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Rv2204c, Rv0753c and Rv0009 antigens specific T cell responses in latent and active TB – a flow cytometry-based analysis

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ABSTRACT

High global prevalence of latent TB infection (LTBI) is a key challenge in distinguishing patients with active pulmonary TB (PTB) from those with LTBI. The functional profile of $CD4^+$ and $CD8^+$ T cell cytokines produced as a response to *Mycobacterium tuberculosis* antigens vary during the course of tuberculosis (TB) infection. We evaluated antigen-specific $CD4^+$ and $CD8^+$ T cell cytokine response after overnight *in vitro* stimulation of peripheral blood with mycobacterial antigens ESAT-6, CFP-10, Rv2204c, Rv0753c and Rv0009 by flow cytometry. A significantly higher frequency of antigen-specific $CD4^+$ or $CD8^+$ IFN-γ^+ T cells were found in LTBI than in PTB. Among all the antigens used, Rv2204c-specific $CD8^+$ IFN-γ^+ displayed the positivity of 72% and 24% in LTBI and PTB respectively. In contrast to IFN-γ, the frequencies of $CD4^+$ or $CD8^+$ secreting TNF-α^+ cells were significantly high in PTB compared to LTBI. $CD8^+$TNF-α^+ analysis showed 60% positivity in PTB and 13.6% positivity in LTBI against Rv0753c antigen stimulation. We also predicted Rv2204c specific $CD8^+$ T cells secreting IL-10 or IL-4 showed maximum differentiation between LTBI and PTB. In conclusion, altered expression of Rv2204c-specific $CD4^+$IFN-γ^+ and $CD8^+$IL-4^+ T cells in LTBI and PTB might be a useful biomarker to differentially diagnose LTBI and active TB.

1. Introduction

Identifying and treating latent TB (LTBI) cases are one of the crucial global strategies for successful TB control (World Health Organization, 2015). This is the primary concern in low TB endemic and developed countries like USA (American Thoracic Society/Centers for Disease Control and Prevention, 2005). Such recommendation is not implemented in high endemic developing countries like India, yet LTBI identification remains the high priority. Successful therapy of latent TB reduces the risk of re-activation of dormant bacteria and subsequent progression to active TB disease. In addition, early treatment might decrease the emergence of multidrug-resistant strains (Klopper et al., 2013).

New immune-based diagnostic method, interferon gamma release assay (IGRA) has been widely used in TB diagnosis since it shows higher sensitivity and specificity compared to tuberculin skin test (TST). In IGRA (TSPOT™.TB and QFT), IFN-γ is measured upon stimulating with Early Secreted Antigen Target (ESAT-6) and Culture Filtrate Protein (CFP-10) antigens, both present within the RD1 (Region of Deletion 1) genomic segment of *M. tuberculosis*. However, both TST and IGRA do not efficiently differentiate latent infection and active tuberculosis (Pai, 2010, 2015) that might lead to inappropriate/biased anti-TB regimens or chemoprophylaxis. Hence, developing efficient diagnostic methods to discriminate LTBI and active TB is of high priority.

Flow cytometry is not only a useful research tool but also valuable as a clinical test, due to its advantages over the presently available immune-based tests. It gives information on the numbers of cells producing a given cytokine and allows the phenotypic differentiation between antigen-specific lymphocyte subsets at various stages of TB infection (Sester et al., 2011). Nemeth suggested quantification of *M. tuberculosis*-specific cytokines derived from different T cells by flow cytometry is a promising new tool for immune-based diagnosis of LTBI (Nemeth et al., 2009). However, whether flow cytometry-based test is better than the TST and IGRA in the diagnosis of LTBI remains unclear and requires further investigations.

