Immunological evaluation of a novel *Mycobacterium tuberculosis* antigen, Rv3117, absent in *Mycobacterium bovis* BCG

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Abstract. Tuberculosis (TB) remains a global infectious disease. To investigate the value of a novel *Mycobacterium tuberculosis* (*M. tuberculosis*) region of difference 5 (RD5)-encoded antigen, Rv3117, in the development of effective immunodiagnostics and vaccines against TB, the immune responses to the antigen were examined in human subjects, as well as in C57BL/6 mice. The results showed that Rv3117 was able to evoke specific humoral and cellular immune responses. Consistent with the results from the RD1-encoded antigens, culture filtrate protein 10 kDa (CFP-10) and early secreted antigenic target 6 kDa (ESAT-6), the immunoglobulin G (IgG), IgM and IgA antibody responses to Rv3117 were able to statistically distinguish between the 65 patients with active pulmonary TB and the 59 healthy controls ($P<0.01$, respectively). In addition, higher levels of Rv3117-specific interferon-γ (IFN-γ) were observed in immunized C57BL/6 mice than in the negative control mice ($P<0.05$). Furthermore, high titers of total IgG, IgG1 and IgG2a antibodies were present in the sera from immunized mice, even six weeks subsequent to the immunization. In conclusion, the present results suggested that Rv3117 may be used as a candidate for the development of TB immunodiagnostics and vaccine design.

Introduction

Tuberculosis (TB) continues to be an increasing cause of morbidity globally and is a leading cause of human mortality in the developing world. According to the most recent World Health Organization statistics, there were an estimated 8.7 million new cases of TB and 1.4 million deaths from the disease in 2011. Geographically, the burden of TB is highest in Asia and Africa. India and China together account for ~40% of the global cases of TB (1). Over the past two decades, drug-resistant TB and TB/human immunodeficiency virus (HIV) co-infection have further threatened to undermine the control of TB (2). Although TB diagnostics have improved (3), they are not suitable to be used in regions with low and middle incomes. Moreover, there are no new efficient drugs to treat drug-resistant TB. Thus, the development of effective immunodiagnostics and vaccines against TB remains a global priority.

Recently, interferon-γ (IFN-γ) release assays (IGRAs) have been introduced into clinical practice for the diagnosis of *Mycobacterium tuberculosis* (*M. tuberculosis*) infection, and two licensed IGRAs are commercially available: QuantiFERON®-TB Gold In-Tube (Cellestis, Carnegie, Victoria, Australia) and T-SPOT®.TB (Oxford Immunotec, Abingdon, UK). Furthermore, novel antigens incorporating these assays may improve detection sensitivity (4). The only TB vaccine presently used in humans, Bacillus Calmette-Guérin (BCG), is widely used and has been available since 1921; however, it provides partial and inconsistent protection (5). A number of hypotheses have been suggested to explain the inconsistent efficacy of the BCG vaccine, including differences among BCG vaccine strains, modulation of the immune responses by previous exposure to environmental mycobacteria and host genetics (6-8). As a result, the antigenic components that are absent in the BCG vaccine, which elicit critical protective immune responses to TB, has been an area of intense investigation (9,10).

Comparative genomic studies have identified several *M. tuberculosis*-specific genomic regions of difference (RDs) that are absent in the avirulent *Mycobacterium bovis* BCG strains (11), which may be useful for TB diagnosis and vaccine design. For example, early secreted antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10) from RD1 (12), Rv0222 from RD4 (13) and Rv3425 from RD11 (14,15) have been identified as useful biomarkers for the immunodiagnosis of TB. Moreover, ESAT-6 and Rv3425 have also been used in TB vaccine studies (9,16,17), respectively. In a previous study, we identified a novel B-cell antigen, Rv3117, which was encoded by DNA segment RD5

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of *M. tuberculosis* (18). Therefore, in this study, we evaluated the humoral and cellular immune responses to this antigen in human subjects, as well as in immunized C57BL/6 mice, and compared them using two RD1-encoded antigens, CFP-10 and ESAT-6.

