

DNA immunization of *Mycobacterium tuberculosis* resuscitation-promoting factor B elicits polyfunctional CD8⁺ T cell responses

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Purpose: T cell-mediated immune responses, and particularly activation of polyfunctional T cells that simultaneously produce multiple cytokines, are necessary for the control of *Mycobacterium tuberculosis*. In the present study, we examined if DNA immunization of *Mycobacterium tuberculosis* resuscitation-promoting factor B (RpfB) elicits polyfunctional T cell responses in mice.

Materials and Methods: C57BL/6 mice were immunized intramuscularly three times, at 3-week intervals, with RpfB-expressing plasmid DNA. For comparison, protein immunization was performed with recombinant RpfB in control mice. After immunization, RpfB-specific T cell responses were assessed by interferon- γ (IFN- γ) enzyme-linked immunosorbent spot assay and intracellular cytokine staining (ICS), and T cell polyfunctionality was assessed from the ICS data.

Results: RpfB DNA immunization induced not only humoral immune responses, but also CD8⁺ and CD4⁺ T cell responses. Immunodominant T-cell epitopes were identified within RpfB by assays with overlapping peptides. RpfB DNA immunization elicited a polyfunctional CD8⁺ T cell response that was dominated by a functional phenotype of IFN- γ ⁺/TNF- α ⁺/IL-2/CD107a⁺.

Conclusion: RpfB DNA immunization elicits polyfunctional CD8⁺ T cell responses, suggesting that RpfB DNA immunization might induce protective immunity against tuberculosis.

Keywords: Tuberculosis, Resuscitation-promoting factor B, Polyfunctional T cells, DNA vaccines

Introduction

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis*. It is estimated that about one-third of the world's population is latently infected with *M. tuberculosis* and that TB causes the death of 1.5 million people per year [1,2]. Unfortunately, the efficacy of the current TB vaccine is limited, thus development of an effective vaccine against TB is urgently required.

The *M. bovis* bacillus Calmette-Guérin (BCG) strain is used in the current TB vaccine, which has been used since 1921 [3,4]. Whereas BCG is effective for the prevention of severe forms of TB in children, such as miliary TB and TB meningitis [5,6], reports of its efficacy for the prevention of adult pulmonary TB are inconsistent [7,8]. In fact, the efficacy varies from 0% to 80% between studies, and it is the lowest in develop-

ing countries where protection against TB is the most needed [7,8].

For effective control of *M. tuberculosis* infection, T cell-mediated immune responses are essential [9,10] and thus, T cell activation following vaccination is considered to be a key requirement for generating protective immunity against TB. In particular, CD4⁺ T helper 1 (Th1) cells play a major role in immune responses against TB by secreting cytokines such as interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) [11-14], which activate macrophages. In addition, CD8⁺ T cells also make important contributions to the protective immune responses against TB, as they not only secrete IFN- γ and TNF- α , but also lyse infected macrophages and epithelial cells [15-17].

Recently, the importance of activating polyfunctional T cells, which simultaneously produce multiple cytokines, has been emphasized in vaccine development. Initially, the significance of polyfunctional T cells was proven by a study that showed that the degree of vaccine protection against *Leishmania major* was predicted by the frequency of polyfunctional Th1 cells simultaneously secreting IFN- γ , TNF- α , and interleukin-2 (IL-2) [18]. In TB, the presence of polyfunctional CD4⁺ Th1 cells correlates with protection against *M. tuberculosis* challenge in mice [19,20]. In addition, the presence of polyfunctional CD8⁺ T cells simultaneously secreting IFN- γ and IL-2 was found to be associated with natural protection against TB and protection following anti-mycobacterial therapy in TB patients [21,22]. In view of these facts, it is expected that effective TB vaccines will also elicit polyfunctional T cell responses.

