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A novel vaccine p846 encoding Rv3615c, Mtb10.4, and Rv2660c elicits robust immune response and alleviates lung injury induced by Mycobacterium infection

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Keywords: *M. tuberculosis*, BCG, multifunctional T cells, cellular immunity, subunit vaccine, Rv3615c, Mtb10.4, Rv2660c

Abbreviations: AP, Streptavidin-alkaline phosphatase; BCG, bacillus Calmette-Guérin; CFU, colony-forming unit; ELISPOT, enzyme-linked immunosorbent spot; *M. bovis*, *Mycobacterium bovis*; MDR, multidrug-resistant; *M. tuberculosis*, *Mycobacterium tuberculosis*; PPD, purified protein derivative; PBS, phosphate buffered saline; TB, tuberculosis; TFP846, triple-antigen fusion protein 846; XDR, extensively drug-resistant

Development of effective anti-tuberculosis (TB) vaccines is one of the important steps to improve control of TB. Cell-mediated immune response significantly affects the control of *M. tuberculosis* infection. Thus, vaccines able to elicit strong cellular immune response hold special advantages against TB. In this study, three well-defined mycobacterial antigens (Rv3615c, Mtb10.4 [Rv0228], and Rv2660c) were engineered as a novel triple-antigen fusion DNA vaccine p846. The p846 vaccine consists of a high density of $CD4^+$ and $CD8^+$ T-cell epitopes. Intramuscular immunization of p846 induced robust T cells mediated immune response comparable to that of bacillus Calmette-Guérin (BCG) vaccination but more effective than that of individual antigen vaccination. After mycobacterial challenge, p846 immunization decreased bacterial burden at least 15-fold compared with individual antigen-based vaccination. Notably, the lungs of mice immunized with p846 exhibited fewer inflammatory cell infiltrates and less damage than those of control group mice. Our data demonstrate that the potential of p846 vaccine to protect against TB and the feasibility of this design strategy for further TB vaccine development.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), is one of the leading causes of morbidity and mortality in humans,[1] and it is becoming increasingly serious with the prevalence of HIV co-infection and the emergence of multidrug–resistant (MDR) or extensively drug–resistant (XDR) strains of *M. tuberculosis*.[2,3] Despite the availability of specific anti-TB drugs and the worldwide administration of the bacillus Calmette–Guérin (BCG) vaccine,[4] one third of the global population is estimated to be infected with *M. tuberculosis*, with 9 million newly diagnosed cases and up to 2.5 million deaths each year. Although in most cases, TB can be successfully treated with multidrug combinations of antibiotics, vaccination is a better, preventive approach to interrupting *M. tuberculosis* infection and transmission. BCG, an attenuated *M. bovis* strain introduced almost a century ago, is currently the only approved TB vaccine and routinely administered to infants in many countries to effectively protect against severe forms of TB. However, the protective efficacy of BCG in adults is inconsistent and inadequate.[5] There is therefore an urgent need to develop new TB vaccines.

The effective host control of *M. tuberculosis* critically depends on a T cell mediated response characterized by the secretion of IFN-γ and other cytokines. Studies of mouse model have shown that both $CD4^+$ and $CD8^+$ T cells are required for protective immunity against TB and for vaccine–induced protection.[6-8] Due to the complexity of the host immune response against TB and the genetic restrictions imposed by the major histocompatibility complex, it is likely that an effective subunit vaccine comprising multiple epitopes should be required to ensure broad coverage of a genetically heterogeneous population.[9,10] For example, protein fusion vaccines constructed from two or three highly immunogenic, protective antigens, i.e.Ag85B and Esat6,

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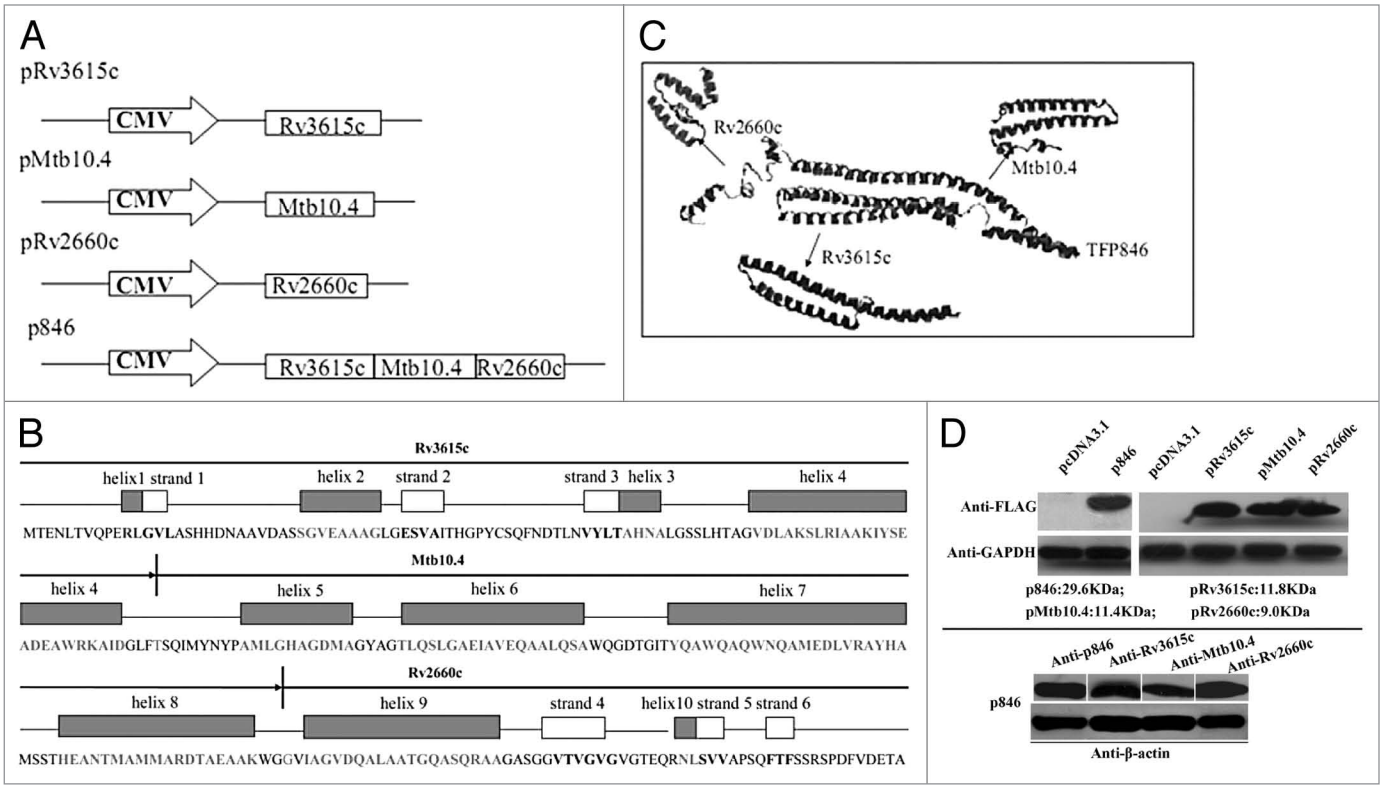

Figure 1. Construction and identification of p846 vaccine. (**A**) Schematic of p846 and its individual-antigen vaccines pRv3615c, pMtb10.4, and pRv2660c. The fusion of Rv3615c-Mtb10.4-Rv2660c triple-antigen was constructed through overlap extension PCR method and the triple-antigen fusion gene was then incorporated into pcDNA3.1 plasmid (+) driven by a cytomegalovirus (CMV) promoter. (**B**) Secondary structure of the triple-antigen fusion protein TFP846. The helix structure was indicated by gray box and stand structure was indicated by open box. (**C**) The three-dimensional structure of TFP846 that available on the I-TASSER server. (**D**) The expression of p846, pRv3615c, pMtb10.4 and pRv2660c in 293T cells. These recombinant plasmids were transfected into 293T cells and then the cell lysis were analyzed by western blotting with anti-Flag antibodies 48 h post transfection (upper panel). The expression of fusion protein was also detected by anti-Rv3615c, anti-Mtb10.4, anti-Rv2660c and anti-p846 antibodies (lower panel).

were more efficacious than protein vaccines of the individual components alone.[11-14] Vaccines including combination of three plasmids encoding individual *M. tuberculosis* secreted proteins,[15] expression of multiple antigens with 2A or linker sequence,[16,17] and a synthetic scrambled antigen comprised of overlapping peptides from four *M. tuberculosis* proteins[18] also have been shown the great potency against TB in experimental model.