Materials and methods

Generation of recombinant antigen Rv3117. The procedures used for the cloning, expression and purification of *M. tuberculosis* RD5-encoded antigen Rv3117 were as described previously (18). The endotoxin was removed using Polymyxin affinity chromatography, in accordance with the manufacturer's instructions (Bio-Rad Laboratories Ltd., Shanghai, China), prior to the recombinant protein being concentrated using an Amicon Ultra 10K device (Millipore, Billerica, MA, USA). The protein purity and content were then assessed using Coomassie blue staining, western blotting and Bradford's assay, using bovine serum albumin (BSA) as the protein standard, respectively. The level of endotoxin was measured using the commercially available Quantitative Chromogenic Endpoint Tachypleus Amebocyte Lysate reactivity endotoxin kit (Chinese Horseshoe Crab Reagent Manufactory Co., Xiamen, China).

Animals and immunization. C57BL/6 mice were purchased from the Shanghai SIPPR/BK Experimental Animal Co., Ltd. (Shanghai, China). The immunization procedure was performed as previously described (19) and the experiments performed adhered strictly to the 1986 Scientific Procedures Act. Three groups of 25-g, 6-week-old, female C57BL/6 mice were used for the experiments. The mice received free access to food and water throughout the study. The six mice of each group were injected subcutaneously with 100 μg purified recombinant protein Rv3117, CFP-10 or phosphate-buffered saline (PBS) mixed with Incomplete Freund's adjuvant (IFA; Sigma, St. Louis, MO, USA), respectively, with two boosters, two weeks apart. Sera were collected from the caudal vein of the immunized mice every two weeks for antibody analysis. Two weeks subsequent to the final immunization, the animals were sacrificed to harvest splenocytes for cytokine analysis.

Human sera samples. A total of 65 serum samples (n=65) from patients with active pulmonary TB (PTB; age range, 15-66 years) and 59 serum samples (n=59) from healthy control subjects (age range, 8-30 years) were collected from Wuxi No. 5 People's Hospital (Wuxi, China). The patients with active PTB were diagnosed by the isolation and identification of *M. tuberculosis*, as well as by clinical and radiological findings. Mycobacterial isolates were obtained from Lowenstein-Jensen cultures, identified to the species level by biochemical procedures (20) and then confirmed using genotyping based on the 16S-23S rRNA gene internal transcribed spacer sequence (21). None of the patients had received anti-TB chemotherapy when the serum samples were collected. The healthy controls had not previously suffered from TB and had negative chest X-rays and sputum culture results for *M. tuberculosis*. The sera collected were stored at -20˚C. The study was approved by the Research Ethics Committees of Shanghai Jiao Tong University School of Medicine (Shanghai, China) and written informed consent was obtained from all the participants after a full explanation of the study.

Enzyme-linked immunosorbent assay (ELISA). In order to assess the antibody responses, ELISA was performed as in our previous studies (15,18). Briefly, 96-well polystyrene flat-bottomed microtiter plates (Costar, Cambridge, MA, USA) were coated with 1-5 μg/ml of Rv3117, CFP-10 or ESAT-6 (Linc-Bio Science Co., Ltd., Shanghai, China) and incubated overnight at 4˚C. Subsequent to being washed four times with PBST [0.05% (v/v) Tween-20 in PBS], the plates were blocked with 200 μl blocking buffer (3% BSA in PBST) for 2 h at 37˚C. The plates were then washed a further four times with PBST. Following this, 200-fold diluted human serum samples or serial dilutions of mouse serum samples (100 μl) in blocking buffer were added to the wells and incubated for 30 min at 37˚C. The pooled sera from the patients with active PTB and healthy donors were used as positive and negative controls, respectively. All the samples were tested in duplicate. The plates were thoroughly washed with PBST and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-human immunoglobulin G (IgG), IgM and IgA antibodies or HRP-conjugated goat anti-mouse IgG, IgG1 and IgG2a antibodies (Southern Biotech, Birmingham, AL, USA), at certain dilutions recommended by the manufacturer, respectively. Following this, the plates were incubated at 37˚C for 30 min and then thoroughly washed a further four times, prior to 100 μl 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (0.04% TMB and 0.04% urea peroxide in 0.1 M sodium acetate-citric acid buffer, pH 4.0) being added. Following 10 min incubation in the dark at room temperature, the reaction was stopped by the addition of 50 μl of 2 M H_2SO_4 to each well. The optical densities were subsequently measured at 450 nm (OD_{450}).

