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LONGITUDINAL STUDY OF CD4 AND CD8 T CELLS PRODUCING CYTOKINE DURING DOTS THERAPY IN TB PATIENTS AND HEALTHY HUMANS

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ABSTRACT

Pulmonary tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis (Mtb)*. Immune response to *M. tuberculosis* is complex and several T cells, cytokines could play a role in protective response. In the present study CD4, CD8 and their cytokines such as (IL-4, IFN-ā) were analysed in response to *Mycobacterium tuberculosis* antigen (ESAT-6 and PPD). Sixty seven active pulmonary TB patients were recruited 0 MF (Month follow up), followed up for 2MF and patients had completed 6 months of chemotherapy and patients had completed treatment at 6 months but further followed up after 3 months . The 45 control group consisted of PPD positive household contacts (HHC). In addition, IFN-ā production in PBMC cultures from 24 patients were measured following stimulation with the *M.tb* specific protein ESAT-6 and PPD. We observed significantly higher $CD4^+$ and $CD8^+$ T cells producing IFN-ā in HHC as compared to patients and this finding suggests that there were defective IFN-ā production in patients and which remained low after 6 month chemotherapy, but after 9 month, levels again increased. These findings suggest the better performance of IFN-ā in HHC as compared to patients, may be the useful TB disease biomarkers in monitoring treatment success.

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KEY WORDS : CD4, CD8, *Mycolsacterium tuberculesis*, T.B. patients, T-cells.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* is one of the most prevalent and commonest serious infectious diseases responsible for high incidences of morbidity and mortality. Control of TB depends on early identification and proper treatment of individuals with active disease. The lack of an accurate diagnostic technique and immunological imbalance has contributed to the emergence of TB as a threat to global health and environment. One third of the world population is infected with *M. tuberculosis* but only 10% of the infected individual would develop active TB during their life time. Approximately 6.1 million new TB cases were notified to national authorities and reported to World Health Organization. Notified TB cases increased from 2013–2015, mostly due to a 34% increase in notifications in India[10]. *M. tuberculosis* is an intracellular pathogen that infects macrophages and triggers a cascade of cell mediated immune responses. T-cell responses are critical components of the protective immunity against *M. tuberculosis*. In humans and mice, adaptive

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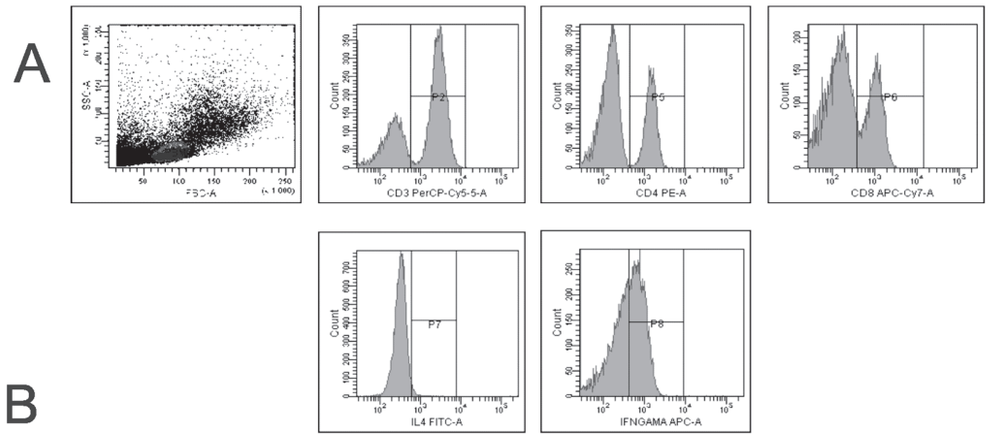