About 170 *M. tuberculosis* antigens containing 800 human T cell epitopes have been identified in immune epitope databases.[19] Understanding of the immunogenicity and the selection of appropriate antigens are crucial for the vaccine design. Rv3615c, originally identified as an ESAT-6-like protein (Esx-1 substrate protein C, EspC), is a small protein (103 amino acids) similar in length to ESAT-6, CFP-10, and other members of the ESAT-6 family.[20] The high density of the multiple $CD4^+$ and $CD8^+$ epitopes in Rv3615c is determined by the dominance of $CD4^+$ responses that include functional T cell subsets secreting both IFN-γ and IL-2.[21] Mtb10.4 (Rv0288), which belongs to a subfamily of the ESAT-6 family,[22] promotes strong T cell immune response in TB patients and BCG-vaccinated donors. It is even more strongly recognized than ESAT-6 in TB patients.[23] Mtb10.4 has been reported to induce specific $CD8^+$ T cells recruited to the site of infection and upregulates FasL and LAMP-1/2, which are correlated with significant in vivo cytolytic activity.[24] Rv2660c, a nutrient stress-induced antigen, is stably expressed in the early and late stages of *M. tuberculosis* infection.[25] This antigen significantly enhances protective immunity characterized by a high proportion of multifunctional CD4+ T cells against *M. tuberculosis* in mice and cynomolgus macaques.[9,26]

In present study, we demonstrated that a DNA plasmid (p846) encoding a fusion protein of three well-defined *M .tuberculosis* antigens (Rv3615c, Mtb10.4, and Rv2660) could be an effective vaccine against *M. tuberculosis* infection. Intramuscular immunization of p846 elicited strong T cells mediated immune response and effectively prevented mice lung injury against mycobacterial infection which was comparable to BCG vaccination. Our results suggest that this novel triple-antigen fusion DNA vaccine can be further developed as TB vaccine.

Results

Generation and identification of triple-antigen fusion vaccine p846

Cellular immune responses are crucial for defending against intracellular pathogens, such as *M. tuberculosis*.[27] To design a new

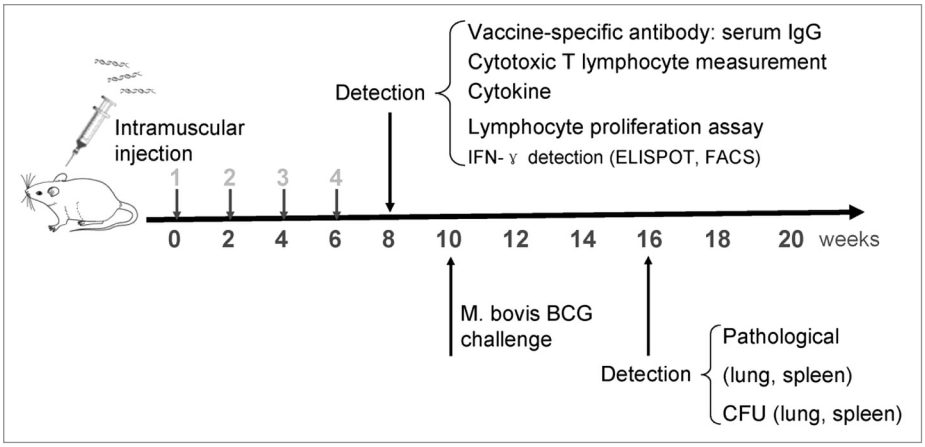

Figure 2. Timeline of the animal vaccination, infection and detection. Groups of BALB/c mice (n = 12 in each group) were immunized by receiving of 50 μg of plasmids DNA vaccine in thigh muscles. BCG group was performed with 10^6 CFU viable bacilli once on the first day. The immune response detection, BCG challenge, bacterial burdens and pathological detection were applied as indicated time points.

vaccine against *M .tuberculosis* infection, three immunodominant antigens containing potent $CD4^+$ and $CD8^+$ T-cell epitopes from *M. tuberculosis* (Rv3615c, Mtb10.4, and Rv2660c) were selected. The triple-antigen Rv3615c-Mtb10.4-Rv2660c gene was amplified through a simple fast overlap extension PCR method and the recombinant plasmid with insertion of this triple-antigen gene in pcDNA3.1(+) was named p846. Plasmids pRv3615c, pMtb10.4 and pRv2660c were also constructed as individual controls. The three antigens were arranged within one single ORF while there was no any separating sequence between them (**Fig. 1A**). The secondary structure (α-helix and β-strand) of the triple-antigen Rv3615c-Mtb10.4-Rv2660c protein was predicated by DNAMAN software (Lynnon Biosoft) (**Fig. 1B**) and the three-dimensional structures of the triple-antigen protein was modeled by I-TASSER (http://zhanglab.ccmb. med.umich. edu/I-TASSER /output/ S135133), which indicated that the functional domains in the fusion protein were similar to those of the corresponding individual antigens (**Fig. 1C**). Following transfection of 293 T cells, the expression of plasmid p846 and the individual antigens were confirmed by the anti-flag antibody in the presence of 30.0, 11.8, 11.4, and 9.0 kDa bands, respectively (**Fig. 1D,** upper panel). Moreover, the p846 fusion protein was recognized by the individual anti-Rv3615c, anti-Mtb10.4, anti-Rv2660c or anti-p846 antibodies, indicating the specific binding of antibodies to each antigen of the fusion protein (**Fig. 1D,** lower panel).

Specific antibody response elicited by p846 vaccination

To test whether vaccination with recombinant plasmids could induce the expression of fusion protein in vivo and induce humoral response, groups of BALB/c mice were injected with one dose of BCG or multiple doses of p846 plasmid, or control plasmids, respectively (**Fig. 2**). The level of antigen-specific IgG was evaluated at indicated time points during vaccination (**Fig. 3A**). The specific IgG detected in BCG, p846, pRv3615c, and pRv2660c groups gradually increased with three-time boosting, reaching its peak in week 12. ELISA results in week 12 detected improved anti-p846 IgG antibody levels (OD_{450nm}, 2.84) in the serum of the p846 immunized mice. This level was significantly higher than those of pMtb10.4 (OD_{450nm}, 0.65) and pRv2660c (OD_{450nm}, 1.79) ($P < 0.05$), but was similar to that of pRv3615c (OD_{450nm}, 2.32). The IgG titer measured in the p846 immunized group, however, was comparable to those of other groups, except that of the pMtb10.4 group (**Fig. 3B**). Furthermore, the affinity indices of the serum IgG elicited by p846, BCG, pRv3615c, pMtb10.4, and pRv2660c vaccination were turned to be statistically no difference (**Fig. 3C**). These data suggest that the humoral immune response could be triggered but not highly promoted by the fusion of triple-antigen Rv3615c-Mtb10.4-Rv2660c.

