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The Role of *Mycobacterium tuberculosis* Rv1986 and Rv3823c in Stimulating Humoral and Cell-Mediated Immune Responses

Fuchao Chen[1,2], Tingting Shi[1], Quan Zhu[1], Lijia Xiao[1], Fuyan Wang[1], Yunhong Tan[3], Kanglin Wan[4], Qizhi Luo[1]* and Ping Yu[1]

[1]Department of Immunology, School of Basic Medical Science, Xiangya School of Medicine, Central South University, China

[2]Department of Immunology, Zunyi Medical College, China

[3]Clinical Laboratory, Hunan Chest Hospital, China

[4]National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, China

Abstract

Background: *Mycobacterium tuberculosis* (*M. tb*) infection is a major global threat, the protective effect of traditional vaccine BCG is poor for adults, and therefore, an effective vaccine against this pathogen is urgently required. Base on this background, we determined to explore the new vaccine for prevent the infection by *M. tb*, so we expressed and purified two M. tb recombinant proteins, Rv1986 and Rv3823c, to investigate their role in induction of immunity.

Methods: Peripheral blood samples were collected from 100 patients diagnosed with TB and admitted to the Hunan Provincial Chest Hospital (Changsha, China) for treatment. The TB diagnosis was confirmed by positive M. tb culture and subsequent biochemical tests. All patients underwent chest radiography. Blood samples were also obtained from 100 healthy individuals and 100 patients with non-TB respiratory diseases. Subjects from both sexes were included and the female to male ratio was 49:51. The age range was from 14 to 81 years with the average age of 42 years. This study was reviewed and approved by the Institutional Ethical Committee of the Xiangya Hospital. Written informed consent was obtained from each participant.

Results: Serum antibodies recognized Rv1986 and Rv3823c in 24% and 32% patients with TB, respectively, in an Enzyme-Linked Immunosorbent Assay (ELISA) using the sera of 100 patients with Tuberculosis (TB) and 200 individuals without TB. Both proteins stimulated the expansion of effector memory T cells and central memory T cells as well as plasma cells in mouse vaccination experiments. Furthermore, Rv1986 induced higher serum interferon-γ level in immunized mice.

Conclusion: The results suggested that Rv1986 and Rv3823c were highly immunogenic antigens and could stimulate humoral and cell-mediated immune responses.

Keywords: ***Mycobacterium tuberculosis*; Memory T cell; Inflammatory cytokine; ELISA; Specific antibody**

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Correspondence:
Qizhi Luo, Department of Immunology, School of Basic Medical Science, Xiangya School of Medicine, Central South University, 172 Tongzipo Road, Changsha, Hunan, 410013, P.R China, Tel: 8673182355010; E-mail: luoqz1025@163.com

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Background

It is reported that approximately one-third of the global population are infected by *Mycobacterium tuberculosis* (*M. tb*) and is a major public health problem. About 10% of the infected population develops Tuberculosis (TB). According the data from WHO, the annual deaths caused by TB are 1.5 million worldwide [1]. Currently, the only available TB vaccine is the Bacillus Calmette-Guérin (BCG) vaccine [2], which is widely used and provides protection in infants and children against TB meningitis. However, the role of BCG in preventing *M. tb* transmission is limited since BCG is considered to be less effective in preventing respiratory TB [3]. Therefore, it is necessary to study the *M. tb* proteins that are absent in BCG and are able to elicit specific humoral responses, cell-mediated responses, and proper innate responses in the host.

Our previous genome-wide analysis of 200 *M. tb* strains and 12 *M. bovis* BCG strains suggested that the proteins Rv1986 and Rv3823c were present in most epidemic strains but were absent in

BCG, and both proteins carried relatively more T cell epitopes and B cell epitopes. For example, based on comparative genomic analysis, Rv3823c contains 16 T cell epitopes and 22 B cell epitopes. Rv1986 is an amino acid transporter, whereas Rv3823c is an integral membrane sulfoglycolipid transporter responsible for mycolic acid transport [4]. The gene copy numbers and location of these proteins vary among different strains. Rv1986 has been reported for its diagnostic potential [5]. Moreover, Rv1986 is present in the virulent *M. bovis* but is absent in the BCG strain [6]. Studies suggested that Rv1986 is an immuno-dominant target of memory T cells.

