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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

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, over-expressed 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

before the termination of the culture. After the incubation period, BD FACS lysing solution was added to the cell pellet and RBCs were lysed in order to remove RBC.

2.4. Cell markers immunostaining

After the RBC lysis, the cells were washed and the cell suspension was made with PBS. The cells were stained with T cell-specific surface markers (PerCP CD3, APC−Cy7 CD4 and PE-Cy7 CD8) and incubated for 30 mins at 4 °C. Following the surface staining, cells were washed with PBS, fixed and permeabilized with cytofix/cytoperm buffer (BD Biosciences, San Diego, CA, USA). After fixing, the cells were washed with Perm/Wash solution (BD Bioscience, San Diego, CA, USA), and then stained for intracellular Th1 (FITC IFN-γ, PE TNF-α, APC IL-2), Th2 (FITC IL-4, PE IL-10) and APC IL-12 cytokines. Cells without staining served as an unstained control. Stained cells were acquired in FACS Canto II flow cytometer (BD and Company, Cockeysville, MD, USA) and at least 100,000 cells in the lymphocyte gate were recorded. Percentage of cytokine-secreting $CD4^+$ and $CD8^+$ cells was analyzed using FlowJo software (Tree Star Inc., Ashland, Oregon, version 7.1.1). First $CD3^+$ lymphocytes were gated to analyse the expression of CD4 and CD8. The frequency of intracellular cytokines was performed on CD4+ and CD8+ population. To assess the quality of T-cell response, we analyzed all possible combinations of intracellular expression of Th1 cytokine-producing $CD4^+$ and $CD8^+$ T cells in subjects with active and latent TB. We followed Boolean gating strategy to assess the combination of IFN-γ, TNF-α, IL-2 cytokines. The positive boundaries were selected based on fluorescence-minus-one (FMO) controls for all the antibodies used. Compensation was calculated from fluorochrome-conjugated antibodies coupled with CompBeads (BD Biosciences). Background cytokine production from the control tube was subtracted from each stimulated condition.

2.5. Statistical analysis

All analysis was performed using GraphPad Prism 5 software (GraphPad Software 5.00, San Diego, CA, USA) for statistical analysis. Non-parametric Mann-Whitney *U* test was applied to compare T-cell frequencies and the percentage of cytokine-secreting cells between LTBI and PTB groups. We performed ROC and determined the cut-off value for each antigen-specific cytokine based on Youden' index (Cut-offs were derived at various sensitivities and specificities and at the maximum Youden's index (YI), i.e. sensitivity + specificity − 1). The value above the cut-off point was considered as positive in LTBI and PTB. A *p*-value < 0.05 was considered as statistically significant.

3. Results

3.1. Clinical characterization of study subjects

A total of 47 study subjects were recruited for this study. Among them, 22 were LTBI and 25 were PTB patients. All LTBIs were positive for QFT-GIT indicating they are infected with *M. tuberculosis*, they did not develop any active disease symptoms and hence in our study, we considered them as latently infected individuals. The demographic profile of recruited study subjects was depicted in Table 1.

3.2. Functional signature of antigen-specific $CD4^+$ and $CD8^+$ T cells in LTBI and PTB

In this study, we measured the percentage of antigen-specific $CD4^+$ and $CD8^+$ secreting cytokines in LTBI and PTB. We evaluated the multi-color flow cytometric data of antigen-specific $CD4^+$ and $CD8^+$T cell cytokine recall responses, such as IFN-γ, TNF-α, IL-2, IL-4, IL-10 and IL-12 after 18 h *in vitro* stimulation with ESAT-6, CFP-10, Rv2204c, Rv0753c and Rv0009 antigens. We first compared the expression of

Table 1
Demographic characteristics of study subjects enrolled in the study.

Category	Latent TB (LTBI)	Pulmonary tuberculosis (PTB)
Total number of Subjects (N)	22	25
Median age (range)	41 (19–55)	45 (22–60)
Sex:		
Male, N (%)	12 (54.6)	15 (60)
Female, N (%)	10 (45.4)	10 (40)
Positivity of smear test, N (%)	–	25 (100)
Smear grade 3+, N (%)	–	7 (28)
Smear grade 2+, N (%)	–	5 (20)
Smear grade 1+, N (%)	–	8 (32)
Scanty	–	5 (20)
QFT-GIT:		
Positives, N (%)	22 (100)	25 (100)
Negatives, N (%)	0 (0)	0(0)
Indeterminate, N (%)	0 (0)	0 (0)

N = Number of individuals; percentage (%) are indicated in brackets, QFT-GIT: Quantiferon TB gold in tube assay.

intracellular cytokines produced by $CD4^+$ and $CD8^+$ T cells from patients with active TB and LTBI subjects. The frequency of antigen-specific cytokines secreting $CD4^+$ and $CD8^+$ was higher in both LTBI and PTB compared to unstimulated cytokine response. The general gating strategy followed for selecting $CD4^+$ and $CD8^+$ T cells were shown in Fig. 1.

