

Loss of Cellular Immune Response against Shared Mycobacterial Antigens is Associated with Active Pulmonary Tuberculosis in Adults

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Abstract

Background: Devising strategies for prevention of pulmonary tuberculosis is critical to halt onward transmission of tuberculosis. Since BCG has been shown to be ineffective in preventing pulmonary tuberculosis, studies for determining protective mechanisms are warranted for effective vaccine designing. We conducted a study in latently infected healthy individuals and healthy contacts of pulmonary tuberculosis for understanding protective immune responses against adult pulmonary tuberculosis caused by reactivation and re-infection, respectively.

Methods: We enrolled healthy latently infected individuals (LTB, n=22), household contacts (HCTB, n=16) of pulmonary tuberculosis patients and sputum positive pulmonary TB patients (ATB, n=19). Latent infection was determined by IFN- γ release assay against ESAT-6 and CFP-10. Cytokines secreted against shared and *M. tuberculosis* specific antigens were determined by IFN- γ ELISPOT and multiplex assays. *Mycobacteria* specific T cells were identified using MHC-I restricted ESAT-6 and Ag85B tetramers. Expression of different chemokine receptors associated with Th1 and Th17 responses was determined by flow cytometry.

Results: LTB group showed highest IFN- γ response and significantly higher response against 38 kDa and Ag85C than ATB group. IFN- γ responses against ESAT-6 correlated negatively with those against 38 kDa in HCTB group ($r=-0.51$, $p=0.021$). Levels of IL-17A, IL-17F and IL-6 were highest in HCTB group against 38 kDa. ATB group showed significantly lower ratios of Ag85B to ESAT-6 tetramer positive CD8⁺ T cells as compared to LTB group. ATB patients had lower frequencies of T cells expressing CCR5 and CCR6 than LTB and HCTB groups, respectively, indicating loss of Th1 and Th17 immune responses.

Conclusion: The study suggested a role of Th1 and Th17 responses in mediating protection against reactivation versus re-infection type of adult pulmonary TB, respectively. It also highlighted importance of shared mycobacterial antigens like 38 kDa and Ag85 in mediating these protective responses indicating their role in effective TB vaccine designing.

Keywords: Pulmonary tuberculosis; Latent infection; Household contacts; Re-infection; Reactivation; Immune responses; Shared antigens

Introduction

Tuberculosis (TB) is a global health problem with around 10.4 million new TB cases and 1.4 million deaths all over the world as reported in 2015 [1]. These TB cases represent only of a minority of individuals among almost one-third of the global population reported to be infected with *Mycobacterium tuberculosis* [2]. Evidence of the infection determined by tuberculin skin test has also been shown only in 30% of the exposed individuals [3] indicating the presence of protective immune mechanisms in the majority of the exposed or infected individuals which help them to control the infection. Active tuberculosis in adults has been thought to occur either by reactivation

of endogenous mycobacteria or re-infection by exogenous mycobacteria [4]. Although initially endogenous reactivation was thought to be the main cause of recurrent tuberculosis in adults, many of the studies done using DNA fingerprinting methodologies stressed the significant contribution of exogenous re-infection to the total tuberculosis burden in areas of high endemicity [5,6] indicating need for devising preventive strategies even against re-infection type of tuberculosis. Hence immune responses seen in healthy individuals with latent tuberculosis and in contacts of the open tuberculosis cases are crucial to infer correlates of protection against reactivation and re-infection types of tuberculosis, respectively.

Th1 and Th17 responses have been shown to mediate of protection in mouse models and in human tuberculosis infection [7]. Deficiency of interferon-gamma (IFN- γ) has been associated with heightened susceptibility to tuberculosis [8,9] and anti IFN- γ or tumor necrosis

factor-alpha (TNF- γ) treatments in latently infected mice have been shown to result in an increase in bacterial count and reactivation of latent tuberculosis [10,11]. Th17 response, an important protective barrier at the mucosal surfaces, has been shown to be critical in controlling tuberculosis disease [12]. During re-infection, there are newly formed granulomas as against the pre-existing chronic granulomas present in latent TB infection [13]. IL-17 has been

suggested to play role in early stages of granuloma formation [14] and Th17 responses have been shown to precede a strong Th1 response in the lungs during tuberculosis infection in mice [15] suggesting its protective role early after exposure. Expression of chemokines receptors like CCR5 and CXCR3 representing Th1 immune response [16], and CCR6, a functional marker of Th17 cells [17] is also crucial in determining protective immune responses against tuberculosis [18].