Enhanced T-cell immune responses induced by p846 vaccination

Next, we examined the impact of immunization with p846 on antigen specific T-cell response in spleens of all immunized mice two weeks after the last immunization. Analysis of antigen specific T-cell response revealed that significantly elevated T-cell proliferation, in response to triple-antigen fusion protein 846 (TFP846) or inactivated H37Rv, was observed in the p846-vaccinated mice (**Fig. 4A**, $P < 0.05$) as compared with those of the mice vaccinated with pRv3615c, pMtb10.4, and pRv2660c. The specific T-cell proliferation level in the mice immunized with BCG was also significantly elevated but not significantly different from that in the mice immunized with p846.

IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays was performed to determine whether the p846 vaccine can induce high level of antigen specific IFN-γ^+ T cells. The results revealed that the number of IFN-γ^+ T cells in the p846-immunized mice was higher than those in the individual antigen–immunized mice when incubated with TFP846 protein or inactivated H37Rv lysate (**Fig. 4B**, $P < 0.05$), similar to the number of IFN-γ+ T cells in the BCG-immunized mice. However, the T cell immune responses specific to individual antigen were similar between p846 immunized mice and single antigen immunized mice (data not shown). Specific CTL activity was also assessed by using SP2/0 cells as target cells (**Fig. 4C**). These results show

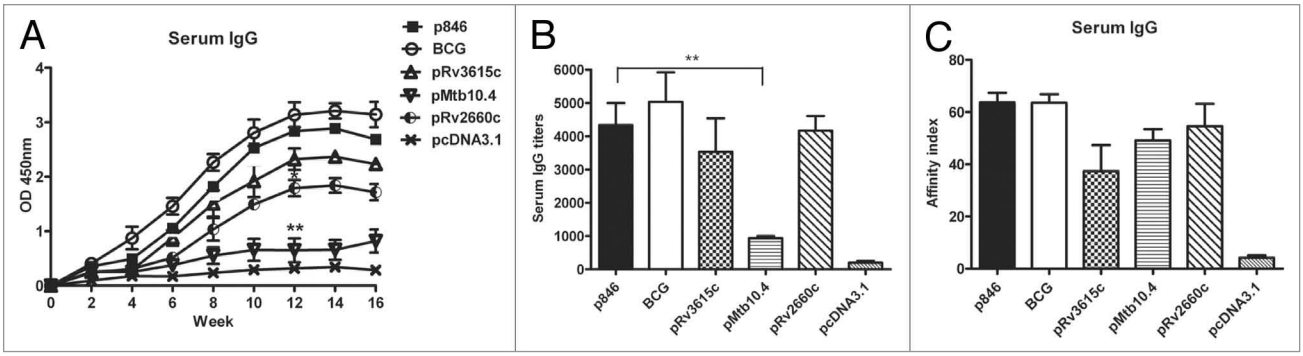

Figure 3. Specific antibody responses induced by p846 and its individual antigen vaccines. (**A**) Specific serum IgG level was measured by ELISA at indicated time points. (**B**) The antibody titers and (**C**) IgG affinity were determined at two weeks after last immunization by ELISA. Data are from one representative experiment of three performed and presented as the mean ± SD *$P < 0.05$, **$P < 0.01$.

that p846 vaccination induced robust specific T-cell immune response similar to that of BCG vaccination but significantly higher than that of individual antigen vaccination.

Vaccination with p846 favored robust Th1 immune response in mice

We further evaluated the functional phenotypes of antigen specific T-cell immune response elicited by p846 vaccination. The splenocytes from p846 or individual antigens immunized mice were isolated 2 weeks after the last immunization and stimulated for 72 h. The amounts of IFN-γ, TNF-α, IL-2, IL-4, and IL-10 in the supernatant were detected by ELISA two weeks after the last immunization. The levels of Th1 cytokines (IFN-γ, IL-2, and TNF-α) were significantly higher in the p846 group than in the individual antigen immunized groups (**Fig. 5**, $P < 0.05$). By contrast, the levels of Th2 cytokines (IL-4 and IL-10) in both groups were only slightly different among these groups. These results demonstrate that the triple-antigen vaccine p846 significantly enhanced the levels of Th1 immune response and T-cell proliferation which are beneficial to *M. tuberculosis* pathogen clearance.

p846 increased the magnitude of multifunctional CD4⁺ and CD8⁺ T cells compared with individual antigen-based vaccines

Multifunctional CD4⁺ and CD8⁺ T cells that simultaneously produce IFN-γ, IL-2, and TNF-α are associated with a good clinical outcome of TB.[28,29] Therefore, we assessed the phenotype and proportion of multifunctional CD4⁺ and CD8⁺ T-cell response elicited by p846 immunization through intracellular cytokine staining. The cells were first gated on CD4⁺ and CD8⁺ T cells, and then the combination expressions of IFN-γ, IL-2, and TNF-α was analyzed. Consistent with the cytokine ELISA results in **Figure 5**, the percentage of the single positive IFN-γ, IL-2, or TNF-α CD4⁺ / CD8⁺ T producer was higher in the p846 group than in the individual antigen vaccine groups (**Fig. 6A**–6F).

Analysis of the IFN-γ⁺TNF-α⁺IL-2⁺ triple-positive, IFN-γ⁺TNF-α⁺ double-positive, IFN-γ⁺IL-2⁺ double-positive, and TNF-α⁺IL-2⁺ double-positive CD4⁺ / CD8⁺ T cells revealed that the proportion of cells producing IFN-γ⁺TNF-α⁺ and IFN-γ⁺IL-2⁺ double-positive cells was the lowest among the eight subsets, ranging from 1.14% to 1.38%, and from 0.71% to 1.04%, respectively for CD4⁺ T cells, and almost undetectable for CD8⁺ T cells. However, the proportion of TNF-α⁺IL-2⁺ double-positive cells were much higher ranging from 16.71% to 26.15% for CD4⁺ T cells and from 6.60% to 13.70% for CD8⁺T cells. The percentages of the IFN-γ⁺TNF-α⁺IL-2⁺ triple-positive multifunctional cells were lower than those of the TNF-α⁺IL-2⁺ double-positive cells: 4.12% to 6.70% for CD4⁺ T cells and 0.89% to 1.77% for CD8⁺ T cells (**Fig. 6G**–**6I**). In other words, p846 immunization particularly increased the frequency of the two subsets of multifunctional T cells (TNF-α⁺IL-2⁺ and IFN-γ⁺TNF-α⁺IL-2⁺) compared with individual antigen-based vaccines ($P < 0.05$), suggesting that p846 immunization may provide more effective protection by inducing these multifunctional T cells.

p846 immunization reduced bacterial burden

Next, we explored the protective effect of p846 vaccination on the mycobacterial replication in mice. Groups of mice were intranasally challenged with 1×10^7 colony-forming unit (CFU) of BCG four weeks after the final immunization and the bacterial burden was determined by colony formation assay six weeks after the challenge. We noticed that the replication of BCG was more efficient in the lung than in the spleen (about 10-fold). The p846 immunization significantly reduced the bacterial burden by $10^{3.2}$-fold in lung tissue, which was $10^{1.2}$-, $10^{1.6}$-, and $10^{1.8}$-fold lower than that with pRv3615c, pMtb10.4, and pRv2660c immunization. A similar observation was detected in the spleen (**Fig. 7A and B**).

p846 immunization alleviated lung injury after BCG challenge

Finally to ask whether the p846 vaccination could improve the tuberculosis pathology after BCG challenge, we performed histopathological study. Lung tissues from the vector- or individual antigen vaccine-immunized mice exhibited widespread and severe interstitial pneumonia, intense inflammation, and diffuse granuloma-like responses as well as excessive lymphocyte and macrophage infiltration after *M. bovis* BCG infection. However, the p846-immunized mice exhibited much less inflammation and intact alveolar morphology, indicating dramatically attenuated tuberculosis pathology (**Fig. 8A**). The pathology of lung in vaccinated mice was also quantified with inflammation

