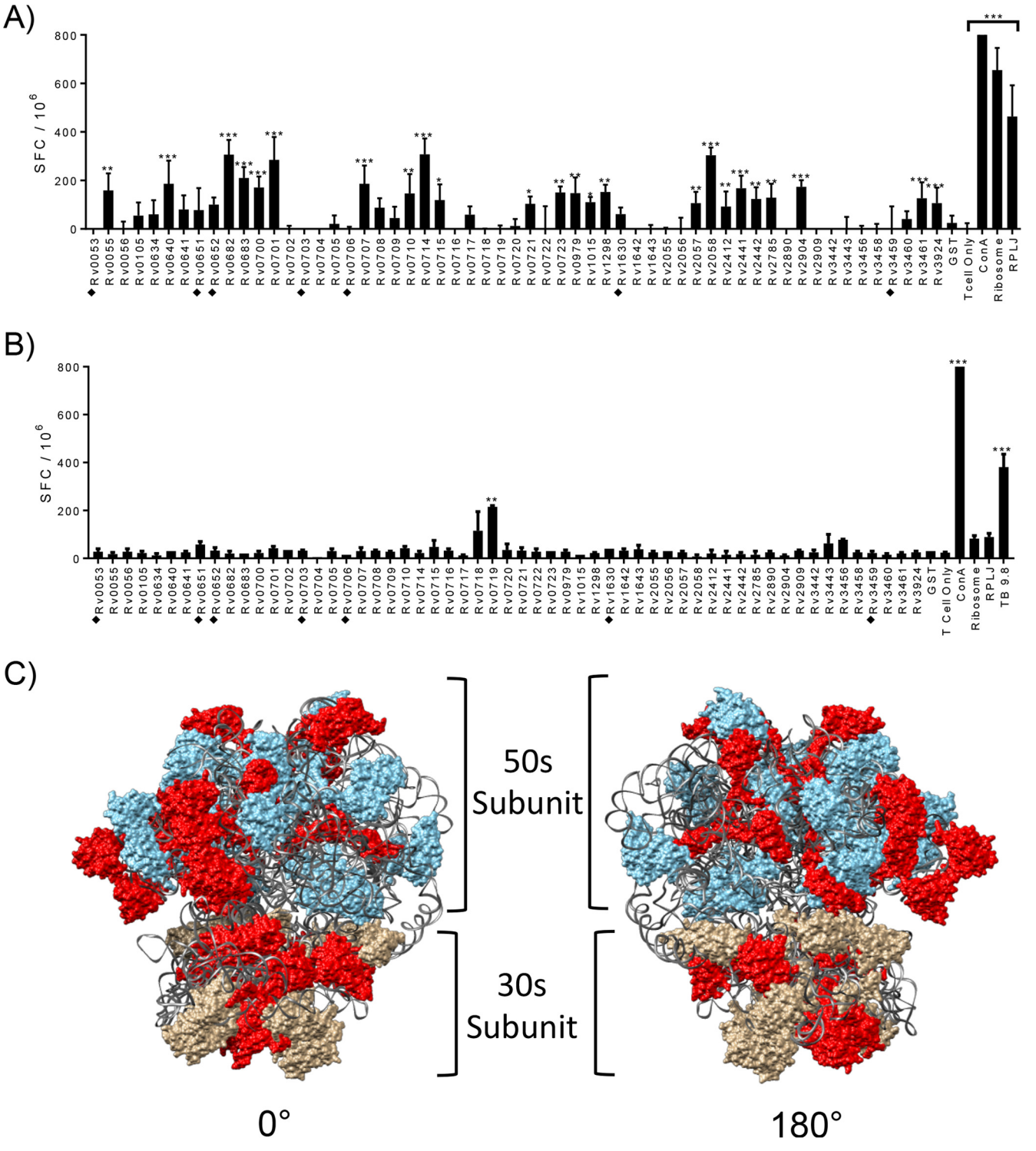

FIG 1 Identification of *M. tuberculosis* ribosomal proteins recognized by CD4$^+$ T cells from IKEPLUS- and BCG-immunized animals. (A and B) Mice (C57BL/6) ($n = 3$) were immunized with 5×10^7 CFU IKEPLUS i.v. (A) or 5×10^6 CFU BCG-Danish s.c. (B). Two weeks later, CD4$^+$ T cells were purified from splenocytes and tested by an ELISPOT assay for IFN-γ production in response to *ex vivo* stimulation with purified recombinant preparations of each of the 57 *M. tuberculosis* ribosomal proteins (10 μg/ml). Responses that are significantly different from those with the negative-control stimulation with purified glutathione *S*-transferase (GST) by two-way ANOVA with FDR correction and a 1% cutoff are indicated (*, $q < 0.01$; **, $q < 0.001$; ***, $q < 0.0001$). Positive-control wells were stimulated with concanavalin A (ConA) (5 μg/ml). Additional positive controls were purified *M. smegmatis* ribosomes (Ribosome) (5 nM) or $RplJ_{TB146-160}$ peptide (RPLJ) (10 μg/ml) (A) and TB9.8 (10 μg/ml) (B). Proteins that were poorly expressed and of lower purity in our system (i.e., potential false negatives) are marked with filled diamonds. Data shown are representative of results from two independent experiments and are displayed as mean numbers of spot-forming cells (SFC) per well, with error bars representing standard errors for quadruplicate (A) and duplicate (B) samples. (C) Proteins that achieved statistical significance for stimulation of CD4$^+$ T cell responses in IKEPLUS-immunized mice in panel A were mapped in red onto the reported structure of the mycobacterial ribosome (PDB accession number 5O61) (28). The 50S subunit is shown in blue, the 30S subunit is in tan, and the rRNA is in gray.