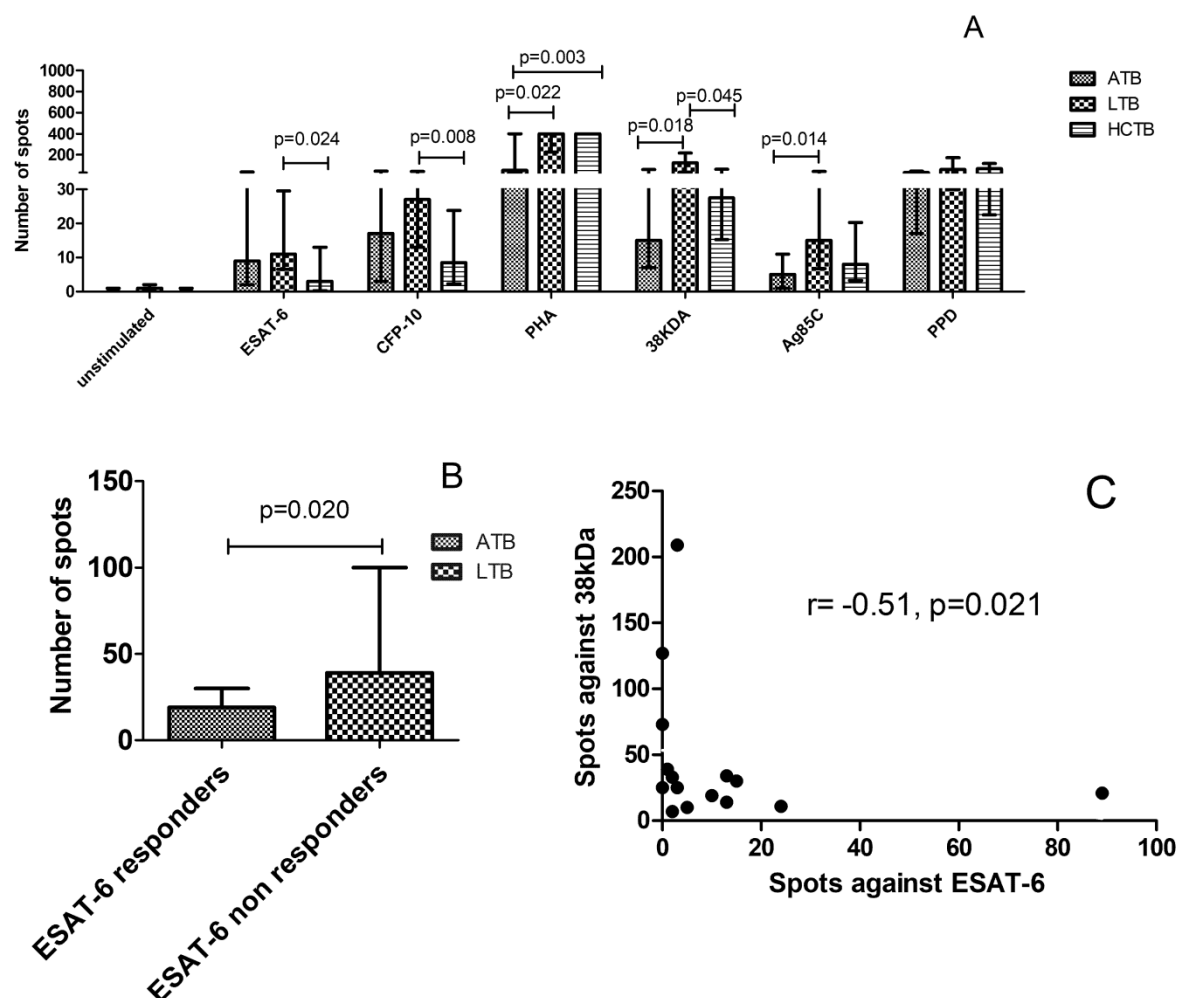


Figure 1: IFN- γ secretory response by ELISPOT assay. (A) Shows number of spots (Y axis) in IFN- γ secretory ELISPOT assay against different mycobacterial antigens (X axis). (B) Shows number of spots (Y axis) in IFN- γ secretory ELISPOT assay against 38 kDa antigen by ESAT-6 responders and non-responders (X axis). ESAT-6 responders had shown more than 5 spots in the IFN- γ secretory ELISPOT assay. The bars represent medians and error bars indicate interquartile ranges for the values. P values showing significant differences ($p < 0.05$) between the groups as calculated by Mann Whitney test are shown in the figure. (C) Shows correlation of magnitude of IFN- γ secretory response against ESAT-6 with that against 38 kDa antigen. The correlation coefficient (r) and p value as assessed by Spearman test are mentioned in the figure.

