Immunogenicity and therapeutic effects of a Mycobacterium tuberculosis rv2190c DNA vaccine in mice

Yan Liang†, Xiaoyan Zhang†, Xuejuan Bai†, Li Xiao, Xiaomei Wang, Junxian Zhang, Yourong Yang, Jinying Song, Lan Wang and Xueqiong Wu*

Abstract
Background: Tuberculosis (TB) is a major global public health problem. New treatment methods on TB are urgently demanded. In this study, Mycobacterium tuberculosis (MTB) rv2190c DNA vaccine was prepared and its immunogenicity and therapeutic effects were evaluated.

Results: Non-infected mice immunized with rv2190c DNA or ag85a DNA showed higher levels of interferon-gamma (IFN-γ) in stimulated spleen lymphocyte culture supernatants, and had more Th1 cells and an elevatory ratio of Th1/Th2 immune cells in whole blood, indicating that Th1-type immune response was predominant. Compared with the saline group, ag85a DNA group and rv2190c DNA group in the infected mice decreased the lung colony-forming units (CFUs) by 0.533 and 0.283 log10, and spleen CFUs by 0.425 and 0.321 log10, respectively, and pathological lesion.

Conclusions: The rv2190c DNA had some immunotherapeutic effect on TB.

Keywords: DNA vaccine, rv2190c DNA, Immunotherapy, Mycobacterium tuberculosis

Background
Tuberculosis (TB) is a severe respiratory infectious disease in which protective immunity and pathological hypersensitivity to an intracellular bacterium coexist [1, 2]. In 2013, there were 9 million incident cases and 1.5 million deaths [3]. It is difficult to cure with anti-TB chemotherapy because a long treatment duration (a minimum of six-month chemotherapy) with multiple drugs was used on active TB, many TB patients cannot complete a full period of treatment, which lead to failure of treatment and acquired drug resistance with eventual creation of multi-drug-resistant TB (MDR-TB) [4]. Accordingly, effective vaccines are needed in the face of failing drug treatments, and this may include therapeutic vaccines.

Protective immunity against TB is largely attributed to a cellular immune response in which the production of the Th1-type cytokines (for example interferon-gamma, IFN-γ) predominates over the production of the Th2-type cytokines (for example interleukin-4, IL-4) [5–7]. DNA vaccination has been found to establish and boost antigen-specific cellular immunity in the direction of responses. Furthermore, immunotherapy with plasmid DNA has been found to be an effective adjunctive treatment in combination with antibacterial chemotherapy in mice. It both shortened the period of treatment and improved the therapy outcome of latent TB infection [8, 9]. Furthermore, we and others have shown that ag85a DNA vaccine can also have immunotherapeutic effects against MDR-TB in mice [10–12]. Here we report that the rv2190c gene of Mycobacterium tuberculosis (MTB) is similarly effective as a therapeutic DNA vaccine. The open reading frame (ORF) contains 1177 nucleotides and encodes a hypothetical protein with an NlpC/P60 domain [13]. Målen et al. [14] identified the protein product of rv2190c in MTB...
culture filtrate, showing that it could be expressed and secreted in vitro. Parthasarathy et al. [15] showed that the gene and its protein product were essential for normal growth and virulence of MTB in vivo and McMurry et al. [16] found that Rv2190c peptide could stimulate peripheral blood mononuclear cells to secrete IFN-γ in persons with latent TB infection. Beyond that, MTB Rv2190c antigen has not been extensively studied and thus therefore presents new possibilities for developing drug targets, diagnostic reagents and vaccines. Accordingly we evaluated its immunogenicity and immunotherapeutic effects as a DNA vaccine in mice.

**Methods**

**Mice**

One hundred 6-8 week age of female BALB/c mice without specific pathogen were purchased from the Academy of Military Medicine and Science, Beijing, China, maintained under infection barrier conditions in a negative pressure animal room in the 309th Hospital of Chinese PLA, Beijing, China, and fed a sterile commercial mouse diet (Beijing KeAoXieLi Company Limited, China). The study procedures were approved by the 309th Hospital of the Chinese PLA Research Animal Ethics Committees.

**MTB strain**

MTB H₃₇Rv was provided by National Institutes for Food and Drug Control, Beijing, China.