The potential role of distinct T cell subsets as biomarkers of active TB and/or LTBI has been studied in the past (Harari et al., 2006; Pantaleo et al., 2006; Chen et al., 2009; Rozot et al., 2013, 2015). These subsets were co-related with antigen load in chronic viral infections (Luarti Ruiz et al., 2006) and tuberculosis (TB) patients (Millington

Abbreviations: M. tuberculosis, Mycobacterium tuberculosis; LTBI, latent tuberculosis infection; TB, tuberculosis; TST, Tuberculin skin test; IGRA, Interferon-gamma release assay; HHC, Healthy household contacts; PTB, pulmonary tuberculosis; QFT-GIT, Quantiferon TB gold in-tube assay; PFT, polyfunctional T cells; PHA, phytohemagglutinin; RPMI, Rosewell Park Memorial Institute; ESAT-6, early secretory antigenic target-6; CFP-10, culture filtrate antigen

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et al., 2007). All $CD4^+$ or $CD8^+$ T subsets co-operate or interfere with each other to control the infection, and the dominant subset might differ between active and LTBI cases (Harari et al., 2006; Pantaleo et al., 2006; Rozot et al., 2013, 2015). One such T cell subset, multifunctional T cells that co-express all 3 cytokines (IFN-γ, TNF-α and IL-2) simultaneously, showed a more comprehensive diagnostic yield by flow cytometry than IGRA in detecting TB infection (Leung et al., 2009; Won and Park 2010) and have shown improved discrimination between active TB and LTBI (Sutherland et al., 2009; Biselli et al., 2010; Harari et al., 2011; Streitz et al., 2012). These different cytokine signatures have been proposed to be associated with disease stages, mycobacterial load or treatment (Caccamo et al., 2010). Therefore, determination of the T cell subsets/cytokine profiles at specific stages of infection, disease and recovery is critical for the development of new diagnostics strategies. Therefore, in this study, we evaluated flow cytometry-based functional characterization of different $CD4^+$ and $CD8^+$ T cells subsets for identifying a biomarker for LTBI and active TB discrimination.

To date, many studies have focused on the response to *M. tuberculosis* antigens such as ESAT-6, CFP-10 and Ag–85 B for TB diagnosis. However, their discriminatory potential is compromised in endemic settings. Therefore, identifying new antigens that are expressed at different phases of TB infection is important to develop antigen-based immuno-diagnostic tests (Zvi et al., 2008; Ottenhoff and Kaufmann, 2012).

Earlier, we have identified 7 proteins from culture filtrate fractions as having a potential to induce T cells *in vitro* and we termed them as "novel human T-cell antigens". For the present analysis, we have selected 3 antigens (Rv2204c, Rv0753c and Rv0009) as they showed very high ($p < 0.0005$) IFN-γ response in latent TB compared to active TB (Deenadayalan et al., 2010). Rv2204c is a hypothetical protein predicted to have a role in the pathogenesis of TB by interacting with host cell macrophages (Dosanjh et al., 2005). Functional genomics analysis had shown that Rv2204c was one of the highly expressed genes during the log phase of *M. tuberculosis* growth (Fu and Fu-Liu, 2007) and also predicted as up-regulated during thiol oxidative stress condition (Dosanjh et al., 2005). Nevertheless, no reports are available for the functional characterization and immunological role of Rv2204c during TB infection and addressed in the present study. The second antigen, Rv0753c is a secreted antigen, identified as a probable methyl malonate-semialdehyde dehydrogenase (MmsA) and predicted to be involved in reactivation of dormant *M. tuberculosis* to active replicative form (Kassa et al., 2012; Serra-Vidal et al., 2014). In addition, it also plays a crucial role in the activation of innate immune response and the initiation of the Th1 phenotype in the adaptive immune response (Kim et al., 2015). However, the immunological function of MmsA has not been studied in detail, particularly with respect to the biomarker for TB diagnosis. The third protein, Rv0009 (Peptidyl-prolyl cis-trans isomerases A or PpiA) considered being crucial for protein folding as it catalyses the inter-conversion of cis and trans peptide bonds. Rv0009 was reported as the weak B cell antigen (Weldingh et al., 2005) and predicted as dormancy associated gene in clinical *M. tuberculosis* strain based *in vitro* hypoxic model in our earlier study (Devasundaram et al., 2015). Thus, we are interested in evaluating the value of these three *M. tuberculosis*-secreted antigens in discrimination of LTBI and active TB.