Identification of antigens mediating protective immune responses is important for designing effective vaccine candidates. *M. tuberculosis* specific antigens like early secreted antigen target-6 kilodalton (kDa)/ ESAT-6, culture filtrate protein-10 kDa (CFP-10), as well as shared mycobacterial antigens like 85 complex/Ag85C, 38 kDa are considered to be important in inducing protective immune responses against tuberculosis and are being evaluated as vaccine candidates [19]. Hence, we used these antigens to assess cellular immune responses in healthy

individuals with latent tuberculosis and those with recent exposure to *M. tuberculosis* to understand their role in control of reactivation and re-infection types of tuberculosis, respectively. We hypothesized that immune responses mediating protection from reactivation versus re-infection types of tuberculosis would differ and Th17 response would be important in mediating protection against re-infection type of tuberculosis as it involves exposure to the bacteria through mucosal surfaces.

Parameter	ATB (n=19)	LTB (n=22)	HCTB (n=16)
Age-Years, Median (Range)	29 (18-65)	35 (19-45)	41.5 (20-60)
Gender- Male: female	13: 6	16: 6	5: 11
No. of IGRA responders to ESAT-6 and CFP-10 (%)	16 (84)	22 (100)	10 (62.5)
CxR Abnormality-Number (lung involvement)	9 (mild-moderate), 10 (severe)	No abnormality detected	No abnormality detected
Sputum positivity-Number (Grade)	13 (1+), 4 (2+), 2 (3+)	Not indicated	Not indicated

Table 1: Characteristics of the study participants.

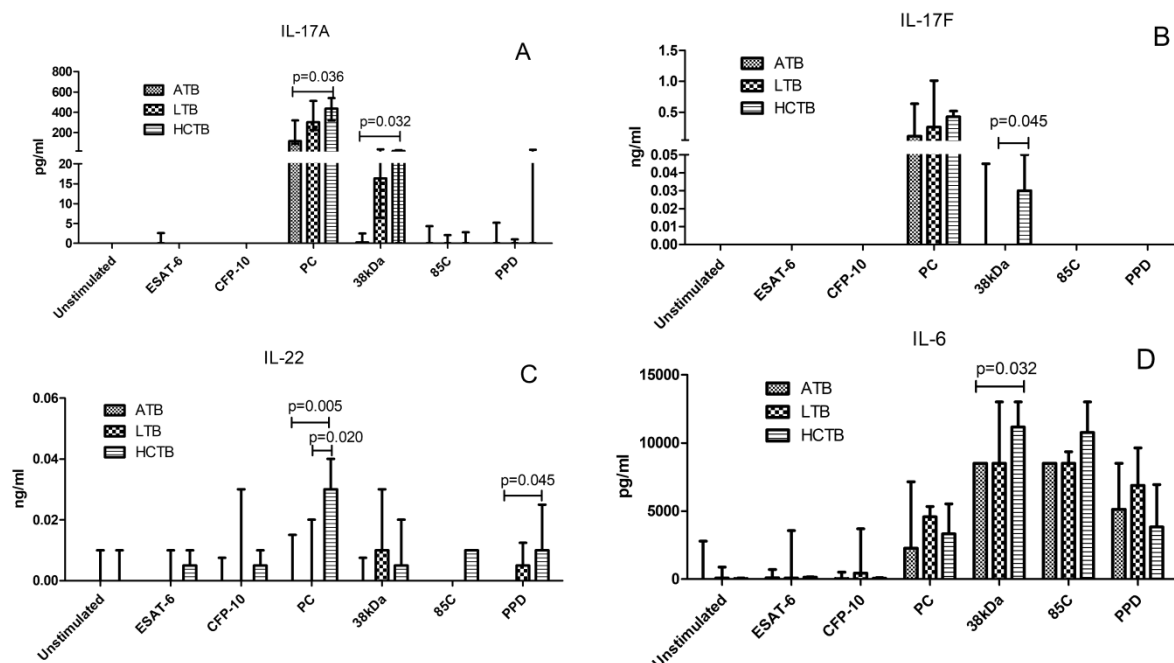


Figure 2: Cytokine response in different groups of the study participants by Multiplex assay. The figure shows levels of cytokines (Y axis) in supernatants of cells of the study participants stimulated with different mycobacterial antigens (X axis) by multiplex assay. The bars represent medians and error bars indicate interquartile ranges for the values. P values showing significant differences ($p < 0.05$) between the groups as calculated by Mann Whitney test are shown in the figure.