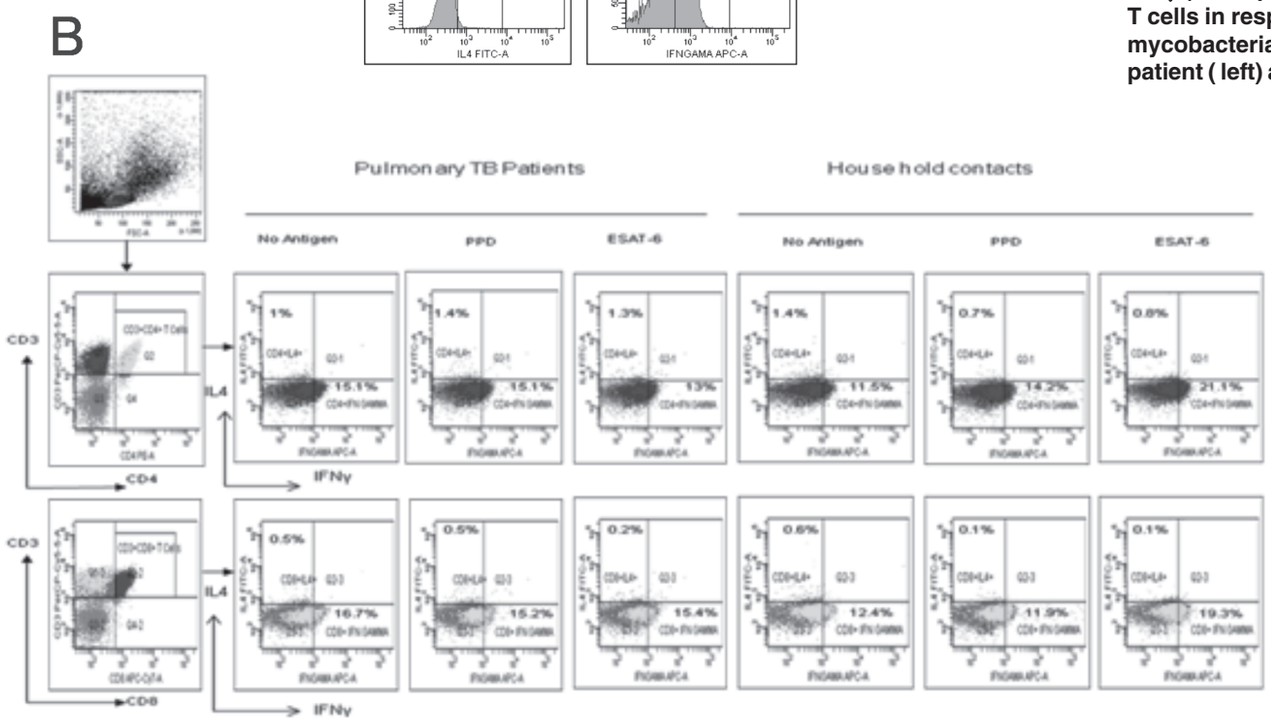

Fig. 1 : *M.tuberculosis* specific CD4 and CD8 T cells expressing IL4/IFNγ in Pulmonary TB patients (P) and their PPD^{+}ve house hold contact (HHC),.- Representative figure A.- Histogram B.- Flow cytometric detection of mean percentage CD4^{+}IL4/IFNγ (upper plots) and CD8^{+}IL4/IFNγ (lower plots) expressing T cells in response to shown mycobacterial antigens in patient (left) and HHC (right).

immune responses to *Mycobacterium tuberculosis* involve $CD4^+$ and $CD8^+$ T-cells[8] and the essential cytokine IFNγ[1,4,5]. Therefore, in the present study we have evaluated T-cells subsets CD4, CD8 T cells in the blood of TB patients when stimulated *in vitro* by PPD and ESAT-6 before and after 2 and 6 months of DOTS therapy: Cytokines such as IFNγ, IL-2 and IL-4 in response to *M. tuberculosis* antigen (ESAT-6 and PPD. All the responses were compared with PPD positive house hold contacts (HHC). This is a follow up study and will help us in understanding the role of T cells in the disease progression and in the recovery and it might also shed light in discriminating protected versus diseased person by identifying immunological markers.

Materials and Methods

Blood samples of clinically confirmed pulmonary TB patients from the State Tuberculosis Demonstration center (STDC), and Shanti Manglick Hospital, Agra were studied from 2010-2015. All these patients were examined by clinicians and microbiological (Ziehl Nielsen staining/culture) examination of their sputum was done. All the pulmonary TB patients were smear positive for AFB (Table-1). None of the patients were HIV^+ or on immunosuppressive drugs. All the patients were given Directly observed treatment short course (DOTS) as per the guidelines of RNTCP.