In this study, recombinant *M. tb* proteins, Rv1986 and Rv3823c, were expressed in *Escherichia coli* to assess their efficacies in the induction of immune responses. We tested the abilities of the recombinant proteins to stimulate innate and adaptive immune cell propagation and cytokine release. Circulating levels of the cytokines including Interleukin-2 (IL-2), IL-4, IL-6, IL-17A, Interferon-γ (IFN-γ), and Tumor Necrosis Factor-α (TNF-α) were elevated in immunized mice. The cytokine secretion and T cell population data suggested that Rv1986 and Rv3823c might boost vaccine-induced T-cell responses.

Methods and Materials

Sample and subjects

Peripheral blood samples were collected from 100 patients diagnosed with TB and admitted to the Hunan Provincial Chest Hospital (Changsha, China) for treatment. The TB diagnosis was confirmed by positive *M. tb* culture and subsequent biochemical tests. All patients underwent chest radiography. Blood samples were also obtained from 100 healthy individuals and 100 patients with non-TB respiratory diseases. Subjects from both sexes were included and the female to male ratio was 49:51. The age range was from 14 to 81 years with the average age of 42 years. This study was reviewed and approved by the Institutional Ethical Committee of the Xiangya Hospital. Written informed consent was obtained from each participant.

Strains and plasmids

The genes encoding full-length Rv1986 and Rv3823c were amplified by Polymerase Chain Reaction (PCR) from the genomic DNA of *M. tb* strain H37Rv. Rv1986 cloning was performed with the forward primer (5'-AAG GAA TTC CAT CCA GTG GCG ATT CTG C-3') and the reverse primer (5'-CGC CCA AGC TTT GGT GAT CGG ATT CCC GTA G-3'); the primers used to clone the first 507 bp of Rv3823c were as follows: the forward primer 5'-AAG GAA TTC CAT CCA GTG GCG ATT CTG C-3' and the reverse primer 5'-CGC CCA AGC TTT GGT GAT CGG ATT CCC GTA G-3'. The C-terminal half of the gene encoding Rv3823c was not included since our previous tests indicated that the C-terminal part affected the solubility of the recombinant protein. Both Rv1986 and Rv3823c contained 6 histidine (His) residues at the C termini. PCR was performed in a 20 μL reaction system comprising 1 μL DMSO, 2 × Taq MasterMix (Cwbio, Beijing, China), 1 U DNA polymerase, 0.5 μM of each primer, and 1 μM template DNA. Amplification was performed in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) using a thermal program of pre-denaturation at 94°C for 10min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 60°C for 30s, and extension at 72°C for 30s. The PCR products were sequenced to verify sequence accuracy and inserted into pET32a plasmid.

Protein expression and purification

Plasmids carrying Rv1986-His or Rv3823c-His were transformed into *E. coli* BL21 (DE3) strain per manufacturer's instructions. After the inoculation of a single *E. coli* colony into 5 mL of Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin, the culture was grown overnight at 37°C with shaking. Then, 1L of LB medium was inoculated from a 5 mL culture. Protein expression was induced by adding 1 mmol/L Isopropyl β-D-1-Thiogalactopyranoside (IPTG) when the Optical Density ($OD_{600\ nm}$) of the cell culture reached 0.8.

After 6 hr of culture, cell pellets were collected by centrifugation and resuspended in lysis buffer containing 0.5 M NaCl, 8.5 mmol/L PMSF (Phenylmethane Sulfonyl Fluoride), and 50 mM Phosphate Buffered Saline (PBS) (pH7.4). After cell lysis, the cell debris was removed by centrifugation and filtration. The filtered lysate was loaded onto a pre-equilibrated Ni-NTA purification column. (Thermo Fisher Scientific, Waltham, MA, USA). The column was washed with buffer containing 30 mM imidazole, and then the bound recombinant proteins were eluted with buffer containing 100 mM imidazole, 100 mM KCl, 50 mM potassium phosphate pH 7.8, 1 mM CaCl2, 10% glycerol, and 5 mM β-mercaptoethanol. Purified protein was concentrated using a centrifugal filter with 30 kDa cut-off (Millipore, Billerica, MA, USA). Protein concentration was measured using the Bradford assay.