In the development of new TB vaccines, resuscitation-promoting factor (Rpf) is considered a promising target antigen [23,24]. Rpfs, first identified in *Micrococcus luteus*, are bacterial proteins that promote the recovery of bacteria from latency to a replicating phase [25]. *M. tuberculosis* expresses five different Rpf proteins, RpfA, B, C, D, and E [26], and they have resuscitation activity [27,28]. Indeed, deletion of *rpf* genes results in delayed reactivation of *M. tuberculosis* in a murine dormancy model [29]. Given that Rpf proteins are expressed during growth of *M. tuberculosis*, Rpfs-specific T cell immunity was expected to be effective in the control of *M. tuberculosis* growth [23,24]. Moreover, Rpfs were shown to be immunogenic in protein [23] or DNA [24] vaccination of mice.

In the present study, we examined if polyfunctional T cell responses are elicited by DNA immunization of *M. tuberculosis* RpfB. Specifically, we immunized mice with RpfB-encoding plasmid DNA and analyzed the polyfunctionality of T cells.

Materials and Methods

RpfB DNA plasmid

The *rpfB* gene of *M. tuberculosis* H37Rv (NCBI Gene ID: 886048) was codon-optimized for mammalian expression and synthesized by GenScript (Piscataway, NJ, USA). The synthesized *rpfB* gene was cloned into the pcDNA3.1(+) mammalian expression vector (Invitrogen, Carlsbad, CA, USA), where the cloned gene is expressed under the control of the cytomegalovirus IE-1 promoter. The sequence of the cloned *rpfB* gene was confirmed by sequencing. Large-scale production of plasmid DNA was performed using the Plasmid Giga Kit (Qiagen, Valencia, CA, USA).

Recombinant RpfB protein

The *rpfB* gene was amplified by polymerase chain reaction from genomic DNA of *M. tuberculosis* H37Rv and cloned into the pET-28a bacterial expression vector (Novagen, Darmstadt, Germany), which carries an N-terminal His-tag for protein purification. The sequence of the cloned *rpfB* gene was confirmed by sequencing. The RpfB protein was overexpressed in BL21 (DE3) *Escherichia coli* and purified using nickel-nitrioltriacetic acid agarose resin. Protein size and purity were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purity was >90%.

Mouse immunization

Female C57BL/6 mice were housed in a specific pathogen-free facility in accordance with our institutional guidelines and used for immunization at the age of 5-6 weeks. For RpfB DNA vaccination, mice were immunized three separate times, at 3-week intervals, intramuscularly in both quadriceps muscles with 100 μ g of RpfB plasmid DNA. For RpfB protein vaccination, mice were immunized three separate times, at 3-week intervals, subcutaneously with 50 μ g of recombinant RpfB (rRpfB) protein. In protein immunization, incomplete Freund's adjuvant (Sigma, St. Louis, MO, USA) was used. For comparison, control mice were vaccinated subcutaneously with 2×10^5 colony-forming unit of BCG (Pasteur 1173).

Synthetic peptides

Seventy one pentadecamer peptides (Mimotopes, Clayton, Australia), overlapping by 10 amino acids each and spanning the complete *M. tuberculosis* (H37Rv) RpfB protein sequence (NCBI Gene ID: 886048) were used. The overlapping peptides (OLPs) were resuspended at 20 mg/mL in dimethyl sulfoxide

and further diluted to 1 mg/mL with phosphate-buffered saline (PBS). RpfB OLPs were mixed into two separate aliquots. The first aliquot included peptides from OLP-1 to OLP-35 and was designated as OLP mix 1. The other aliquot included peptides from OLP-36 to OLP-71 and was designated as OLP mix 2. In T-cell assays, either each single OLP or OLP mixes were used for T-cell stimulation.

Indirect enzyme-linked immunosorbent assay

Serum was collected 4 weeks after the final immunization, and anti-RpfB antibody was assessed by indirect enzyme-linked immunosorbent assay (ELISA). A Maxisorp microtiter plate (Nunc, Roskilde, Denmark) was coated with 10 µg/mL of rRpfB protein and blocked with 5% fetal bovine serum (FBS)-0.05% Tween-phosphate buffered saline (PBST). Sera of the immunized mice were serially diluted and dispensed into each well. After 2 hours of incubation, horseradish peroxidase-conjugated goat anti-mouse IgG (Millipore, Bedford, MA, USA) was added. One hour later, color reaction was performed with 3,3', 5,5'-tetramethylbenzidine (Sigma) for 15 minutes. The reaction was stopped by 2 M H₂SO₄, and optical density was read at 450 nm.