Flow cytometry

For flow cytometry study Peripheral blood mononuclear cells (PBMCs) were stained with cell surface anti CD3, CD4, CD8, cells in staining buffer for 30 minute at 4°C and cells were fixed with 4% formaldehyde before permeabilization for intracellular staining (ICS). ICS for cytokine was done by using anti- IFN-γ, IL-4, suspension was incubated for 30 minutes at 4 ºC, cells was then washed with staining buffer and was suspended in staining buffer till acquisition. Flow cytometry analysis was performing on FACS Aria (BD Bioscience) instrument and data were analyzed using FACS Diva software. Detection of cytokine IFNã in 5 days culture supernatant by Cytokine Bead Array (CBA).

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

Mycobacterial specific $CD4^+/CD8^+$T cells producing IL-4 and IFNγ in patients and HHC.

We observed significantly high $CD4^+$ and $CD8^+$ T cells producing IFNγ in HHC as compared to patients (Figs. 2A,B) and this finding suggests that there were defective IFNã production in patients and which remained low after 6 month chemotherapy, but when we compared the cytokine levels after 9 month, levels again increased (Figs. 2C, D). Higher IL-4 expressing $CD4^+$ T-cells were noted which decreased after the therapy in patients (Fig. 2 D) suggesting role of IL-4 in the suppression of IFNγ or survival of bacteria and progression of diseases. In other words higher Th2 type of response might be leading to suppression of Th1 immunity in patients.

Cytokine IFNγ in the culture supernatant of PBMCs in patients and HHC

We observed higher secretion of IFNã in HHC as compared to pulmonary TB patients confirming the role of IFNã in protection against TB as seen in house hold contacts by restricting the infection inspite of living with the same exposure to *M. tuberculosis* (Fig.3 A). Similar finding was seen with CD4/CD8 producing IFNã in flow cytometry data.

Discussion

One of the major concerns of immunologists is to find out the biomarkers or the protective correlates of the disease and monitoring treatment mediated clearance of *M. tuberculosis*. Mycobacterium being an intracellular pathogen T helper type 1 of immune response 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 and other showing no or lower IFNã production in patients than controls[9]. Lowering of IFNã response after treatment with individual variations has been reported[6]. 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.

Interestingly in our study significantly higher $CD4^+$ and $CD8^+$ T cells producing IFNγ were noted in HHC as compared to patients and this finding