3.3. Flow cytometry analysis of $CD4^+$ and $CD8^+$ T cell cytokines at the single-cell level

3.3.1. IFN-γ^+ $CD4^+$ and $CD8^+$ T cells

The expression of $CD4^+$ T cells producing any of the 3 cytokines (IFN-γ or IL-2 or TNF-α) were assessed in LTBI and PTB after stimulation with *M. tuberculosis*-specific antigens. The percentage of IFN-γ producing $CD4^+$ T cells was high in LTBI for all antigenic stimulation, whereas no such response was observed in PTB (Fig. 2). The median levels and the range of $CD4^+$ secreting IFN-γ were shown in Fig. 2. Only significant results are presented. By ROC analysis, we determined the cut-off value for each antigen-specific cytokine based on Youden' index. The values above the cut-off were considered as positive in both the groups.

A significantly higher frequency of ESAT-6, Rv2204c and Rv0753c specific $CD4^+$ IFN-γ^+ T cells was found in LTBI than in PTB patients comparable to that of ESAT-6. Though both ESAT-6 and Rv2204c antigen showed same positivity in LTBI, positivity was lower in PTB against the test antigen Rv2204c. Therefore, Rv2204c antigen-specific $CD4^+$ IFN-γ^+ T cells could serve as a better biomarker, when compared to ESAT-6, for discrimination of LTBI and PTB (Table 2). Despite this higher $CD8^+$ IFN-γ^+ levels in LTBI, the difference between LTBI and PTB was significant only for ESAT-6 stimulation. Taken together, antigen-specific $CD4^+$ IFN-γ^+ T cells allow discrimination between LTBI and active TB in our settings/experiments.

3.3.2. TNF-α^+ $CD4^+$ & $CD8^+$ T cells

The frequencies of $CD4^+$ and $CD8^+$ secreting TNF-α^+ were high in PTB compared to LTBI with all antigens stimulation. The median percentage of $CD4^+$ and $CD8^+$ secreting TNF-α^+ cells was depicted in Fig. 3. Regarding $CD4^+$ TNF-α^+, statistical significance attained only for CFP-10, Rv2204c and Rv0753c antigens. (Fig. 2). The diagnostic potential of these antigens was shown in Table 2. Among the tested antigens, CFP-10 showed maximum positivity of 64% in PTB whereas it has shown 18% positivity in LTBI. Thus, in contrast to IFN-γ, antigen-specific $CD4^+$ secreting TNF-α are associating with active TB compared to LTBI. Similiar to $CD4^+$, the frequencies of $CD8^+$ T-cells producing TNF-α cytokine was higher in PTB when compared to LTBI (Fig. 3)
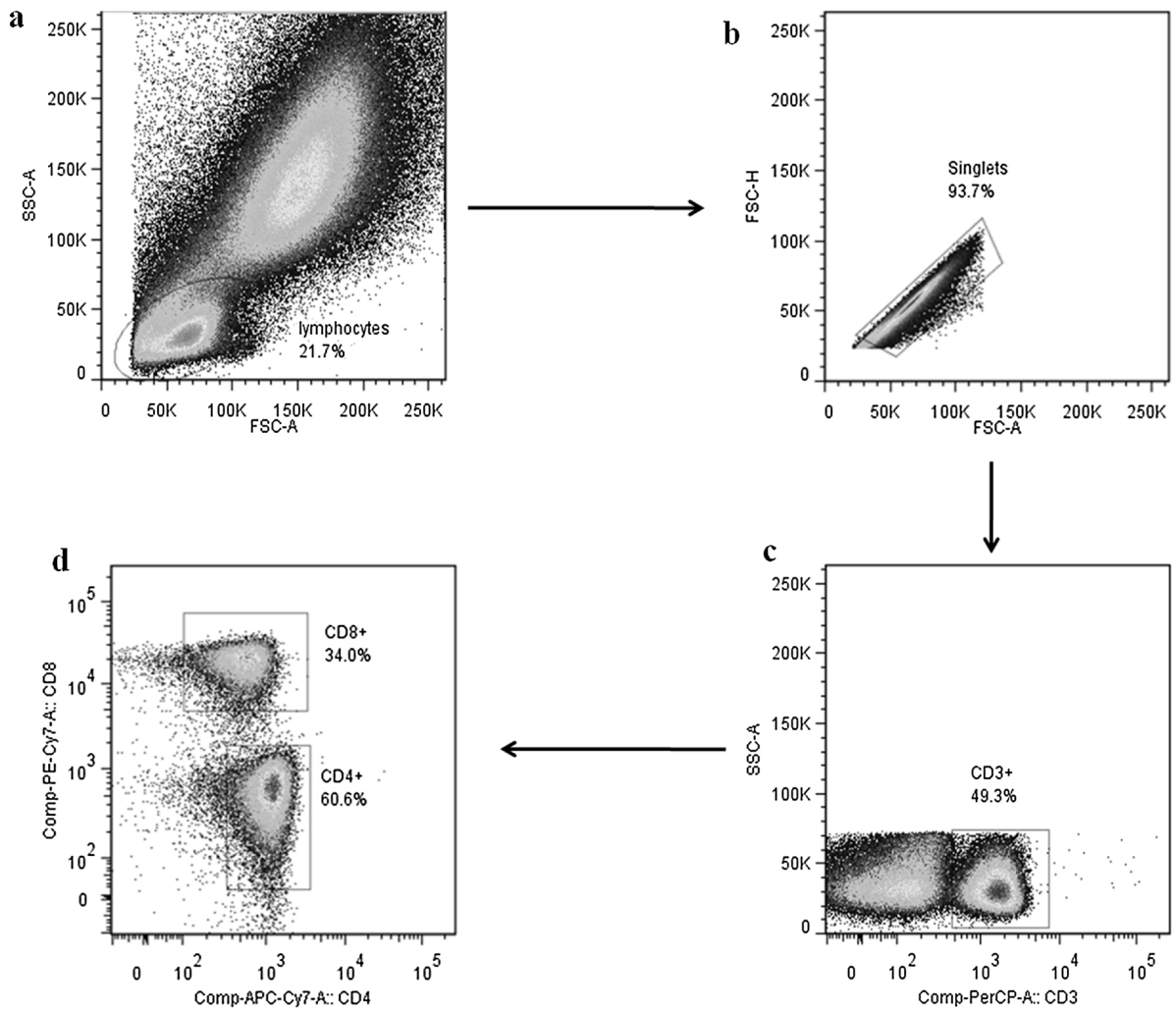
Fig. 1. Gating strategy of CD4$^+$ and CD8$^+$ T cells. Lymphocytes were gated based on size (forward scatter, FSC) and granularity (sideward scatter, SSC). Cell doublets were excluded using forward FSC-area and FSC-height parameters to gate singlets (b). From singlet cells, T-lymphocytes were selected by the expression of CD3 and from CD3$^+$ population (c), CD4 and CD8 population were selected (d).