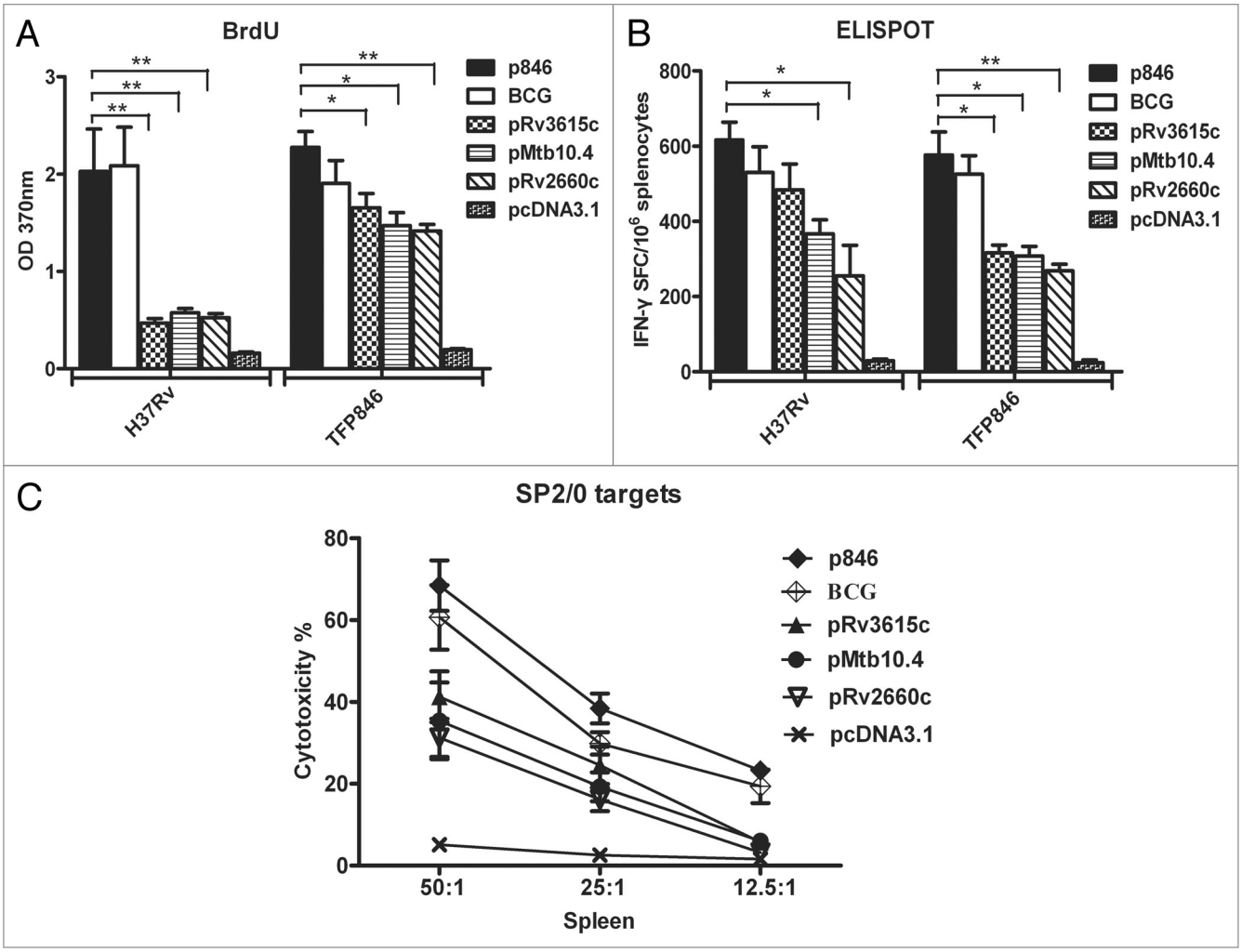

Figure 4. *M. tuberculosis* specific cellular responses induced by p846 and its individual antigen vaccine immunization. Two weeks after the last immunization, splenocytes were harvested and stimulated with TFP846 protein or inactivated H37Rv in vitro. (**A**) Lymphocyte proliferation was measured by BrdU assay. (**B**) Frequency of IFN-γ-secreting cells in the spleens of mice was measured by ELISPOT assay. (**C**) *M. tuberculosis*-specific CTL activity was evaluated by lactate dehydrogenase assay. The effector /target cell ratio was between 50:1 and 12.5:1. Data in the graph are from one representative experiment of three independent experiments performed. Error bars represent the means plus standard deviations (n = 6). Values that are statistically significantly different are indicated by $*P < 0.05$, $**P < 0.01$.

score as shown in **Figure 8B**. These results indicate that the triple-antigen vaccine p846 improved the protective efficacy against mycobacterial infection compared with individual antigen vaccines, to a level similar to the well-established attenuated bacterial vaccine BCG in mice.

Discussion

Tuberculosis has long been a major infectious disease giving rise to a significant global health problem. Approximately one third of world population is considered infected with *M. tuberculosis*, the principal causative agent of TB. The prevalence of HIV and the emergence of the MDR and XDR strains have ranked TB among the top two causes of mortality.[1] BCG is the only available vaccine against TB. However, the protective efficacy of BCG varies among adults, and the precise immune mechanisms that protect BCG-vaccinated infants remain unclear because of their complexity, thus hindering the development of TB vaccines.[27] Therefore, current research focuses on developing an effective and well-defined vaccine.

Subunit vaccines have the advantages of excellent safety profile, and, as they are comprised of defined components, allow standardized production. It is particularly valuable in those HIV high burden countries because reports showed that the utility of BCG in infants with HIV increased their risk of developing disseminated BCG-osis,[30] similar to the effect on those immune compromised persons.[31,32] In our study, we found that p846 vaccination was less toxic than BCG because mice immunized with BCG exhibited several clinical symptoms, such as hair loss and skin lumps. In contrast, the p846-immunized mice appeared to have the same characteristics as healthy mice, implying the safety of p846 which is particular important in regard to the immuno-compromised persons.