Material and Methods

Study population

Adult patients of active pulmonary tuberculosis (ATB: n=19), healthy contacts of sputum positive active pulmonary TB (HCTB: n=16) and individuals with latent tuberculosis infection (LTB: n=22) were recruited from Sassoon General hospital and NARI clinics in Pune, India. Patients with positive sputum smear for *M. tuberculosis* before initiation of anti-tuberculous therapy were selected as cases of active pulmonary tuberculosis. Individuals with latent tuberculosis were healthy, asymptomatic and had normal chest X-ray. They showed positive response in Interferon Gamma Release Assay (IGRA) as tested by T-SPOT TB kit (Oxford Immunotec Ltd, UK) and did not have any recent history of exposure to tuberculosis. House-hold contacts of

patients with sputum positive pulmonary tuberculosis were also enrolled in the study. They had contact of at least one month with the cases. They were also healthy, asymptomatic and had normal chest X-ray. The study was approved by Ethics committees of NARI and Sassoon General Hospital. The samples were collected after obtaining written informed consent from the patients. All the participants tested negative for HIV serology and did not have any obvious immune-suppressive aetiologies like malignancies, immunosuppressive drug intake, and diabetes. Characteristics of the study participants were as shown in Table 1.

IFN- γ secretory ELISPOT assay

IFN- γ secretory response to ESAT-6 and CFP-10 was assessed using T-SPOT TB kit (Cat no.:TB.300, Oxford Immunotec Ltd, UK) as per

the manufacturer's instructions. Additionally IFN- γ secretory response to other TB antigens like 38 kDa, Ag85C (BEI resources, USA) and purified protein derivative (PPD-Span diagnostics, India) was also assessed by an ELISPOT assay as described elsewhere [20]. Briefly, 0.25×10^6 PBMCs (per well) were incubated with a panel of TB antigens (3 μ g/ml) or phytohemagglutinin (PHA- 3 μ g/ml) in a pre-wetted anti-human IFN- γ antibody-coated plate at 37°C in 5% CO₂ for 16 to 20 h. Detection antibody conjugated with alkaline phosphatase and BCIP/NBT substrate were added sequentially after the overnight

incubation with intermittent washing steps. The reaction was stopped by washing the plate with distilled water thoroughly and the plate was read on an automated ELISPOT reader (AID, Germany) after complete drying of the plate. Unstimulated control wells showed less than 5 spots in all the participants and PHA stimulated wells showed 20 or more spots in all the samples. The samples showing 5 spots or more were considered as responders for IGRA as per the T-SPOT TB kit criteria.