Enzyme-linked immunosorbent assay (ELISA)

Micro ELISA plates were first coated with *M. tb* recombinant protein (250 ng/well in 100 μL PBS) overnight at 4°C and then blocked using the Odyssey blocking buffer containing 5% skim milk. Human serum samples (1:80 dilution) were incubated in the coated ELISA plates for 45 min at 37°C. Bound Rv1986-specific or Rv3823c-specific IgG antibodies were detected using HRP (horseradish peroxidase)-conjugated anti-human IgG (BD Biosciences, San Jose, CA). The final results were recorded as mean OD (Optical Density) since all tests were performed in triplicates. All above-mentioned blood samples were also examined using the tuberculosis antibody IgG detection ELISA kit (Rongsheng Biotech, Shanghai, China).

Western blot

The *M. tb* recombinant proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel and then electroblotted onto a Polyvinylidene Fluoride (PVDF) membrane. The membrane was blocked for 1 hr with blocking buffer (2% w/v skimmed milk in PBS) and washed thrice with 0.1% Tween-20 in PBS. Sera from immunized mouse (1:1,000 or 1:2,000 dilutions in PBS) were incubated with the PVDF membranes for 45 min at room temperature. The membranes were washed again and incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (Auragene, Changsha, China) for 45 min at room temperature. The membranes were further washed with 0.1% Tween-20 in PBS, and signals were detected using a chemiluminescence reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Animal immunization experiments

Pathogen-free Balb/c mice (5 to 7 weeks-old) were randomly assigned to vaccinated and control groups (n=6 per group). Mice were immunized with the Rv1986-adjuvant, Rv3823c-adjuvant, and Rv1986-Rv3823c-adjuvant combination by intradermal vaccinations. Each mouse was injected with 60 μg recombinant proteins/mouse/injection (60 g Rv1986, 60 μg Rv3823c, or 30 μg Rv1986 plus 30 μg Rv3823c) emulsified in incomplete Freund's adjuvant (50% v/v, total

Table 1: Detection of antigen specific antibodies in the sera from patients with tuberculosis.

ELISA methods	Detection results				Detectable rate %
	PPD ELISA				
		+	-	Total	
PPD	+	66	0	66	66%
	-	0	34	34	
Rv1986	+	20	4	24	24%
	-	44	32	76	
Rv3823c	+	28	4	32	32%
	-	40	28	68	

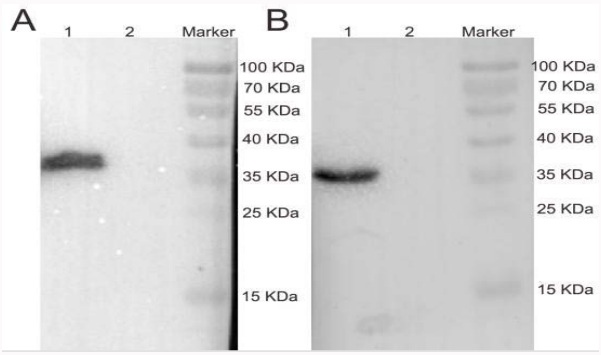

Figure 1: Western blot reactivity of sera from patients with tuberculosis to Rv1986 and Rv3823c. One-thousand fold dilution of serum from patients with TB was used in the Rv1986 or Rv3823c blotting assay. (A) Lane 1: Purified *Mycobacterium tuberculosis* protein Rv1986; Lane 2: negative control (vector only transfected E. coli lysate). (B) Lane 1: Purified *Mycobacterium tuberculosis* protein Rv3823c; Lane 2: negative control (vector only transfected *E. coli* lysate). The experiments were repeated thrice, and one of the results is presented as a merged image with protein markers.

volume of 50 μL). The recombinant proteins were diluted in PBS. Each mouse was immunized four times in an interval of two weeks. PBS or complete Freund's adjuvant was injected in the control group. All animals were raised in sterile, isolated cages to prevent spontaneous infection. Two weeks after the last dose, the mice were anaesthetized and sacrificed by cervical dislocation. Animal experiments were conducted in accordance with the rules and guidelines of the Animal Care Committee of Xiangya School of Medicine.