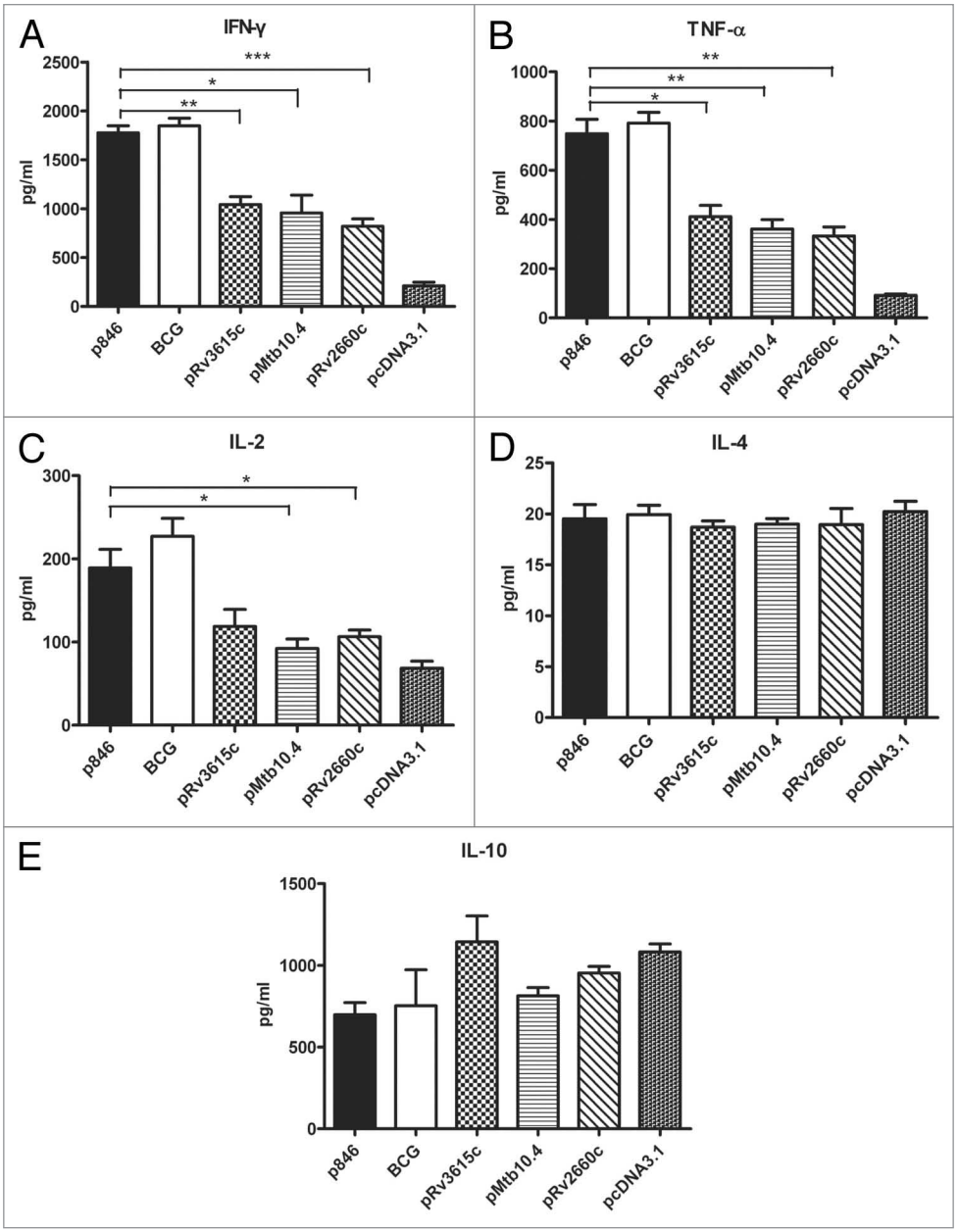

Figure 5. The enhancement of Th1 immune response by p846 immunization. Two weeks after the last immunization, splenocytes of the mice immunized with different vaccines were cultured and stimulated with inactivated H37Rv proteins (10μg/ml) for 72 h, and the Th1 cytokines (**A**) IFN-γ, (**B**) TNF-α, (**C**) IL-2, and the Th2 cytokines (**D**) IL-4, (**E**) IL-10 in the culture supernatant were measured by ELISA.

Recently, a number of subunit vaccines has been moved to clinical trial, these include fusion proteins based on Ag 85B and ESAT-6 (HyVac1),[33] Ag85B and TB10.4 (HyVac4),[34] Ag85B and ESAT-6 and Rv2660c(H56),[9] and Rv1196 and Rv0125 (Mtb72f)[35] as well as the non-replication Modified Vaccinia Ankara expressing Ag85A (MVA85A)[36] and adenoviral systems Ad 35 expressing Ag85A, Ag85B, and TB10.4.[37] Other reports have described secreted lipoprotein such as Mpt83;[38] dormancy antigens such as HspX[12] and 16 kDa heat shock protein,[12,39] and the Rpf-regulated gene Rv3407[40] as subunit vaccines against TB. We showed the three antigens (Rv3615c, Mtb10.4, Rv2660c) used in this study are all well-defined and effective. The fusion of these three antigens results in the expression of 30 kDa triple-antigen, a proper size for immunogens. Notably, the triple-antigen fusion p846 covers diverse $CD4^+$ and $CD8^+$ T cell epitopes to ensure the broad recognition by T cells. It turned out that the p846 immunization induced both efficient specific CD4+ and CD8+ T-cell activation in terms of the frequency of cytokine secreting $CD4^+$ and $CD8^+$ T cells. However, the CD4+ T cell might proliferate more efficiently than CD8+ T cell because we observed the increased CD4+/CD8+ T-cell ratio of 2.5. The protective immunity elicited by triple-antigen fusion was significantly improved compared with individual antigen vaccination and each of the three antigens partially contributes to