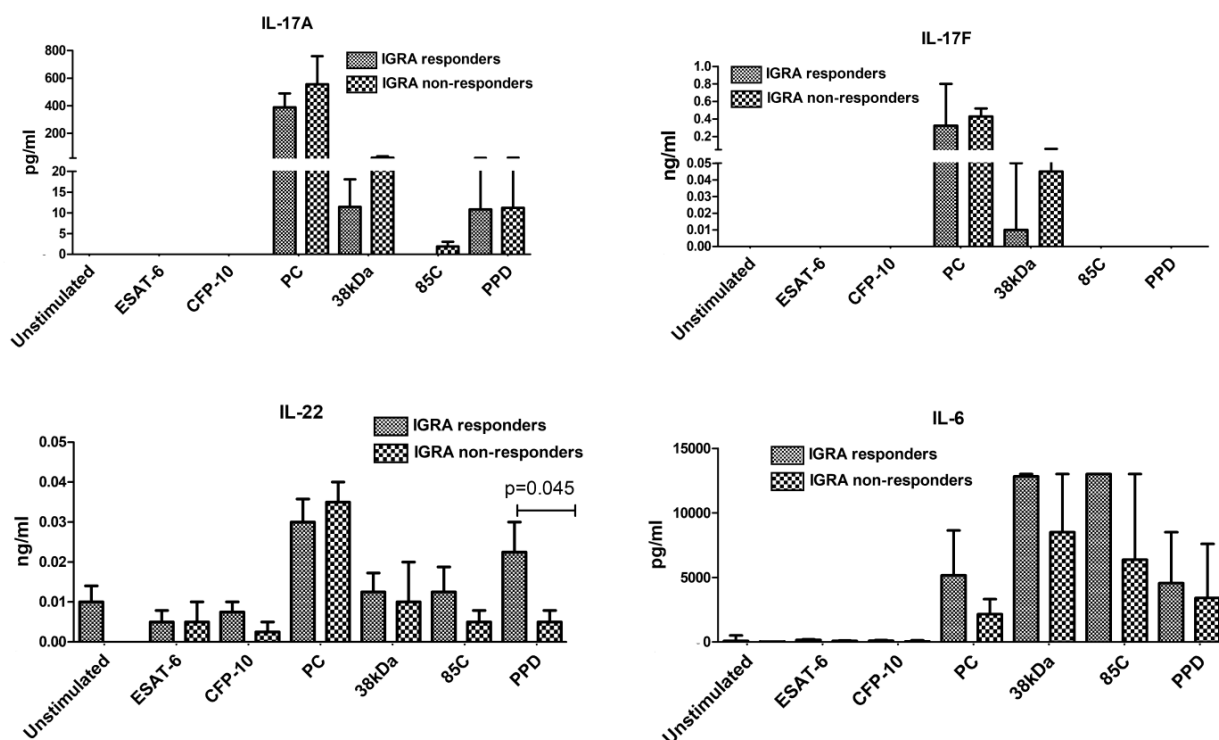


Figure 3: Cytokine response in IGRA responders and non-responders among HCTB group participants by Multiplex assay. The figure shows levels of cytokines (Y axis) in supernatants of cells of the IGRA responders and non-responders among HCTB group participants stimulated with different mycobacterial antigens (X axis) by multiplex assay. The bars represent medians and error bars indicate interquartile ranges for the values. P value showing significant difference ($p < 0.05$) calculated by Mann Whitney test is shown in the figure.

Multiplex cytokine assay

The supernatants of the stimulated cells in the ELISPOT assay after overnight incubation were assessed for cytokines like IL-17A, IL-17F, IL-22, and IL-6 by a protein array system using a customized kit (Cat no.: HTH17MAG-14K, Millipore, USA). Assays were performed according to the manufacturer's instructions. Data were collected and analyzed using Bio-plex 200 system and Bio-Plex Manager software (Bio-rad Laboratories, USA).

Flow cytometry

Frequency of TB antigen specific cells was determined using fluorochrome labeled HLA-A*0201 restricted tetramers loaded with ESAT-6-AMASTEGNV and Ag85B-GLPVEYLQV (synthesized by NIH Tetramer core facility). T cells expressing chemokine receptors such as CCR5, CXCR3 and CCR6 were estimated by flow cytometry. PBMC from the 3 groups were stained with the tetramers, anti CD4 PE-Texas Red (Cat No.: MHCD0417, Invitrogen), anti CD3 APC-Cy7,

Cat No.: 557832; anti CCR5 PE-Cy5, Cat No.: 556889; anti CXCR3 APC, Cat No.: 550967 and anti CCR6 PE-Cy7, Cat No.: 560620 (all from BD Biosciences, USA) for 30 minutes at room temperature in dark. The cells were washed and fixed by adding 3% Formaldehyde and were acquired within 24 h to get 30,000 gated CD4 events on FACS Aria I (BD Biosciences, USA). The data were analyzed using FACSDiva software version 4.0.

Statistical analysis: Statistical analysis was done and graphs were plotted using GraphPad Prism software version 5. Two groups were compared using Mann Whitney test using one-tailed analysis and correlation was done using Spearman nonparametric test. p values of < 0.05 were considered significant.

Results

ATB group showed low IFN- γ secretory responses against shared mycobacterial antigens

IFN- γ secretory ELISPOT assay showed a loss of IFN- γ secretory response to all *M. tuberculosis* antigens in ATB group as compared to the LTB group (Figure 1A). The loss of the response was significant against 38 kDa ($p=0.018$) and Ag85C ($p=0.014$). The LTB group showed significantly higher IFN- γ secretory responses to ESAT-6 ($p=0.024$), CFP-10 ($p=0.008$) and 38 kDa ($p=0.045$) in comparison to HCTB group. The patients with active tuberculosis also showed significant loss of the response to PHA as compared to the other two groups ($p=0.022$ and 0.003). ESAT-6 responders among HCTB group showed significantly lower IFN- γ secretory response against 38 kDa ($p=0.020$) as compared to ESAT-6 non responder (Figure 1B) and magnitude of IFN- γ secretory response against ESAT-6 correlated negatively with that against 38 kDa antigen ($r=-0.51$, $p=0.021$) in HCTB group (Figure 1C).