**Preparation of recombinant Rv2190c protein**

The procedures preparation of Rv2190c protein were briefly as follows: a 1168 bp gene fragment of rv2190c was amplified by polymerase chain reaction (PCR). The forward primer with a Nhe I enzyme site: 5’-CTAGGCTAGCCACCATTGGGGCTCG ACCAGGTTGGTT-3’; the reverse primer with an EcoR I enzyme site: 5’-CCGGAATTCGTAACCGGCC GGGCGTCG -3’ (synthesized by Shanghai Sangon Ltd. Beijing, China). The PCR product fragment was 1177 bp. Recombinant plasmid rv2190c DNA was sequenced by Huada gene Ltd. Beijing, China and was found to conform to the sequence designed using BLAST analysis. EndoFree plasmid purification kit (Qiagen, Hilden, Germany) was used to purify rv2190c DNA vaccine.

**Immunogenicity of rv2190c DNA vaccine**

Fifty female BALB/c mice were divided into 5 groups as follow: (1) saline as a negative control (100 μl); (2) vector pVAX1 as a negative control (100 μg in 100 μl saline); (3) M. vaccae (22.5 μg in 100 μl saline, Longcome Biological Pharmacy, Anhui, China) as a positive control; (4) ag85a DNA (100 μg in 100 μl saline), as a positive control; (5) rv2190c DNA (100 μg in 100 μl saline), immunized intramuscularly three times at two-week intervals.

**Cytokine production in vitro**

The mice were sacrificed at three weeks after the third immunization. The mouse splenocytes were isolated and cultured (5 x 10⁵ cells/well) with Ag85A or Rv2190c protein (20 μg/ml) or phytohaemagglutinin (PHA; 20 μg/ml) for 72 h. The levels of IFN-γ and IL-4 in the splenocytes culture supernatants were detected using enzyme-linked immunosorbent assay (ELISA) kit (BD PharMingen, San Diego, California, USA) according to the manufacture’s procedures.

**Determination of CD4⁺ T cell subsets expressing intracellular IFN-γ or IL-4**

The operation procedure was described previously [20]. Briefly, Th1 and Th2 cells responding to Ag85A or Rv2190c proteins were calculated. Cells expressing IFN-γ and IL-4 were presented as a percentage of the total population of CD3⁺ cells. The data were collected using a fluorescence-activated-cell-sorting (FACS) Calibur flow cytometer (BD Pharmingen) and analyzed using CellQuest software.

**Treatment of TB-infected mice**

Fifty mice were challenged with 6.4 x 10⁵ colony-forming units (CFUs) of MTB H₃₇Rv through trail vein injection, randomly divided into five groups mentioned above, and then treated at the third day after infection.

**Bacterium counts**

The mice were sacrificed by cervical dislocation under anesthetic with 5 ml dethyl ether (Beijing Chemical
Reagents Company, Beijing, China) at two weeks after the third immunization. The tissue suspensions of mouse lungs and spleens were serially diluted 10-fold, and 100 μL suspension dilution were inoculated in duplicate on Lowenstein-Jensen medium plates and cultured at 37 °C for 4 weeks. MTB colonies on medium were counted and the results were showed as CFUs per organ.

**Lung histopathological examination**
The mouse lungs tissues paraffin-embedded were sliced into 3-μm thick tissue sections, which were dyed with hematoxylin and eosin, and then examined by a certified and veteran pathologist.

**Statistical analyses**
Data are shown as means and standard deviations. Statistical analyses were performed using one-way ANOVA followed by Dunnett’s multiple comparison test, and a P-value of < 0.05 was considered as significant difference.

**Results**
**Preparations of rv2190c DNA and recombinant Rv2190c protein**
The nucleotide sequence in pVAX1-rv2190c plasmid had 100% identity with MTB rv2190c sequence as designed and the fragment size from restriction-enzyme-
digested recombinant plasmid pVAX1-rv2190c was 1.177 kb on 0.8% agarose gel electrophoresis, and that confirmed the successful construction (Additional file 1: Figure S1).

The recombinant Rv2190c protein was soluble in expression and amounted to 30% of total bacterial protein. The molecular mass of the purified Rv2190c protein was around 42 kDa by SDS-PAGE, its purity was higher than 90% (Additional file 2: Figure S2).

Specific cytokine production levels in splenic lymphocyte culture supernatants
The IFN-γ level in splenic lymphocyte culture supernatants in the rv2190c DNA group was obviously higher than those in the saline, plasmid vector and M. vaccae groups (P < 0.05), but had no significant difference from that in the ag85a DNA group. The production of IL-4 was not significantly different between groups (Fig.2, Additional file 3).