2. Materials and methods

2.1. Study subjects

This study was approved by the Institutional Ethics Committee of National Institute for Research in Tuberculosis (NIRT), India. All the volunteers were informed about the study procedure and written consent was obtained. Adults (> 18 years) with active pulmonary TB and LTBI were recruited for this study. Individuals with previous history of TB, those who underwent anti-TB treatment or those under immunosuppressive therapy were excluded from the study.

2.1.1. Healthy household contacts: (HHC) (N = 22)

This study group was recruited from the families where there is at least one sputum positive pulmonary tuberculosis patient (PTB) (Index case) sharing the same quarters, for at least 3 months immediately preceding the start of treatment of the index case. They include parents, spouse, children or siblings who have > 10 h contact with active TB patients per day, and thus have a high probability of *M. tuberculosis* infection. These study subjects were identified by visiting the households of adult smear-positive PTB patients. The presence of latent TB infection (LTBI) in HHC was confirmed by Quantiferon TB Gold-in-tube assay (QFT-GIT) test (Cellestis, Qiagen, Venlo, Netherlands). Active TB disease among LTBI was ruled out by chest x-ray and smear microscopy. Further, none of the LTBI showed clinical symptoms of active TB. The male to female ratio is 12 and 10. After recruitment, subjects were followed up to 6 months and those who developed active TB during this period were also excluded from the analysis.

2.1.2. Pulmonary TB patients: (PTB) (N = 25)

This group comprised of adult patients who are recently diagnosed positive for *M. tuberculosis* sputum smear. The study subjects for this group were recruited from pulmonary TB patients enrolled for treatment at Government Thiruvotteeswarar Hospital of Thoracic Medicine (GTHTM), Otteri, Chennai, India. The male and female ratio is 15 and 10. The presence of active TB was confirmed by positive sputum smear microscopy (fluorescent microscopy-Auramine O phenol staining) and culture. As per WHO guidelines, we classified the PTB patients 3+, 2+, 1+ and scanty based on the number of bacilli observed. Amongst, 7 were 3+, 5 were 2+, 8 1+ and 5 patients showed scanty. All the PTB subjects were subjected to QFT-GIT.

2.2. Quantiferon TB gold-In tube (QFT-GIT) assay

The IGRA was performed using QFT-GIT kit, which contains 3 tubes. 1 mL of blood was added in each of the three tubes pre-coated with *M. tuberculosis* antigens for test sample tube, phytohemagglutinin (PHA) for positive control tube and no antigen or saline-coated tube for the negative control. The tubes were incubated for 16–24 h at 37 °C, 5% atmospheric CO_2 and plasma were collected after centrifugation. The collected supernatant was tested for IFN-γ levels according to the manufacturers' instructions. The test results were analyzed and interpreted using software supplied by the manufacturer (Cellestis, Qiagen, Venlo, Netherlands).

2.3. 2.3 In vitro antigen stimulation of whole blood

The proteins Rv2204c, Rv0753c and Rv0009 were cloned, overexpressed and purified by recombinant technology which was described earlier (Prabhavathi et al., 2015; Pathakumari et al., 2015a, 2015b). The standard recombinant proteins, ESAT-6 and CFP-10 were the kind gift from Colorado State University (CSU), Fort Collins, CO, USA. Endotoxin concentration in all recombinant protein preparation was quantified by LAL assay and ranged from 1 EU to 10 EU per mg of protein, which is acceptable for immunological assays (Coler et al., 2001). To minimize sample consumption, the blood was diluted to 1:1 with Rosewell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with glutamine (0.29 g/L), penicillin (100 IU/L) and streptomycin (0.1 mg/mL). The cultures were stimulated with recombinant proteins (ESAT-6, CFP-10, Rv2204c, Rv0753c and Rv0009) at a final concentration of 5 μg/mL along with the mitogen (PHA) as a positive control. Cells cultured under similar conditions without any stimulation served as a control. The culture medium was supplemented with the co-stimulatory antibodies, anti-CD28 and anti-CD49d (Becton Dickinson, San Jose, CA, USA) at a final concentration of 0.5 μg/mL and culture plate was incubated for 18 h at 37 °C, 5% CO_2. We added 1 μL of Brefeldin A (BD Biosciences, San Diego, CA, USA) per stimulation from the stock of 10 mg/mL, 16 h