Mycobacterial Diseases
ISSN: 2161-1068

Patel et. al, Mycobact Dis 2016, 6:1
DOI: 10.4172/2161-1068.1000198

Research Article open access journal

Differential Cytokine Response and IgG Subclasses in Pulmonary TB Patients, Household Contacts and BCG Vaccinated PPD-ve Healthy Controls

Rakesh Patel[1], Rajni Upadhyay[1], Bharat Bajaj[2], V.S.Yadav[3], Nalini Srivastava[4], D.S.Chauhan[5], Santosh K Kar[6] and Beenu Joshi[1*]

[1]*Department of Immunology, National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Taj Ganj, Agra, India*

[2] *State TB Demonstration Center, S.N. Medical College, Agra, India*

[3]*Department of Biostatistics, National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Taj Ganj, Agra, India*

[4]*School of Studies in Biochemistry, Jiwaji University, Gwalior, India*

[5]*Department of Microbiology & Molecular Biology, National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Taj Ganj, Agra, India*

[6]*School of Biotechnology, Kalinga Institute of Industrial Technology, Bhubaneswar, India*

[*]**Corresponding author:** Beenu Joshi, Department of Immunology, National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Taj Ganj, Agra, India; Tel: +91-562-2331756; Fax : +91-562-2331755; E-mail: beenuj2002@yahoo.co.in

Received date: December 14, 2015; **Accepted date:**March 3, 2016; **Published date:** March 13, 2016

Abstract

Immune response to *Mycobacterium tuberculosis* is complex and some cytokines are believed to be involved in protective response in humans. In the present study the levels of IFNγ, IL-17, IL-10 and IL-2 stimulated by PPD and ESAT-6 in the PBMCs of active untreated pulmonary TB patients (PTB), their PPD+ve household contacts (HHC) and PPD-ve healthy controls (H) has been estimated at the start and 2 months and 6 months after the start of treatment. IgG subclass antibodies to ESAT-6 and Ag85 complex circulating in the serum of the study subjects on the same days were also measured. Significantly elevated levels of cytokines were secreted by the unstimulated PBMC of patients and HHC in comparison to healthy individuals. PPD significantly increased the secretion of all the measured cytokines by the PBMC of healthy individuals and patients. Higher levels of IL-2 and lower levels of IL-10 were secreted by the PBMC of healthy individuals than the patients whereas that between HHC and patients did not differ. Ratio of IFNγ/IL-10 was also compared among study subjects and significantly higher ratio was observed in healthy subjects than patients and HHC. Significantly higher IgG1 response to ESAT-6 and Ag85 complex was detected in patients' sera than HHC. No effect of treatment could be observed in cytokine and IgG1 levels in all the groups on all the days studied. Our findings suggest preactivated state of TB patients and their PPD+ household contacts and significant cellular immune reactivity by PPD-ve healthy individuals. Further, our findings suggest that IFNγ/IL-10 ratio could differentiate infection from cure.

Keywords: IFNγ; IL-10; IL17; IL-2; IgG subclasses; PPD; ESAT-6; TB

Introduction

Tuberculosis (TB) is one of the major infectious diseases worldwide infecting approximately 3 million individuals with 1.5 million deaths in 2013 [1]. One of the major concerns of immunologists is to find out the protective correlates of the disease and monitoring changes due to mediated clearance of *Mycobacterium tuberculosis*. Mycobacterium being an intracellular pathogen immune response by Th1 cells plays a pivotal role in restricting infection. Measurement of IFNγ has been done to decipher Th1 response in TB against various *M. tuberculosis* antigens. However, results of various studies are conflicting with some reporting higher IFNγ in patients than controls while other showing no or lower IFNγ production in patients than controls [2]. Lowering of IFNγ response after treatment with individual variations has been reported [3]. Moreover, IFNγ detection alone could not differentiate active TB cases form latent tuberculosis infection, which suggests that immune response in TB is complex and other cytokine/immune parameters need to be investigated. Importance of IL-17A, a recently described pro-inflammatory cytokine in granuloma formation and host defence in TB has been suggested [4]. Anti-inflammatory cytokines such as IL-10, IL-4 and TGFβ are also important biomarkers of disease progression as they suppress Th1 cytokine secretion and are up regulated in pulmonary TB [5].

Detection of antibody using specific antigens has also been considered as a diagnostic marker for TB. However, variability in the humoral responses in different population has been indicated by estimating total IgG antibodies. Selective increase in IgG1 and IgG3 antibodies in TB patients with advanced pulmonary disease has been indicated [6]. A predominant IgG1 response to ESAT-6 and CFP-10 along with IFNγ, TNFα and IL-6 has been reported in Brazilian TB patients before treatment which declined after 6 months of therapy suggesting dominant Th1 phenotype in active TB patients [7]. On the other hand in other study from Brazil no differences in IgG1 response in active TB and 3 months treated patients was noted [8]. Population wise difference in antibody reactivity has also been described [9]. Not many reports are available from Indian subcontinent on biomarker signatures for distinguishing patients and healthy individuals and how they change on treatment.