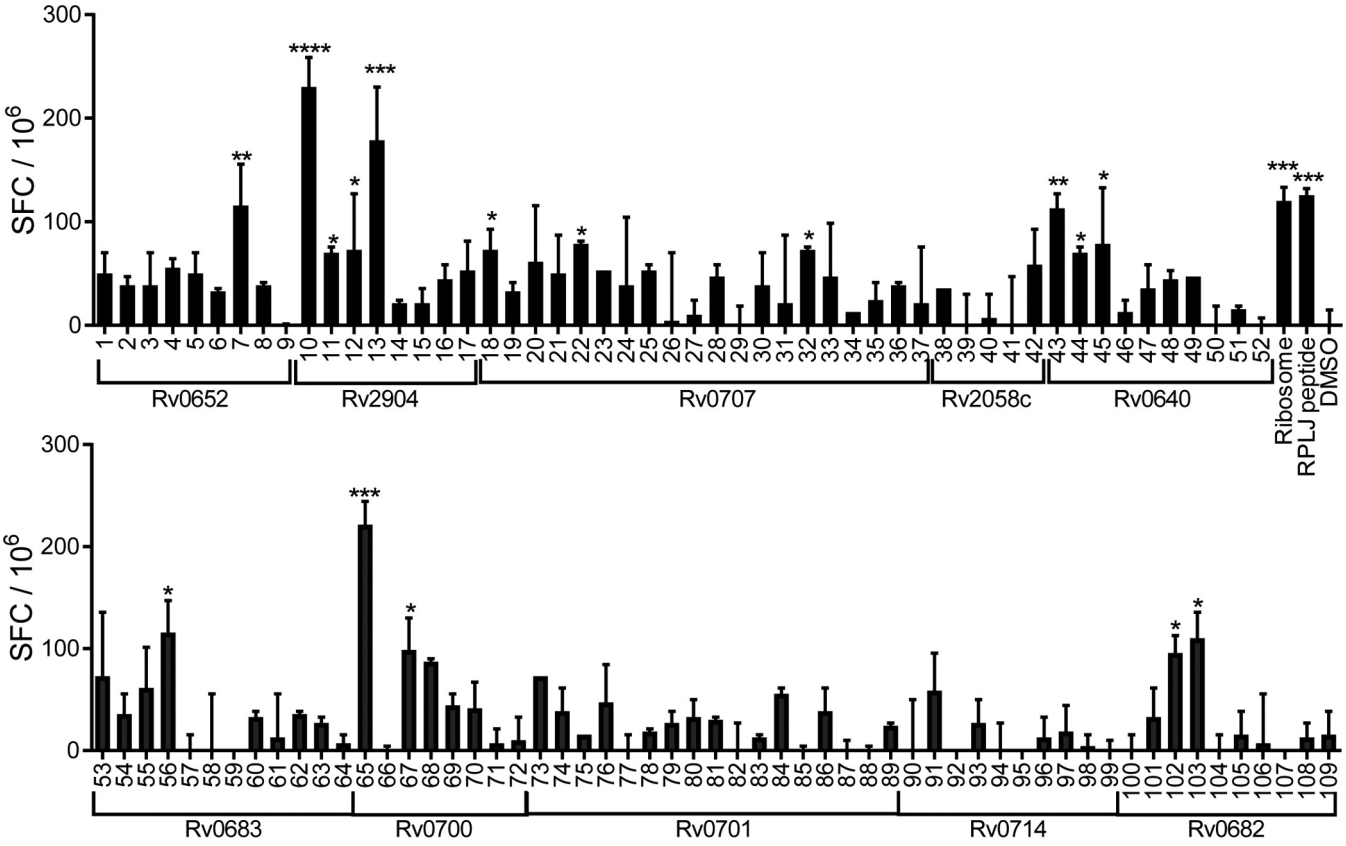

FIG 2 Identification of 15-mer epitopes from immunogenic *M. tuberculosis* ribosomal proteins. Mice (C57BL/6) ($n = 3$) were immunized with 5×10^7 CFU IKEPLUS i.v. Two weeks later, $CD4^+$ T cells were purified from splenocytes and tested for IFN-γ production by an ELISPOT assay in response to *ex vivo* stimulation with 109 mycobacterial ribosomal peptides (10 μg/ml) derived from 10 *M. tuberculosis* ribosomal proteins, as indicated. Responses that are significantly different from those under the control DMSO conditions by ANOVA are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Positive-control wells were stimulated with purified *M. smegmatis* ribosomes (Ribosome) (5 nM) or $RplJ_{TB146-160}$ peptide (RPLJ) (10 μg/ml). Data shown represent mean values and standard errors for duplicate samples and are representative of results from two independent experiments.

a response to mycobacterial ribosomes by $CD4^+$ T cells. These results indicated that prior exposure to BCG did not preclude the stimulation of subsequent priming of responses against ribosomal proteins, suggesting the possibility of using such proteins as immunogens to broaden antimycobacterial immunity induced by BCG vaccination.

To test the ability of an individual ribosomal antigen to supplement BCG immunization, mice were immunized subcutaneously (s.c.) with either BCG or recombinant RplJ protein or primed with BCG followed by subsequent administrations of RplJ protein. At 56 days postimmunization, mice were sacrificed, and splenic $CD4^+$ T cells were isolated and incubated with naive APCs in the presence of the *M. tuberculosis* sonicate from H37Rv, $RplJ_{TB146-160}$ peptide, recombinant RplJ protein, or TB9.8 peptide (all at 10 μg/ml). Responses were quantitated by an IFN-γ ELISPOT assay (Fig. 4B). Mice immunized with BCG alone produced a significant $CD4^+$ T cell response to the *M. tuberculosis* sonicate as well as the TB9.8 peptide, while mice immunized with recombinant RplJ protein alone produced $CD4^+$ T cell responses to the *M. tuberculosis* sonicate and RplJ protein, as expected. Most notably, mice immunized sequentially with BCG boosted by RplJ protein elicited significant $CD4^+$ T cell responses to the *M. tuberculosis* sonicate, RplJ protein, and $RplJ_{TB146-160}$ peptide (Fig. 4B). The magnitude of the responses to RplJ protein and peptide in the BCG-primed and RplJ protein-boosted group suggested that BCG may have had some ability to weakly stimulate $CD4^+$ T cell responses to the ribosomal antigen, although these responses rose to a measurable level only after boosting with the purified protein. These results also suggested that the immuno-

TABLE 1 Immunogenic epitopes identified by peptide screens of mycobacterial ribosomal proteins[a]

Peptide	Residues	Sequence	*P* value
Rv0652 (RplL)			
7	93–107	AKDLVDGAPKPLLEK	0.0022
127	91–105	KEAKDLVDSAPKPLL	0.0001
Rv2904 (RplS)			
10	69–83	VERTFPVHSPNIDHI	0.0001
11	15–29	DIPAFNPGDTINVHV	0.0290
12	65–79	YGVGVERTFPVHSPN	0.0240
13	73–87	FPVHSPNIDHIEVVT	0.0002
Rv0707 (RpsC)			
16	210–224	GKRELAAAAPAGADR	0.0240
20	206–220	DIVGGKRELAAAAPA	0.0167
32	182–196	IDYGLYEAKTTFGRI	0.0240
Rv0640 (RplK)			
43	12–26	KLQIVAGQANPAPPV	0.0025
44	67–81	SFTFTLKTPPAAKLL	0.0290
45	16–30	VAGQANPAPPVGPAL	0.0167
Rv0651 (RplJ)			
123	146–160	KAAGLFNAPASQVAR	0.0001
124	151–165	FNAPASQVARLAAAL	0.0001
Rv0683 (RpsG)			
56	16–30	PVYGSQLVTQLVNKV	0.0188
Rv0700 (RpsJ)			
65	32–46	TGASVVGPVPLPTEK	0.0006
67	28–42	TVVRTGASVVGPVPL	0.0401
172	31–45	RTGASVVGPVPLPTE	0.0001
Rv0682 (RpsL)			
102	18–32	KVKTAALKGSPQRRG	0.0458
103	30–44	RRGVCTRVYTTTPKK	0.0240
Rv1630 (RpsA)			
154	431–445	QMEKFAAAEAEAANA	0.0001
Rv3458c (RpsD)			
162	1–15	MARYTGPATRKSRRL	0.0018

[a]Peptides identified in screens for immunogenic epitopes (Fig. 2 and 3) are listed with their locus tag (name), library identification, peptide number, residue location in the intact protein, amino acid sequence, and statistical significance in the screen conducted. Proteins identified as being immunogenic using the approach illustrated in Fig. 1 are highlighted in boldface type.

dominant antigen TB9.8 may be inhibited, or at least less dominant, in the context of boosting with ribosomal antigen.

Induction of strong multifunctional Th1 CD4$^+$ T cell responses in RplJ-boosted mice. To determine the strength and multifunctionality of mycobacterial ribosome-specific CD4$^+$ T cell responses, mice were immunized with BCG or with BCG followed by recombinant RplJ protein. On day 70 after the initial BCG administration, mice were sacrificed, and spleens were harvested for intracellular cytokine staining and flow cytometry. Mice immunized with BCG alone showed significant numbers of IFN-γ- and tumor necrosis factor alpha (TNF-α)-producing CD4$^+$ T cells when restimulated with the *M. tuberculosis* sonicate but not with the other antigens tested. In marked contrast, BCG-immunized mice boosted with RplJ protein showed significant responses to RplJ protein and peptide for each of the three cytokines tested as well as responses to the *M. tuberculosis* sonicate and TB9.8 (Fig. 5A; see also Fig. S2 in the supplemental material). Whereas BCG-immunized RplJ-boosted mice responded to RplJ-specific stimuli with significant TNF-α, IFN-γ, and interleukin-2 (IL-2) responses, the BCG-specific