Enzyme-linked immunospot (ELISPOT) assay. The mouse spleens were removed aseptically and gently ground through a 70-μm cell strainer. Following this, single-cell suspensions were prepared using mouse lympholyte EZ-Sep™ density gradient centrifugation (Dakewe Biotech Co., Ltd., Beijing, China). IFN-γ ELISPOT kits (eBioscience, San Diego, CA, USA) were used in accordance with the instruction manual. In brief, 96-well polyvinylidene difluoride (PVDF; Millipore, Beverly, MA, USA) plates were coated with 100 μl anti-IFN-γ monoclonal antibody overnight at 4˚C. The plates were then washed twice with 200 μl ELISPOT coating buffer and blocked with 200 μl complete RPMI-1640, supplemented with penicillin, streptomycin and 10% fetal calf serum, at room temperature for 1 h. The blocking solution was subsequently discarded from the plates, and freshly isolated splenocytes were plated in duplicate at a density of $5x10^4$ cell/well in 200 μl and stimulated with Rv3117 or CFP-10 (0.1, 1 or 10 μg/ml) for 24 h at 37˚C in 5% CO_2, using phytohemagglutinin (PHA) and medium as positive and negative controls, respectively. Following this, the cells and medium were decanted from the plates and the plates were washed three times with PBST. A total of 100 μl diluted biotinylated detection antibodies were then added to each well and the plates were incubated for 2 h at room temperature. Subsequently, the antibody solution was decanted and washed four times with PBST, with the wells

Table I. Sensitivities and specificities for antibody responses to the region of difference 5 (RD5)-encoded protein Rv3117.

	Rv3117			CFP-10			ESAT-6		
Items	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
Cut-off[a]	0.371	0.386	0.250	0.273	0.213	0.253	0.378	0.210	0.177
Sensitivity[b] (n=65) (%)	26.2 (17)	26.2 (17)	43.1 (28)	24.6 (16)	23.1 (15)	33.8 (22)	24.6 (16)	13.8 (9)	20.0 (13)
Specificity[c] (n=59) (%)	98.3 (1)	96.6 (2)	94.9 (3)	94.9 (3)	96.6 (2)	93.2 (4)	96.6 (3)	96.6 (3)	93.2 (4)

[a]Cut-off value was calculated from the mean optical density value plus two standard deviations for healthy controls. [b]Sensitivity was determined by dividing the number of positive cases by the total number of patients with pulmonary tuberculosis. [c]Specificity was determined by dividing the number of negative controls by the total number of healthy controls. CFP-10, culture filtrate protein 10 kDa; ESAT-6, early secreted antigenic target 6 kDa; Ig, immunoglobulin.

being left to soak for 1 min per wash, in order to reduce the background staining. Following this, 100 μl diluted streptavidin-HRP-conjugated solution was added to each well and the plates were incubated for 45 min at room temperature. The two sides of the membrane were then washed five times with PBST and the plates were washed twice with PBS, without Tween-20. Thawed 3-amino-9-ethylcarbazole (AEC) substrate solution (100 μl/well) was subsequently added. Following this, the plates were incubated for a further 30 min at room temperature in the dark. Once spots were able to be observed in the wells under an inverted microscope, the reaction was stopped by thoroughly rinsing the two sides of the membrane with distilled water. The plates were then air dried, and the spots were counted using an immunospot image analyzer.