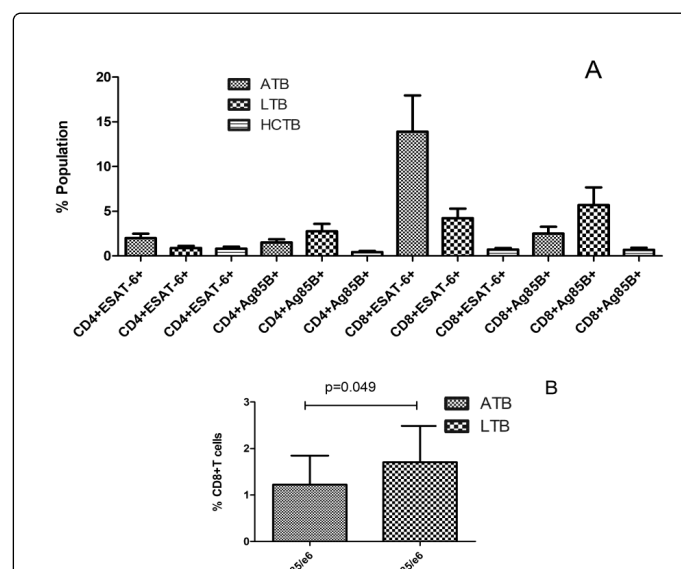


Figure 4: Tetramer assay for identification of TB specific T cells. (A) shows CD4⁺ and CD8⁺ T cells from different study groups binding to ESAT-6 and Ag85B MHC-I restricted tetramers. (B) Shows ratio of Ag85B to ESAT-6 tetramer positive CD8⁺ T cells in ATB and LTB groups. The bars represent medians and error bars indicate interquartile ranges for the values. P values showing significant differences ($p<0.05$) between the groups as calculated by Mann Whitney test are shown in the figure.

Predominant Th17 Cytokine response in HCTB group

Supernatants of PBMCs stimulated with different *M. tuberculosis* antigens were assessed for levels of Th17 cytokines by a multiplex assay (Figure 2). Levels of IL-17A, IL-17F, IL-22 and IL-6 were highest in HCTB group as compared to the other 2 groups. HCTB group had significantly higher IL-17A and IL-6 ($p=0.032$) levels as compared to ATB group against 38 kDa. IL-17F levels were significantly higher in HCTB groups as compared to latent tuberculosis group against 38 kDa ($p=0.045$). PHA induced IL-22 levels were significantly higher in HCTB group than LTB ($p=0.020$) and ATB (0.005) groups. There was

no significant difference in the levels of these cytokines in IGRA positive and IGRA negative healthy contacts except for IL-22 levels which were significantly higher ($p=0.045$) in IGRA positive contacts as compared to IGRA negative contacts against PPD (Figure 3).

Altered ratio of Ag85B to ESAT-6 tetramer positive CD8⁺ T cells in ATB group

ESAT-6 and Ag85B tetramers were used to determine expression of these receptors on TB antigen specific CD4⁺ and CD8⁺ T cells. Frequency of ESAT-6 tetramer positive CD4⁺ and CD8⁺ T cells was higher in ATB and that of Ag85B tetramer positive CD4⁺ and CD8⁺ T cells was higher in LTB group, although it did not show any statistical significance (Figure 4A). The ratio of Ag85B to ESAT-6 tetramer positive CD8⁺ T cells was found to be significantly higher in LTB group (Figure 4B).

Lower Chemokine receptor expression on CD4⁺ and CD8⁺ T cells in ATB group

Chemokines receptor expression on CD4⁺ and CD8⁺ T cells was determined by flow cytometry (Figure 5A). CCR5 expression was found to be significantly higher on CD4⁺ and CD8⁺ T cells in LTB group as compared to ATB ($p=0.025$ and 0.007 , respectively) and HCTB ($p=0.025$ and 0.03 , respectively) groups. CXCR3 expression on CD4⁺ and CD8⁺ T cells was significantly lower in ATB group as compared to LTB ($p=0.001$ for both the types of cells) and HCTB ($p=0.004$ and 0.019 , respectively) groups. CCR6 expression was found to be significantly higher in HCTB group as compared to ATB group on CD4⁺ ($p=0.009$) and CD8⁺ T ($p=0.023$) cells. There was no significant difference in the expression of these chemokines receptors in IGRA positive and IGRA negative healthy contacts. Expression of these markers was also determined on tetramer positive CD8⁺ T cells. Frequency of CXCR3+ ($p=0.004$) and CCR5+ ($p=0.022$) Ag85B tetramer+ as well as CXCR3+ESAT-6 tetramer+ ($p=0.020$) CD8⁺ T cells were significantly higher in LTB group (Figure 5B). Frequency of tetramer positive cells in HCTB group was very low and hence was not considered in this analysis.