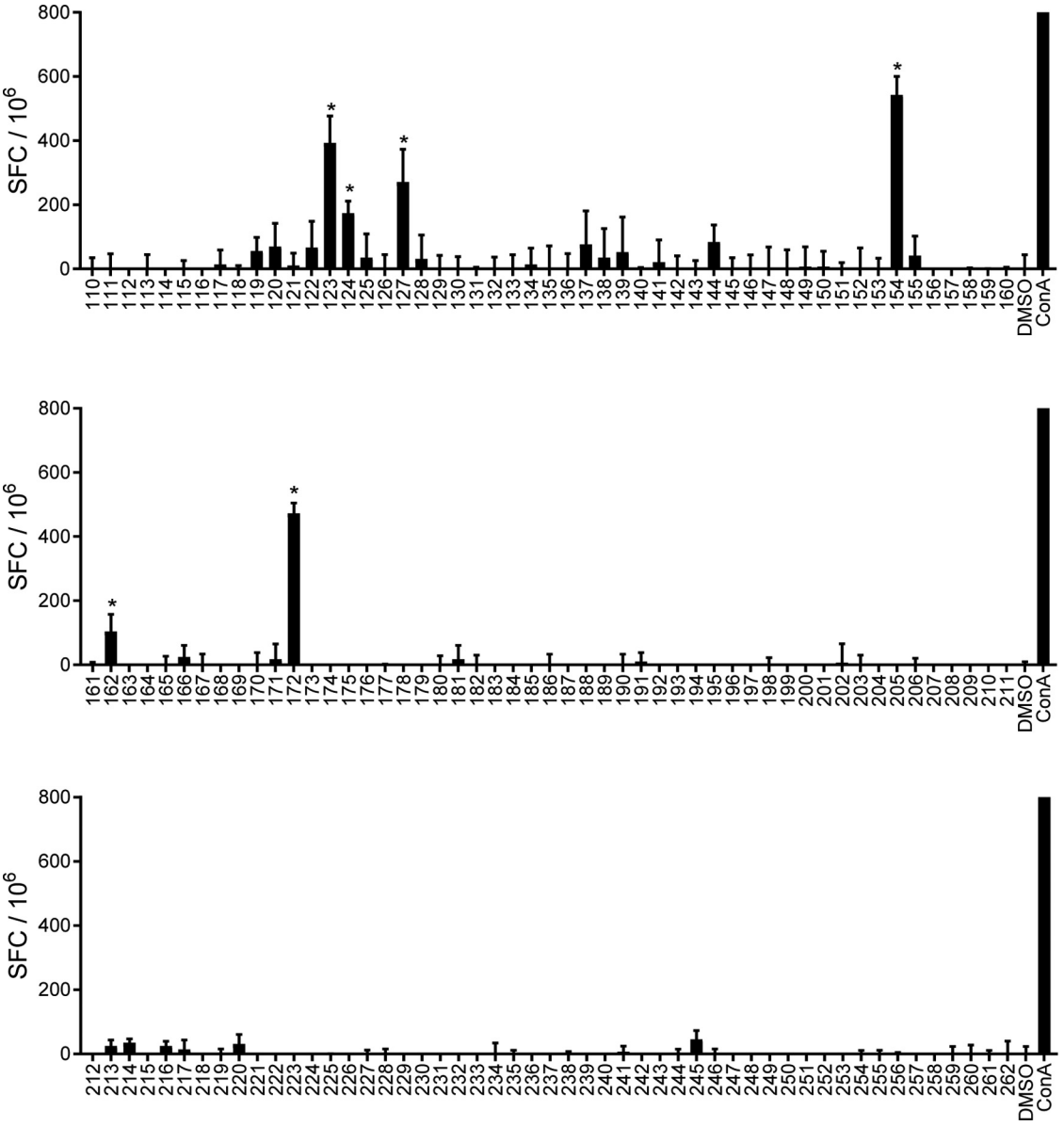

FIG 3 Identification of epitopes predicted from the total *M. tuberculosis* ribosomal proteome. Mice (C57BL/6) ($n = 3$) were immunized with 5×10^7 CFU IKEPLUS i.v. Two weeks later, $CD4^+$ T cells were purified from splenocytes and tested for IFN-γ production by an EPLISPOT assay in response to *ex vivo* stimulation with 153 mycobacterial ribosomal peptides, as indicated (10 μg/ml). Responses that are significantly different from those under the control DMSO conditions are indicated (*, $P < 0.05$). Positive-control wells were stimulated with concanavalin A (ConA) (5 μg/ml). Data shown represent mean values and standard errors for quadruplicate samples and are representative of results from two independent experiments.

stimuli (*M. tuberculosis* sonicate and TB9.8) seemed to stimulate only TNF-α- and IFN-γ-producing cells (Fig. 5A). These results suggest that 70 days after BCG immunization, the mice had a waning $CD4^+$ T cell response to antigens delivered only by the initial priming, while BCG-immunized mice boosted with RplJ protein created strong $CD4^+$ T cell memory populations that maintained responses to mycobacterial ribosomal antigens and to antigens originally primed by BCG.

The immunized $CD4^+$ T cells were also examined for their average levels of cytokine production based on mean fluorescence intensity (MFI) values for intracellular cytokine staining. Although the populations were small, BCG-immunized mice that responded to antigen restimulation produced a large amount of TNF-α and a moderate amount of IFN-γ (Fig. 5B and Fig. S2). This was in contrast to the BCG-immunized RplJ-boosted