In the present study levels of IFNγ, IL-17, IL-10, IL-2 secreted by the peripheral blood mononuclear cells (PBMC) and IgG subclasses antibodies against ESAT-6 and antigen85 complex of pulmonary TB patients at the initiation of anti- tuberculosis therapy and after 2 and 6 months of treatment were investigated and their response was compared with the response of PPD+ve household contacts and PPD-

ve healthy individuals. By this study we hoped that we will find out biomarker that could distinguish between healthy PPD-ve individuals, PPD+ve household contacts of TB patients and TB patients from each other and also determine treatment efficacy in patients.

Material and Method

Study subjects

Untreated active pulmonary TB patients were selected from the State Tuberculosis Demonstration and Training Center (STDC), Agra. All these patients had undergone clinical, microbiological (Ziehl Nielsen staining/culture) and chest X-ray examinations as per the guideline of Revised National TB Control program (RNTCP), Central TB Division (CTD), and Government of India. All the patients were sputum positive and AIDS, diabetes, hepatitis, hypertension, pregnancy, malnutrition, malignancies, treatment failure, corticosteroids use and alcoholism were exclusion criteria. All the patients were given direct observed short course treatment (DOTS) as per the guidelines of RNTCP. Patients were followed up for 6 months and blood was collected at the initiation of therapy (0 month), 2 and 6 months post treatment. PPD+ve household contacts (HHC) that were in direct contact with patients with no prior history of mycobacterial disease were also included in the study. Students who come for M.Sc dissertation and short term training having no prior history of mycobacterial disease were included as healthy controls (H). All the healthy controls were PPD-ve and were BCG immunised. All the patients, their HHC and H included in the present study were over 18 and under 50 years of age. None of the controls develop any clinical symptoms during the study period. The study was approved by Institutional human ethics committee and blood sample was collected after written informed consent. Serum samples were stored at -20°C till used.

Antigens: Purified Ag85 complex and r-ESAT6 were obtained from Colorado State University (Colorado USA) through a TB Research Material and vaccine Testing contract (NIH Contract HHSN 266200400091C/ADB Contract NOI-AI-40091. PPD was obtained from Statens Serum Institute, Denmark.

Enzyme linked immunosorbent assay: Polystyrene 96-well micro plates (Maxisorp, Nunc U.S.A) were coated with 100 µl per well of Ag85 complex (25 ng/ml) and ESAT-6 (12.5 ng/ml) diluted in carbonate bicarbonate buffer (pH 9.6) overnight. Plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) and the remaining protein-binding sites were blocked by adding 200 µl blocking buffer, 2% BSA in PBS per well. Serum samples diluted (1:100) in 1% BSA PBS-T were added in duplicate (100 µl per well). Plates were washed after 2 hours of incubation at 37°C and mouse anti-human IgG1 (1:1000), IgG2 (1:2000), IgG3 (1:4000), and IgG4 (1:1000) conjugated with HRP (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) were added. Plates were incubated at 37°C for 1 h and after washing 100 µl of substrate solution containing 0.5 mg ortho-phenylene diamine per ml of distilled water with 5 µl/ml of H_2O_2 was added. Plates were kept at room temperature for 20 min in the dark. The reaction was stopped by adding 50 µl of 7% H_2SO_4 each well and the optical density was measured at 492 nm (Spectramax-M2 Reader, Molecular Devices, Sunny vale, CA, USA).

Separation and culture of PBMCs: Peripheral blood Mononuclear cells (PBMCs) were isolated from blood samples of patients, their household contacts and healthy individuals by Fycoll Hypaque density gradient centrifugation method. Cells were counted and adjusted to 2 $\times 10^6$ cells/ml in 5% FCS containing culture medium (RPMI 1640) and 100 µl of suspension was added per well in 96 well U bottom culture plates. Viability of cells as tested by trypan blue stain was ≥ 95%. Cells were stimulated with the optimal dose of antigens, PPD 5 µg/ml and ESAT-6 10 µg/ml. Culture plate was incubated for 5 days at 37°C in 5% CO_2 humidified incubator. Culture supernatants were harvested and stored at -20°C till use.

Detection of cytokine by Cytokine Bead Array (CBA): Cytokines were detected in culture supernatants of PBMC (5 µl per sample) using the human BD CBA Human Soluble Protein Flex Set, as described by the manufacturer (Becton Dickinson, San Jose, CA, USA). Acquisition of standards and samples were performed using the FACS ARIA flow cytometer and data was analysed using FCAP Array software (Becton Dickinson).

Statistical analysis: The comparison between groups was carried out through the non-parametric Mann-Whitney U test. Prism software version 3 was used for the analysis of data. Statistical difference was considered significant at 0.5% ($p<0.05$).

Results

Total 67 active untreated pulmonary TB patients, 45 PPD+ve household contacts and 11 PPD-ve healthy controls were recruited for the study. However, only 24 patients and 20 household contacts could be followed up longitudinally upto 6 months and data of these subjects were considered. Detail of the subjects is presented in table 1.