IFN-γ enzyme-linked immunosorbent spot assay

A 96 well MultiScreen enzyme-linked immunosorbent spot (ELISpot) plate (Millipore) was coated with anti-mouse IFN-γ antibody (clone AN-18, eBioscience, San Diego, CA, USA). The antibody-coated plate was blocked with 1% bovine serum albumin (Bovogen Biologicals, Essendon, Australia). Freshly-isolated splenocytes were dispensed into each well (500,000 cells/well), and 10 µg/mL of each single OLP was added. After 20 hours of incubation, the plate was washed with PBS and 0.05% PBST. Biotin-conjugated anti-mouse IFN-γ antibody (clone RA-6A2, eBioscience) and alkaline phosphatase-streptavidin (BD Pharmingen, San Diego, CA, USA) were sequentially added. A color reaction was then performed using AP color reagent (Bio-Rad, Hercules, CA, USA). The number of IFN-γ spot forming units was counted using an ELISpot reader (Cellular Technology Ltd., Cleveland, OH, USA). To evaluate CD8⁺ and CD4⁺ T cell responses separately, T cells were isolated from splenocytes using CD8 or CD4 microbeads (Miltenyi Biotec, Auburn, CA, USA), and IFN-γ ELISpot assays were performed with isolated T cells and T cell-depleted splenocytes [30].

Intracellular cytokine staining and polyfunctional T cell assay

Freshly-isolated splenocytes were resuspended in RPMI 1640 containing 10% FBS and 2 mM L-glutamine and stimulated with a single immunodominant OLP (10 µg/mL), OLP mix (1 µg/mL for each OLP) or rRpfB protein (5 µg/mL). To evaluate cytotoxic degranulation activity of T cells, anti-CD107a-PE-Cy7 (BD Biosciences, San Jose, CA, USA) was added into the culture medium. Brefeldin A (GolgiPlug, BD Biosciences) and monensin (GolgiStop, BD Biosciences) were added 2 hours later. After another 10 hours of incubation, splenocytes were first stained with ethidium monoazide (Sigma) and then stained with anti-CD3-V500, anti-CD4-V450 and anti-CD8-APC-H7 (all from BD Biosciences). The stained cells were permeabilized using Cytofix/Cytoperm kit (BD Biosciences) and then stained with anti-IFN-γ-APC, anti-TNF-α-PE and anti-IL-2-FITC (all from BD Biosciences). Fluorescence-activated cell sorting (FACS) analysis was performed using an LSRII flow cytometer (BD Biosciences) and the data were analyzed using FlowJo software (Treestar, San Carlos, CA, USA). T cells positive for the various combinations of cytokines and degranulation were analyzed and quantified using a Boolean gating function in FlowJo software [31].

Results

Humoral immune responses induced by RpfB immunization

First, we assessed the RpfB-specific humoral immune responses after RpfB immunization. RpfB-specific immunoglobulin (Ig) was quantified in serially-diluted sera by indirect ELISA, and both DNA and protein immunization of RpfB were found to significantly increase the anti-RpfB Ig titer, though protein immunization was more potent (Fig. 1). In contrast, anti-RpfB Ig was not induced by BCG immunization (Fig. 1). These data show that both RpfB DNA and protein are immunogenic and capable of eliciting humoral immune responses.

Identification of the immunodominant T-cell epitopes in RpfB

Next, we identified the immunodominant T-cell epitopes in RpfB. Splenocytes were isolated from RpfB DNA-immunized mice and stimulated with each of 71 OLPs spanning RpfB followed by assessment using an IFN-γ ELISpot assay. IFN-γ production was observed by direct *ex vivo* stimulation following stimulation with OLPs including OLP-14, OLP-30, and OLP-51 (Fig. 2A). To identify which T cell subset is responsible for IFN-γ production following stimulation with each epitope, CD8⁺ or CD4⁺ T cells were isolated from splenocytes,