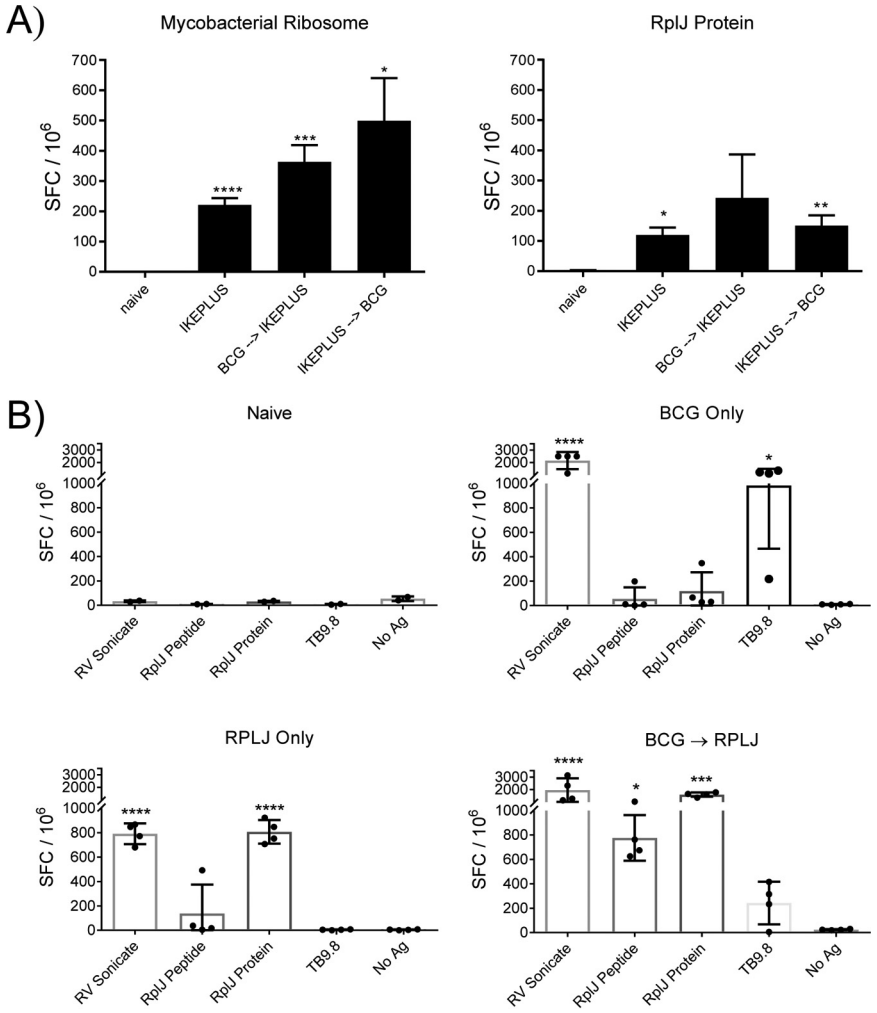

FIG 4 BCG immunization does not impede ribosome-specific priming of $CD4^+$ T cells. (A) Mice (C57BL/6) ($n = 3$) were immunized with 5×10^7 CFU IKEPLUS i.v., 5×10^6 CFU BCG s.c. followed by 5×10^7 CFU IKEPLUS i.v. 4 weeks later, or 5×10^7 CFU IKEPLUS i.v. followed by 5×10^6 CFU BCG s.c. 2 weeks later. Two weeks after the final immunization, $CD4^+$ T cells were purified from splenocytes and tested for IFN-γ production by an ELISPOT assay in response to *ex vivo* stimulation with recombinant RplJ protein (10 μg/ml) or purified mycobacterial ribosomes (5 nM). Responses that are significantly different from those in control naive mice by ANOVA are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Data shown represent mean values and standard errors for quadruplicate samples and are representative of results from three independent experiments. (B) Mice (C57BL/6) ($n = 4$) were immunized with 5×10^6 CFU BCG s.c., 5×10^6 CFU BCG s.c. followed 4 weeks later by two administrations of RplJ protein (25 μg) and CpG-ODN 1826 (20 μg) s.c. 2 weeks apart, or RplJ protein (25 μg) and CpG-ODN 1826 (20 μg) twice 2 weeks apart s.c. Two weeks after the final immunization, $CD4^+$ T cells were purified from splenocytes and tested for IFN-γ production by an ELISPOT assay in response to *ex vivo* stimulation with recombinant RplJ protein (10 μg/ml), $RplJ_{TB146-160}$ peptide (10 μg/ml), or TB9.8 peptide (10 μg/ml). Positive-control wells were stimulated with the H37Rv sonicate (10 μg/ml). Responses that are significantly different from those of the no-antigen (No Ag) control by ANOVA are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Data shown represent mean values and standard errors for groups of four mice and are representative of results from two independent experiments.

mice, in which the $CD4^+$ T cells produced large amounts of all three cytokines analyzed in response to mycobacterial ribosomal antigens. Interestingly, the BCG-specific antigens in BCG-immunized RplJ-boosted animals produced a dominant TNF-α $CD4^+$ T cell response with moderate levels of IFN-γ and almost no IL-2 (Fig. 5B). These results suggested that ribosomal antigens may prime a qualitatively different $CD4^+$ T cell response than those produced by BCG immunization.

Identification of polyfunctional $CD4^+$ T cell populations from the same group of mice was also performed. The $CD4^+$ T cell populations from mice immunized with BCG

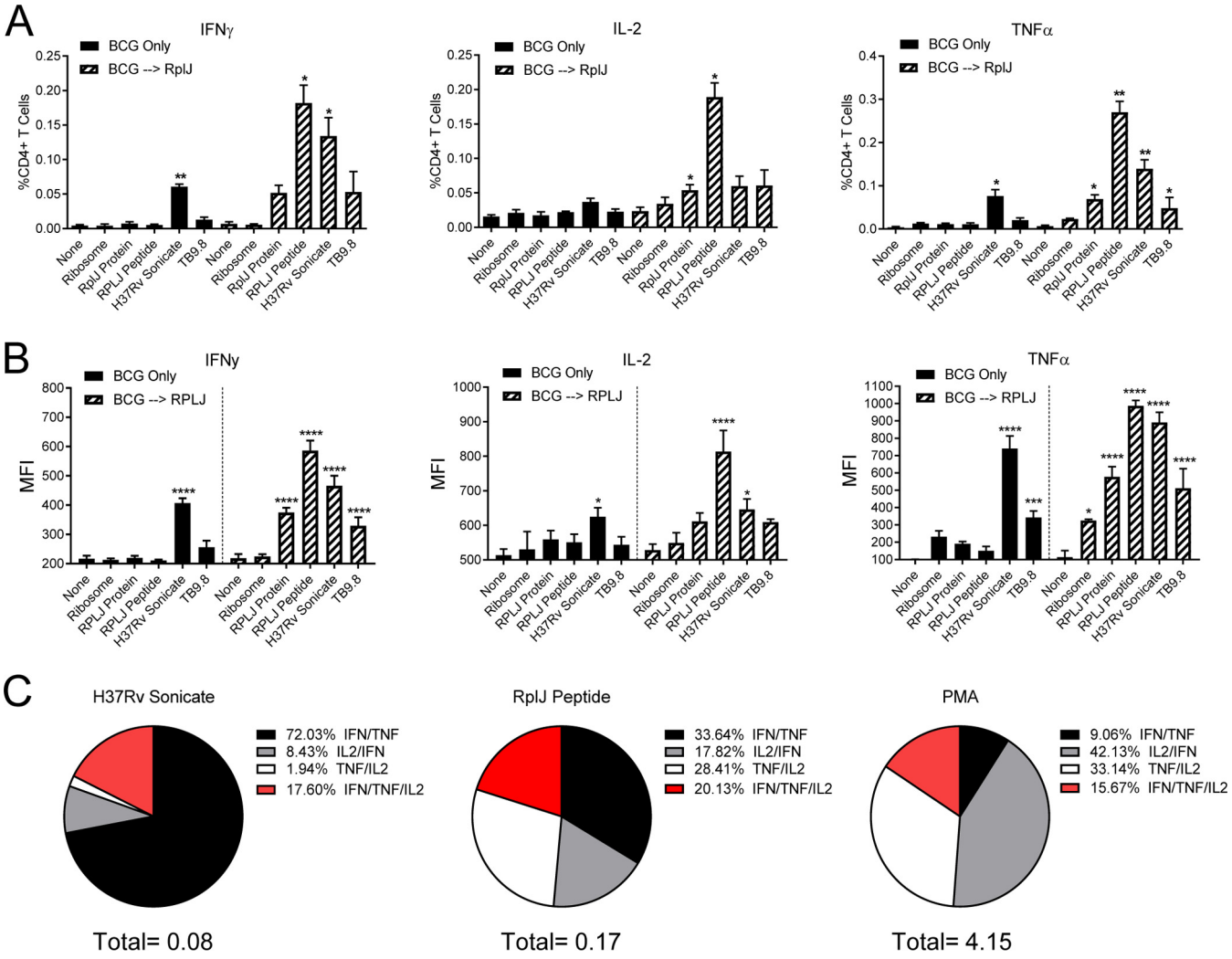

FIG 5 Induction of Th1 multifunctional CD4$^+$ T cells by boosting with RplJ protein. Mice (C57BL/6) ($n = 4$) were immunized with 5×10^6 CFU BCG s.c. or 5×10^6 CFU BCG s.c. followed 4 weeks later by two administrations of RplJ protein (25 μg) and CpG-ODN 1826 (20 μg) s.c. 2 weeks apart. Four weeks after the final immunization, single-cell suspensions of splenocytes were restimulated with recombinant RplJ protein (10 μg/ml), $RplJ_{TB146-160}$ peptide (10 μg/ml), TB9.8 peptide (10 μg/ml), H37Rv sonicate (10 μg/ml), or purified *M. smegmatis* ribosomes (5 nM). After 6 h of restimulation, cells were fixed and stained as described in Materials and Methods and analyzed by FACS analysis. Positive-control cells were stimulated with PMA plus ionomycin (10 μg/ml) (not shown). (A) Frequencies of CD4$^+$ T cells producing each cytokine in immunized mice in response to antigen restimulation. (B) Mean fluorescence intensity (MFI) as an indication of the average level of cytokine production by CD4$^+$ T cells in response to antigen restimulation. (C) Pie charts showing the relative fractions of dual- and triple-cytokine-producing CD4$^+$ T cells following restimulation with the H37Rv sonicate, RplJ peptide, or PMA-ionomycin. The total below the pie charts is the fraction of total CD4$^+$ T cells producing two cytokines. Percent values in the key represent the percentages of the total dual- and triple-cytokine-producing CD4$^+$ T cells in the sample. Data shown represent mean values and standard errors for groups of four mice; responses that are significantly different from those with the no-antigen control by ANOVA are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