Study subject	Total Number	Sex		Mean Age Years	Age Range
		Male	Female		
Patients	24	11	13	24 ± 6.50	18-40
Household contact	20	10	10	35.4 ± 8.32	25-47
Healthy control	11	7	4	22 ± 0.63	21-23

Table 1: Baseline demographic details of study subjects

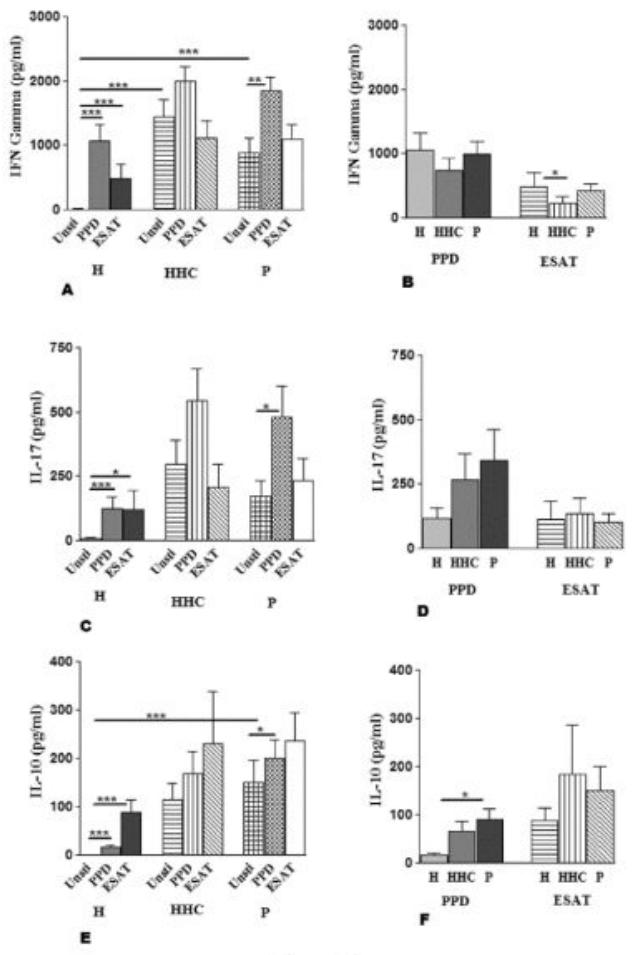

Figure 1

Figure 1: Secretion of cytokines in the culture supernatant of PBMCs of healthy PPD-ve individuals (H), PPD+ve household contacts (HHC) and untreated active pulmonary TB patients (P). PBMCs from study groups were stimulated with or without PPD & ESAT-6 and cytokines were estimated in the culture supernatants harvested after 5 days by cytometric bead array (CBA). IFNγ levels (A) IL-17 (C) and IL-10 (E) in unstimulated (unsti) PBMCs and after antigenic stimulation. Comparison of IFNγ (B) IL-17 (D) and IL-10 (F) values among study subjects after stimulation with PPD & ESAT-6 (antigen specific cytokine levels were normalised after subtracting unstimulated value from stimulated ones). Results are expressed as Mean± 1SEM. Only significant values are indicated *** = p<0.0001, ** =p<0.001, * = p<0.01.

Cytokine measurement was done in culture supernatant of the PBMCs of untreated active pulmonary TB patients (P), their household contacts (HHC), PPD-ve healthy individuals (H) and treated patients after 2 and 6 months of therapy by flowcytometry using BD CBA Human Soluble Protein Flex Set for IFNγ, IL-2, IL-4, IL-17 and IL-10.

Basal levels of IFNγ in the culture supernatant were significantly higher (p<0.0001) in HHC and patients than healthy individuals. PPD and ESAT-6 significantly increased (p<0.0001) the levels in PPD –ve healthy individuals. On the other hand M.tb antigens did not have any effect on this cytokine level in HHC however, PPD significantly increased (p<0.001) the levels in patients (Figure 1A). Levels of IFNγ were also compared among healthy, HHC and patients after stimulation with antigens. No difference was noted among the groups after PPD stimulation however, ESAT-6 induced significantly higher (p<0.01) levels of IFNγ in healthy individuals than HHC (Figure 1B).

IL-17 a pro inflammatory cytokine which has been implicated to have a role in host defence in TB was also measured before and after stimulation with antigens. Significantly higher basal IL-17 levels were noted in culture supernatant of PBMCs of patients and their HHC (p<0.001) than healthy individuals. Stimulation with PPD and ESAT-6 both significantly increased (PPD p<0.0001, ESAT p<0.01) the levels of this cytokine in healthy (H). Antigenic stimulation did not change the levels of IL-17 in HHC but PPD increased the levels in patients (p<0.01) though ESAT-6 had no effect (Figure 1C). No difference in IL-17 levels was noted after antigenic stimulation among three groups (Figure 1D).