Cytokine expression

Levels of IL-2. IL-4, IL-6, IL-10, IFN-γ, and TGF-α in the plasma of immunized mice were determined using a customized Cytometric Bead Array (CBA) kit. This assay provided a way of capturing and detecting groups of analytes using flow cytometry. The cytokine bead array measurement was conducted according to the manufacturer's instructions. Results were obtained on a BD FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The final results were the average of data from six subjects.

Flow cytometry and cell sorting

Flow cytometry was performed at the Laboratory Platform of the Xiangya School of Medicine. APC-Cy™7 anti-mouse CD3e, BV510-anti-mouse CD4, FITC-anti-mouse CD8, Phycoerythrin (PE)-anti-mouse CD11b, PerCP-Cy5.5-anti-mouse CD19, BV421-anti-mouse CD44, PE-Cy™7-anti-mouse CD45R, FITC-anti-mouse CD49b, PE-anti-mouse CD62L, and APC anti-mouse CD138 antibodies were purchased from BD Biosciences (San Jose, CA, USA). Blood cells from immunized mice were stained with fluorochrome-conjugated monoclonal antibody mentioned above for 20 min on ice and then analyzed on a FACS Calibur flow cytometer. Cell populations were identified by light scatter signals and fluorescent signatures. The detailed gating strategy is mentioned in (Figure 1). Data were processed using the FlowJo v10 software (Tree Star, San Carlos, CA, USA).

Statistical analysis

Averages of triplicate measurements with standard deviations are presented. Analysis of Variance (ANOVA) was used to analyze the data from experimental groups and control groups. *p* value lower than 0.05 was considered statistically significant.

Results

Mycobacterium tuberculosis recombinant proteins are recognized by antibodies from patients with tuberculosis or immunized mice

The predicted molecular weights of Rv1986 and Rv3823c are 43.9 kDa and 39.6 kDa, respectively. Recombinant proteins Rv1986 and Rv3823c could be detected using specific serum antibodies from immunized mice (Rv1986-adjuvant or Rv3823c-adjuvant immunization) as shown in (Figure 1).

Rv1986 and Rv3823c were specifically recognized by serum antibodies of certain patients in an ELISA-based serodiagnostic test using sera from 100 patients with TB. In the Rv1986-based ELISA, 24% of the blood samples from patients with TB yielded positive results (Table 2). In the Rv3823c-based ELISA, 32% of the blood samples from patients with TB produced positive results (Table 3). Based on the detection results of the tuberculin (PPD) ELISA test presented in (Table 1), the false-positive rates for the detection of *M. tb*-specific antibodies using Rv1986 and Rv3823c were 4/24 and 4/32, respectively. Furthermore, these two recombinant proteins failed to detect *M. tb* specific antibodies in some serum samples that were positive by PPD ELISA. The false-negative rates of Rv1986- and Rv3823c-based ELISA were 44/76 and 10/68, respectively. Although *M. tb* proteins Rv1986 and Rv3823c might not be suitable candidates for diagnosis of *M. tb* infection, our results demonstrated that Rv1986 or Rv3823c elicited humoral response in patients with TB.

According to the measurements of 100 patients with TB, 100 patients with non-TB respiratory diseases, and 100 healthy volunteers using Rv1986- and Rv3823c-based ELISA, the overall specificities of Rv1986- and Rv3823c-based ELISA were 92% and 96%, respectively. The overall sensitivities of Rv1986- and Rv3823c-based ELISA were 24% and 32%, respectively.

Mycobacterium tuberculosis recombinant proteins induced inflammatory cytokines and cytokines promoting cellular or humoral immune response

Cytokine secretion in response to the Rv1986-adjuvant, Rv3823c-adjuvant, and Rv1986-Rv3823c-adjuvant combination, or adjuvant alone was measured. Rv1986 and Rv3823c significantly elevated IL-4 production, indicating its role in stimulating humoral immunity (Figure 2). Similar stimulatory effects on IL-4 levels were observed in the group vaccinated with both Rv1986 and Rv3823c. PBS or adjuvant alone did not increase the levels of IL-4.