Except for CFP-10 antigen stimulation, the frequency of all other antigen-specific CD8$^+$ TNF-α^+ T cells significantly differs between LTBI and PTB. The test antigen Rv0753c showed 60% and 13.6% positivity in PTB and LTBI respectively (Table 2). The standard antigen ESAT-6 showed 64% (16/25) positivity in PTB and 22.7% (5/22) positivity in LTBI (Table 2). This indicates the standard antigen ESAT-6 and test antigens showed almost similar positivity in LTBI and PTB for TNF-α. Overall, the phenotype CD4$^+$ CD8$^+$ TNF-α^+ T cells were associated

more with PTB compared to LTBI.

We also assessed the CD4$^+$ and CD8$^+$ cells producing IL-2 in LTBI and PTB upon overnight *in vitro* antigenic stimulation. Though all antigens showed high IL-2$^+$ T cells in LTBI compared to PTB, the difference was not statistically significant.

3.3.3. IL-4, IL-10 and IL-12 secreting CD4$^+$ and CD8$^+$ T cells

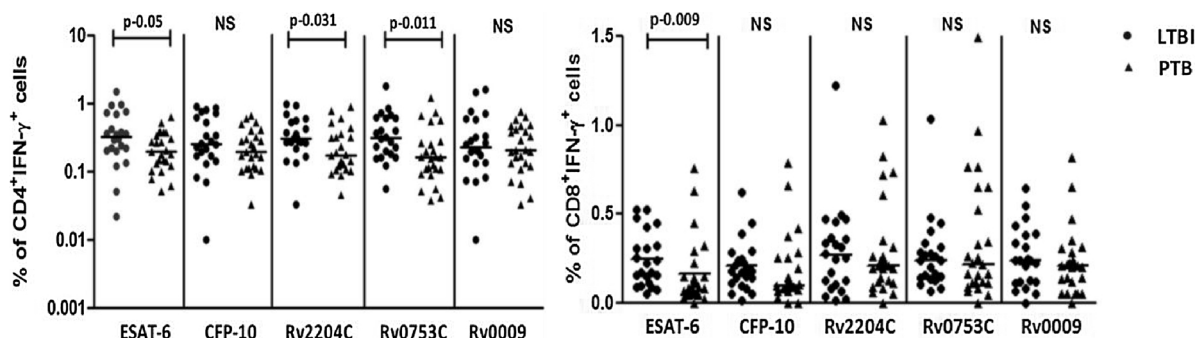In another set of analyses, we compared the frequency of antigen-

Fig. 2. Frequency of IFN-γ^+ CD4$^+$ and CD8$^+$ T cells in LTBI and PTB. Graphical summary of immunophenotypic analyses of 22 LTBI and 25 PTB subjects. The scatter plot represent percentage of IFN-γ cytokine secreting CD4$^+$ and CD8$^+$ T cells in LTBI and PTB. The phenotypes of CD4$^+$ and CD8$^+$ IFN-γ^+ T cells were associated with latent TB. Each dot represents an individual and horizontal line indicates median. Differences were compared using a Mann-Whitney *U* test. Statistical differences with *p*-values were shown. NS- Non Significant.

Table 2
Positivity for CD4 and CD8 secreting cytokines in LTBI and PTB.