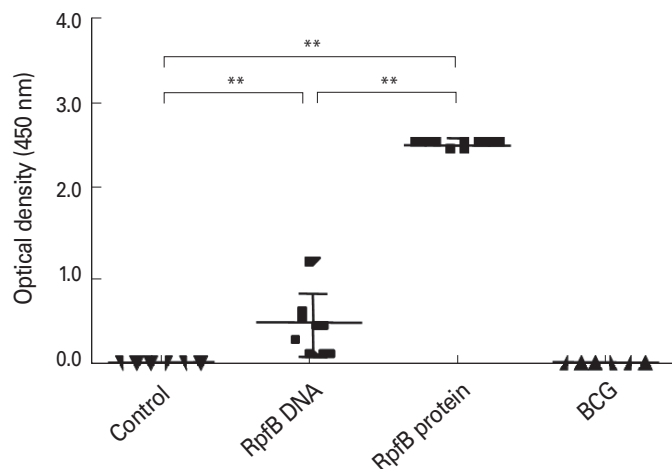


Fig. 1. Resuscitation-promoting factor B (RpfB)-specific antibody response induced by RpfB immunization. Mice were immunized as described in Materials and Methods, and serum collected. The sera were serially diluted (1:5,000), and RpfB-specific Ig was measured by indirect enzyme-linked immunosorbent assay. BCG, bacillus Calmette-Guérin. The graph represents mean \pm SEM (n=6). Mann-Whitney U test was performed (**p<0.01).

and isolated T cells and T cell-depleted splenocytes were subjected to IFN- γ ELISpot assays. OLP-14-stimulated IFN- γ production was observed in isolated CD8⁺ T cells (Fig. 2B), while OLP-30- and OLP-51-stimulated IFN- γ production was observed in CD4⁺ T cells (Fig. 2C). Taken together, OLP-14 (AGV-QVHDADTIVLRR) is a dominant epitope for CD8⁺ T cells, and OLP-30 (GGLVRTVHLPAPNVA) and OLP-51 (LPVANV-VVTPAHEAV) are dominant epitopes for CD4⁺ T cells; however, it should be noted that the minimal epitope within each peptide was not determined. Thus, we used these OLPs for T cell stimulation in further analyses.

T cell immune responses elicited by RpfB DNA immunization

We evaluated T cell responses after RpfB immunization by intracellular cytokine staining (ICS). In ICS, splenocytes were stimulated with OLP mixes or dominant-epitope OLPs, and CD4⁺ and CD8⁺ T cell responses were separately analyzed by gating each subset during FACS analysis. CD4⁺ T cell responses to OLP mix 1 and OLP mix 2 were potently induced by RpfB DNA immunization as shown by IFN- γ ICS whereas they were scarcely induced by BCG or RpfB protein immunization (Fig. 3A). Similar results were observed when cells were stimulated with the CD4⁺ T-cell epitopes, OLP-30 and OLP-51 (Fig. 3A). RpfB DNA immunization also strongly induced RpfB-specific TNF- α production by CD4⁺ T cells (Fig. 3B). Moreover, cytotoxic degranulation activity (represented by CD107a)