only were too small to permit reliable identification of polyfunctional cells with confidence. However, BCG-immunized mice boosted by RplJ protein had large enough CD4$^+$ T cell populations producing two and three cytokines to allow analysis of their profiles in response to restimulation conditions (Fig. 5C). Stimulation with phorbol myristate acetate (PMA)-ionomycin was used to activate the total CD4$^+$ T cell population in these mice and identify the range of possible polyfunctional CD4$^+$ T cells. This showed that cells with the potential to produce both IFN-γ and IL-2 made up half of the total dual-cytokine-producing CD4$^+$ T cells, followed by 40% of CD4$^+$ T cells producing TNF-α and IL-2 and 10% producing IFN-γ and TNF-α. When stimulated with BCG-specific antigens using a crude *M. tuberculosis* (H37Rv) sonicate, the CD4$^+$ T cell population was skewed heavily toward dual IFN-γ- and TNF-α-producing cells, with fewer IL-2-producing cells being present overall. In contrast, specific restimulation with

RplJ peptide revealed CD4$^+$ T cell populations that were much more diverse in their dual-cytokine-producing profile. Overall, the numbers of triple-cytokine-producing cells were approximately the same among the three stimulations. These results suggested that boosting BCG-primed animals with RplJ protein and adjuvant established a strong multifunctional Th1-like CD4$^+$ T cell population that was not produced by BCG priming alone.

Reduction of lung pathology and bacterial burden following *M. tuberculosis* challenge in RplJ-boosted mice. The effects of RplJ protein boosting of BCG-primed mice during challenge with *M. tuberculosis* were examined by immunizing mice as described above and challenging them with low-dose aerosol delivery of virulent *M. tuberculosis* (H37Rv) 4 weeks later. Ten weeks after challenge, mice were sacrificed, and lungs were fixed and evaluated for granulomatous inflammation. Naive mice receiving no vaccination had the most severe disease, with extensive coalescing granulomatous inflammation, and this was also the case in BCG-immunized mice, although it was less severe than in naive control mice (Fig. 6). BCG-immunized mice that were boosted with RplJ protein exhibited the least severe disease, with moderate granulomatous inflammation. Mice boosted with RplJ protein showed significantly reduced lung pathology compared to naive mice or BCG-immunized animals. These results suggested that supplementing BCG with ribosomal antigens may improve disease pathology and outcomes.

The effects of RplJ boosting of BCG-primed animals on the bacterial burden in the lungs and mediastinal lymph nodes was also assessed 10 weeks following low-dose aerosol infection. In the lungs of both BCG-primed and BCG-primed RplJ-boosted mice, there were significant reductions in bacterial burdens compared to naive control mice. In the mediastinal lymph node, BCG immunization did not significantly reduce the *M. tuberculosis* burden, but BCG-primed and RplJ-boosted mice significantly reduced the *M. tuberculosis* burden compared to both naive and BCG-immunized mice. These results indicated that RplJ boosting had benefits in controlling *M. tuberculosis* in the lymph nodes but had less ability to augment the effects of BCG in reducing the bacterial burdens in the lungs of challenged mice (Fig. 7).

DISCUSSION

The discovery of new antigens for effective immunization against *M. tuberculosis* is a daunting task due to multiple factors, such as the complex life cycle of the bacterium, its relatively large genome, multiple immune evasion mechanisms, and the prevalence of previous exposure to mycobacteria prior to immunization (31–34). Although there is relatively limited knowledge of the correlates of protection for an *M. tuberculosis* vaccine, it is generally accepted that CD4$^+$ T cell responses are critical for controlling *M. tuberculosis* infections. Recent studies have highlighted that many of the known T cell epitopes in *M. tuberculosis* and BCG are hyperconserved (35), suggesting a unique survival strategy that overactivates the host immune system and leads to the exhaustion of CD4$^+$ T cells (36). Priming of CD4$^+$ T cell responses to cryptic or subdominant antigens not recognized during natural infection has been shown to promote a less differentiated CD4$^+$ T cell memory phenotype marked by IL-2 secretion and the absence of the marker KLRG1 (37, 38), and there are data that suggest that these T cells may express functions that can reprogram the T cell response to be more protective (39). Taken together, these findings suggest that the incorporation of cryptic antigens into vaccination strategies may be a useful strategy to promote CD4$^+$ T cells that enhance protection against *M. tuberculosis* and to imprint the protective function of these cells on T cells previously primed by BCG immunization (36).

Immunization of mice with the genetically modified *M. smegmatis* strain IKEPLUS was previously shown to significantly enhance the survival of mice when challenged with *M. tuberculosis*, compared to standard BCG immunization (26). IKEPLUS immunization led to the development of a Th1-like immune response that conferred protection from *M. tuberculosis* and was transferable to naive animals primarily by CD4$^+$ T cells (26). A recent follow-up study identified the mycobacterial ribosome as a prominent