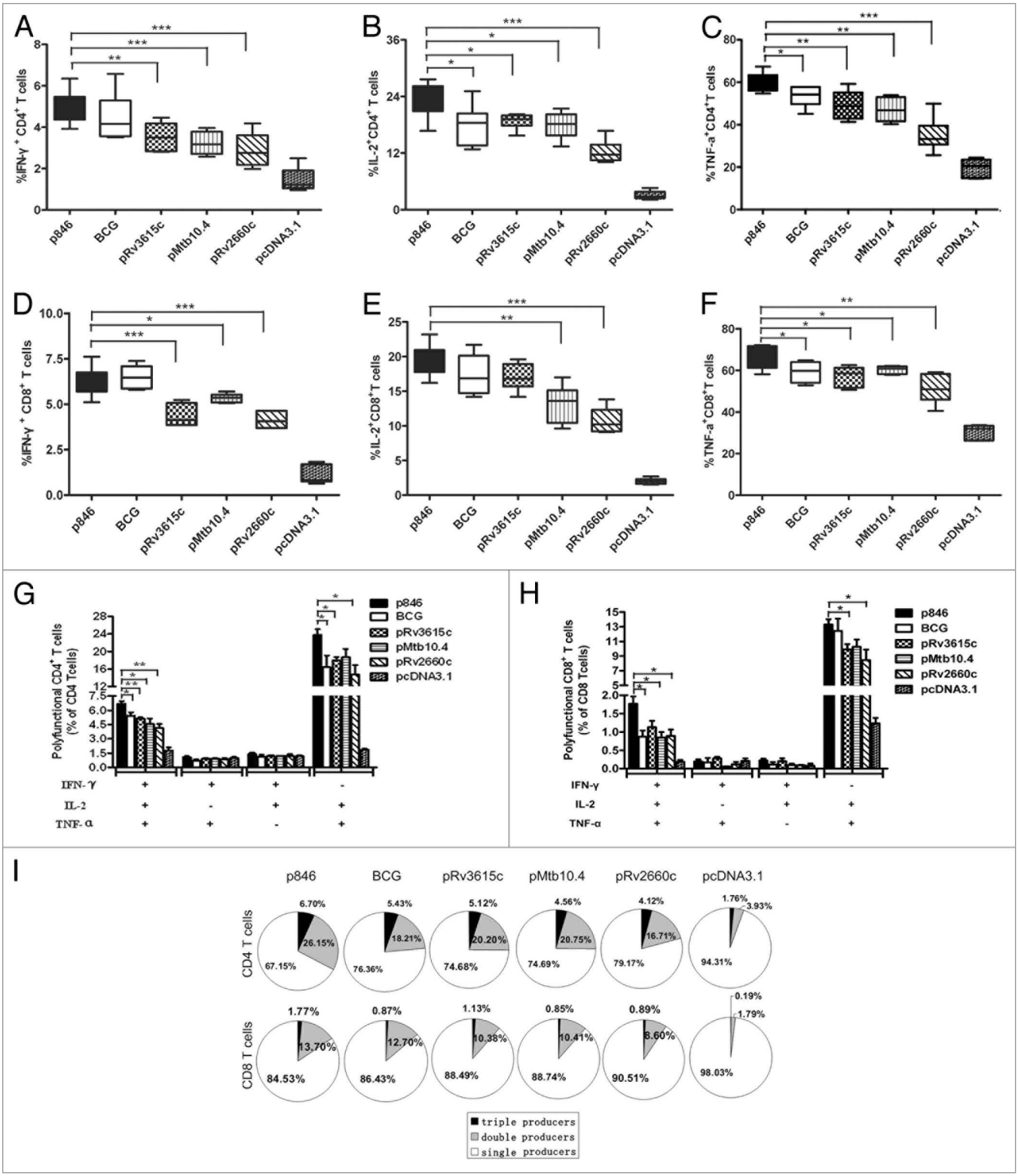

Figure 6. Frequencies of IFN-γ-, TNF-α- and IL-2-producing CD4+ or CD8+ T cells. Mouse splenocytes 2 wk following the last immunization were isolated and stimulated with 10 μg/ml inactivated H37Rv for 24 h. Following extra 4 h re-stimulation, the cells were stained with specific cytokine antibodies and subjected to flow cytometry. Plots were gated on CD4+ and CD8+ lymphocytes and analyzed for (**A**) Frequency of single positive IFN-γ producing CD4+ T cells, (**B**) Frequency of single positive IL-2 producing CD4+ T cells, (**C**) Frequency of single positive TNF-α producing CD4+ T cells, (**D**) Frequency of single positive IFN-γ producing CD8+ T cells, (**E**) Frequency of single positive IL-2 producing CD8+ T cells, (**F**) Frequency of single positive TNF-α producing CD8+ T cells, (**G**) Frequency of double or triple positive IFN-γ, IL-2 and TNF-α producing CD4+ T cells and (**H**) Frequency of double or triple positive IFN-γ, IL-2 and TNF-α producing CD4+ T cells. (**I**) Pie chart analysis total of IFN-γ, IL-2 and TNF-α single producing, double producing and triple producing T cells two weeks after last immunization.

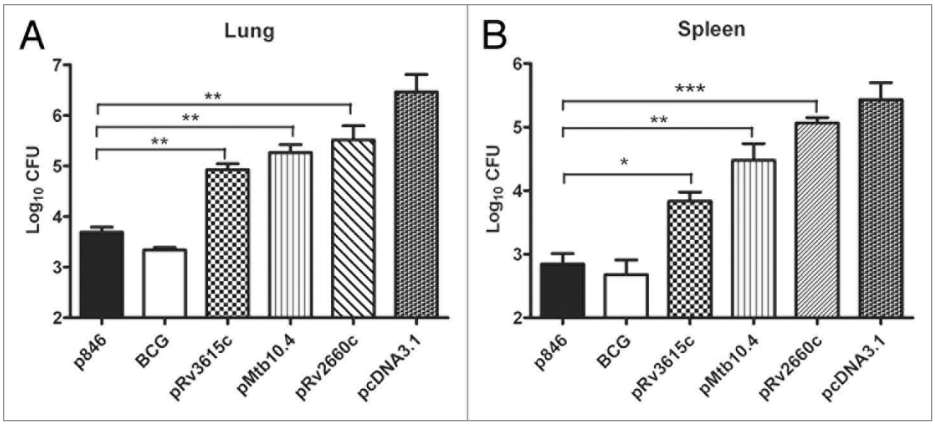

Figure 7. Vaccination with the p846 inhibited *M. bovis* BCG replication after challenge. Four weeks after the last immunization, mice were intranasally infected with 1×10^7 CFU BCG. Six weeks post infection, the mice were sacrificed and the numbers of BCG in the lungs and spleens of mice were determined by in vitro colony formation assays. (**A**) The numbers of BCG in the lungs. (**B**) The numbers of BCG in the spleens. Data are presented as the mean ± SD (n = 6) and are one representative of three separate experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

the enhanced T-cell immune responses of p846 to the protection upon mycobacterial challenge..

For a successful subunit vaccine, late stage antigens induced during the dormant or the resuscitation stages are worth incorporating to achieve the maximum protection on all stages of *M. tuberculosis* infection. We took the advantage of multi-stage antigen Rv2660c in p846 and showed the potent capacity of p846 to induce *M. tuberculosis* specific protection in mice as prophylactic vaccine. However, considering that the success of *M. tuberculosis* as a pathogen lies in its ability to persist within humans for long periods in a latent state,[41,42] the potential of p846 to prevent the reactivation of dormant bacilli after exposure warrants investigation.

Some factors may influence the efficacy of subunit vaccines. One is the number of antigens used in subunit vaccines. Increasing the diversity of antigens in subunit vaccines may enhance their efficacy against *M. tuberculosis* and also ensure their recognition by T cells from diverse human populations.[12,14] It has been demonstrated that vaccines based on a combination of three antigens can induce levels of protection similar to or even better than those induced by BCG in mice.[43,44] We therefore decided to combine three antigens in our study to get powerful protection against TB. Indeed, in line with the previous reports we were able to induce the *M. tuberculosis* specific protective response comparable to BCG. Whether this protective response can be further elevated by fusion of more than three antigens is of interest for future investigation.

Attention to the combination fashion of multiple antigens during delivery is also needed to be paid. We constructed the p846 plasmid through the overlap extension PCR to directly connect the multiple antigens.[45] It may differs from the previous

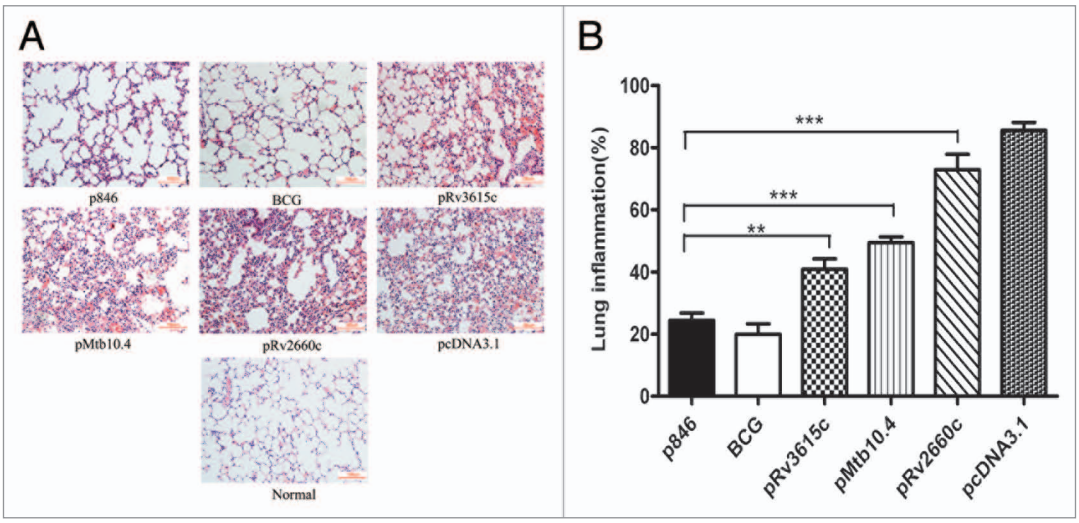

Figure 8. Vaccination with the p846 alleviated lung injury induced by BCG challenge. Groups of mice were vaccinated with vehicle control, individual antigen, p846, or BCG and four weeks after the last vaccination, the mice were challenged with 1×10^7 CFU BCG. Six weeks post infection, the mice were sacrificed and (**A**) pathology of H&E-stained lung tissues and (**B**) inflammation scoring were evaluated. Data shown are representative images (×10) from each group of mice (n = 6 per group) from three separate experiments, Scale bar = 100 μm.