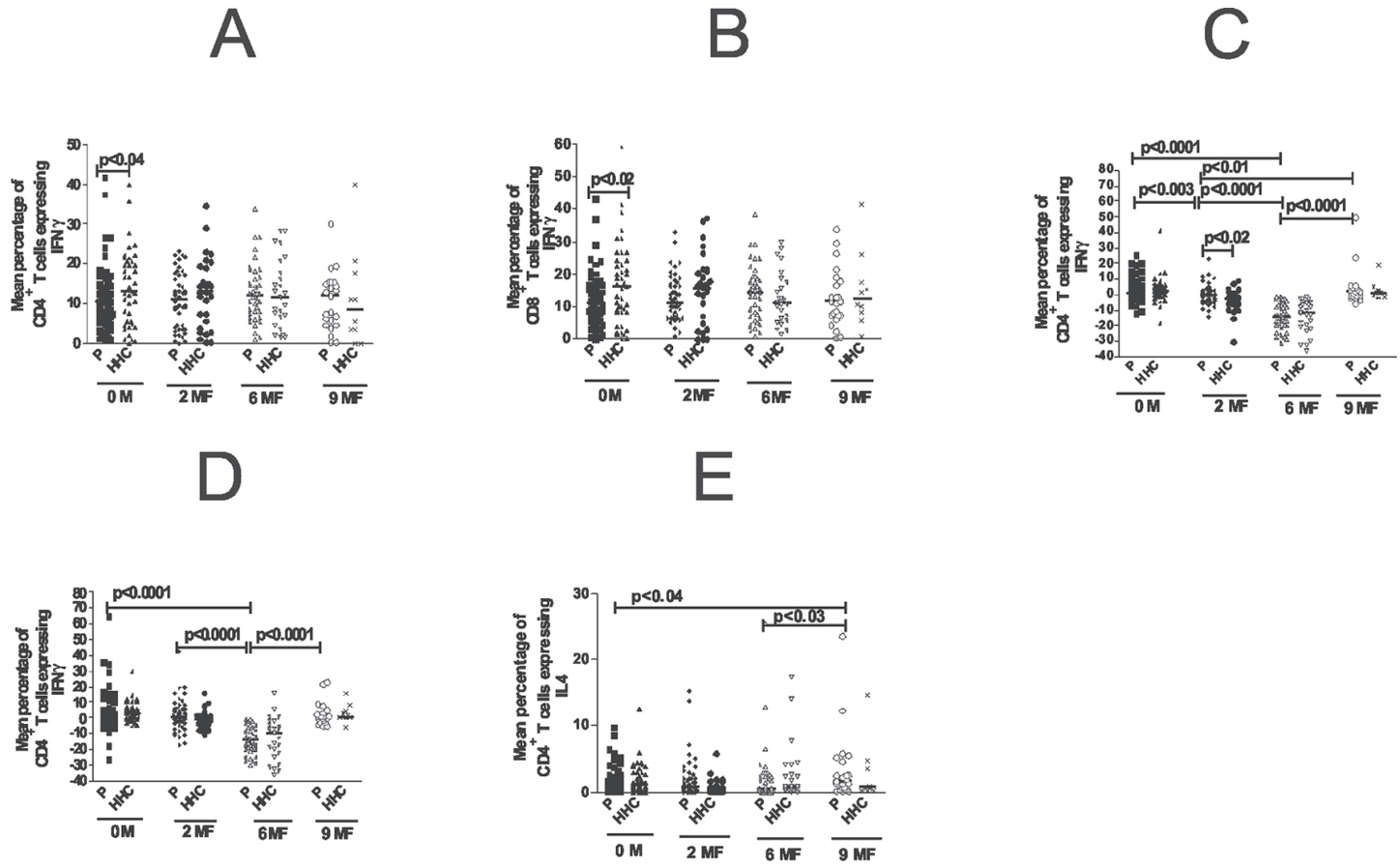

Fig. 2 : *M. tuberculosis* specific CD4⁺ /CD8⁺ T cells expressing IL4/IFNγ in PTB patients (P) and their PPD⁺ve house hold contact (HHC), A.- Mean percentage of CD4⁺ IFNγ expressing T cells in unstimulated PBMCs before and after chemotherapy, B.- Mean percentage of CD8⁺ IFNγ expressing T cells in unstimulated PBMCs before and after chemotherapy, C.- Mean percentage of CD4⁺IFNγ expressing T cells in patients and HHC after stimulation with PPD (The value is after subtracting unstimulated value from stimulated value), D.- Mean percentage of CD4⁺IFNγ expressing T cells in patients and HHC after stimulation with ESAT-6 (The value is after subtracting unstimulated from stimulated value). E. - Mean percentage of CD4⁺IL4 expressing T cells in unstimulated PBMCs before and after chemotherapy, Black bar represents the median value.

