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Research Article

Increased Frequency of Antigen-Specific Polyfunctional T Cells in Tuberculosis Patients

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This study assessed the polyfunctional T cells in healthy household contacts (HHCs) and TB patients. This study also assessed the memory subsets responsible for the secretion of IFN-γ during the short-term culture with *Mycobacterium tuberculosis* antigens. Frequencies of $CD4^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells and $CD8^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells specific to *M. tuberculosis* antigens were significantly higher in TB patients compared to HHC. IFN-γ-secreting T cells, during overnight stimulation with *M. tuberculosis* antigens, belonged to effector memory subset with a $CD45RA^-CD27^-$ phenotype. However, the number of IFN-γ-secreting effector memory cells did not differ between HHC and TB patients.

1. Introduction

Tuberculosis (TB) is a global health problem with 2 billion people infected with the causative agent *Mycobacterium tuberculosis* (*M. tuberculosis*). Of these, only 10 percent will progress to active TB disease resulting in almost 2 million deaths per year [1]. This provides compelling evidence that the human immune system is capable of controlling the pathogen. However, the precise mechanisms contributing to the loss of immune control and progression of active TB disease are not known. A better understanding of these processes is critical for the development of improved diagnostics, treatment protocols, and vaccines.

It has been long recognized that Interferon gamma (IFN-γ) producing T cells provide the major effector response in TB [2–5]. However, assessment of IFN-γ producing T cells alone may not be sufficient. Further analysis of various T cell memory subsets, such as central memory and effector memory subsets, may provide some light in understanding the T cell protection in TB.

T cells are able to produce multiple factors simultaneously and they are termed polyfunctional T cells. The evidence from animal and human models of chronic viral infections indicates that high levels of chronic antigen stimulation lead to functional impairment of antigen-specific T cell responses, with reduced cytokine production, cytotoxicity, and proliferative capacity [6–11]. The capacity of antigen-specific T cells to produce multiple cytokines simultaneously has been associated with superior functional capacity [12] and has been correlated with control of human chronic viral infections such as human immunodeficiency virus (HIV) [13–15] and hepatitis C virus (HCV) [16]. Moreover, polyfunctional T cells have been associated with protection against disease progression in mouse models of *Leishmania major* [17] and *M. tuberculosis* [18]. Polyfunctional T cells, which produce IFN-γ, IL-2, and TNF-α, have been described in studies of *M. tuberculosis* infected adults [19, 20], but they ended up with contradicting results.

In this study, we have enumerated the polyfunctional T cells in QuantiFERON-TB Gold in-tube (QFT-IT) positive HHC and QFT-IT positive TB patients. We also looked into the contribution of different memory T cell subset in the secretion of IFN-γ during short-term culture with TB antigens.

2. Material and Methods

This study protocol was approved by the Institutional Ethical Committee of the National Institute of Research in

Tuberculosis (NIRT), India. NIRT was formerly known as Tuberculosis Research Center. An informed written consent was obtained from study subjects before the blood was drawn.

2.1. Study Subjects. A total of 50 study subjects, who were positive for QFT-IT, were selected for this study. Among them, 25 were healthy household contacts (HHCs), and 25 subjects were newly diagnosed active pulmonary TB patients. All the study subjects were recruited at the National Institute for Research in Tuberculosis (NIRT), Chennai, India. Active TB was confirmed by identification of *M. tuberculosis* by culture from at least one of the 3 sputum samples. None of the TB patients had previous history of TB, and all were naïve for the antituberculous therapy. The age range of TB was from 28 to 52 years with a median of 36 years. Of them, 75%, were males.

HHCs were identified from families, where there was at least one case of sputum positive pulmonary TB living in the same household, for at least 3 months immediately preceding the start of treatment of the index case. Among the 25 HHCs, 62% were males, and their age ranged from 25 to 55 years, with a median of 32 years. All were naïve for TB prophylaxis treatment.

2.2. Isolation of Peripheral Blood Mononuclear Cells (PBMCs). PBMCs were isolated by density gradient centrifugation by carefully layering heparinized blood over an equal volume of Histopaque (Sigma-Aldrich Corp., St. Louis, MO, USA) and spinning at 18°C for 30 minutes at 2000 rpm. The buffy coat containing peripheral blood mononuclear cells was removed, washed twice using Hanks' balanced salt solution (HBSS) (Sigma-Aldrich Corp, St. Louis, MO, USA), and resuspended in complete medium consisting of RPMI 1640 (Sigma-Aldrich Corp., St. Louis, MO, USA), 2 mM L-glutamine, 1% 100X Antibiotic Antimycotic Solution (Invitrogen Corp., CA, USA), and 10% heat, inactivated human AB-serum (Sigma-Aldrich Corp. MO, USA).