Anti-inflammatory cytokine IL-10 was also measured before or after stimulation with PPD and ESAT-6. Similar to IFNγ and IL-17, basal level of IL-10 in patients was significantly higher (p<0.0001) than healthy individuals but no difference was noted with HHC. Both PPD and ESAT-6 significantly increased (p<0.0001) the levels in healthy individuals. On the other hand no effect of antigenic stimulation was noted in HHC but PPD induced significant IL-10 levels (p<0.01) in patients (Figure 1E). Interestingly on comparing the IL-10 levels among the groups after antigenic stimulation significantly higher levels were noted in patients than healthy individuals (p<0.01) after stimulation with PPD but no difference was observed in HHC and healthy individuals (Figure 1F). ESAT-6 was not shown to be affecting IL-10 levels in any group. IL-2 and IL-4 levels were also evaluated with or without PPD and ESAT-6 stimulus. IL-4 levels were very low under the condition it was measured (data not shown). Basal levels of IL-2 was significantly high in patients as compared to healthy individuals (p<0.01) but PPD significantly rose (p<0.01) these levels in healthy individual. No effect of antigenic stimulus was noted on IL-2 levels in patients and HHC (Figure 2A). On comparing the levels after PPD stimulus significantly high levels (p<0.01) were observed in healthy than patients and HHC (Figure 2B).

IFNγ / IL-10 ratio has been suggested to be marker of disease severity in pulmonary and extra pulmonary TB but we wished to see the difference in IFNγ/IL-10 ratio in our study subjects. IFNγ/IL-10 ratio without stimulus was significantly high in patients (p<0.01) and HHC (p<0.001) than healthy individuals. On the contrary, after PPD stimulus IFNγ/IL-10 ratio was significantly higher in healthy than HHC (p<0.001) and patients (p<0.0001). No difference among groups was observed with ESAT-6 stimulus (Figure 3).

Patients were followed up longitudinally and levels of various cytokine were measured after 2 and 6 months of therapy (ATT). All the patients improved clinically and were sputum negative after 6 months of treatment. No effect of PPD on IFNγ levels was noted (Suppl fig.1B). The levels of this cytokine rose after 2 months of therapy after ESAT-6 stimulation and declined after 6 months of ATT but this change was not significant (Supplementary Figure.1).

In unstimulated cultures the levels of IL-17 increased after 6 months whereas after PPD stimulation the levels declined after 6 months but the differences are not significant. After ESAT-6 stimulus IL-17 levels increased after 2 months of ATT but again decreased after completion

of 6 months of therapy though these changes are not significant (Supplementary Figure 2).

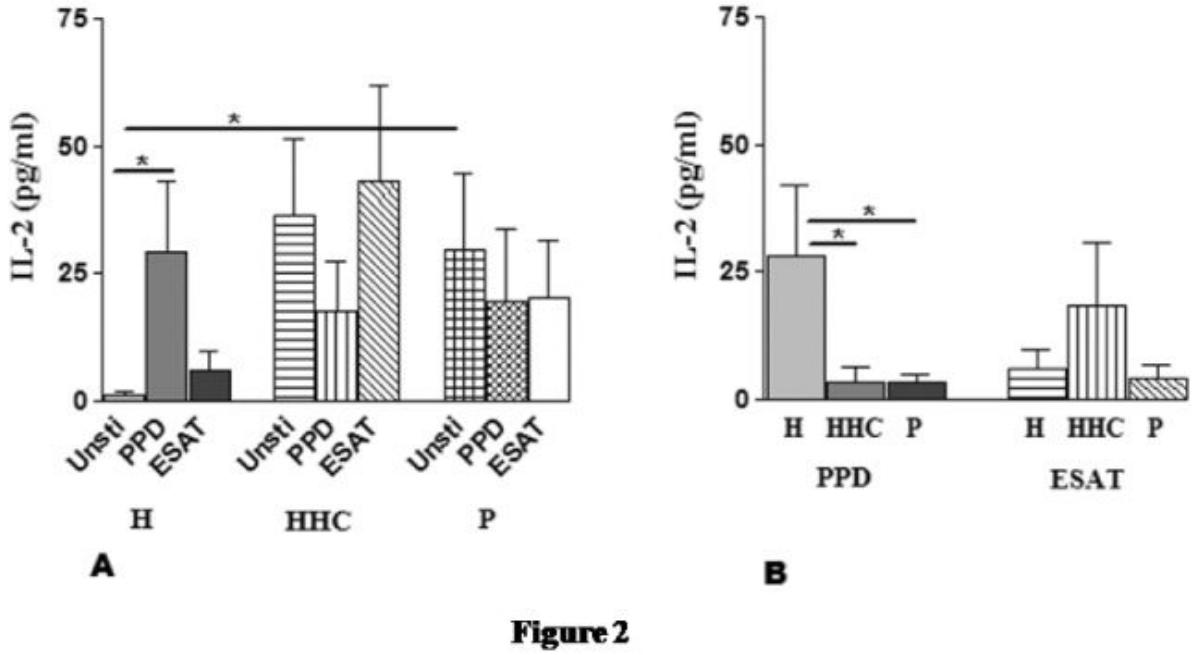

Figure 2

Figure 2: Secretion of IL-2 in the culture supernatant of PBMCs of healthy PPD-ve individuals (H), PPD+ve household contacts (HHC) and untreated active pulmonary TB patients (P) after stimulation with or without PPD & ESAT-6. Cytokines was estimated in the culture supernatants harvested after 5 days by cytometric bead array (CBA). (A) levels in unstimulated (unsti) PBMCs and after antigenic stimulation. (B) Comparison of values among study subjects after stimulation with antigens (antigen specific cytokine levels were normalised after subtracting unstimulated value from stimulated ones). Results are expressed as Mean± 1SEM. Only significant values are indicated. * =p<0.01.

