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T cell recall response of two hypothetical proteins (Rv2251 and Rv2721c) from *Mycobacterium tuberculosis* in healthy household contacts of TB — Possible subunit vaccine candidates

D. Santhi, Alamelu Raja*

Department of Immunology, National Institute for Research in Tuberculosis (ICMR), (Formerly Tuberculosis Research Centre), No.1, Mayor Sathyamoorthy Road, Chetpet, Chennai 600 031, India

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Summary The demonstrated variable efficacy of the only licensed TB vaccine *Mycobacterium bovis* bacillus Calmette—Guérin (*M. bovis* BCG) encourages the need for new vaccine candidates against TB. Antigen specific cellular immune response is often considered imperative during *Mycobacterium tuberculosis* (*M. tuberculosis*) infection and antigens that are strongly associated with the latent phase of infection are drawing increasing attention for anti-TB vaccine development. Here, we investigated the phenotypic and functional profiles of two novel mycobacterial antigens Rv2251 and Rv2721c during T cell recall response via multi-color flow cytometry. Healthy household contacts of TB (latent/HHC) and active pulmonary TB (PTB) patients were recruited to investigate the difference in antigen specific T cell recall response. These two antigens induced expansion of $CD45RA^{-}$ $CCR7^{+}$ central memory subtypes and $CD45RA^{-}$ $CCR7^{-}$ effector memory cells in latent population which suggests their possible association with HHC. Rv2251 and Rv2721c antigen specific IFN-γ, TNF-α and IL-2 response was also significantly high in HHC when compared to the PTB ($p < 0.005$, $p < 0.05$ and $p < 0.05$ respectively). The frequency of multifunctional T cells also was high in HHC compared to the PTB with statistical significance only for the antigen Rv2251. Often, the dominant Th1 immune response in HHC is correlated with the protection against the active TB disease. Collectively, we report the first insights into Rv2251 and Rv2721c antigen specific immune response in human donors of TB and provide the immunologic rationale for selecting them for vaccine development against TB.
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* Corresponding author. Fax: +91 (044) 2836 2528.
E-mail address: alamelur@nirt.res.in (A. Raja).

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Introduction

Despite the availability of the only licensed vaccine BCG, tuberculosis (TB) is still the leading microbial cause of death.[1] Though, the administration of BCG conferred protection against TB from its first oral administration in 1921, it later showed little or no protection during large field trials.[2] BCG might diminish the most severe forms of TB, such as TB meningitis in children. But, it has had varying impacts on the occurrence of pulmonary TB in adults, which represents the transmissible form of this disease. Hence, an improved vaccine as a replacement to BCG or a subunit boosting vaccine to administer after BCG vaccination is needed. Subunit vaccine approaches hold a number of advantages, like increased safety, stability and ability to boost prior BCG immunization.[3] Pools of proteins obtained from *Mycobacterium tuberculosis*, the causative agent of tuberculosis, are often screened for subunit vaccine development and several promising candidates have been targeted.

The role of $CD4^+$ Th1-cells in TB is best understood and many researchers concluded that anti-TB immunity is predominantly mediated by $CD4^+$ Th1 cells.[4,5] To date, many mycobacterial antigens have been screened for their ability to induce cellular immune responses with the goal of subunit vaccine application. These include Ag85 complex,[6] ESAT-6,[7] CFP-10,[8] Rv0577,[9] HspX[5] etc., but their T cell responses are not homogenous.[10] We hypothesize that, one of the reasons for variation in T cell response to these antigens might be due to the absence of specific MHC alleles amongst different ethnicities for presentation of these antigenic peptides. This hypothesis is well supported by our earlier published work that showed comparatively low population coverage and MHC binding for ESAT-6 and CFP-10 peptides relative to other screened antigens.[11] Speculations about the reasons for the differences in BCG efficacy also linked the role of genetic differences in different ethnicities.[12] In this light, we have selected two mycobacterial antigens, predicted to have high MHC binding affinity and high percentage of population coverage in lieu of immunodominant antigens ESAT-6 and CFP-10. We evaluated T cell immune responses with the aim of subunit vaccine targetings.

These two antigens are Rv2251 (Possible flavoprotein) and Rv2721c (Possible conserved transmembrane alanine and glycine rich protein). Rv2251 was predicted to be an outer membrane protein[13] and the Rv2721c was identified by mass spectrometry in the membrane fractions of *M. tuberculosis*.[14] In general, membrane localized proteins are targeted for the vaccination studies since they are readily available for immune processing within the host.[15] In the field of TB, we can consider latently infected individuals who remain healthy as protected against active TB disease due to their ability to control the infection, unlike active TB patients. Their cellular immune response would reflect the type of immunity responsible for their efficient disease control and serve as a good experimental model.[16] Thus, we are interested in evaluating these two antigens' specific T cell responses to make a better correlation either with protection or pathology. This human based *in vitro* study would minimize the variations with other existing animal based *in vitro* TB models. Our analysis is bringing the first insight into Rv2251 and Rv2721c antigens' specific immune response in human models.

Materials and methods

In vitro cloning of Rv2251 and Rv2721c, over-expression and purification

The recombinant plasmid encoding Rv2251 antigen was obtained by *in vitro* cloning.[11] The same methodology was adapted for cloning and purification of Rv2721c. Briefly, encoding gene sequences of Rv2721c were amplified from *M. tuberculosis* H37Rv genomic DNA with gene specific primers by Phusion High Fidelity DNA polymerase (New England Biolabs, MA). The amplified Rv2721c gene and pRSET-A plasmid DNA were digested with restriction enzymes Sac-I and Hind-III (NEB, MA). The digested gene and plasmid DNA were ligated by T4 DNA ligase (NEB, MA) and transformed into a cloning strain of *Escherichia coli* (*E. coli*) DH5α. Positive recombinants confirmed by DNA sequencing were then transformed into the *E. coli* BL21 (DE3) (Invitrogen, USA) strain for protein induction and purification. The purified Rv2721c antigen was used for *in vitro* blood stimulation along with recombinant Rv2251 antigen.

Study participants

Informed written consent was obtained from all the donors and the study was approved by an Institutional Ethical committee of the National Institute for Research in Tuberculosis, Chennai, India. Thirteen milliliters (mL) peripheral blood samples were collected from a total of thirty nine individuals. Among the thirty nine donors, twenty two were latently infected individuals in our case healthy household contacts (HHCs) of TB and seventeen had active pulmonary tuberculosis (PTB). The criteria for HHC recruitment was based on sharing the living shelter minimum for three months with at least one sputum positive active TB patients (index TB case) who are naive for anti-tubercular therapy. HHCs had more than ten hours per day of close contact with adult smear-positive PTB patients. They were identified by visiting the households of adult smear positive pulmonary TB patients, who were enrolled for treatment at the Government Thiruvatteeswarar Hospital of Thoracic Medicine, Otteri, Chennai, Tamil Nadu, India. HHCs had no active TB symptoms which were ruled out by chest X-ray and smear microscopy. PTB individuals were selected based on positive sputum microscopy results of three sputum samples.

The infection state of all the study participants was assessed by the Interferon gamma release assay (IGRA). No active TB symptoms were found in HHCs which was confirmed by negative sputum smear microscopy. PTB study participants who had symptoms of immunosuppressive disease such as HIV infection were excluded from this study.

Interferon gamma release assay (IGRA)

QuantiFERON-TB Gold In-Tube (QFT-GIT) kit (Cellestis, Qiagen) contains three pre-coated tubes coated with TB-

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