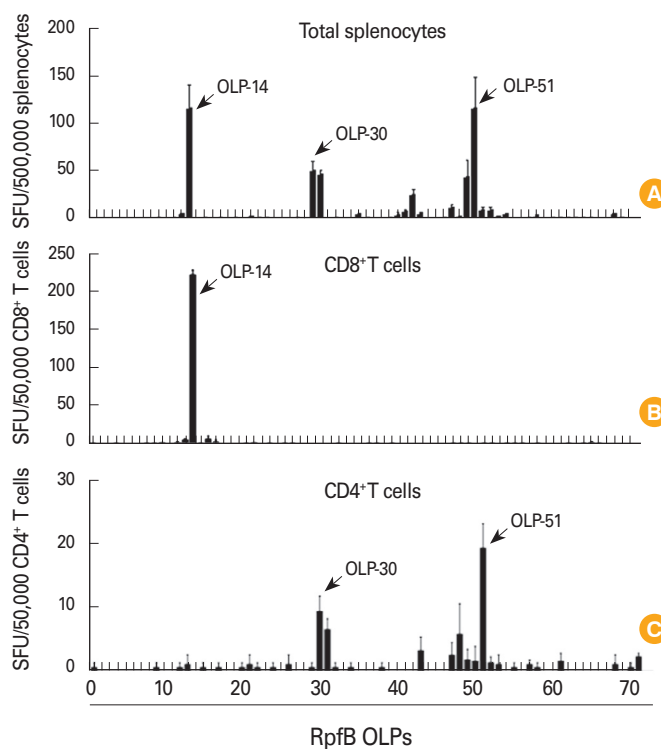


Fig. 2. Identification of immunodominant epitope overlapping peptides (OLPs) recognized by T cells. Mice were immunized with resuscitation-promoting factor B (RpfB) plasmid DNA as described in Materials and Methods. Splenocytes were obtained from immunized mice, and CD8⁺ and CD4⁺ T cells were further isolated. Interferon- γ (IFN- γ) enzyme-linked immunosorbent spot (ELISpot) assays were performed with 500,000 total splenocytes per well (n=6) (A), 50,000 CD8⁺ T cells per well (n=3) (B), and 50,000 CD4⁺ T cells per well (n=3) (C). In IFN- γ ELISpot assays, cells were stimulated with each of 71 OLPs. SFU, spot forming units. Each bar graph represents mean \pm SEM.

of CD4⁺ T cells was increased following RpfB DNA immunization (Fig. 3C). However, an IL-2 response was not elicited (Fig. 3D).

In CD8⁺ T cells, IFN- γ was potently induced in response to OLP mix 1 and the CD8⁺ T-cell epitope OLP-14 following RpfB DNA immunization, but not after BCG or RpfB protein immunization (Fig. 3E). RpfB DNA immunization also strongly induced TNF- α production (Fig. 3F) and cytotoxic degranulation activity (Fig. 3G) of CD8⁺ T cells whereas an IL-2 response was not elicited (Fig. 3H). Cytokine production in response to OLP mix 2 was not detected in CD8⁺ T cells and thus, it is not presented.

Taken together, RpfB DNA immunization induced vigorous responses in both CD4⁺ and CD8⁺ T cells, in particular IFN- γ and TNF- α production and cytotoxic degranulation activity.