Phenotype	Antigens	p Value	AUC	CI 95%	Cut-off	% Positivity in LTBI (22)	% Positivity in PTB (25)
CD4$^+$ IFN-γ$^+$	ESAT-6	0.05	0.66	0.506–0.824	0.1985	81.8 (18)	48 (12)
	Rv2204c	0.031	0.683	0.527–0.839	0.2050	81.8 (18)	40 (10)
	Rv0753c	0.011	0.716	0.568–0.864	0.174	77.2 (17)	40 (10)
CD8$^+$ IFN-γ$^+$	ESAT-6	0.009	0.72	0.571–0.870	0.152	72.7 (16)	24 (6)
CD4$^+$TNF-α$^+$	CFP-10	0.007	0.727	0.578–0.875	0.3655	18.1 (4)	64 (16)
	Rv2204c	0.006	0.732	0.589–0.876	0.397	27.2 (6)	64 (16)
	Rv0753c	0.002	0.758	0.615–0.900	0.198	45.4 (10)	96 (24)
CD8$^+$TNF-α$^+$	ESAT-6	0.012	0.712	0.565–0.860	0.341	22.7 (5)	64 (16)
	Rv2204c	0.004	0.741	0.601–0.882	0.476	18.1 (4)	60 (15)
	Rv0753c	0.004	0.74	0.597–0.882	0.530	13.6 (3)	60 (15)
	Rv0009	0.013	0.71	0.560–0.861	0.395	22.7 (5)	68 (17)
CD4$^+$IL-4$^+$	ESAT-6	0.0016	0.769	0.634–0.903	0.163	50 (11)	96 (24)
	Rv2204c	0.0035	0.749	0.606–0.891	0.2905	22.7 (5)	72 (18)
CD8$^+$IL-4$^+$	ESAT-6	0.016	0.70	0.556–0.854	0.160	50 (11)	80 (20)
	CFP-10	0.017	0.701	0.548–0.855	0.215	22.7 (5)	68 (17)
	Rv2204c	0.0012	0.776	0.632–0.920	0.289	9 (2)	72 (18)
	Rv0009	0.008	0.723	0.578–0.868	0.170	36.3 (8)	72 (18)
CD8$^+$ IL-10$^+$	Rv2204c	0.022	0.69	0.537–0.851	0.428	36.3 (8)	84 (21)

specific CD4$^+$ secreting IL-4, IL-10 and IL-12 in LTBI and PTB. The median levels of CD4$^+$ and CD8$^+$ IL-4$^+$ T cells were high in PTB compared to LTBI for all antigen stimulations (Fig. 4) and only significant results are presented. Compared to CD4$^+$, CD8$^+$ secreting IL-4$^+$ T cells were highly responded to all the antigens. The significant difference in the frequency of CD8$^+$ IL-4$^+$ was higher in response to Rv2204c followed by Rv009 and standard antigens (Table 2). The test antigen Rv2204c showed positivity of 72% (18/25) in PTB and 9% (2/22) in LTBI. This suggests that CD8 T cells could be the major cell population in secreting IL-4 in PTB. Our analysis predicted that Rv2204c specific CD8$^+$ IL-4$^+$ T cells could serve as a biomarker in discriminating LTBI and PTB when compared to standard and other test antigens selected for this study.

With respect to antigen-specific CD4$^+$ IL-10$^+$ response, the minimal difference was observed between LTBI and PTB with no statistical significance in the difference (data not shown). The only antigen that showed a significant difference between LTBI and PTB, when CD8$^+$IL-10$^+$ levels were considered, was Rv2204 (Fig. 5). This antigen exhibited 84% (21/25) positivity in PTB but also showed substantial positivity of 36% in LTBI (Table 2). Regarding CD4$^+$ IL-12$^+$ or CD8$^+$ IL-12$^+$ T cells, the proportion of antigen-specific CD8$^+$ IL-12$^+$ T cells was higher in LTBI than PTB, but no statistically significant differences were derived for any of the antigen stimulations used (Data not shown). Altogether, the frequency of antigen-specific CD4$^+$ IL-4$^+$ and CD8$^+$ secreting IL-4 or IL-10 T-cells at the single-cell level distinguishes LTBI from PTB.

3.3.4. Multi-functional cytokine analysis of CD4$^+$ and CD8$^+$ T cells

To further characterize the quality of T-cell response, we analyzed all possible combinations of intracellular expression of IFN-γ, IL-2 and TNF-α in cytokine-producing CD4$^+$ and CD8$^+$ T cells in subjects with active and latent TB. We followed Boolean gating strategy to assess the combination of IFN-γ, TNF-α, IL-2 cytokines (Fig. 6). The cytokine profile revealed that, in both LTBI and PTB, the proportion of T cells producing the dual cytokines simultaneously was greater compared to triple-cytokine-producing T cells. There was an increase in the expression of dual cytokine-secreting T cells in PTB compared to LTBI. Among the dual secreting T cells, CD8$^+$ TNF-α$^+$ IL-2$^+$ T cells were the most prominent dual positive T-cell subset observed in PTB. The proportion of CD8$^+$ TNF-α$^+$ IL-2$^+$ T cells was higher in PTB than LTBI with a significant difference for ESAT-6, CFP-10 and Rv2204c stimulation (Fig. 7). The discrimination potential of these antigen-specific functional phenotype was shown in Table 3. Taken together these finding suggests that these antigen-specific dual secreting CD8$^+$ TNF-α$^+$ IL-2$^+$ T-cells allowed the better discrimination of LTBI and PTB when compared to other cytokine combinations.