Vaccination with the Rv1986-adjuvant, Rv3823c-adjuvant, or a combination of both proteins significantly increased IL-6 release. IL-6 is considered as a pro-inflammatory cytokine, but it also functions as

Table 2: The positive numbers and rate of Rv1986-based ELISA.

ELISA Methods	Positive numbers	Detectable rate
Rv1986	24	24%
Total	100	100%

Table 3: The positive numbers and rate of Rv3823c-based ELISA.

ELISA Methods	Positive numbers	Detectable rate
Rv3823c	32	32%
Total	100	100%

an anti-inflammatory cytokine for T cells. Furthermore, compared to the PBS group, administration of adjuvant alone also increased IL-6 levels.

The Th1 response is characterized by increased production of IFN-γ and IL-2. Among the immunization groups, significant elevation of INF-γ production was observed in the group administered with 60 μg Rv1986 and the Rv1986-Rv3823c combination (each protein, 30 μg), suggesting the role of Rv1986 in cell-mediated immunity. The Rv1986-adjuvant, Rv3823c-adjuvant, and their combination induced significant amounts of IL-2 compared to that in the control groups.

All groups vaccinated with the recombinant protein showed higher production of pro-inflammatory cytokine IL-17A than that stimulated by the adjuvant or PBS control group (Figure 2). Furthermore, both Rv1986 and Rv3823c could stimulate production of TNF-α, a pro-inflammatory cytokine; however, Rv1986 induced higher level of TNF-α than Rv3823c. We also determined serum IL-10 levels (an anti-inflammatory cytokine) in immunized mice to further investigate the immuno-modulatory role of these *M. tb* proteins. Rv1986, Rv3823c, or Rv1986 plus Rv3823c did not significantly increase the secretion of IL-10.

Mycobacterium tuberculosis recombinant proteins induced the expansion of memory T cells, plasma cells, and innate immune cells

Next, we investigated the effect of recombinant protein vaccination on $CD4^+$ and $CD8^+$ T-cells. Naïve T cells express high levels of CD62L and low levels of CD44, whereas memory T cells exhibit high expression of CD44 and low expression of CD62L. The percentage of effect or memory T helper cell subsets was determined by selecting $CD3^+$, $CD4^+$, $CD8^-$, $CD44^{hi}$, and $CD62L^{hi}$ cells. A significant increase in the number of effect or memory T helper cells was observed after Rv1986 or Rv3823c vaccination (Figure 3). Vaccination with the combination of the two proteins yielded similar results as the single protein vaccinations. The cell surface markers of $CD3^+$, $CD4^+$, $CD8^-$, $CD44^{hi}$, and $CD62L^{lo}$ were used to distinguish central memory T helper cells. Vaccination with Rv1986, Rv3823c or the Rv1986-Rv3823c combination induced the expansion of central memory T helper cells in mice. The levels of effector memory cytotoxic T cells was determined by selecting $CD3^+$, $CD4^-$, $CD8^+$, $CD44^{hi}$, and $CD62L^{hi}$ cells. Similarly, both Rv1986 and Rv3823c could increase the percentage of effector and central memory cytotoxic T cells, and the combination of the two proteins induced more memory cytotoxic T cells than either *M. tb* protein.

The cell surface markers of $CD19^+$, $CD45R^-$, and $CD138^+$ were used to analyze plasma cells from immunized mice. CD45R is expressed on B cells before their terminal differentiation to plasma cells. Mouse CD138 is initially expressed on pre-B and immature B cells in the bone marrow and it disappears when B cells migrate to the periphery. However, CD138 is re-expressed when B cells differentiate into plasma cells. Therefore, $CD19^+$ $CD45R^+$ $CD138^-$ markers were used to select B cells. Both Rv1986 and Rv3823c could stimulate B cell expansion, but the percentage of B cells was only 20% higher than that stimulated by the adjuvant. Rv1986 vaccination increased the proportion of plasma cells in the mouse serum. However, it is interesting that the plasma cell expansion induced by Rv1986 plus adjuvant was much more than that induced by Rv1986-Rv3823c-adjuvant combination.