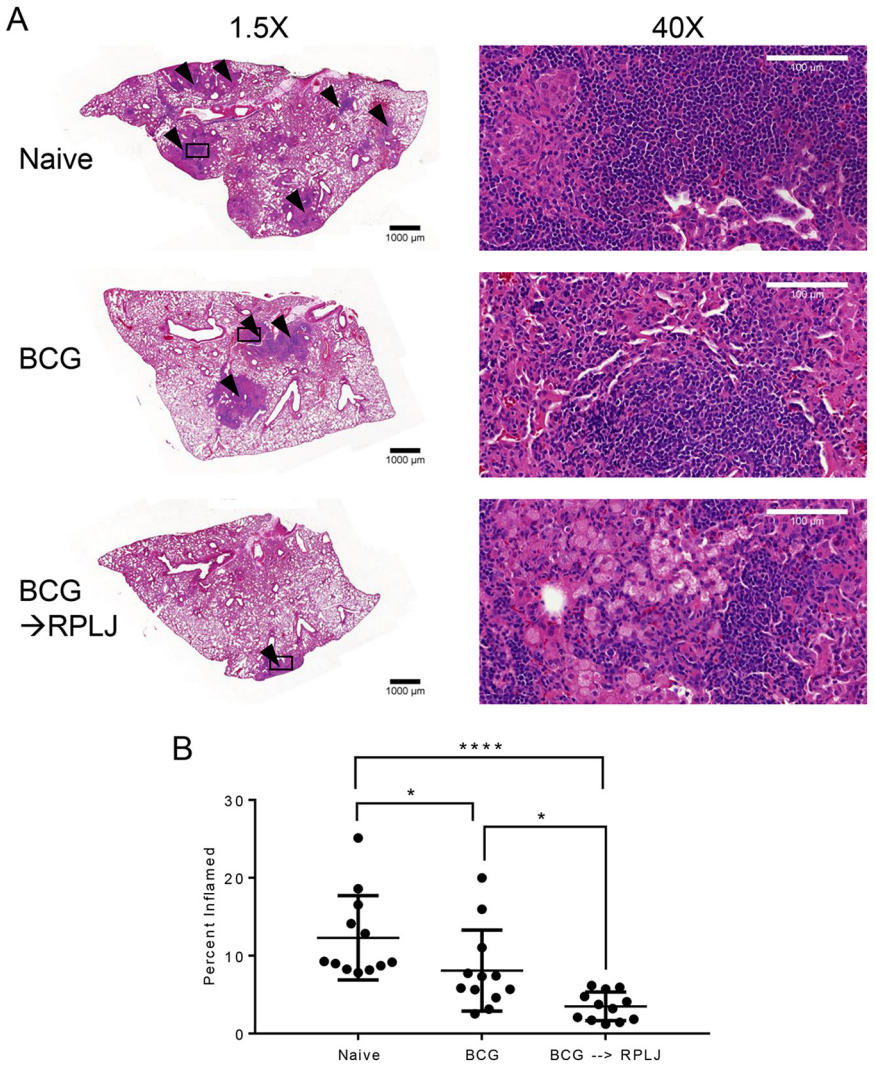

FIG 6 RplJ boosting reduces lung pathology in *M. tuberculosis*-challenged mice. Mice (C57BL/6) ($n = 7$) were immunized with 5×10^6 CFU BCG s.c. or with 5×10^6 CFU BCG s.c. followed 4 weeks later by two administrations of RplJ protein (25 μg) and CpG-ODN 1826 (20 μg) s.c. 2 weeks apart. Four weeks after the final immunization, mice were exposed to low-dose aerosolized *M. tuberculosis* for 20 min, resulting in approximately 100 CFU being deposited in the lungs of the mice. Ten weeks after challenge, mice were sacrificed, and lungs were harvested and fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin and sectioned at a 5-μm thickness. Sections were stained with hematoxylin and eosin. (A) Lung sections imaged at the indicated magnifications. Black triangles indicate granulomatous inflammation in the pictured section. Squares indicate the area enlarged to a ×40 magnification. Images are representative of data from groups consisting of four mice each. (B) Sections were evaluated for granulomatous inflammation by using ImageJ software to identify the percent area of inflammation. Data shown represent mean values and standard errors for groups of four mice; responses that are significantly different from those for naive control mice by ANOVA are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

target of CD4$^+$ T cells during IKEPLUS immunization (27) and specifically defined epitopes within the RplJ and RpsA proteins. The present study extended these findings by more definitively surveying all 57 mycobacterial ribosomal proteins to identify those harboring epitopes for CD4$^+$ T cell responses in appropriately immunized mice. As expected, mice immunized with the rapidly growing *M. smegmatis* IKEPLUS strain formed strong CD4$^+$ T cell responses to ribosomal proteins, recognizing 24 out of the total of 57 proteins, while BCG-immunized CD4$^+$ T cells responded to only one ribosomal protein (Fig. 1). The mechanism for this difference in the priming of antiribosome responses between these mycobacterial species is unknown, although one

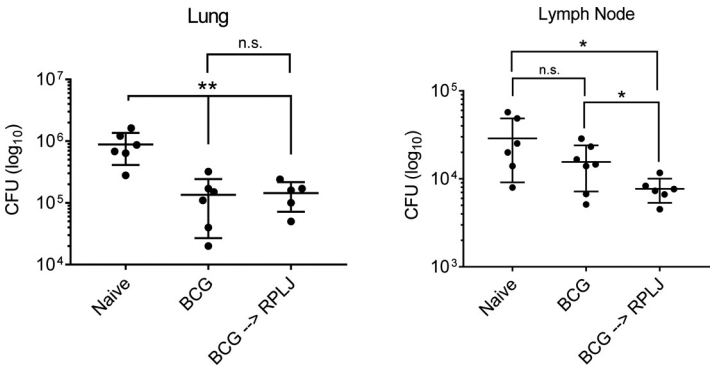

FIG 7 Effect of RplJ boosting on tissue bacterial burden in *M. tuberculosis*-challenged mice. Mice (C57BL/6) ($n = 7$) were immunized with 5×10^6 CFU BCG s.c. or with 5×10^6 CFU BCG s.c. followed 4 weeks later by two administrations of RplJ protein (25 μg) and CpG-ODN 1826 (20 μg) s.c. 2 weeks apart. Four weeks after the final immunization, mice were exposed to low-dose aerosolized *M. tuberculosis* for 20 min, resulting in approximately 100 CFU being deposited in the lungs. Ten weeks after challenge, mice were sacrificed, and single-cell suspensions prepared from lungs and mediastinal lymph nodes were plated onto 7H10 agar. Each symbol is representative of data for one mouse. Data shown represent mean values and standard errors. Responses that are significantly different from another group by a *t* test are indicated (*, $P < 0.05$; **, $P < 0.01$; n.s., not significant).

possibility is that it is related to the larger number of ribosomes in fast-growing mycobacteria like *M. smegmatis*, versus slow-growing mycobacteria like BCG or *M. tuberculosis* (40, 41). Thus, the relatively large number of bacterial ribosomes present during a transient *M. smegmatis* infection may skew the $CD4^+$ T cell response toward the mycobacterial ribosome.

To our knowledge, this is the first study to examine the entire complement of mycobacterial ribosomal proteins for their ability to stimulate $CD4^+$ T cell responses. Early studies suggested that mycobacterial ribosomes were immunogenic in mice and guinea pigs, although the precise identities of the immunogenic components were not definitively established (42–48). In the present study, we identified multiple immunogenic protein antigens present in the mycobacterial ribosome, and for 10 of the 24 proteins identified as being immunogenic, we defined $CD4^+$ T cell epitopes at the 15-mer peptide level (Table 1). Our findings confirmed the immunogenicity of the *M. tuberculosis* $RplJ_{146-160}$ peptide identified in our previous work (27) and extended this by showing that immunization with a rapidly growing *M. smegmatis* strain stimulated a broad response against multiple cross-reactive *M. tuberculosis* ribosomal proteins. In addition, mycobacterial homologues of two ribosomal proteins identified in previous studies as being protective antigens of other microbial pathogens were identified in our comprehensive survey of mycobacterial ribosomal proteins. These proteins were RplL (L7/12), which is a target for protective immune responses against *Brucella abortus*, and RplC (L3), which is a target of protective immunity against *Leishmania major* in animal models (49–53). Overall, our findings provide a rich source of new antigens that potentially can be incorporated into various vaccine delivery systems already in clinical trials or advanced stages of development (11, 35, 54).

We previously reported that BCG was unable to stimulate a significant $CD4^+$ T cell response to mycobacterial ribosomes in mice, even when injected at high doses by the subcutaneous or intravenous (i.v.) route (27). This result raised the possibility that BCG immunization may actively inhibit the presentation of mycobacterial ribosomes and block the formation of ribosome-specific $CD4^+$ T cells. However, our results in the present study suggested that this is not the case, since BCG vaccination actually primed mice to generate stronger ribosome-specific $CD4^+$ T cell responses when followed by immunization with an *M. smegmatis* strain or with purified ribosomal protein (Fig. 4). Mice that were immunized with BCG and boosted with RplJ protein primed a strong Th1-like $CD4^+$ T cell response to the ribosomal protein, and these responses were dominated by cells with substantial polyfunctionality in terms of the production of IL-2,

IFN-γ, and TNF-α (Fig. 5). Analysis of the dual-cytokine-producing cells of mice immunized with BCG and boosted with RplJ protein indicated a preponderance of dual TNF-α- and IFN-γ-producing cells, whereas recall with an epitope of RplJ ($RplJ_{TB146-160}$) revealed a multifunctional $CD4^+$ T cell response that greatly increased the amount of IL-2-producing cells and reduced the number of dual IFN-γ and TNF-α producers. The differences in these responses may be relevant to the protective effects afforded by $CD4^+$ T cells reactive with different antigens, as it has been reported that $CD4^+$ T cells that coproduce IFN-γ and TNF-α are more prevalent in patients with active *M. tuberculosis* infections and not present in patients who successfully control *M. tuberculosis* and have latent infection (55, 56). Recent studies have indicated that cryptic antigens are able to prime a $CD4^+$ T cell response with greater TNF-α and IL-2 coproduction and with a higher proliferative capacity, whereas immunodominant antigens were found to prime mainly IFN-γ/TNF-α-coproducing $CD4^+$ T cell responses (57). Our results suggest that boosting BCG-vaccinated animals with RplJ protein may prime $CD4^+$ T cell responses that resemble those associated with cryptic antigen recognition and, possibly, with better control of infection (57, 58).