Interestingly, there was a slight increase in the dual cytokine IFN-γ$^+$ and IL-2$^+$ CD4$^+$ T cells in LTBI compared to PTB, whereas CD8$^+$ T cells showed a reversed pattern. Similar to CD4$^+$, CD8$^+$ T cells producing both IFN-γ and TNF-α were slightly higher in PTB than LTBI, but there was no statistical significance was derived. Hence, except TNF-α$^+$ IL-2$^+$, the other dual positive (IFN-γ$^+$ IL-2$^+$, IFN-γ$^+$ TNF-α$^+$) and triple positive (IFN-γ$^+$ IL-2$^+$ TNF-α$^+$) secreting CD4 and CD8 phenotypes did not show the significant statistical difference (data not shown).

Overall, the above results showed that single cytokine-producing CD4$^+$ and CD8$^+$ T-cell subsets (either IFN-γ$^+$, TNF-α$^+$ and IL-4$^+$ depending on the stimulus) followed by double producing CD4$^+$ and particularly CD8$^+$ (TNF-α$^+$IL-2$^+$) T cells were the prominent
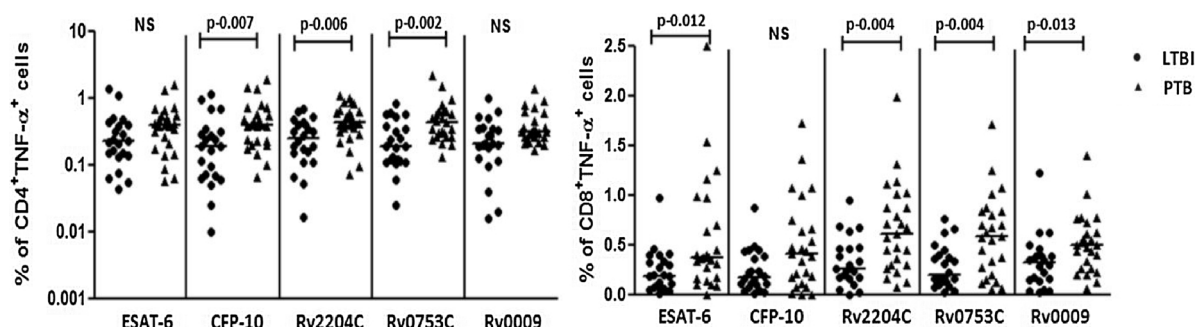
Fig. 3. Frequency of TNF-α$^+$ CD4$^+$ & CD8$^+$ T cells in LTBI and PTB. The scatter plot represents percentage of antigen specific TNF-α cytokine secreting CD4$^+$ and CD8$^+$ T cells in LTBI and PTB. Horizontal line indicates median. Differences were compared using a Mann-Whitney *U* test. Statistical differences with *p*-values were shown. NS- Non Significant.
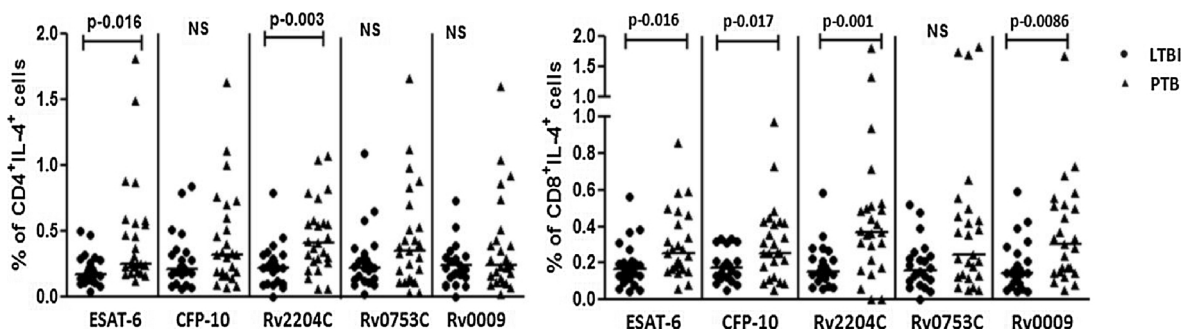
Fig. 4. Percentage of IL-4 secreting CD4^{+} and CD8^{+} T cells. Expression of antigen specific IL-4 levels in LTBI and PTB. The scatter plot represent percentage of cytokine secreting CD4^{+} and CD8^{+} T cells in LTBI and PTB. The phenotypes of IL-4^{+}CD8^{+} T cells were associated with active TB. Horizontal line indicates median. Significant p values are reported. NS- Non Significant.

Fig. 5. Percentage of IL-10 secreting CD8^{+} T cells. The scatter plot represents frequency of IL-10 cytokine producing CD8^{+} T cells in LTBI and PTB. Horizontal line indicates median. Differences were compared using a Mann-Whitney *U* test. Statistical differences with *p*-values were shown. NS- Non Significant.
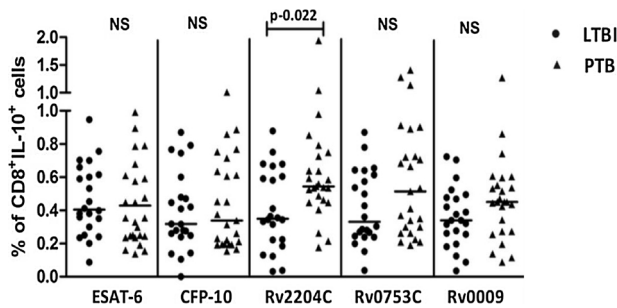
phenotypes that were differentially expressed in LTBI and PTB.