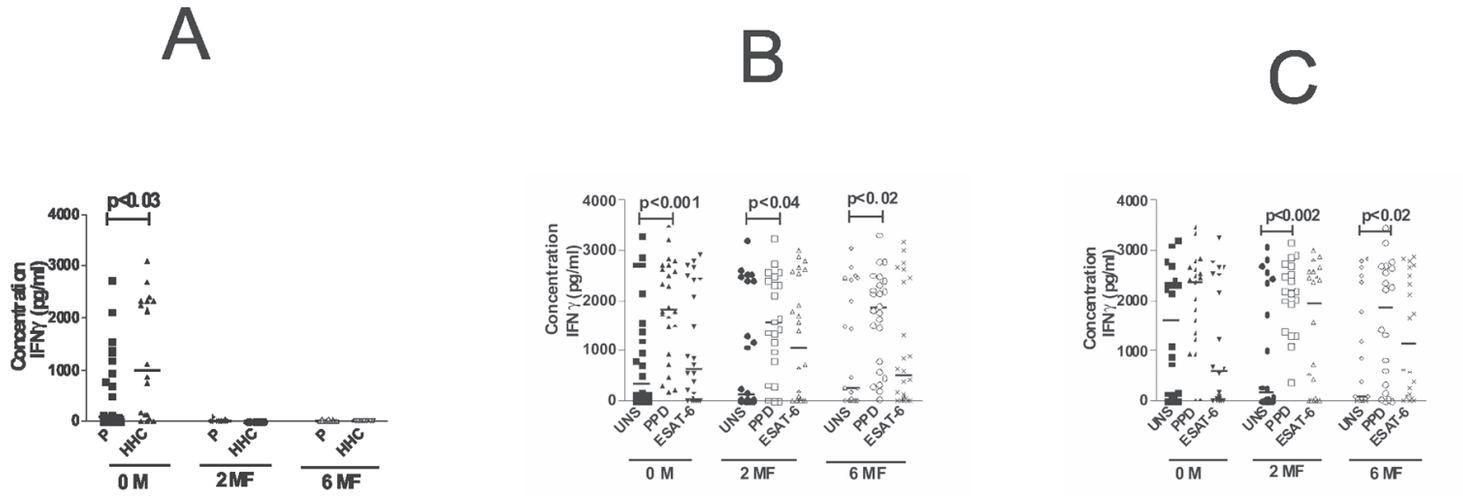

Fig. 3 : ***M. tuberculosis*** **specific peripheral blood mononuclear cells secreting IFNγ in PTB patients (P) and their PPD⁺ve house hold contact (HHC) in 5 days culture supernatant, A.- IFNγ in unstimulated PBMCs before and after chemotherapy, B.- IFNã in patients after stimulation with PPD and ESAT-6 before and after therapy. C.- IFNγ in HHC after stimulation with PPD and ESAT-6.**

TABLE-1 : Study subjects for T-cells in the study

Study subject	Total No.	Sex		Mean age Years	2 MF	6 MF	9 MF
		Male	Female				
Patients	67	35	32	27.76 ± 9.93	46	42	23
HHC	45	20	25	34.40 ± 9.33	28	25	10

suggests that there were defective IFNã production in patients which remained low after 6 month chemotherapy, but when we compared the cytokine levels after 9 months, levels again increased suggesting that patients makeup their immune parameter at the level of IFNã after successful TB treatment[2]. In 2011 it was noted that polyfunctional PPD specific CD4 T cells increased in all subjects following 6 months of TB treatment and cytokine production capacity of *M. tuberculosis* specific CD4 and CD8 T cell responses is associated with mycobacterial load. Higher IL-4 expressing $CD4^+$ T cells suggest that high Th2 type of response might be leading to suppression of Th1 immunity in patients. Overall, there were no significant differences in the production of IL-4 and IL-2 by stimulated cells from TB patients and HHC. We could not come across any longitudinal study on IL-4 expression by CD4 T cells in TB patients during therapy. Significant inter individual variation has also been observed in the evaluation of cytokine pattern in a longitudinal study[3]. Significant impairment of functional capacity of *M. tuberculosis* specific CD4/CD8 T cells has been reported previously with increasing mycobacterial load[2]**.** We observed depleted IFNγ producing $CD4^+$ cells after ESAT-6 and PPD stimulation in patient after 6 months of therapy and the percentage of these cells increased after 9 months follow-up, which suggests that the treatment of patient improves immune mechanism.

We observed higher secretion of IFNã in HHC when compared with PTB patients *ex vivo*. PPD induced IFNã secretion in patients at 0 month and all time point of chemotherapy and in HHC in 2 and 6 month follow up. We also observed higher IFNã expression by CD4/CD8 cells in unstimulated culture at 0 month. Similar finding in culture supernatant confirms a role of IFNã in protection against TB as seen in house hold contacts by restricting the infection inspite of living with the same exposure to *M. tuberculosis.*

Significantly high $CD4^+$ and $CD8^+$ T cells producing IFNγ in HHC as compared to patients and this finding suggests that there were defective IFNã production in patients which remained low after 6 month chemotherapy but when we compared the cytokine levels after 9 month levels again increased. We further confirmed this finding in culture supernatant which confirms role of IFNã in restricting infection, Higher IL-4 expressing $CD4^+$ T cells was noted which decreased after the therapy in patients suggesting role of IL-4 in the suppression of IFNγ (Th1 response) or survival of bacteria and progression of disease. The study gives scope for future study on T-cells mediated suppression in intracellular infection and suggests complex nature of immune mechanism operating in infectious disease like TB. This endeavour would restablish that the function of the immune system combined with anti TB therapy will benefit the clinical outcome in patients with TB.

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