CD11b and the iC3b receptor are expressed on macrophages, Natural Killer (NK) cells, and some B and T cells. CD49b, an integrin alpha subunit, is expressed on dendritic cells, NKT cells,

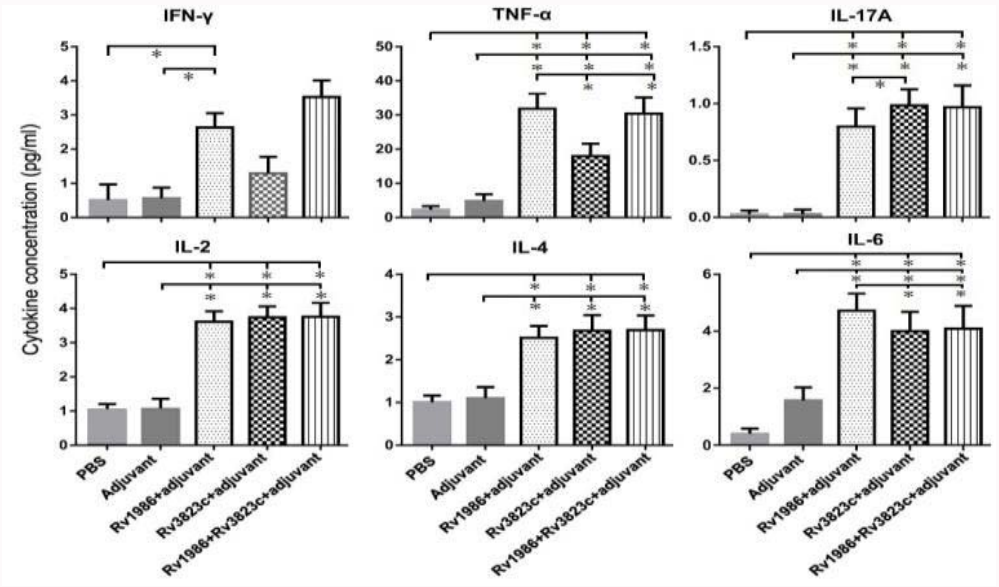

Figure 2: Measurement of the levels of *Mycobacterium tuberculosis* protein-induced cytokines. The mice were randomly grouped and immunized with the Rv1986-adjuvant, Rv3823c-adjuvant, Rv1986-Rv3823c-adjuvant, adjuvant alone, or PBS by intradermal injections. Each group contained 12 mice and each sample were measured thrice. The measurement results within each group were averaged. p value lower than 0.05 was considered statistically significant.