4. Discussion

The functionality of *M. tuberculosis*-specific CD4^{+} and CD8^{+} T cells are differentially impacted by disease stage, mycobacterial load, and treatment, (Millington et al., 2007; Casey et al., 2010) suggesting that certain subsets might serve as biomarkers of disease activity, pathogen burden, or treatment response. Therefore, in the current study, we used flow cytometry to characterize *M. tuberculosis* antigen-specific T cells in subjects with LTBI and active TB disease. We also tested the hypothesis that different cytokine profiles of *M. tuberculosis*-specific T cells might discriminate latent and active TB. In our previous study, the antigens Rv2204c, Rv0753c and Rv0009 specific cytokines showed a promising diagnostic marker for LTBI infection (Pathakumari et al., 2015a, 2015b). In this study, we evaluated the antigen-specific functional
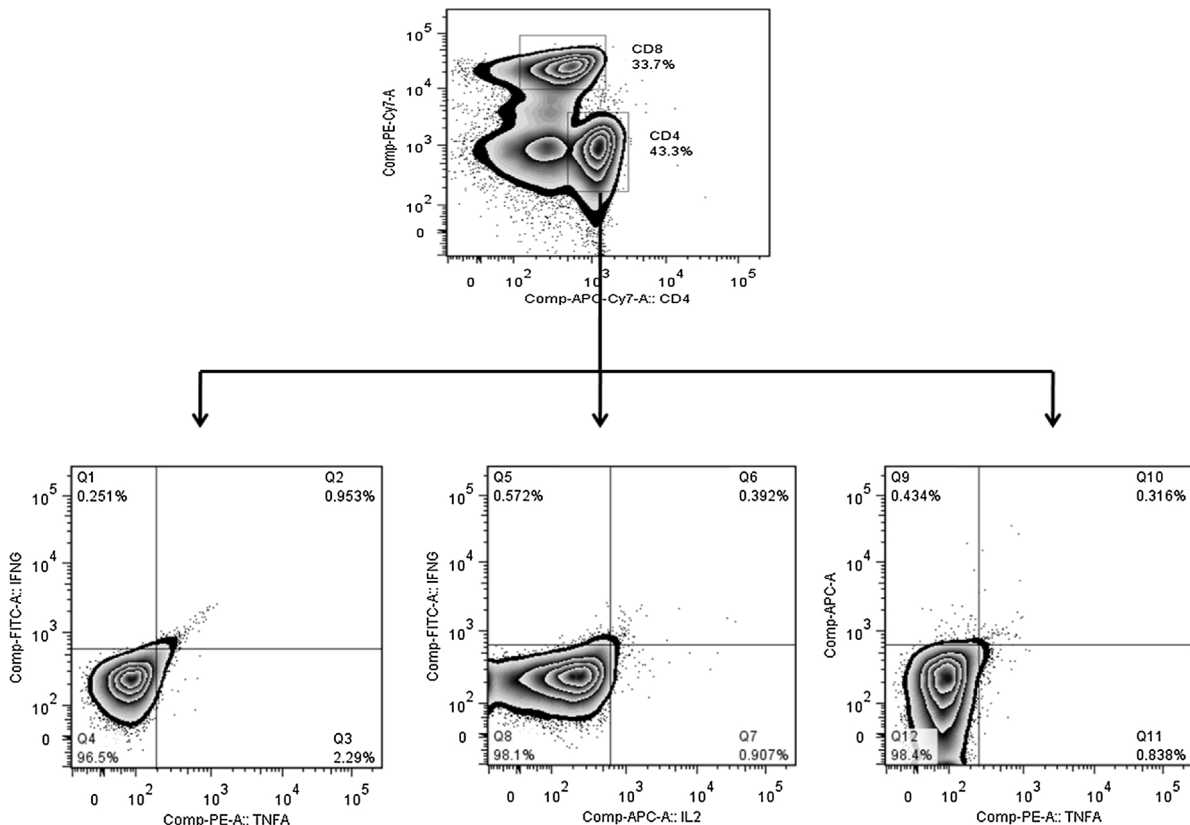
Fig. 6. Multi-functional Cytokine Analysis of CD4^{+} and CD8^{+} T Cells. The frequency of intracellular cytokines was performed on CD4^{+} and CD8^{+} population. We followed Boolean gating strategy to assess the combination of IFN-γ, TNF-α, IL-2 cytokines. This representative figure shows that the combination of IFN-γ TNF-α, IFN-γ IL-2 and TNF-α IL-2 secreted CD4^{+} population. The same gating strategy was also followed for CD8^{+} population.