Discussion

Immune responses play a significant role in protecting against infectious diseases and can help in devising strategies for countering them. The responses in individuals with latent tuberculosis and contacts of active tuberculosis patients would be important in identifying the correlates of protection against active tuberculosis. The present study was conducted to compare cellular immune responses in these populations. Magnitude of IFN- γ response was highest in LTB group as compared to HCTB and ATB groups against most of the mycobacterial antigens used in the assay indicating predominant Th1 type of immune response in these individuals. ATB group showed overall immunosuppression as evident from significant loss of IFN- γ secretory response against PHA as compared to the other two groups as well as significantly lower response to 38 kDa and Ag85C antigens as compared to the LTB group. Reduced IFN- γ secretory responses to Ag85 and 38 kDa have been reported in patients with active tuberculosis as compared to PPD positive healthy individuals [21,22]. However such significant loss was not observed in case of ESAT-6 and CFP-10. Many studies have also reported either no difference in IFN- γ response or even higher response against ESAT-6/CFP-10 in active tuberculosis [23]. Tetramer assay also showed significantly higher

ratios of frequency of Ag85B to ESAT-6 tetramer positive CD8⁺ T cells in LTB group as compared to ATB group indicating protective role of immune responses against shared mycobacterial antigens. Vaccine constructs expressing Ag85 were also shown to mediate more efficient protection than those expressing ESAT-6 in a mouse model [24]. Also decreasing IFN- γ reactivity against Ag85 was shown to be associated

with the development of clinical tuberculosis in HIV infection [21], highlighting the protective potential of IFN- γ response against this antigen. We detected 0.1%-9.3% of MHC-I tetramer positive CD4⁺ T cells indicating existence of MHC-I restricted CD4 cytotoxic cells in tuberculosis as also reported in other infections [25,26].

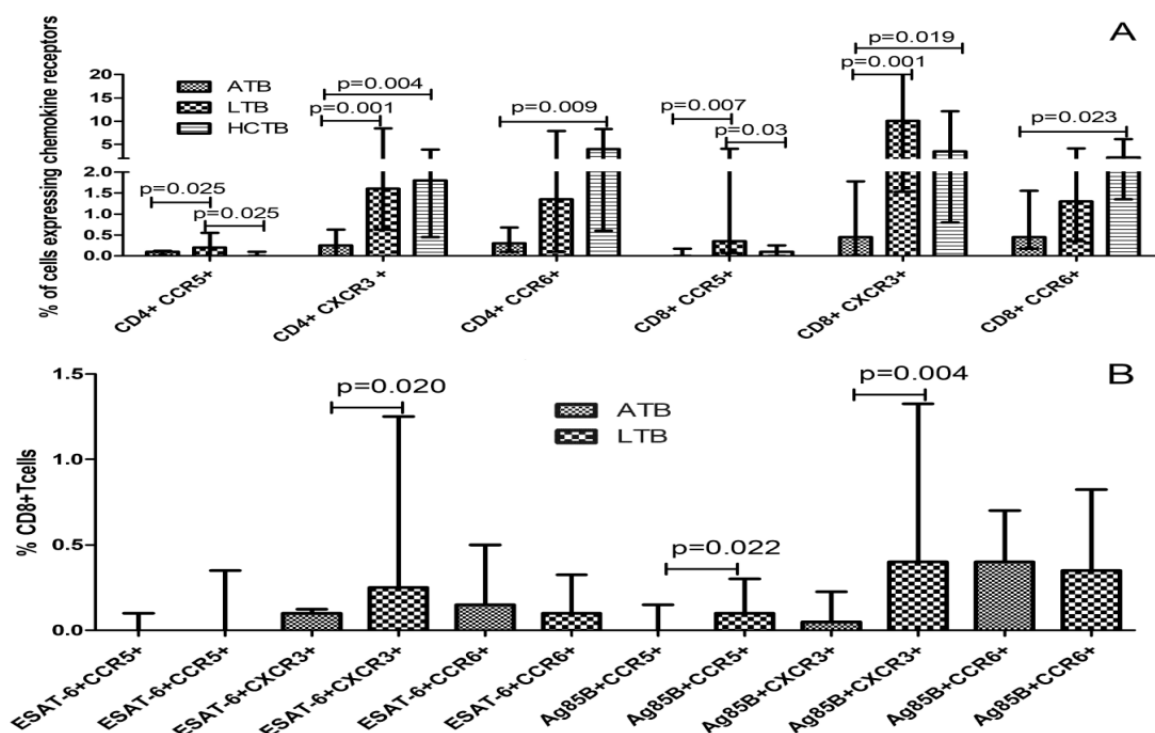


Figure 5: Chemokine receptor expression by flow cytometry. (A) Shows frequency of T cells (Y axis) expressing chemokines receptors, CCR5, CXCR3, CCR6, as shown on X axis. (B) Shows frequency of ESAT-6 and Ag85B tetramer+CD8⁺ T cells expressing these chemokine receptors. The bars represent medians and error bars indicate interquartile ranges for the values. P values showing significant differences ($p < 0.05$) between the groups as calculated by Mann Whitney test are shown in the figure.