CD4+ T cell subsets expressing intracellular IFN-γ or IL-4
The proportion of CD4+ T cells expressing IFN-γ (Th1) in response to Ag85A or Rv2190c proteins was significantly higher in the whole blood from the rv2190c DNA group than those from the saline group and vector group by flow cytometry (P < 0.001), but there was no significant difference from the M. vaccae group or ag85a DNA group. The proportion of cells expressing IL-4 (Th2) was significantly higher in the blood from the rv2190c DNA group than those from the control groups (P < 0.05), but there was no significant difference from the M. vaccae group or ag85a DNA group. Th1/Th2 ratio in the blood from the rv2190c DNA group was significantly decreased than that in the ag85a DNA group, and increased than those in the saline, vector and M. vaccae groups, but the differences were not significant (Fig. 3, Additional file 3).

Mouse survival
There was one mouse death in vector group, one in M. vaccae group and one in rv2190c DNA group at 29 days after infection (90% survival). The other mice were all alive.

Bacterial counts in the lungs and spleens
The live bacteria in mouse lungs and spleens were determined at two weeks after third of immunotherapy. The lung CFUs from saline, vector, M. vaccae, ag85a DNA and rv2190c DNA groups were 7.334 ± 0.180, 7.233 ± 0.102, 7.081 ± 0.369, 6.801 ± 0.407 and 7.051 ± 0.154 log10, respectively (Fig.4a, Additional file 3), and the spleen CFUs were 6.919 ± 0.117, 6.808 ± 0.067, 6.652 ± 0.345, 6.494 ± 0.211 and 6.598 ± 0.143 log10, respectively (Fig.4b, Additional file 3). Compared with saline group, rv2190c DNA and ag85a DNA decreased the lung CFUs by 0.283 (P > 0.05) and 0.533 log10 (P < 0.05) and the spleen CFUs by 0.321 and 0.425 log10 (P < 0.05), respectively.

Histopathological changes
The lung sections from the saline group and plasmid vector group showed extensive lung lesions, in which hyperemia and congestion in alveoli with many lymphocytes and destructive structure caused by severe inflammation. The lung sections from treatment groups showed more foamy macrophages but less lymphocyte infiltration, the alveoli were relatively clear and had normal structure. Representative histopathological changes of five groups were shown in Fig.5.

Fig. 2 IFN-γ (a) and IL-4 (b) levels in the culture supernatants of splenocytes were detected by ELISA. The production of IFN-γ from rv2190c DNA group was significantly higher than from the saline group, vector group and M. vaccae group (P < 0.05), but the production of IL-4 was not significantly different between the groups.
Discussion
In this study, a DNA sequence encoding MTB Rv2190c protein was inserted into plasmid vectors pET30a and pVAX1, thereby recombinant Rv2190c protein and rv2190c DNA vaccine were prepared and subsequently used to compare the therapeutic effects of rv2190c DNA with those of ag85a DNA as vaccines against TB in mice.

The cellular immune responses to rv2190c DNA were measured as increase in frequency of circulating T cells that produced either IFN-γ or IL-4 in response to cognate vaccine antigen since these cytokines are markers of protective Th1 responses and non-protective Th2 responses respectively. A balance between Th1 and Th2 in which Th1 predominates is essential for the control of TB and mycobacterial infection in mice and human [2, 4]. In this study, the significantly increased IFN-γ level in the spleen lymphocyte culture supernatant, the abundance of Th1 cells and elevatory ratios of Th1 /Th2 cells in antigen-specifically stimulated whole blood from the rv2190c DNA group were all similar to the results obtained in the ag85a DNA group. Thus both vaccines induced a predominantly Th1 immune response [5, 12–14]. This finding was consistent with evidence from McMurry et al. [16] showing that Rv2190c peptide can induce IFN-γ production in cells from persons with latent TB infection, and indicated a potential utility of Rv2190c antigen in therapeutic vaccination.