IL-10 levels were also evaluated during and after therapy. In the culture supernatant when no stimulus was added levels of this cytokine declined after 2 months of ATT which again increased after completion of therapy however, these differences were not significant. IL-10 levels after PPD and ESAT-6 stimulation though reduced after 2 months of therapy and again increased after 6 months but it was not significant (Supplementary Figure 3).

IgG subclasses to ESAT-6 and Ag85 complex: Elevated IgG1 levels to ESAT-6 have been reported earlier in TB patients. We have also reported significant IgG antibody response to Ag85 complex in TB patients in our previous study [10]. In the present study IgG subclasses (IgG1, 2, 3 &4) were measured in the sera of pulmonary TB patients (0 month, 2 and 6 months), their PPD+ve contacts and healthy PPD –ve individuals. IgG1 levels of patients were significantly higher ($p<0.01$) than HHC using both ESAT-6 and Ag85complex (Figure 4A and 4E). No difference was observed in the IgG2, IgG3 and IgG4 response in patients and HHC using both the antigens (Figures 4B,C, D and F data of Ag85 complex IgG2 and IgG3 not shown)). IgG1 response against ESAT-6 increased in patients after 2 months of therapy which declined after 6 months of therapy but this difference was not statistically significant (Figure 4A). IgG2 levels against ESAT-6 increased ($p=0.0513$) after 6 months of therapy in patients as compared with the response at 0 month (Figure 4B). IgG3 response against ESAT-6 significantly increased ($p<0.01$) after 2 months of therapy (Figure 4C). However, IgG4 response significantly decreased ($p<0.01$) after 6 months of therapy (Figure 4D).In contrast, no effect of therapy was noted on the IgG1, IgG2 and IgG3 response against Ag85 complex in patients, whereas IgG4 response increased ($p<0.01$) after 2 months of ATT (data of IgG2&IgG3 not shown).

Discussion

Understanding the host immune response to *M. tuberculosis* antigens in TB endemic areas is important for the development of efficacious vaccine and diagnostic test. In the present study cytokine IFNγ, IL-17, IL-10, IL-4, IL-2 and IgG subclasses to ESAT-6 and Ag85 were evaluated in TB patients, their household contacts and healthy PPD-ve individuals. Patients were also followed longitudinally for their cytokine and IgG responses after 2 and 6 months of ATT. In our study secretion of IFNγ and IL17 by the unstimulated PBMC were significantly elevated in patients and household contacts (HHC) than healthy individuals. This shows the activated state of PBMC in patients and HHC ex vivo. Significant increase of IFNγ and IL17 levels were noted when PBMCs were stimulated with PPD and ESAT-6 in healthy individuals whereas PPD raised the levels in the PBMC of patients but not in HHC. No difference in IFNγ and IL17 levels was noted in three groups after antigenic stimulation.

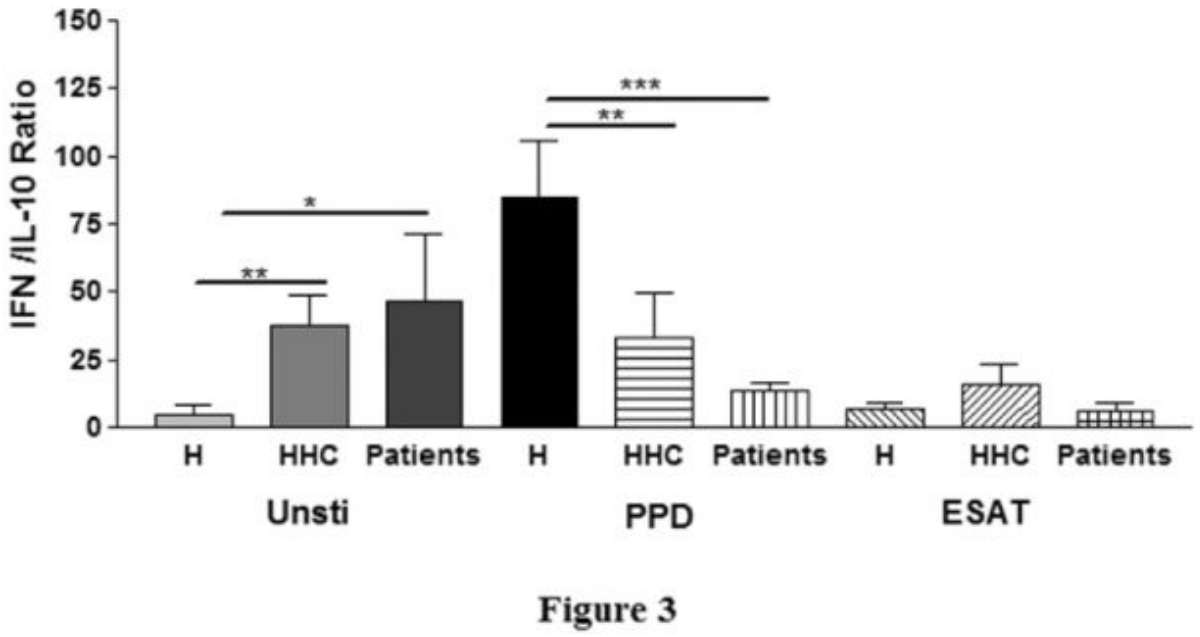

Figure 3

Figure 3: Ratio of IFNγ/IL10 values in the supernatant of PBMCs with or without stimulation with PPD & ESAT-6. Results are expressed as Mean± 1SEM. Antigen specific cytokine values are given after subtracting values of unstimulated culture (Unsti) from stimulated ones. (H) healthy PPD-ve individuals, (HHC) PPD+ve household contacts and (P) untreated active pulmonary TB patients. Only significant values are indicated *** = p<0.0001,** = p<0.001, * = p<0.01.