Statistical analysis. The differences were compared using a Student's t-test and a non-parametric test within the SPSS 13.0 data analysis program (SPSS, Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Rv3117 was expressed as a His-tagged fusion protein in Escherichia coli (E. coli). The gene *Rv3117* was expressed in *E. coli* BL21 (DE3) PlysS cells and purified as a 6xHis-tag fusion protein in the soluble fraction under non-denaturing conditions. The purified recombinant protein Rv3117 was fractionated using electrophoresis on a 12% polyacrylamide gel. A single band corresponding to the 48.1 kDa (including the mass of the N-terminal fusion domain of pET32a) protein was observed (Fig. 1) and confirmed using anti-His monoclonal antibody (data not shown). A total of <5.0 EU/mg endotoxins was typically observed and subsequently used for animal immunization.

Rv3117 elicited strong, specific antibody responses, consistent with CFP-10 and ESAT-6. To evaluate the immunological nature of the B-cell antigen Rv3117, two RD1-encoded antigens of *M. tuberculosis*, CFP-10 and ESAT-6, were selected and the antibody responses to these antigens in individual sera were measured using ELISA (Fig. 2). Consistent with the results from the RD1-encoded antigens, CFP-10 and ESAT-6, the IgG, IgM and IgA antibody responses to Rv3117 were able to statistically distinguish between the 65 patients with active PTB and the 59 healthy controls (P<0.01, respectively).

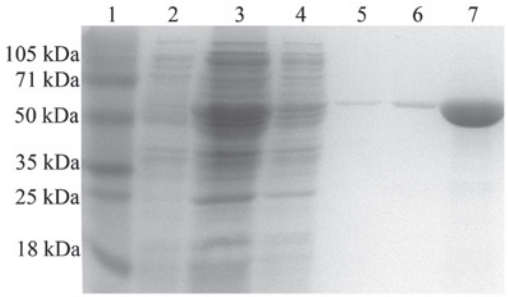

Figure 1. The recombinant Rv3117 was purified. The cleared cell lysate and various immobilized-metal affinity chromatography fractions were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were visualized using Coomassie blue staining. Lane 1, molecular weight markers; lane 2, non-induced cell lysate; lane 3, isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced cell lysate; lane 4, flow-through; lanes 5-7, wash with 5, 10 and 250 mM imidazole, respectively.

When the ELISA results were determined by the cut-off values, equal to the mean OD value for the healthy control serum samples plus two standard deviations, the sensitivity of the IgG antibody responses to Rv3117 (26.2%) were no lower than those to CFP-10 (24.6%) and ESAT-6 (24.6%) in the 65 patients with active PTB. Moreover, in these patients, the sensitivities of the IgM and IgA responses to Rv3117 (26.2 and 43.1%) were higher than those to CFP-10 (23.1 and 33.8%) and ESAT-6 (13.8 and 20.0%), respectively. Of note, the specificities of the antibody responses to any one of the three antigens remained ≥93.0% (Table I).

Rv3117 evoked high levels of antigen-specific IFN-γ in C57BL/6 mice. Twenty-four hours subsequent to stimulation, single-splenocyte suspensions from immunized mice were obtained and assayed for IFN-γ. An ELISPOT assay was used to determine the relative number of IFN-γ-expressing mouse cells in the single-splenocyte suspensions. The number of such cells were shown by spot-forming units. Levels of antigen-specific IFN-γ were evaluated in the supernatants of the *in vitro* cultures of immune splenocytes restimulated with the proteins. As shown in Fig. 3, the RD1-encoded antigen CFP-10 evoked high levels of antigen-specific IFN-γ in C57BL/6 mice. This was consistent with the results from the RD5-encoded antigen Rv3117, in which it was observed that the IFN-γ production in response to Rv3117 (10.0 μg/ml) was statistically significant (P<0.05) in the cultures of splenocytes