This study also examined the ability of boosting BCG immunization with RplJ protein to protect mice against *M. tuberculosis* challenge. Most notably, mice primed with BCG and boosted with RplJ protein also showed significantly reduced pulmonary pathology and lower bacterial burdens in their mediastinal lymph nodes following challenge (Fig. 6 and 7). However, our findings so far have been restricted to proof-of-concept studies using only a single ribosomal protein to boost BCG-induced immunity, and future studies are planned to assess the effects of other immunogenic ribosomal proteins identified in this study as potential vaccines for boosting protective immunity against *M. tuberculosis* challenge. While we observed no general preference for components of the large or small ribosomal subunits in the $CD4^+$ T cell responses in our studies, understanding the kinetics of expression of these proteins during *M. tuberculosis* infection, and variations in their posttranslational modifications and associations with cytosolic proteins (59–61), might be critically important in designing future vaccination studies. During normal growth of the bacteria and establishment of infection, ribosomes are relatively abundant (62), and all 57 ribosomal proteins should be available during this stage of infection, with one protein, RplL (L7/L12), present at a higher level than all others due to its formation of multimers (49–51). In contrast, during the latent phase of infection, it is believed that ribosomal silencing factors act on the ribosome, preventing the association of the small and large ribosomal subunits (52, 60). The large ribosomal subunits may be kept stable and present during this phase, while small ribosomal subunits will be subject to degradation and turnover. Thus, we hypothesize that the proteins of the large ribosomal subunit would be more abundant during the latent phase of *M. tuberculosis* infection. Many vaccine formulations currently in clinical trials are formulated with both active- and latent-phase antigens (11, 53). This raises the likelihood that the proteins of the large ribosomal subunit would act as both active and latent antigens during immunization and that the 15 antigenic proteins of the *M. tuberculosis* large ribosomal subunit identified in our study are strong candidates for incorporation into future experimental vaccines against *M. tuberculosis*.

MATERIALS AND METHODS

Mice. Six- to eight-week-old female C57BL/6J (B6) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice were housed in pathogen-free facilities according to procedures and regulations established by the Albert Einstein College of Medicine Institute for Animal Studies and Biosafety Committees. Mice were allowed to acclimate to their housing for 1 week before experiments. All procedures performed were in accordance with protocols approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee (IACUC).

Cell culture. Mammalian cells were cultured in RPMI 1640 medium or Dulbecco's modified Eagle's medium (DMEM) (ThermoFisher Scientific, Grand Island, NY) containing 0.5% 2-mercaptoethanol (ThermoFisher Scientific, Grand Island, NY), 1% penicillin-streptomycin (ThermoFisher Scientific, Grand Island, NY), 1% HEPES (ThermoFisher Scientific, Grand Island, NY), 1% minimal essential medium (MEM) nonessential amino acids, and 10% fetal calf serum (FCS) (Atlanta Biologicals). Cultures were maintained in a humidified 37°C incubator with 5% CO_2 (for RPMI) or 10% CO_2 (for DMEM) in air.

Mycobacterial cultures and immunizations. *M. smegmatis* strains mc²155 and IKEPLUS were described previously (26) and were grown in liquid Sauton medium (63). Bacillus Calmette-Guérin (BCG)-Danish was obtained from the Statens Serum Institute (Copenhagen, Denmark) and was grown in Middlebrook 7H9 medium (Difco Laboratories, BD Diagnostic Systems, Sparks, MD) with oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco Laboratories, BD Diagnostic Systems, Sparks, MD) and 0.05% tyloxapol (Sigma-Aldrich, St. Louis, MO). Bacteria were grown to mid-log phase from low-passage-number frozen stocks and then frozen in medium containing 5% glycerol at −80°C. Prior to immunization, bacterial stocks were thawed, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS containing 0.05% Tween 80. The bacteria were then sonicated in a bath sonicator for 5 min to achieve single-cell suspensions. Mice were immunized with 5×10^7 CFU IKEPLUS via the tail vein or 5×10^6 CFU BCG-Danish subcutaneously (s.c.) in the scruff of the neck. For immunization with purified ribosomal proteins, aliquots of the proteins were thawed, diluted with adjuvant and double-distilled water (ddH_2O) to a final concentration of 25 μg of protein per 200 μl, and sterilized by passage through a 0.22-μm filter. The adjuvant CPG-ODN 1826 (InvivoGen, San Diego, CA) was added to ribosomal proteins at a concentration of 20 μg per 200 μl. Protein-plus-adjuvant mixtures were injected s.c. in the scruff of the neck with a volume of 200 μl per injection.

Quantification of granulomatous inflammation in lung histology sections. Lung sections were stained with hematoxylin and eosin, and images were acquired by using the Panoramic 250 Flash II slide scanner (3DHistech, Budapest, Hungary). Images were analyzed by color threshold to determine the total area of lung sections at a ×1.5 magnification. The color threshold was adjusted to isolate areas containing a high density of nuclei indicative of granulomatous inflammation, and the area was recorded. The inflamed area measured was divided by the total area measured and expressed as a percentage to represent the extent of granulomatous inflammation.

Preparation of bacterial sonicates and purified ribosomes. The H37Rv sonicate was produced by sonicating irradiated *M. tuberculosis* H37Rv (BEI Resources, Manassas, VA) in lysis buffer (30 mM Tris-HCl, pH 8.0) containing 0.05% tyloxapol and protease inhibitors (cOmplete, Mini, EDTA-free protease inhibitor cocktail [Roche], at 1 tablet per 10 ml) for 20 min on ice. The resulting crude sonicate was centrifuged at 1,500 × *g* for 12 min at 4°C. The supernatant was collected and retained, and the pellet was resuspended and sonicated as described above. This step was repeated three times, and the supernatants were combined. The combined supernatant was centrifuged at 14,000 × *g* for 30 min at 4°C and filtered through a 0.22-μm filter. The protein concentration was determined by using a bicinchoninic acid (BCA) protein assay kit (ThermoFisher Scientific, Grand Island, NY).

Ribosomes from IKEPLUS were purified and quantitated as described previously (27).