In contrast to our report IFNγ response to ESAT-6 and ESAT-6/CFP-10 fusion proteins has been reported in TB patients and similar levels were found in PPD+ve and PPD-ve healthy individuals. In a recent report significantly higher IFNγ levels were reported in individuals with latent TB (LTBi) compared to active TB (ATB) in response to PPD which was not noted in our study [11]. In an endemic study area like ours similar exposure of patients and their PPD+ve HHC both to mycobacterial and nontuberculous mycobacterial antigens could lead to T cell activation thereby resulting in significant IFNγ response in unstimulated cultures. Therefore, further stimulation with PPD and ESAT-6 could not increase the levels upto the extent that their values could be differentiated among the groups. Basal IL-10 levels were also significantly high in patients than healthy individuals but after PPD stimulation IL10 levels were significantly higher in patients than healthy individuals. Interestingly, though basal ratio of IFN/IL-10 was significantly higher in patients and HHC than healthy but PPD induced ratio was significantly higher in healthy individuals. Higher M.tb specific IFNγ and lower IL-10 in healthy individuals is suggestive of protective correlates for anti-mycobacterial immunity in an endemic setting. Similar to our findings higher IFNγ/IL-10 in healthy controls than patients has been reported by Priya et al [12]. Ratio of mycobacterium induced IFNγ /IL-10 has been suggested as a possible biomarker for disease severity in both pulmonary and extra pulmonary TB [13]. Up regulated levels of IL-10 have been reported in PTB than endemic controls [14-16].

Sutherland et al reported lower level of IL-17 and IL-10 in TB patients in response to PPD but higher levels of IFN γ in response to EC antigens but in their study supernatant was taken after short term culture of 1 day [17]. IL17 and IFNγ levels were also evaluated in the culture supernatant of *M. bovis* stimulated PBMCs of healthy PPD-ve, LTBi and TB patients. In contrast to our results this study reported significantly higher IFNγ levels after stimulation with antigen in LTBi as compared to healthy individuals but no difference in IL-17 levels was seen [18]. Difference in this study could be due to the ethnicity of the population studied.

Similar to other cytokines IL-2 levels without any stimulus was very low in healthy individuals but interestingly IL-2 levels were significantly higher in healthy individuals when compared with patients and HHC after PPD stimulation. Higher IFNγ, TNFα and IL-2 expressing CD4+ T cells were earlier reported in TB patients than healthy contacts when multiple cytokine were evaluated whereas percentage of CD4+ T cells expressing IL-2 alone were lower [17]. Low IL-4 levels were observed under the condition of our study hence no correlation could be done.

We also followed up the same TB patients longitudinally after 2 and 6 months of ATT; however we did not find any effect of therapy on the level of any cytokine in the absence or presence of antigenic stimulus. Similar to our study no effect of ATT was noted on IFNγ response after 6 months of therapy by Sauzullo et al [3]. On the other hand increase in IFNγ and IFNγ/IL-10 ratio in patients after 2-4 and 6 months of therapy has been reported by Priya et al [12,18]. The difference seen in our study could be due to small numbers of patients followed by these authors. Mattos et al. have reported lower IFNγ levels after 6 months of therapy in TB patients [7]. IFNγ production by CD4+ T cells was also evaluated before and after 26 weeks of treatment. Similar to our study preactivated state at diagnosis was observed in patients with high IFNγ +CD4+ T cells which normalised after treatment. The difference in our study could be due to the longer period of incubation of PBMCs with antigens as we measured cytokine in 6 days culture. Short term culture

would detect mainly effector memory T cells responses of recently activated lymphocytes whereas; central memory T cells are expanded and activated in long term cultures. Similar to our study no difference in CD4+ IFNγ+ cells were noted after 24 weeks of treatment though significant reduction was noted after 8 weeks post treatment by Feruglio et al. These researchers also reported higher cytokine response by PPD induced CD4 T cells at 6 day compared to CFP. This also explains higher response seen in our study groups by PPD than ESAT-6. Significant inter individual variation has also been observed in the evaluation of cytokine pattern in a longitudinal study [19].