Table 1. Overview of the primers

Protein and Primer name	Primer sequences	Restriction enzyme sites
Rv3615c	P1 5′-CG***GCTAGC***GC CACCATGACG GAAAACTTGA CCGTCCAG-3′	NheI
	P2 5′-CC***CTCGAG***TC ACTTATCGTC GTCATCCTTG TAATCGGTAA ACAACCCGTC GATAGC-3′	XhoI
	P3 5′-TAGTTGTACA TGATTTGCGA GGTAAACAAC CCGTCGATAG CCTT-3′	
Mtb10.4	P4 5′-CG***GCTAGC***GC CACCATGTCG CAAATCATGT ACAACTACCC C-3′	NheI
	P5 5′-CC***CTCGAG***TT ACTTATCGTC GTCATCCTTG TAATCGCCGC CCCATTTGGC GGCT-3′	XhoI
	P6 5′-TCGCAAATCA TGTACAACTA CCCCG -3′	
	P7 5′-CTGGCCTGTT GCTGCAAGCG CCTGGTCGAC GCCCGCTATC ACGCCGCCCC ATTTGGCGG-3′	
Rv2660c	P8 5′-CG***GCTAGC***GC CACCGTGATA GCGGGCGTCG ACCAG-3′	NheI
	P9 5′-CC***CTCGAG***TT ACTTATCGTC GTCATCCTTG TAATCGTGAA ACTGGTTCAA TCCCAGTATC GC-3′	XhoI
	P10 5′-CTTGCAGCAA CAGGCCAGGC AAGCCA-3′	
	P11 5′-CG***CTCGAG***TT ACTTATCGTC GTCATCCTTG TAATCGTGAA ACTGGTTCAA TCCCAGTATC GCGCA-3′	XhoI

Restriction enzyme sites in the 5' terminal of primers were in bold and italics.

reports where multiple mycobacterial antigens were fused with picrornavirus 2A or protein linker sequence.[15-17] We showed that the fusion of three immuno-dominant antigens without any spacing sequence was still able to achieve preferable T cells mediated protective immunity compared with that of BCG. One possible explanation is that the T-cell epitopes are determined by the linear amino acid sequence and our T-cell epitopes are not adjacent to the antigen joints. Therefore the T-cell recognition seems to be not affected by this construction strategy of triple-antigen fusion at least in our study.

One of the important findings in the present study is the potent induction of TNF-α^+IL-2^+ and IFN-γ^+TNF-α^+IL-2^+ $CD4^+/CD8^+$ T cells. It is well established that protective immunity against *M .tuberculosis* depends on the recruitment of antigen-specific T cells and the release of cytokines to interact with macrophage. Although a broad spectrum of cytokines contributes to protection, the type 1 response, which is dominated by IFN-γ, TNF-α, and IL-2 secretion, is considered critical for protective immunity against TB.[30,46] We detected significantly improved level of Th1 cytokine secretion (IFN-γ, TNF-α, and IL-2) by antigen-specific $CD4^+/CD8^+$ T cells after vaccination with p846. Accordantly, intracellular cytokine staining showed increased frequency of multifunctional $CD4^+/CD8^+$ T cells with p846 vaccination. The multifunctional T cells induced by p846 vaccination should establish long lasting immunological memory and give rise to a strong and robust secondary response upon challenge with *M. tuberculosis* based on other studies, where they indicate that such T cells are functionally superior to single-positive counterparts.[47-49] Vaccination with p846 may mimic the nature mycobaterial infection more, as the percentage of multifunctional $CD4^+$ T lymphocytes was found increased in active TB and decreased in TB patients after anti-mycobacteral therapy.[28,29]

In summary, we have developed a novel vaccine expressing fusion of three well-defined *M. tuberculosis* antigens. Vaccination with p846 provided remarkable T cell mediated immune response as well as effective protection after BCG challenge. Our data indicate that the new vaccine p846 can be used against mycobacterial infection.

Materials and Methods

Animals

Six- to eight-week-old female BALB/c mice ($H\text{-}2^d$) were purchased from the experimental animal center of the Chinese Academy of Sciences and maintained in pathogen-free conditions. All animal experiments were performed according to the guidelines for the care and use of laboratory animals of the Laboratory Animal Ethical Commission of Soochow University.

Bacterial strains and culture conditions

Escherichia coli strains DH5α and BL21 (DE3) were grown in a Luria-Bertani medium with or without agar and used for cloning and expression, respectively. *M. bovis* BCG (Denmark strain 1331) was provided by the Center for Disease Control of Suzhou and was cultivated in a Middlebrook 7H9 medium or enumerated on 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase, 0.50% glycerol, and 0.05% Tween 80. A purified protein derivative (PPD) was purchased from the Shanghai Institute of Biological Products. An inactivated *M. tuberculosis* H37Rv strain was provided by the Fifth People's Hospital of Suzhou.

Construction of triple-antigen vaccine p846

The genes encoding Rv3615c, Mtb10.4, and Rv2660c were amplified by PCR using their corresponding primers (**Table 1**) and H37Rv genomic DNA as the template. Primers P1 and P2 were used to amplify the gene encoding the Rv3615c antigen, P4 and P5 the gene encoding Mtb10.4, and P8 and P9 the gene encoding Rv2660c. The DNA fragment encoding the triple antigen of Rv3615c, Mtb10.4, and Rv2660c was generated through overlap extension,[45] with P1/P3 used to amplify the Rv3615F fragment, P6/P7 the Mtb10.4F fragment, and P10/P11 the Rv2660cF fragment. P1/P11 was used to amplify the triple-antigen DNA fragment with the mixture of Rv3615F, Mtb10.4F, and Rv2660cF as the template. The PCR products were digested

with NheI/XhoI and then cloned into the corresponding sites of expression vector pcDNA3.1(+). This process formed a recombinant plasmid named p846 expressing triple-antigen Rv3615c-Mtb10.4-Rv2660c and created individual antigen-expressing vectors named pRv3615c, pMtb10.4, and pRv2660c. Each expressed antigen was tagged with a flag (DYKDDDDK) on the 3′ terminal for detection. Recombinant plasmids p846, pRv3615c, pMtb10.4, and pRv2660c and vector pcDNA3.1 for immunization were transformed into competent *E. coli* DH5α. Endotoxin-free plasmid DNA was purified with the QIAGEN Plasmid Mega Kit according to the manufacturer's instructions.

Expression and purification of mycobacterial antigens

For the prokaryotic expression of TFP846, the triple-antigen DNA fragment was directly excised from the p846 plasmid with NheI/XhoI and cloned into a pET-28a vector (Novagen) named pTFP846. The protein was induced in BL21 at 1 mM at 37 °C for 6 h. Bacterial pellets were collected by centrifugation, prepared in a sample buffer, and subjected to sodium dodecyl sulfate-PAGE (SDS-PAGE). Protein expression was confirmed by western blot. The TFP846 triple-antigen protein was maintained in the insoluble inclusion body fraction and purified by Ni-nitrilotriacetic acid-metal ion affinity chromatography under denaturing conditions according to the manufacturer's instructions (GE Healthcare). The purified proteins were diluted in sterile phosphate buffered saline (PBS), with pyrogen-free reagents and tested to exclude endotoxin contamination. Protein concentration was determined with a Micro BCA™ protein assay kit (Thermo), and the solution was stored at −80 °C for further experiments. The other recombinant proteins (Rv3615c, Mtb10.4, and Rv2660c) were generated in a similar manner.

Western blot

Each of 3 μg p846, pRv3615c, pMtb10.4, or pRv2660c plasmids were transfected to 293 T cells. Cells were then collected and washed with PBS and lysed with 5 × SDS sample buffer two days post tranfection. Proteins were separated on a 12% SDS gel, transferred to a PVDF membrane, incubated with mouse anti-flag antibody (Southern Biotech; 1:2000) and the detection of p846 fusion protein was further incubated with the home-made mouse anti-Rv3615c, Mtb10.4, Rv2660c, or p846 antibodies(1:1000). Following horseradish peroxidase (HRP) conjugated anti-mouse secondary antibody incubation (Southern Biotech, 1:5000), the signals were developed using Super signal West Dura (Thermo).