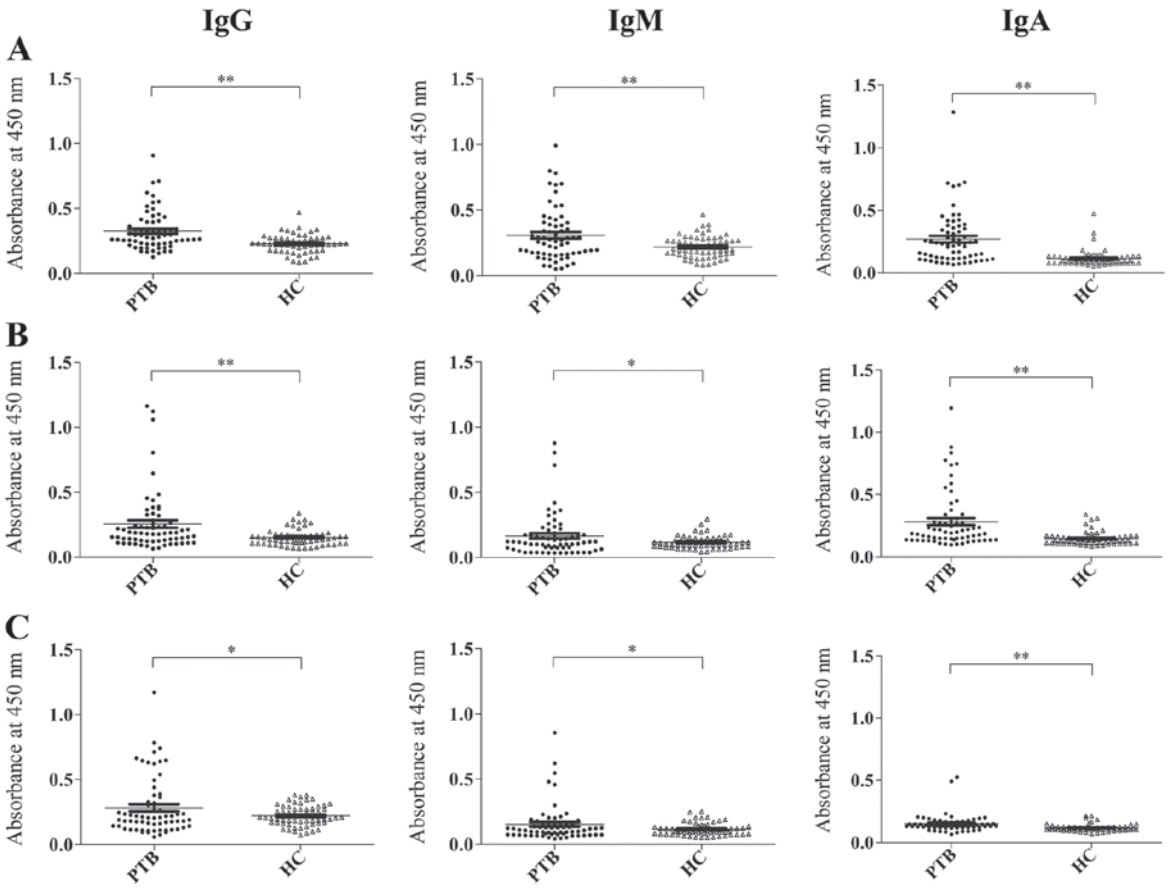

Figure 2. Strong specific antibody responses to Rv3117 (A) were elicited, consistent with those to culture filtrate protein 10 kDa (CFP-10; B) and early secreted antigenic target 6 kDa (ESAT-6; C). The horizontal lines indicate the means and the standard error in each group. P-values were calculated using an independent samples t-test and are shown above the plots. *P<0.05; **P<0.01. PTB, patients with active pulmonary tuberculosis; HC, healthy control subjects; Ig, immunoglobulin.