Generation of an *M. tuberculosis* ribosomal protein library. H37Rv genomic DNA was obtained from BEI Resources (Manassas, VA). By using the primers indicated in Table S1 in the supplemental material, each of the 57 *M. tuberculosis* ribosomal protein genes was amplified by PCR using Q5 high-fidelity polymerase 2× master mix (NEB, Ipswich, MA). Cloning of inserts was accomplished via ligation-independent cloning. The resulting inserts were treated with T4 polymerase (NEB, Ipswich, MA) at room temperature for 30 min to create nucleotide overhangs. The vector pmscg9 (64) was linearized with the SspI restriction enzyme (NEB, Ipswich, MA) and similarly treated with T4 polymerase. The digested vector and insert were incubated together at a molar ratio of 1:3 at room temperature for 5 min. The resulting ligation reaction mixtures were used to transform *E. coli* MAX Efficiency DH5α cells (ThermoFisher Scientific, Grand Island, NY) according to the manufacturer's instructions. Transformed DH5α cells were grown in LB broth (ThermoFisher Scientific, Grand Island, NY), and plasmid DNA was isolated by using the QIAprep spin miniprep kit (Qiagen, Hilden, Germany). Plasmid inserts were validated by sequencing by the Sanger method (Genewiz, South Plainfield, NJ). For the production and purification of recombinant *M. tuberculosis* ribosomal proteins, 5-ml cultures of *E. coli* BL21 harboring plasmids encoding ribosomal proteins were grown overnight in 1 liter of autoinduction medium [Terrific broth (Sigma), 1 mM $MgSO_4$, 25 mM $(NH_4)_2SO_4$, 50 mM KH_2PO_4, 50 mM Na_2HPO_4, 0.9% glycerol, 0.05% glucose, 0.2% α-lactose]. Cultures were centrifuged at 3,000 × *g* for 10 min, and pellets were frozen at −80°C, thawed at room temperature, and resuspended by using 5 ml bacterial protein extraction reagent (BPER) (ThermoFisher Scientific) per g of wet pellet. The mixture was rotated for 15 min at room temperature to lyse the cells and then centrifuged at 16,000 × *g* for 20 min to separate the insoluble and soluble fractions. Insoluble pellets were resuspended with 5 ml per g of wet pellet of inclusion body solubilization buffer (8 M urea, 1% Triton X-100, 0.6 M NaCl, 20 mM sodium phosphate), rotated for 2 h at room temperature, and then centrifuged at 27,000 × *g* for 15 min to remove debris and nonsolubilized proteins. Recombinant ribosomal proteins were purified by using a peristaltic pump (GE Healthcare, Marlborough, MA) and HisTrap HP columns (GE Healthcare). Protein was eluted by using 8 column volumes of elution buffer (500 mM imidazole, 0.5 M NaCl, 20 mM sodium phosphate), and 1-ml fractions were collected for analysis. Fractions that contained protein, as determined by the absorption at 280 nm, were pooled and depleted of imidazole by using a 7,000-molecular-weight-cutoff (MWCO) Zeba desalting column (Thermo). The concentrations of the desalted pure protein fractions were determined by using the Bradford assay (Thermo), and purified proteins were run on Any kD precast protein gels (Bio-Rad) to determine the molecular weight and purity. Purified ribosomal proteins were aliquoted in 150-μg stocks and frozen at −80°C.

Peptide libraries. A peptide library based on the sequences of 10 selected *M. tuberculosis* ribosomal proteins (RplL, RplS, RpsJ, RpmB2, RplK, RpsG, RpsC, RplC, RplN, and RpsL) was designed based on strain H37Rv sequences collected from Tuberculist (http://genolist.pasteur.fr/TubercuList/). The sequences were ranked by their predicted binding affinity for MHC class II (I-A^b). MHC binding affinity was predicted by using the IEDB (http://www.iedb.org/) analysis resource consensus tool (30). Peptides with a consen-

sus rank of ≤33.33 (i.e., top one-third best binders) were selected, and those with an overlap of 3 amino acids or more with a higher-ranking peptide were removed. This analysis resulted in 109 predicted peptides, which were synthesized and isolated by using a standard commercial service for generating peptide batches with 70 to 80% purity (Mimotopes, Mulgrave, Victoria, Australia). A second peptide library was independently constructed based on areas of homology between *M. tuberculosis* and *M. smegmatis* ribosomal proteins. The sequences of all 57 ribosomal proteins from the *Mycobacterium smegmatis* (strain mc²155) and *M. tuberculosis* (H37Rv) strains were collected from UniProt (http://www.uniprot.org/). The sequences were then clustered at a 30% amino acid sequence identity level to group similar sequences into separate clusters with the epitope cluster analysis tool available at the IEDB tools website (65). Each cluster of sequences was then aligned separately by using the ClustalW algorithm as implemented in the MEGA tool (66). For these clusters, 15-mer peptides overlapping by 10 amino acids were generated, and duplicates were removed. The remaining peptides were then ranked based on their hypothetical binding affinity for MHC class II (I-A^b), as predicted by using the IEDB analysis resource consensus tool (30). Peptides with an IEDB consensus percentile rank of ≤10.0 were selected, and variants (peptides from same sequence position but having amino acid mismatches) were removed by eliminating the variant with the higher consensus percentile. This resulted in 153 peptides, which were synthesized on a small scale (~1 mg) and provided at 70 to 80% purity by A&A Labs (San Diego, CA). All peptides were reconstituted in dimethyl sulfoxide (DMSO) and used at a final concentration of 10 μg/ml per peptide.

IFN-γ ELISPOT and flow cytometry analyses. Measurement of IFN-γ-producing $CD4^+$ T cells purified from the spleens of previously immunized animals was done by an ELISPOT assay as described previously (27). For staining and analysis of surface markers by flow cytometry, $CD4^+$ T cells were restimulated with antigens in culture and collected by centrifugation at 300 × *g* for 5 min. Cells were then resuspended in 50 μl of Live/Dead fixable blue reagent (Thermo) diluted 1:500 in PBS and incubated at room temperature for 30 min. After washing with 1× fluorescence-activated cell sorter (FACS) buffer (PBS, 2% FCS, 0.05% NaN_3), cells were resuspended in FACS buffer containing a 1:200 dilution of CD11b-biotin (Thermo), a 1:200 dilution of CD19-biotin (BioLegend), and 5 μg/ml of 2.4G2 antibody (BioLegend). The cells were incubated for 30 min at room temperature and washed once in FACS buffer. A surface marker staining cocktail (CD4-Alexa 488 clone GK1.5 [BioLegend], CD3-peridinin chlorophyll protein [PerCP]/Cy5.5 clone 145-2C11 [Thermo], and CD8α-allophycocyanin [APC]/Cy7 clone 53-6.7 [BioLegend], all at a 1:200 dilution), also containing 5 μg/ml 2.4G2 antibody and a 1:200 dilution of streptavidin-Pacific Blue, was prepared in FACS buffer, and 50 μl was added to each well. The cells were incubated for 30 min at room temperature and washed once in FACS buffer. The cells were then washed once in PBS, resuspended in 50 μl of 1% paraformaldehyde (Sigma), and incubated at 4°C overnight. The next day, cells were washed once and resuspended in 100 μl FACS buffer. For intracellular cytokine staining, fixed and surface-stained cells were washed once with PBS, resuspended in 50 μl of FoxP3 fixation/permeabilization buffer (eBioscience), and incubated at room temperature. After 1 h, 200 μl of intracellular cytokine staining (ICS) permeabilization buffer (Dulbecco's phospate-buffered saline [DPBS] plus Ca^{2+} and Mg^{2+} [Thermo], 1.0% HEPES [Thermo], 2.0% FCS, 0.1% saponin [Sigma], and 0.05% NaN_3 [Sigma]) was added to the wells. The plates were washed once with ICS permeabilization buffer. An intracellular cytokine staining cocktail (1:100 dilution of anti-IFN-γ–phycoerythrin [PE] clone XMG1.2 [BioLegend], 1:400 dilution of anti-IL-2–PE/Cy7 clone JES6-5H4 [BioLegend], 1:100 dilution of anti-TNF-α–APC clone MP6-XT22 [BioLegend]) was prepared in ICS permeabilization buffer with 5% normal mouse serum, and 50 μl was added to each well. Cells were incubated at 37°C for 30 min and transferred to 4°C overnight. The next day, cells were washed twice with permeabilization buffer and resuspended in 200 μl FACS buffer. All analyses and data collection were done by using an LSRII benchtop flow cytometer (BD Biosciences) and FlowJo software (BD Biosciences).

Statistical analyses. The significance of ELISPOT responses to proteins of the total library of 57 mycobacterial ribosomal proteins was determined by using two-way analysis of variance (ANOVA) with a false discovery rate (FDR) correction. The cutoff for significance was a 1% FDR. All data reported are represented as mean values ± 1 standard error of the mean (SEM). Statistical significance was determined by using one-way ANOVA in the case of multiple comparisons, unless stated otherwise in the figure legends. GraphPad Prism (GraphPad, La Jolla, CA) was used for all statistical calculations.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00009-18.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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