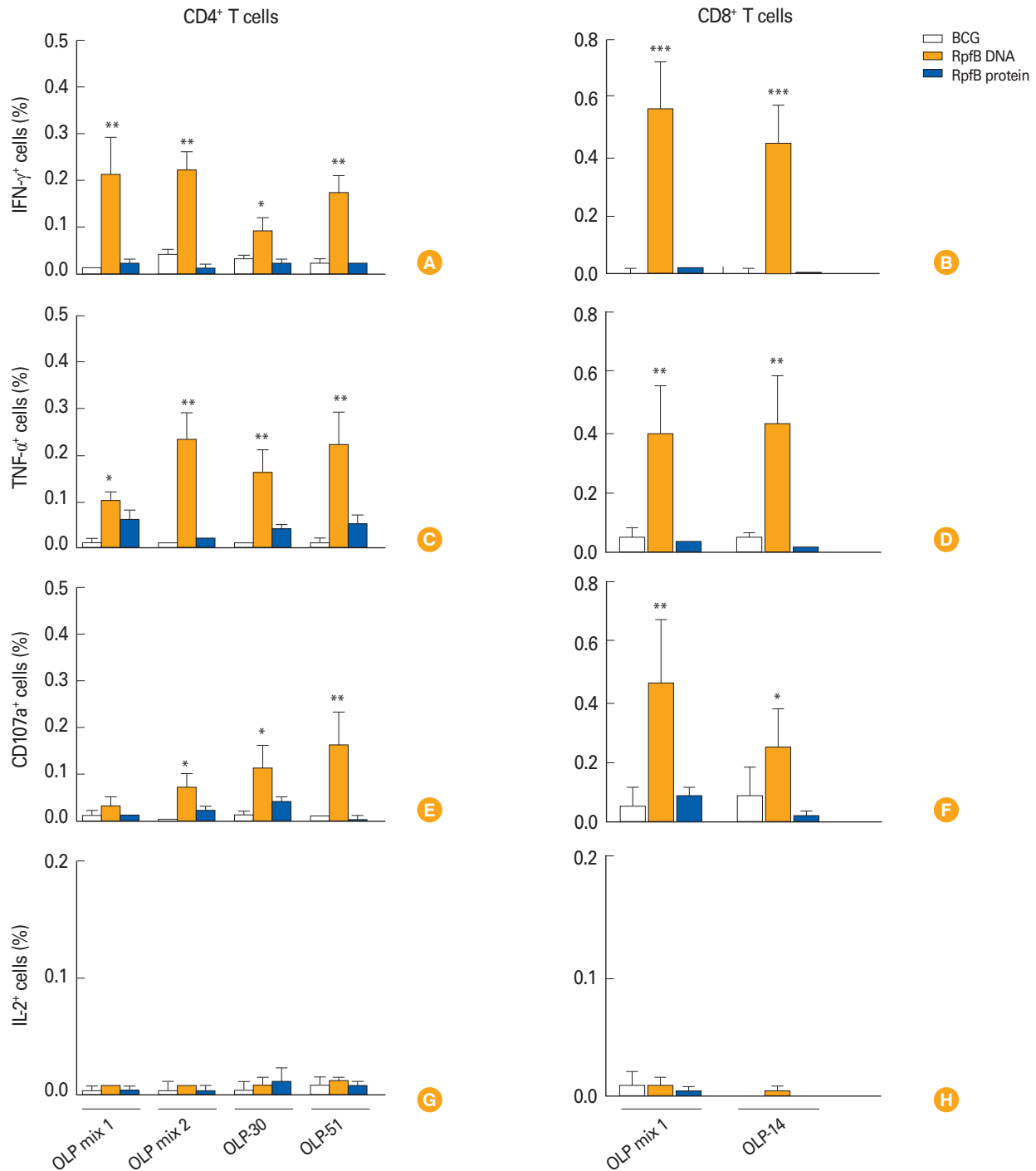


Fig. 3. Resuscitation-promoting factor B (RpfB)-specific T cell responses induced by RpfB immunization. Mice were immunized as described in Materials and Methods, and splenocytes were obtained from immunized mice. Intracellular cytokine staining was performed for interferon- γ (IFN- γ) (A, B), tumor necrosis factor- α (TNF- α) (C, D), CD107a (E, F), and interleukin-2 (IL-2) (G, H) after stimulation with the indicated overlapping peptide (OLP) mix or epitope OLP. CD4⁺ (A, C, E, G) and CD8⁺ (B, D, F, H) T cell subsets were separately analyzed by gating on each subset during fluorescence-activated cell sorting analysis. BCG, bacillus Calmette-Guérin. Each bar graph represents mean \pm SEM (n=6). Mann-Whitney U test was performed (*p < 0.05, **p < 0.01, ***p < 0.001).

Polyfunctionality of RpfB-specific T cells

Finally, we assessed the polyfunctionality of RpfB-specific T cells by Boolean gating analysis of the ICS data presented in

Fig. 3. To assess the polyfunctionality of T cells, we analyzed four different functions: production of IFN- γ , TNF- α , IL-2 and expression of a marker of cytotoxic degranulation activity,