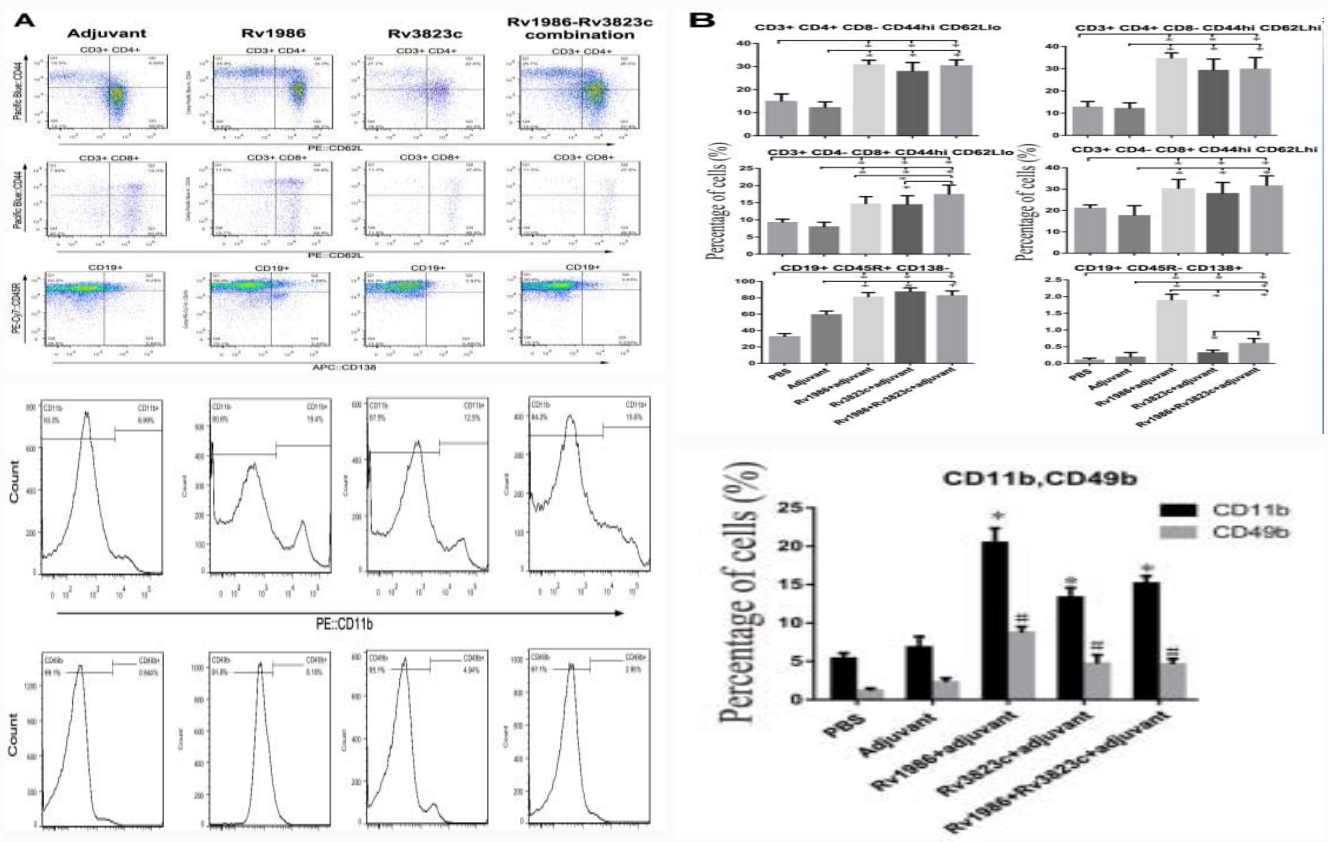

Figure 3: Proliferation of immune cells in mice stimulated by Rv1986 and Rv3823c. (A) Blood samples from mice immunized with Rv1986-adjuvant, Rv3823c-adjuvant, Rv1986-Rv3823c-adjuvant, adjuvant alone, or PBS were analyzed by flow cytometry for assessment of effector/central memory $CD4^+$ or $CD8^+$ T cells, plasma cells, macrophages, and pan-NK cells. The cells were stained with anti-mouse CD3, CD4, CD8, CD11b, CD19, CD44, CD45R, CD49b, CD62L, and CD138 monoclonal antibodies. Flow cytometry plot gated on $CD4^+$ T cells, $CD8^+$ T cells, or $CD19^+$ B cells. (B) Representative plots of cell percentages were the average of six independent experiments.

NK cells, and regulatory T cells. Therefore, CD11b and CD49b were used as markers of macrophages and pan-NK cells (macrophages, natural killer, and natural killer T cells). In the Rv1986 vaccinated group, a higher percentage of macrophages and pan-NK cells were observed. Rv3823c also stimulated macrophages and pan-NK cells, but its stimulation was weaker than that of Rv1986 (Figure 3). The combination of Rv1986 and Rv3823c did not enhance the stimulation effects.

Discussion

Our results indicated that both Rv1986 and Rv3823c were highly immunogenic antigens. The immune cell proliferation and cytokine induction suggested their role in induction of immunity. Rv1986 and Rv3823c could be recognized by antibodies from the sera of patients with TB. In mouse immunization experiments, both proteins could promote the development of cell-mediated immunity and humoral immunity response.