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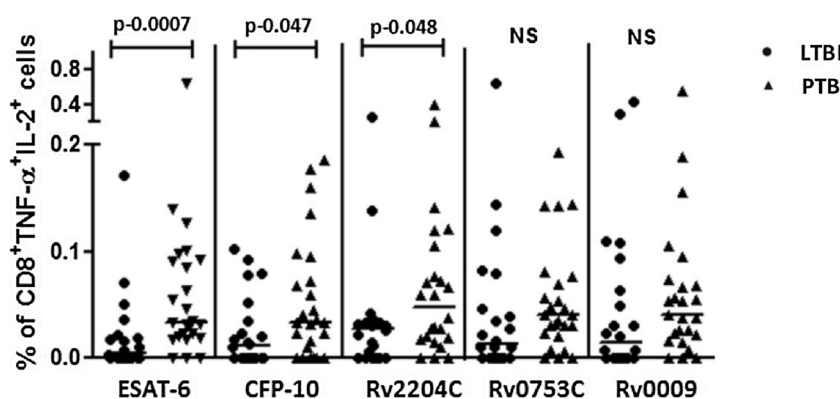
Fig. 7. Frequency of $CD8^+$ TNF-α^+ IL-2^+ T cells in LTBI and PTB. Frequency of antigen specific $CD8^+$ TNF-α^+ IL-2^+ T cells in LTBI and PTB. The horizontal line indicates median and each dots represents in the plot shows the frequency of $CD8^+$ TNF-α^+ IL-2^+ T-cells for single subject. Comparisons of two groups were done using a Mann-Whitney U test. The numbers above the horizontal bars represent the significant p values are reported. NS – not significant (p > 0.05).

profile of $CD4^+$ and $CD8^+$ T cell response to these *M. tuberculosis* antigens. The differences in the cytokine profiles between active disease and latent infection were expressed as a frequency of cytokine-producing $CD4^+$ and $CD8^+$ T cells.

Upon antigen stimulation, we identified higher $CD4^+$ and $CD8^+$ IFN-γ^+ response in LTBI compared to PTB. Few *M. tuberculosis* antigen-specific T cells are recruited and often retained in the lung tissues of TB patients due to the active replication of bacilli and hence these antigens specific cells are low in circulation and could be used to discriminate the disease state (Nemeth et al., 2009). Interestingly, CD4 secreting IFN-γ^+ T cells exhibited a significant difference between LTBI and PTB by the test antigens such as Rv2204c and Rv0753c. On the other hand, the phenotype $CD8^+$ IFN-γ^+ T cells showed significant difference only with a standard antigen such as ESAT-6. Thus, test antigens' specific $CD4^+$ secreting IFN-γ^+ T cells and standard antigens' specific $CD8^+$ secreting IFN-γ^+ T cells might be a useful antigen-biomarker combination to differentiate LTBI and PTB.

In the present study, both CD4 and CD8 TNF-α was significantly high in PTB and Rv0753c antigen showed the highest positivity in PTB, whereas Rv2204c antigen showed the least positivity in LTBI. Thus, the opposite trends of these two antigens could be used in combination for the differential diagnosis of active TB from LTBI. Our results showed a substantial increase in the proportion of TNF-α^+ $CD4^+$ or $CD8^+$ T cells in subjects with active disease, and this parameter might be the strongest predictor for diagnosis of active disease. In agreement with the present study, earlier studies also reported the association of TNF-α^+ level with active TB and this phenotype could be widely used in the differential diagnosis of latent TB and active TB (Sutherland et al., 2009; Harari et al., 2011; Streitz et al., 2012). The population of $CD4^+$ and $CD8^+$ T cells producing TNF-α expanded under inflammatory conditions of high mycobacterial load that might be a component of active TB (Harari et al., 2011).

IL-2 is a T-cell growth factor that promotes proliferation and differentiation of antigen-specific T cells. We observed the frequencies of IL-2 secreting T cells higher in LTBI than in those with active disease. However, none of the antigens used here showed a significant difference between LTBI and PTB. The IL-2 response in the present study is consistent with an earlier study where IL-2 levels were observed at equal levels in LTBI compared to active TB (Wilkinson and Wilkinson, 2010).

In this study, we showed that PTB patients have increased capacity

for IL-4 production by circulating T lymphocytes. It is unclear whether increased production of IL-4 active in TB causes, or merely reflects the severity of the disease. Lucey and co-workers reported that IL-4 might reduce the killing of mycobacteria by phagocytes through inhibition of IFN-γ production (Lucey et al., 1996). Therefore, IL-4 has the ability to suppress the protective inflammatory response by inhibiting production of Th1 cytokines. We reported, compared to CD4, CD8 secreting IL-4 might be a better marker in the differential diagnosis of active TB and LTBI. Similar to IL-4, IL-10 was also primarily secreted in PTB compared to LTBI. Earlier studies suggested that the increased IL-10 levels in active TB regulates the production of inflammatory cytokines and causes deleterious effects to the patient during active disease (Harris et al., 2007; Redford et al., 2010). In the present study, the only antigen, Rv2204c specific $CD8^+$ IL-10^+ T cells displayed the significant difference between LTBI and PTB with the maximum positivity of 84% in PTB and 36.3% positivity in LTBI. Taken together, we demonstrated the functional profile of CD8 secreting IL4 and IL-10 might be useful for better discrimination of LTBI and active TB.