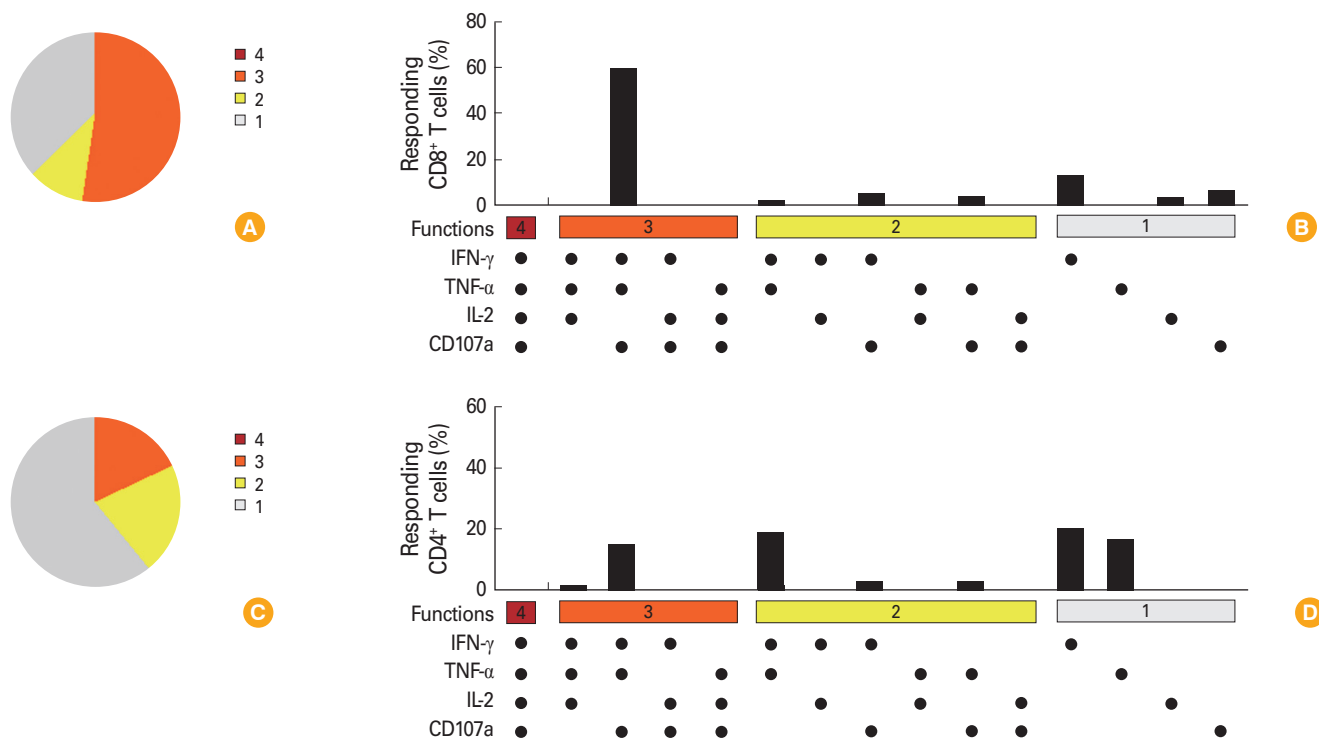


Fig. 4. Analysis of the polyfunctionality of resuscitation-promoting factor B (RpfB)-specific T cells induced by RpfB DNA immunization. Representative examples of the analysis of polyfunctionality of CD8⁺ T cells (A, B) and CD4⁺ T cells (C, D) from an RpfB DNA-immunized mouse are presented. Cells were stimulated with overlapping peptide (OLP) mix 1 for the analysis of CD8⁺ T cells (A, B), and with OLP mix 2 for the analysis of CD4⁺ T cells (C, D). Polyfunctionality for interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), interleukin-2 (IL-2), and CD107a was assessed. The data are represented by the pie graphs showing the fraction of T cells positive for a given number of functions (A, C). Detailed analysis of polyfunctionality is presented with every possible combination of functions (B, D).

CD107a. First, the OLP mix 1-stimulated CD8⁺ T cell response was analyzed after RpfB DNA vaccination, and these CD8⁺ T cells were determined to be polyfunctional as more than half of the cells exerted three functions simultaneously (representative data from a single mouse are presented in Fig. 4A and B). The most predominant functional phenotype was that of IFN- γ ⁺/TNF- α ⁺/IL-2⁺/CD107a⁺ (Fig. 4B), and this polyfunctional T cell response was observed only following RpfB DNA immunization, and not following BCG or RpfB protein immunization (data not shown). A similar pattern of polyfunctionality was observed against the CD8⁺ T cell epitope, OLP-14 (data not shown).