Immunization protocol

The mice were randomly divided into six groups of 12: vector control, pRv3615c, pMtb10.4, pRv2660c, p846, and BCG. The mice were injected with 30 μl of 0.25% bupivacaine into the quadricep of each hind leg 3 d before DNA immunization. Plasmid DNA (50 μg) was intramuscularly injected into the same area, and immunization was repeated 3 times at 2-week intervals., The BCG group was subcutaneously immunized with 10^6 CFU of BCG only once on the first day. Two weeks after the immunization, 6 mice from each group were used for analyzing T-cell immune response, while the other 6 were reserved for the BCG challenge experiment. Serum was collected every 2 weeks by retro-orbital bleeding and stored at −80 °C for further analysis.

IFN-γ enzyme-linked immunosorbent spot assay

An assay to determine IFN-γ-producing T cells was performed with ELISPOT kit (BD PharMingen). Plates were coated with captured anti-IFN-γ monoclonal antibodies (mAb) at 4 °C overnight and then blocked with a complete medium at room temperature for 2 h. Splenocytes from immunized mice were isolated 2 weeks after last immunization, plated (5×10^5 cells/well) in stimulation of TFP846 protein, or inactivated H37Rv (each protein at a final concentration of 10 μg/ml) at 37 °C for 60 h. After the plates were washed with de-ionized water and PBS with Tween 20, biotinylated anti-IFN-γ mAb was added at room temperature for 2 h. Streptavidin–alkaline phosphatase (AP) was added to the plates, which were incubated for 1 h. The color was developed by an AP colorimetric substrate. An immunospot analyzer (Cellular Technology) was used to enumerate spots.

Enzyme-linked immunosorbent assay of cytokines

Two weeks after last immunization, freshly isolated spleen cells from vaccine-immunized mice were plated in duplicate in 24-well plates at 5×10^6 cells/well in 500 μl RPMI 1640 supplemented with penicillin, streptomycin, and 10% newborn calf serum (Shangbao Biotech). These cells were stimulated with inactivated H37Rv (10 μg/ml) at 37 °C and 5% CO_2 for 72 h. The concentrations of IFN-γ, TNF-α, IL-4, IL-10, and IL-2 in the culture supernatant were measured with an ELISA kit (eBioscience) according to the manufacturer's procedure.

Lymphocyte proliferation assay

The proliferation of splenocytes from immunized mice was measured two weeks after the last immunization. Viable splenocytes were adjusted to a concentration of 5×10^6 cells/ml and added to 96-well flat-bottomed plates at 5×10^5 cells/well with 10 μg/ml of TFP846 protein or inactivated H37Rv. A BrdU labeling reagent (Roche) was added to each well at a ratio of 1:1000. The culture plates were maintained in the same conditions for another 24 h and then incubated with anti-BrdU peroxidase. The absorption value at 370 nm was measured. Each sample was analyzed in triplicate. The proliferative responses of individual mice were expressed by OD_{370nm}.

Measurement of cytotoxic T lymphocyte

Two weeks after the final immunization, splenocytes were isolated and stimulated in vitro with 10 μg/ml of the recombinant TFP846, Rv3615c, Mtb10.4, and Rv2660c proteins in the vaccinated mice and with PPD in the BCG-treated group at 37 °C in a humidified incubator with 5% CO_2. The processed cells were used as effector cells. Mouse myeloma cell line SP2/0 cells were pulsed with the inactivated *M. tuberculosis* H37Rv for 24 h as target cells. A nonradioactive cytotoxic T lymphocyte (CTL) assay was performed with a lactate dehydrogenase cytotoxicity detection kit (Roche).The effector cells were titrated in U-bottom 96-well plates at effector–target cell ratios of 50:1, 25:1, and 12.5:1; 1×10^4 target cells were then added. After incubation at 37 °C for 72 h, 100 mL of cell supernatant per well was removed and transferred into corresponding wells of the 96-well plate. A reaction mixture (100 mL) was added to each well, which was incubated at room temperature for 30 min. The absorbance value at 492 nm was measured. The percentage cytotoxicity of CTL was calculated as follows:

Cytotoxicity (%) = ([effector and target cell mixture − effector cell control] − low control/[high control − low control]) × 100%

Intracellular cytokine staining and flow cytometry

Splenocytes were harvested from the vaccinated mice two weeks after the last immunization. Isolated lymphocytes (5×10^6) were stimulated with 10 μg/ml inactivated H37Rv in 24-well plates for 24 h and then re-stimulated with 50 ng/ml phorbol myristate acetate (PMA), 500 ng/ml ionomycin (Sigma Aldrich), and 10 μg/ml brefeldin A (eBioscience) for 4 h. Cells were collected and stained with PerCP-anti-mouse CD4 (BD PharMingen) and PE-Cy7-anti-mouse CD8 antibodies (BD PharMingen). The cells were then fixed and permeabilized with Cytofix/Cytoperm Buffer™ (Becton Dickson). Intracellular staining was performed with APC-anti-mouse IFN-γ (Biolegend), PE-anti-mouse IL-2 (BD PharMingen), and FITC-anti-mouse TNF-α antibody (eBioscience). The stained cells were analyzed with a FACSCanto II flow cytometer with FACSDiva software.

Antibody detection

Antigen-specific antibody responses were measured by ELISA. Microtiter plates were coated overnight at 4 °C with the recombinant TFP846, Rv3615c, Mtb10.4, Rv2660c proteins and with PPD in the BCG-treated group at 5 μg/ml in a carbonate/bicarbonate buffer (pH = 9.6). The plates were then washed with 0.05% Tween 20 in PBS and blocked with 1% bovine serum albumin in 0.05% Tween 20/PBS at room temperature for 2 h. Serum samples (1:100 dilution) from immunized mice were added in duplicate in corresponding antigen coated plates and incubated at 37 °C for 2 h with goat anti-mouse IgG conjugate to streptavidin. The plates were washed with 0.05% Tween 20/PBS and developed by using a TMB substrate. The reaction was stopped with 2N H_2SO_4, and data were collected at 450 nM by an ELISA reader. The endpoint titer was defined as the highest serum dilution that gave an absorbance value above the optical density of 0.050 and was twice that of the matched dilution of unvaccinated mouse sera.[50] The avidity of serum IgG was determined through ELISA with a urea elution step as previously described.[51]

Protective efficacy of p846 vaccine

Four weeks after the last immunization, the immunized mice were intranasally challenged with 1×10^7 CFU of *M. bovis* BCG. The number of bacteria in the spleen and lungs was counted six weeks post-challenge according to serial dilutions of individual tissue homogenates in triplicate on a Middlebrook 7H11 medium. The plates were incubated at 37 °C for three to four weeks, and colonies were then counted, calculated, and presented as $\log_{10}$ CFU per organ. For histological analysis, lung tissues were harvested six weeks after mycobacterial challenge, fixed in 10% buffered formalin, and embedded in paraffin blocks. Sections of 3 μm were stained with hematoxylin and eosin and analyzed by a pathologist under light microscopy for treatment allocation. To score lung inflammation and damage, the entire lung section was analyzed with confluent inflammatory infiltration, which was quantified and expressed as a percentage of the lung surface.

Statistical analysis

Statistical analyses were performed with Microsoft Excel or GraphPad Prism. All data were given as mean and standard deviation. The data were statistically analyzed by two-tailed independent the Student *t* test through SPSS 12.0. The level of statistical significance was set to $P < 0.05$.

Disclosure of Potential Conflicts of Interest

The authors declare that there is no conflict of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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