Polyfunctional T cells have a memory function with proliferative capacity and ability to control the replication of *M. tuberculosis* (Day et al., 2011; Kannanganat et al., 2007). Further, it has been suggested that combined analyses of different cytokines produced by multi-functional T cells might aid discrimination of LTBI and active TB (Harari et al., 2011; Streitz et al., 2011, 2012). In the current study, except IFN-γ^+ and IL-2^+ other dual secreting $CD4^+$ and $CD8^+$ T cells were high in PTB compared to LTBI. However, among the dual secreting $CD4^+$ and $CD8^+$ T cells, except TNF-α^+ IL-2^+, none of the other phenotype combinations showed a significant difference between LTBI and PTB. Further, compared to dual secreting $CD4^+$ T cells, antigen-specific $CD8^+$ T cells were predominant in LTBI and PTB. We observed, compared to test antigens ESAT-6 specific $CD8^+$ TNF-α^+ IL-2^+ T secreting might be more associated with active TB. This result suggests that bacterial burden or antigen load may have a quantitative relationship with the frequency of cytokine (TNF-α^+IL-2^+) response of T cells to *M. tuberculosis* antigens. The frequency of $CD4^+$ secreting IFN-γ and IL–2 T cells were slightly high in LTBI, as observed in other studies showing that this phenotype might be correlated protection against TB (Darrah et al., 2007; Abel et al., 2010). However, in our study, this phenotype did not attain significant difference between LTBI and PTB. In line with our study, an increasing proportion of $CD4^+$ T cells that produce both IL-2 and IFN-γ were found in LTBI compared to active TB (Sester et al.,

Table 3
Positivity for $CD4^+$ and $CD8^+$ dual secreting TNF-α^+IL-2^+ in LTBI and PTB.

Phenotype	Antigens	p Value	AUC	CI 95%	Cut-off	% Positivity in LTBI (22)	% Positivity in PTB (25)
CD4	ESAT-6	0.028	0.686	0.525–0.847	0.0160	50 (11)	96 (24)
CD8	ESAT-6	0.00079	0.786	0.648–0.924	0.0165	31.8 (7)	88 (22)
	CFP-10	0.047	0.669	0.512–0.825	0.023	27.2 (6)	64 (16)
	Rv2204c	0.048	0.668	0.509–0.826	0.044	9 (2)	52 (13)

2011; Casey et al., 2010).

Earlier studies proposed that increased proportions of multifunctional T cells in LTBI subjects correlate with the certain level of protection against disease development, while a decreased frequency was expected in those individuals who developed the disease (Caccamo et al., 2010). However, in this study, most of the antigens exhibited an increased frequency of multifunctional T cells in patients with active TB disease and lower levels in LTBI subjects. In line with our observations, another study in the Gambia also demonstrated that TB cases had significantly higher levels of multifunctional CD4$^+$ T cells compared to LTBI (Sutherland et al., 2009).

There are potential contributing factors to increasing the T cell function in TB disease does not equate to protection from disease progression. These include an increase in regulatory T cells in active TB disease (Guyot-Revol et al., 2006), changes in the ratio of Th1/Th2 cytokines (Morosini et al., 2005) and changes in production of other cytokines such as TNF-α and IL-17 (Cooper et al., 2008). In this regard, we observed a significant increase in CD4$^+$ and CD8$^+$ T cells producing TNF-α and the decrease in IL-2 levels in active TB, which may contribute to disease progression, despite the increase in the proportion of multifunctional T cells.

One of the limitations of our study is lack of healthy control group and the small sample size. Since our primary goal is to analyse, whether the selected antigens specific immune response is useful in differential diagnosis of LTBI and PTB, the healthy control group was not recruited at this level. In endemic regions like India, the majority are exposed to non-tubercle mycobacteria (0.7–34%) (Myneedu et al., 2013) or BCG vaccinated (70%) and it is highly necessary to exclude healthy controls of these categories. This requires screening of larger samples in order to reach the desired study subjects and requires additional screening tests to differentiate healthy controls from healthy TB contacts that is a time-consuming process and requires sufficient funding and support. Due to these limitations, the current study was restricted to LTBI and PTB to evaluate the selected novel antigens for discriminating latent and active TB. Efforts are being taken to receive funding to complete additional objectives related to the current results and in very near future, we will be able to communicate.

Recruiting larger samples is required to derive a meaningful conclusion to use these antigens for differential diagnosis of latent and active TB. With the available facility and given contract period with the hospital, we were able to collect 60 samples but in few samples, cell number was not sufficient and results of other screening tests were missing and this led to compromise our sample size. Though a small number of subjects were evaluated, our preliminary data indicate that the functional analysis of specific CD4$^+$ and CD8$^+$ responses to novel antigens and standard antigens might contribute to the characterization of the immune profile of LTBI and active TB patients. Additional studies are highly recommended for validating our observation in larger study populations for using this functional T cell response for the development of diagnostic algorithms. Further, future prospective studies are required to follow up LTBI subjects to estimate their risk of progression to active TB by measuring these antigen-specific functional T cell responses. This could be useful to define who might need preventive therapy in high endemic settings.

5. Conclusion

Compared to multifunctional T cells, single cytokine producing CD4 and CD8 T cells might be useful in discrimination of LTBI and active TB. Our results clearly demonstrated, altered expression of Rv2204c-specific CD4$^+$ IFN-γ$^+$ and CD8$^+$ IL-4$^+$ T cells in LTBI and active TB. Thus, these two phenotypes might be useful in the differential diagnosis of LTBI and active TB. However, additional studies are required to validate our observation in larger study populations for using these markers for differential diagnosis of latent and active TB.

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