Levels of cytokines like IL-17A, IL-17F, IL-22 and IL-6 in the supernatants of PBMCs of the study participants in response to mycobacterial antigens and PHA were also determined. Levels of IL-17A, IL-17F, IL-22 and IL-6 were highest in HCTB group indicating the induction of predominant Th17 type of immune response in them. Increased secretion of Th17 cytokines has been reported in household contacts or mycobacteria exposed healthy individuals in a number of studies [27,28] indicating a possible protective role of this response in healthy contacts. IL-22 has also been recently shown to play a protective role during emerging *M. tuberculosis* infection in mice model [29]. Levels of IL-6, an inducer of Th17 differentiation, have been shown to be raised contacts of active pulmonary TB indicating probable role of IL-6 in inducing Th17 response in them [30]. Although IL-6 and Th17 response have been implicated in pathogenesis of active tuberculosis [31], antigen specificity of these responses might play important role in inducing protective immune responses against mycobacteria. Individuals with persistent IGRA positivity have been shown to have greater Th17 response after mycobacterial exposure [32]. However, we did not find differences in chemokines receptor expressions as well as levels of Th17 cytokines, except for IL-22 levels which were significantly higher in IGRA positive healthy contacts against PPD indicating induction of Th17

response irrespective of the infection status of the individual. Interestingly, ESAT-6 responders among HCTB group had significantly lower 38 kDa specific IFN- γ secretory responses than ESAT-6 non-responders. The response to ESAT-6 signifies presence of *M. tuberculosis* infection whereas the response to 38 kDa antigen, being a shared antigen, could be because of BCG vaccination or prior exposure to other mycobacteria. Negative correlation between the responses against these antigens indicates that the higher immune response against the shared antigen might mediate protection from *M. tuberculosis* infection in the recently exposed individuals.

We looked for expression of chemokines receptors, CCR5, CXCR3, and CCR6, to confirm the findings of predominant Th1 and Th17 responses in latent TB infection and household contacts, respectively. The frequency of CCR5+CD4⁺ T cells was significantly higher in LTB group patients as compared to the other two groups indicating higher number of Th1 cells in them. The frequency of CCR6+CD4⁺ cells was highest in HCTB group indicating predominant Th17 response in them. Higher numbers of CXCR3+CD4⁺ cells were observed in LTB as well as HCTB individuals as compared to active tuberculosis patients possibly indicating induction of both Th1/Th17 cells in them. The lowest frequency of cells expressing these receptors in ATB group

indicated probable loss of Th1 and Th17 response in these patients as also reported in one of the studies [33]. The similar pattern of chemokine receptor expression was also observed on CD8⁺ T cells indicating a homing potential of these cells to the site of infection for mediating protection [34]. We also analysed expression of these receptors on tetramer+CD8⁺ T cells to determine antigen specificity of the response. Frequency of CXR3+ and CCR5+ tetramer+CD8⁺ T cells were found to be significantly higher in LTB group as compared to ATB group. Unfortunately, we could not determine frequency of these cells in HCTB group as tetramer positive cells were found to be low in them.

In conclusion, we characterized immune responses in individuals with latent tuberculosis and household contacts of active pulmonary tuberculosis to determine protective immune responses against reactivation and re-infection types of adult tuberculosis. Th1 type of immune responses were predominant in individuals with latent tuberculosis and Th17 immune responses were observed in the contacts as determined by cytokine response as well as chemokine receptor expression patterns. It would be interesting to study these responses at mucosal surfaces. The study also indicated a possible protective effect of immune responses against shared mycobacterial antigens, suggesting their importance in effective TB vaccine designing.

Competing Interests

All authors declare that they have no competing interests.

Authors' Contribution

All authors conceived and designed the study. VP, PK, SBi, NP acquired the data. MT, JP, and SBa supervised the data collection. AS and VP performed the data analysis, interpretation of data and drafted the manuscript. MT, TD and RG assisted in data interpretation and critically reviewed the manuscript. All authors read and approved the final manuscript.

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