A previous study demonstrated that Rv1986 could stimulate central memory T cells to secrete higher level of IL-2 [6], which is consistent with our data (Figure 2,3). We observed the elevated serum level of IL-2 as well as increased percentage of central memory T cells. We observed that elevated serum level of IL-2 as well as increased percentage of central memory T cells. Some studies showed that certain *M. tb* mutants failed to induce a strong Th1 type immune response because of the decrease in the expression of lipid metabolism-related genes, including the gene for Rv3823c [7]. Our results indicated that Rv3823c did not induce significant IFN-γ release (Figure 2), suggesting that the role of Rv3823c in cell-mediated immunity is less important than other lipid metabolism-related genes. Another research group observed that the *M. tb* mutants with lipid metabolism defects (including mutation in the gene encoding Rv3823c) accumulated larger amount of mycolic acids in their cell walls compared to the cell walls of wild-type strains [8]. Therefore, the loss in the ability to induce cell-mediated immune response might be due to change in cell wall composition in the *M. tb* mutants since evidence show that mycolic acids inhibit the TLR-2 dependent pathway [9]. The suppression of Th1 type immune responses in the *M. tb* strain with disruption in the Rv3823c gene could be ascribed to the immuno-modulatory role of sulfatides that is transported by Rv3823c [10]. Rv3823c is required to assemble tetra-acylated sulfoglycolipids [11]. However, Rv3823c stimulated significant numbers of macrophages and NK cells (Figure 3).

In patients with pulmonary TB, IL-6 increased inflammatory cytokine secretion by T cells [12]. Experiments with the BCG infected cell model indicated that IFN-γ- or IL-17A-stimulated epithelial cells secreted high levels of IL-6 [13]. Therefore, the increase in IL-6 in mice vaccinated with Rv1986 or Rv3823c might be related to the elevated IFN-γ or IL-17A levels (Figure 2). Patients with active TB have not only increased plasma levels of IL-6 but also high plasma TNF-α [14]. Actually, successful host defense against *M. tb* requires the production of pro-inflammatory cytokines, such as IFN-γ, IL-17A, and TNF-α [15,16]. In our study, both Rv1986 and Rv3823c significantly elevated

IL-17A and TNF-α levels. A significant increase in the proportion of IFN-γ in response to Rv1986 was observed, which is consistent with previous results [17]. The expansion of Rv1986-stimulated cytotoxic T cells might be associated with a substantial rise in IFN-γ (Figure 3). Furthermore, high levels of IFN-γ reduced neutrophil recruitment, which suggested the potential role of Rv1986 in vaccine-mediated protection of the lung. Compared to the induction by Rv1986, the combination of two proteins induced less IFN-γ and TGF-α, which might be due to the dose of the injected protein (Figure 2).

However, this study is not without limitations. Rv1986 and Rv3823c could only be recognized by a small percentage of sera from patients with TB, the cause of which is unclear. Differences in *M. tb* strains might lead to varied humoral immune responses. Furthermore, the protective effects against *M. tb* infection are untested.

Conclusion

We show that vaccination with Rv1986 or Rv3823c induces the production of IL-6, IL-17A, and TNF-α, and stimulates the proliferation of memory T cells. Furthermore, Rv1986 also induces IFN-γ, which is critical in anti-TB cell-mediated immune responses. This finding suggested that *M. tb* proteins Rv1986 and Rv3823c have the potential to elicit protective immunity through T cells, plasma cells, and inflammatory cytokines, indicating their roles in future vaccine development.

Declarations

Ethics approval and consent to participate

This study was reviewed and approved by the Institutional Ethical Committee of the Xiangya Hospital. Written informed consent was obtained from each participant.

Consent for Publication

All participants or their legal guardians give their consents for their or their minor child's data to be published in this journal.

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Authors' Contributions

FC performed the experiments. LX and QZ managed and coordinated the research activity. YT provided study materials, reagents, materials. FW verified the overall reproducibility of results. YW oversighted the research activity planning and execution; wrote the initial draft. PY formulated the overarching research goals and aims; developed and designed methodology. KW acquired the financial support for the project leading to this publication; provided critical review and revision.

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