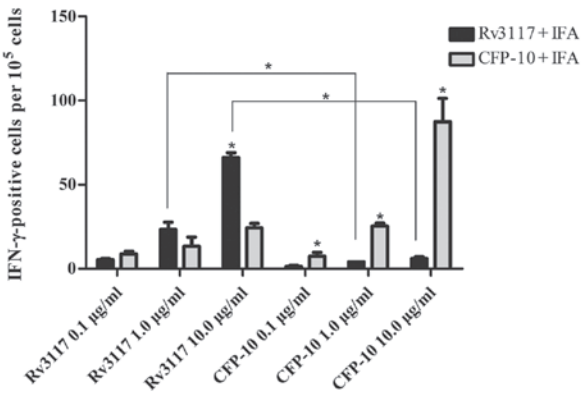

Figure 3. Antigen-specific interferon-γ (IFN-γ)-producing T cells in immunized C57BL/6 mice. The mice were immunized with protein (100 μg/ml) Rv3117 in Incomplete Freund's adjuvant (IFA) and culture filtrate protein 10 kDa (CFP-10) in IFA, respectively. The cellular immune responses were measured using an enzyme-linked immunospot (ELISPOT) assay with splenocytes isolated from C57BL/6 mice immunized with purified proteins. Splenocytes ($5x10^4$ cell/well) were stimulated with 0.1, 1.0 and 10 μg/ml protein Rv3117 or CFP-10, respectively. The number of cells secreting IFN-γ per $1x10^5$ cells following stimulation with Rv3117 or CFP-10 is shown. The data shown are from the pooled cells from >5 mice and are expressed as the mean ± standard deviation. *P<0.05.

from Rv3117-immunized C57BL/6 mice when compared with control CFP-10-immunized C57BL/6 mice (Fig. 3). Moreover, splenocytes from the Rv3117-immunized mice produced high levels of IFN-γ when stimulated *in vitro* with Rv3117, whereas low levels of IFN-γ were produced when the splenocytes were stimulated *in vitro* with the control protein CFP-10 (Fig. 3).

Rv3117 induced a specific humoral response in C57BL/6 mice. Sera were collected from the caudal vein of mice 0-6 weeks subsequent to immunization in order to perform antibody analyses. Specific IgG, IgG2a and total IgG antibody responses were measured using ELISA. Fig. 4 shows the level of antibody responses in the sera of mice immunized with IFA-emulsified protein Rv3117 or emulsified PBS against the protein in different weeks subsequent to the immunization. Compared with the PBS control group, the mice immunized with Rv3117 produced higher levels of antibodies against Rv3117 protein. High titers of total IgG and IgG1 antibodies were detected in the sera of the mice immunized with the Rv3117 antigen during the 2-6-week period and the antibody levels of IgG2a isotype against the protein Rv3117 increased from the 2-week time-point following the immunization. The mice immunized with PBS showed lower detectable titers of IgG, IgG1 and IgG2a antibodies.

Discussion

The causative agent of TB, *M. tuberculosis*, is an intracellular pathogen, and Type 1 T helper (Th1) cell-mediated immune