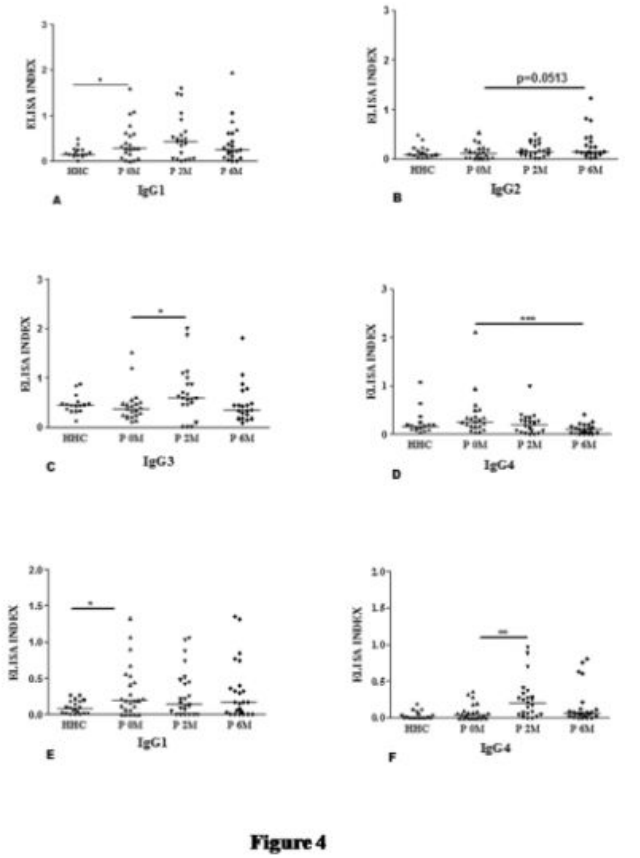

Figure 4

Figure 4: IgG subclasses against ESAT-6 and Ag85 antigens in untreated active pulmonary TB patients (P) and their PPD+ve house hold contact (HHC). Values are given as ELISA Index (Described in materials and methods). Values of IgG subclasses in patients at the initiation of therapy (0M), during follow up after 2 months (2M) and 6 months (6M) of therapy (ATT) are also shown. Each dot represents one individual, bar represents the median value. (A) IgG1, (B) IgG2, (C) IgG3, (D) IgG4 response against ESAT-6, (E) IgG1, (F) IgG4 response against Ag85. Only significant values are indicated. *** = p<0.0001,** = p<0.001,* = P<0.01.

Role of IgG1 and IgG3 in bacterial uptake and clearance due to the presence of Fc receptor of these antibodies on macrophages has been suggested [20]. IgG response to protein antigen is T cell dependent and induction of human IgG1 and IgG3 have been shown to be helped by IFNγ produced by Th1 cells [21,22]. We noted significantly higher IgG1 response to ESAT-6 and Ag85 complex in TB patients than HHC. However, no change in IgG1 levels to both the antigens was noted after ATT. IgG3 levels to ESAT-6 increased after 2 months of therapy but decreased after 6 months of therapy. IgG4 levels against ESAT-6 significantly declined after 6 months of therapy whereas with Ag85 complex IgG4 levels significantly increased after 2 months of therapy. Other studies have also demonstrated predominance of IgG1 in TB patients as compared to healthy individuals however, contrary to our finding Macedo et al. have reported higher IgG3 response to ESAT-6 and Ag 85 A & B in TB patients than endemic controls and they have correlated their findings with higher Th1 response of patients. Follow up studies have been done taking whole IgG response to mycobacterial antigens which showed decline in the levels however, we have studied all four IgG subclasses which suggests that the decrease could be due to IgG3 and IgG4 which we observed using ESAT-6.

In conclusion, our study demonstrates pre activated state of TB patients and their household contacts. Further, higher levels of antigen specific IL-2 and IFNγ/IL-10 ratio were observed in healthy controls than patients which could be a correlate of cure and protective immunity. Significantly higher IgG1 response was noted in patients than household contacts. Significant impairment of functional capacity of *M.tuberculosis*-specific CD4/CD8+ T cells has been reported previously with increasing mycobacterial load [23]. Since our study was done in a small cohort, follow up of larger group and investigation of polyfunctional T cells and the suggested potential markers need to be done in future studies to understand the immune correlates in endemic setting like our study area.

Acknowledgement

Authors thank Drs. K.K.Mohanty and Bhawna Sharma for their help in manuscript preparation. Dr. Bhawna Sharma and Ms. Bhavyata Dua are acknowledged for their help in flowcytometry. Authors are also thankful to Mr. M.M. Alam for sample collection and field visits and Microbiology Department for smear microscopy and mycobacterial culture. Authors sincerely acknowledge Indian Council of Medical Research, New Delhi for financial assistance.