In CD4⁺ T cells, the response to the OLP mix 2 was assessed for polyfunctionality. Compared to CD8⁺ T cells, CD4⁺ T cells were less polyfunctional (Fig. 4C). In fact, the most predominant functional phenotype was that of IFN- γ ⁺/TNF- α ⁺/IL-2⁺/CD107a⁺ (Fig. 4D), and more than half of OLP mix 2-specific CD4⁺ T cells were monofunctional (Fig. 4C). However, RpfB DNA immunization induced more triple-positive and double-positive CD4⁺ T cells than did BCG or RpfB protein im-

munization (data not shown). A similar pattern of polyfunctionality was observed against OLP mix 1 and the CD4⁺ T cell epitopes, OLP-30 and OLP-51 (data not shown).

Thus, all mice immunized with Rpf DNA showed similar patterns of T cell polyfunctionality: a highly polyfunctional CD8⁺ T cell response and a less polyfunctional CD4⁺ T cell response.

Discussion

In the present study, we investigated the immunological response to *M. tuberculosis* RpfB DNA immunization. RpfB DNA immunization induced not only an RpfB-specific antibody response, but also CD8⁺ and CD4⁺ T cell responses in mice. Further, the induced CD8⁺ T cell response was determined to be polyfunctional as the major population of RpfB-specific CD8⁺ T cells were triple-positive (IFN- γ ⁺/TNF- α ⁺/CD107a⁺). Previously, T cell immunogenicity of Rpf DNA immunization was reported [24]; however, T cell polyfunctionality has never been studied after Rpf DNA immunization.

Polyfunctional T cells simultaneously exert multiple functions such as cytokine secretion and cytotoxic activity. Recently, T cell polyfunctionality has been investigated in several infectious diseases of human and mice. It was reported that polyfunctional human immunodeficiency virus (HIV)-specific CD8⁺ T cells were maintained in HIV long-term nonprogressors [32]. Furthermore, the degree of Th1 cell polyfunctionality correlated with vaccine efficacy in a *Leishmania* vaccination study [18]. In addition, the efficacy of the smallpox vaccine has been attributed to polyfunctionality of virus-specific CD8⁺ T cells [33]. Further, in a recent primate study, polyfunctional hepatitis C virus (HCV)-specific T cells were associated with vaccine-induced control of HCV [34]. Taken together, the generation of polyfunctional T cells has been emphasized in vaccine development.

Polyfunctional T cells have been also studied in TB. The presence of polyfunctional CD8⁺ T cells was associated with natural protection against TB and protection following antimycobacterial therapy in TB patients [21,22]. Furthermore, vaccine-induced polyfunctional CD4⁺ Th1 cells correlated with protection against TB in mice challenged by *M. tuberculosis* [19,20]. Induction of polyfunctional T cells was also proven by experimental TB vaccines in human studies [35-37]. Altogether, polyfunctional T cells are considered to provide vaccine-induced immunity and mediate protection against TB.

In the present study, RpfB DNA immunization elicited a polyfunctional CD8⁺ T cell response; however, the CD4⁺ T cell response was somewhat less polyfunctional with more than half of RpfB-specific CD4⁺ T cells being monofunctional (Fig. 4D) in RpfB DNA-immunized mice. Although RpfB DNA immunization induced more triple-positive or double-positive CD4⁺ T cells than did BCG or RpfB protein immunization (Fig. 4F), this level of polyfunctionality of CD4⁺ T cells might not be sufficient for protection against TB considering the importance of the CD4⁺ Th1 response during TB protection [11-14]. Thus in future studies, increasing the polyfunctionality of CD4⁺ T cells will need to be addressed in order to improve RpfB DNA vaccination. This might be achieved by *in vivo* electroporation during RpfB DNA vaccination as it is known that electroporation enhances DNA vaccine-induced immune responses [38-40].

In summary, RpfB DNA immunization elicits polyfunctional T cell response, especially in the CD8⁺ T cell subset, and this polyfunctional CD8⁺ T cell response was dominated by the IFN- γ ⁺/TNF- α ⁺/IL-2⁺/CD107a⁺ phenotype. These results suggest that RpfB DNA immunization might induce protec-

tive immunity against TB, and *M. tuberculosis*-challenging studies are warranted in the future.

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