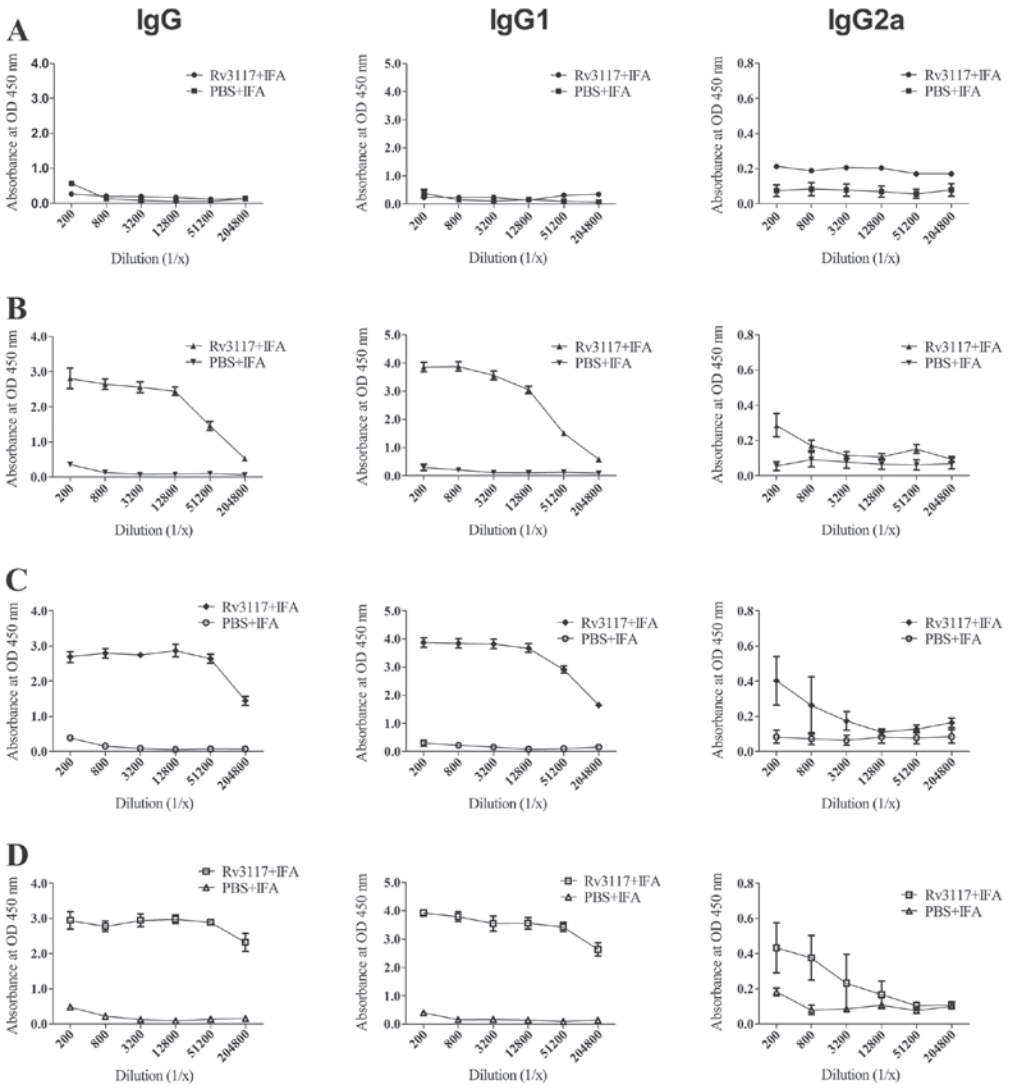

Figure 4. High titers of immunoglobulin G1 (IgG1), IgG2a and total IgG antibody responses to Rv3117. C57BL/6 mice were immunized subcutaneously with protein Rv3117 (100 μg/ml) emulsified with Incomplete Freund's adjuvant (IFA). The control animals were immunized with phosphate-buffered saline (PBS) and IFA without protein. Serum samples were collected following each immunization for a number of weeks and analyzed using enzyme-linked immunosorbent assay (ELISA) for the presence of anti-Rv3117 IgG1, IgG2a and IgG. Each point is the mean of data from five mice. The data are expressed as the mean ± standard deviation. (A) 0 weeks; (B) 2 weeks; (C) 4 weeks; (D) 6 weeks.