When used to treat infected mice, rv2190c DNA vaccine did indeed reduce the numbers of live bacteria found in the organs sampled after 6 weeks of treatment when compared to control mice. However, the reduction in lung bacterial load was small, not statistically significant, and less than that obtained after ag85a DNA treatment. In contrast, the reduction of load in the spleen was significant, but again less than that observed after ag85a DNA treatment. Apparently, although the rv2190c protein enhanced antibacterial immunity it was in this respect a less effective vaccine antigen than the
Ag85A protein. Nevertheless, in a practical therapeutic vaccine for human use, more than one antigen is likely to be required and Rv2190c is clearly a candidate for inclusion. The greater effects in spleen than in lung suggest that the DNA vaccines were more active against bacteria in extra-pulmonary sites than in the lung, but whether this was due to differential effects on bacterial dissemination, growth inhibition or killing was not investigated. Although organ bacterial load is one of the important indicators to evaluate curative effects on animal TB experiments [21], evaluation of the impact upon pathology is also essential. Strikingly, the lesions in the lungs of rv2190c DNA and ag85a DNA vaccinated groups were similarly lessened, suggesting that rv2190c DNA and ag85a DNA provided similarly efficient immunotherapy for TB disease in this model.

Conclusions
We successfully constructed a MTB rv2190c DNA vaccine that could induce Th1-type cellular immune reactions in mice and had some immunotherapeutic effects on tuberculosis in mice. It may have potential for use in an immunotherapeutic DNA vaccine against TB.

Additional files

**Additional file 1: Figure S1.** Fragment sizes of restriction-enzyme-digested recombinant plasmid pVAX1-rv2190c. 1. PCR amplification product of the recombinant plasmid pVAX1-rv2190c colony; 2. The recombinant plasmid pVAX1-rv2190c digested by restriction endonuclease Nhe I and EcoR I; 3. Digest of the pVAX1 vector DNA with restriction endonuclease Nhe I and EcoR I; 4. The recombinant plasmid pVAX1-rv2190c DNA digested by restriction endonuclease EcoR I; 5. Digest of the pVAX1 Vector DNA with restriction endonuclease EcoR I; M: DM5000 DNA Marker. (TIF 45 kb)

**Additional file 2: Figure S2.** Expression and purification of recombinant Rv2190c protein as determined by SDS-PAGE electrophoresis. The gel was subjected to electrophoresis followed by Coomassie blue staining. Lane M, protein molecular weight marker. Lane 1, E. coli lysates engineered before isopropylthiogalactopyranoside (IPTG) induction. Lane 2, E. coli lysates engineered after induction with IPTG. Lane 3, the purified recombinant Rv2190c protein. (TIF 39 kb)

**Additional file 3: The Excel data file [FOLT] Figshare. [DOI:10.6084/m9.figshare.4668148 and https://figshare.com/s/2b4d6c228965735769bb6] includes all datasets supporting the conclusions of this article: IFN-γ in spleen lymphocyte culture supernatants, IL-4 in spleen lymphocyte culture supernatants, CD4+ T cell subsets expressing intracellular IFN-γ or IL-4, CFU in the lungs and spleens. (XLS 143 kb)

**Abbreviations**
CFUs: Colony-forming units; ELISA: Enzyme-linked immunosorbent assay; FACS: Fluorescence-activated-cell-sorting; IFN-γ: Interferon-gamma; IL-4: Interleukin-4; MDR-TB: Multi-drug-resistant tuberculosis; MTB: Mycobacterium tuberculosis; ORF: Open reading frame; PCR: Polymerase chain reaction; TB: Tuberculosis.

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**Availability of data and materials**
The data that support the findings of this study are included in the main manuscript and supplementary file.

**Authors’ contributions**
YL participated in designing and performing the experiments, writing the manuscript. XZ participated in the construction of rv2190c DNA vaccine, tested immunogenicity of rv2190c DNA vaccine and drafted the manuscript. XB carried out the preparation of recombinant Rv2190c protein. LX carried out the immunoscans. XW and LW participated in the animal experiments. JZ and YY carried out the statistical analysis. JS performed the lung histopathological examination. XW designed the study, directed the experiments and revised the manuscript. All authors approved this manuscript.

**Competing interests**
The authors declare that they have no competing interests.

**Consent for publication**
The authors have stated that it is not applicable.

**Ethics approval**
The study procedures were approved by the 309th Hospital of the Chinese PLA Research Animal Ethics Committees.

**Author details**
1. Army Tuberculosis Prevention and Control Key Laboratory, Beijing Key Laboratory of New Techniques of Tuberculosis Diagnosis and Treatment, Institute of Tuberculosis Research, the 309th Hospital of Chinese PLA, Beijing 100091, People’s Republic of China. 2. Zhengzhou Kingmed Center for Clinical Laboratory, Zhengzhou 450016, People’s Republic of China.

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