References

1. Global Tuberculosis Report (2014) (WHO).
2. Veenstra H, Crous I, Brahmbhatt S, Lukey P, Beyers N, et al. (2007) Changes in the kinetics of intracellular IFN-gamma production in TB patients during treatment. Clin Immunol 124: 336-344.
3. Sauzullo I, Mengoni F, Lichtner M, Massetti AP, Rossi R, et al. (2009) In vivo and in vitro effects of antituberculosis treatment on mycobacterial interferon-gamma T cell response. PLoS One 4: e5187.
4. Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, et al. (2007) IL-17 mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium bovis bacilli* Calmette-Guerin infection. J Immunol 178: 3786-3796.
5. Dlugovitzky D, Bay ML, Rateni L, Urízar L, Rondelli CF, et al. (1999) In vitro synthesis of interferon-gamma, interleukin-4, transforming growth factor-beta and interleukin-1 beta by peripheral blood mononuclear cells from tuberculosis patients: relationship with the severity of pulmonary involvement. Scand J Immunol 49: 210-217.
6. Hussain R, Dawood G, Abrar N, Toossi Z, Minai A, et al. (1995) Selective increases in antibody isotypes and immunoglobulin G subclass responses to secreted antigens in tuberculosis patients and healthy household contacts of the patients. Clin Diagn Lab Immunol 2: 726-732.
7. Mattos AMM, Almeida C de S, Franken KLMC, Alves CCde S, Abramo C, et al. (2010) Increased IgG1, IFN-gamma, TNF-alpha and IL-6

responses to *Mycobacterium tuberculosis* antigens in patients with tuberculosis are lower after chemotherapy. Int Immunol 22:775-782.

8. Macedo GC, Bozzi A, Weinreich HR, Bafica A, Teixeira HC et. al (2011) Human T Cell and Antibody-Mediated Responses tothe *Mycobacterium tuberculosis* Recombinant 85A, 85B, and ESAT-6 Antigens. Clin and Dev Immunol 2011: 351573.
9. Araujo Z, Giampietro F, Cançado LC, Singh M, Wide A (2008) Comparison of serological responses in two different populations with pulmonary tuberculosis. Mem Inst Oswaldo Cruz 103: 661-667.
10. Kumar G, Dagur PK, Singh PK, Shankar H, Yadav VS, et al. (2010) Serodiagnostic efficacy of *Mycobacterium tuberculosis* 30/32-kDa mycolyl transferase complex, ESAT-6, and CFP-10 in patients with active tuberculosis. Arch Immunol Ther Exp (Warsz) 58: 57-65.
11. Marín ND, París SC, Rojas M, García LF (2013) Functional profile of CD4+ and CD8+ T cells in latently infected individuals and patients with active TB. Tuberculosis (Edinb) 93: 155-166.
12. Sai Priya VH, Latha GS, Hasnain SE, Murthy KJ, Valluri VL (2010) Enhanced T cell responsiveness to *Mycobacterium bovis* BCG r32-kDa Ag correlates with successful anti-tuberculosis treatment in humans. Cytokine 52: 190-193.
13. Jamil B, Shahid F, Hasan Z, Nasir N, Razzaki T, et al. (2007) Interferon gamma/IL10 ratio defines the disease severity in pulmonary and extra pulmonary tuberculosis. Tuberculosis 87:279-287.
14. Boussiotis VA, Tsai EY, Yunis EJ, Thim S, Delgado JC, et al. (2000) IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. J Clin Invest 105: 1317-1325.
15. Gong JH, Zhang M, Modlin RL, Linsley PS, Iyer D, et al. (1996) Interleukin-10 downregulates *Mycobacterium tuberculosis*-induced Th1 responses and CTLA-4 expression. Infect Immun 64: 913-918.
16. Hussain R, Kaleem A, Shahid F, Dojki M, Jamil B, et al. (2002) Cytokine profiles using whole blood assays can discriminate between tuberculosis patients and healthy endemic controls in BCG-vaccinated population. J Immunol Meth 264:95–108.
17. Sutherland JS, Adetifa IM, Hill PC, Adegbola RA, Ota MO (2009) Pattern and diversity of cytokine production differentiates between *Mycobacterium tuberculosis* infection and disease.Eur. J. Immunol 39: 723–729.
18. Cowan J, Pandey S, Filion LG, Angel JB, Kumar A, et al. (2009) Comparison of interferon-?-, interleukin (IL)-17- and IL-22-expressing CD4 T cells, IL-22-expressing granulocytes and proinflammatory cytokines during latent and active tuberculosis infection.Eur J Immunol39:723-729.
19. Feruglio SL, Tonby K, Kvale D, Dyrhol-Riise AM (2015) Early dynamics of T helper cell cytokines and T regulatory cells in response to treatment of active *Mycobacterium tuberculosis* infection. Clin Exp Immunol 179: 454-465.
20. Hussain R, Shiratsuchi H, Ellner JJ, Wallis RS (2000) PPD specific IgG1 antibody subclass upregulate tumour necrosis factor expression in PPD-stimulated monocytes: possible link with disease pathogenesis in tuberculosis. Clin Exp Immunol 119: 449–455.
21. Finkelman FD, Katona IM, Mosmann TR, Coffman RL (1988) IFN-gamma regulates the isotypes of Ig secreted during in vivo humoral immune responses. J Immunol 140: 1022-1027.
22. Snapper CM, McIntyre TM, Mandler R, Pecanha LM, Finkelman FD, et al. (1992) Induction of IgG3 secretion by interferon gamma: a model for T cell-independent class switching in response to T cell-independent type 2 antigens. J Exp Med 175: 1367-1371.
23. Day CL, Abrahams DA, Lerumo L, Janse van Rensburg E, Stone L, et al. (2011) Functional capacity of *Mycobacterium tuberculosis*-specific T cell responses in humans is associated with mycobacterial load. J Immunol 187: 2222-2232.