responses are indispensable for protective immunity against TB (22,23). The only vaccine that is used at present, the attenuated *M. bovis* strain BCG, induces Th1 cell responses; however, it has been inconsistent in providing protection from the disease, with low or unmeasurable efficacy in many of those regions with the highest incidence (24). Therefore, additional immune responses are required for a vaccine demonstrating optimal efficacy. Previous studies have suggested a number of hypotheses to explain the inconsistent efficacy, including differences among BCG vaccine strains, modulation of the immune responses by previous exposure to environmental mycobacterium and host genetics (6-8). However, there is still uncertainty and certain hypotheses remain to be tested. Although it has been suggested that the explanation regarding differences in strains may be discounted, as parallel studies in the UK and Malawi using the same strain of vaccine still showed differences in efficacy (8), Keyser *et al* (25) revealed that BCG substrains had the capacity to drive Th2 immunity and induced variable protection against *M. tuberculosis* infection (25). Moreover, further study is required with regard to host genetics, as human Toll-like receptor 1 (TLR1) and TLR6-deficiency altered immune responses to BCG vaccination in South African infants (26). In adults and adolescents, pre-exposure to environmental mycobacteria is likely to influence immune responses (27). However, studies in neonates and infants have demonstrated that the same vaccine may have a different immunogenicity in different populations; therefore, pre-exposure to environmental mycobacteria is probably not a factor in efficacy (28,29).

The low efficacy of BCG may be interrelated with the fact that it lacks important antigens (30). The Rv3117 selected for the present study, located in RD5, is absent in the BCG strains (11). This antigen was revealed to encode a probable rhodanese-like thiosulfate sulfurtransferase and to be involved in intermediary metabolism and respiration (31,32). To the best of our knowledge, most of the important antigens of TB were initially screened and identified in the IgG antibody response studies, as with Rv3117. In our previous study, all five

M. tuberculosis RD5-specific proteins were cloned and then used to screen patients with active TB, and Rv3117 was identified as a novel B-cell antigen (18). In this study, the results of IgG, as well as IgM and IgA, antibody responses to Rv3117, when compared with two RD1-encoded antigens, CFP-10 and ESAT-6, further indicated that Rv3117 was a potential candidate for the development of TB immunodiagnostics or vaccine study. However, the reason for IgA antibody responses to Rv3117, CFP-10 or ESAT-6 being able to distinguish patients with active PTB and healthy controls ($P<0.01$, respectively) has yet to be elucidated, as shown in Fig. 2. The low sensitivity of antibody responses to Rv3117 suggested that Rv3117 was not suitable for the serodiagnosis of TB compared with other B-cell antigens (15,33,34). However, the high specificity of the results suggested that it may be useful in further IGRA studies for TB immunodiagnostics.

The development of a Th1 immune response mediated by IFN-γ is a prerequisite for mounting efficient protection against the *M. tuberculosis* challenge (22). Therefore, we investigated whether Rv3117 was able to evoke antigen-specific INF-γ in C57BL/6 mice. In the study, the cytokine secretion pattern from Rv3117-immunized mice showed statistically higher IFN-γ compared with that from the control group mice (Fig. 3), confirming a Th1-type response to the chosen protein and a potential protective effect against *M. tuberculosis*. Similarly, Mustafa and Al-Attiyah demonstrated that synthetic peptides from RD5-encoded proteins were also able to evoke high levels of IFN-γ in patients with PTB (35). In the present study, we demonstrated that IFA-emulsified antigen Rv3117 was able to increase the production of IFN-γ (Fig. 3) and induce increasing concentrations of antigen-specific IgG2a antibodies subsequent to immunization (Fig. 4). These results were consistent with those of the RD11-encoded antigen Rv3245 (19), which is now in further and preclinical studies in China (9,36-38). These data indicated that IFA may be used as one of the most optimal adjuvants for Rv3117-based vaccine studies in animal models. Studies in mouse models of TB have shown that BCG efficacy may be enhanced through supplementation with other RD antigens (39). Further studies by the authors are planned to investigate the cellular immune response induced by an Rv3117-modified BCG in C57BL/6 mice and its protective efficacy prior to or subsequent to *M. tuberculosis* H_{37}Rv challenge.

In conclusion, we evaluated the humoral and cellular immune responses to a novel *M. tuberculosis* RD5-encoded antigen, Rv3117, and compared the results with those from two RD1-encoded antigens, ESAT-6 and CFP-10. The results of the immunological characterization suggested that Rv3117 may be used as a candidate for the study of TB immunodiagnostic development and vaccine design.

Acknowledgements

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