2.3. In Vitro Culture. PBMCs were stimulated overnight in the presence of PPD, ESAT-6, and CFP-10 proteins at the concentration of 5 μg/mL or absence of any stimulant in a 37°C, 5% CO_2 incubator. Just 4 hours prior to stopping the reaction, 5 μg/mL of Brefeldin A was added to each well. After overnight incubation, the cells were washed with HBSS and used for downstream process.

2.4. Intracellular Cytokine Assay. Cytokine-(IFN-γ, TNF-α and IL-2) secreting T cells were enumerated by intracellular cytokine staining method. Briefly, cells were washed and labelled with fluorochrome-conjugated antibodies for surface proteins. Then, the cells were fixed using Cytofix/Cytoperm buffer (BD Biosciences, San Jose, CA, USA) and Washed with Perm/wash buffer (BD Biosciences, San Jose, CA, USA). The cells were then stained for intracellular cytokines. After washing, cells were fixed using 1% paraformaldehyde. The following combination of antibodies is used. To identify multiple cytokine-secreting polyfunctional T cells, cells were stained with CD4 PE-Cy5 or CD8 PE-Cy5, IL-2 APC, IFN-γ Alexa Fluor 488, and TNF-α PE. To identify the memory cell subset, the cells were stained with CD4 APC or CD8 APC, CD27 PE, CD45RA PE-Cy5, and IFN-γ Alexa Fluor 488 (BD Biosciences, San Jose, CA, USA).

Table 1: Enumeration of antigen-specific IFN-γ, IL-2, and TNF-α T cells in HHC and TB patients.

Cytokine	Antigen	HHC median (range)	TB median (range)	P value
CD4 IFN-γ	PPD	0.30 (0–4.4)	0.9 (0–5.6)	0.137
	ESAT-6	0.39 (0.09–3.4)	0.8 (0.1–3.8)	0.101
	CFP-10	0.36 (0.1–6.7)	0.67 (0.13–2.4)	0.747
CD8 IFN-γ	PPD	0.35 (0.1–2)	0.52 (0–1.4)	0.901
	ESAT-6	0.43 (0.04–1.9)	0.56 (0–1.8)	0.989
	CFP-10	0.24 (0.1–1.1)	0.48 (0.06–2.0)	0.089
CD4 TNF-α	PPD	0.37 (0.1–1.3)	0.57 (0–2.1)	**0.036**
	ESAT-6	0.39 (0.2–2.4)	0.53 (0–2.1)	0.651
	CFP-10	0.38 (0.1–6.7)	0.48 (0.1–5.9)	0.826
CD8 TNF-α	PPD	0.54 (0.1–2.0)	0.62 (0–4.0)	0.505
	ESAT-6	0.47 (0.16–3.1)	0.62 (0.18–5.1)	0.722
	CFP-10	0.47 (0–2.8)	0.64 (0–3.1)	0.639
CD4 IL-2	PPD	0.47 (0.0–2.1)	0.58 (0–5.9)	0.181
	ESAT-6	0.32 (0–4.1)	0.53 (0.1–1.9)	0.798
	CFP-10	0.22 (0–4.6)	0.74 (0–2.3)	0.639
CD8 IL-2	PPD	0.55 (0.1–2.3)	0.38 (0–4.0)	0.730
	ESAT-6	0.42 (0.1–2.7)	0.49 (0–4.9)	0.791
	CFP-10	0.44 (0–2.2)	0.24 (0–3.2)	0.836

2.5. Flow Cytometric Acquisition and Analysis. The sample tubes containing paraformaldehyde-fixed cells were acquired using a FACSCalibur Flow cytometer buffer (BD Biosciences, San Jose, CA, USA). The acquired flow cytometric data were analyzed using FlowJo software (Tree Star Inc., San Carlos, CA, USA) version 7.1.1.

2.6. Statistical Analysis. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Mann-Whitney "*U*" test was carried out to calculate the differences between the groups. Receiver operator curve (ROC) analysis was performed to derive the cut-off points.

3. Results

3.1. Frequency of Single Cytokine-Secreting T Cells. Table 1 shows the number of PPD-, ESAT-6-, and CFP-10 specific $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ T cells upon overnight stimulation of PBMC from HHC and the TB patients. Although, antigen-specific $CD4^+IFN\text{-}\gamma^+$ T cells were slightly higher in TB patients than HHC, the difference did not reach statistical significance. Similarly, the frequency of antigen-specific $CD8^+IFN\text{-}\gamma^+$ T cells also did not differ significantly between HHC and TB.

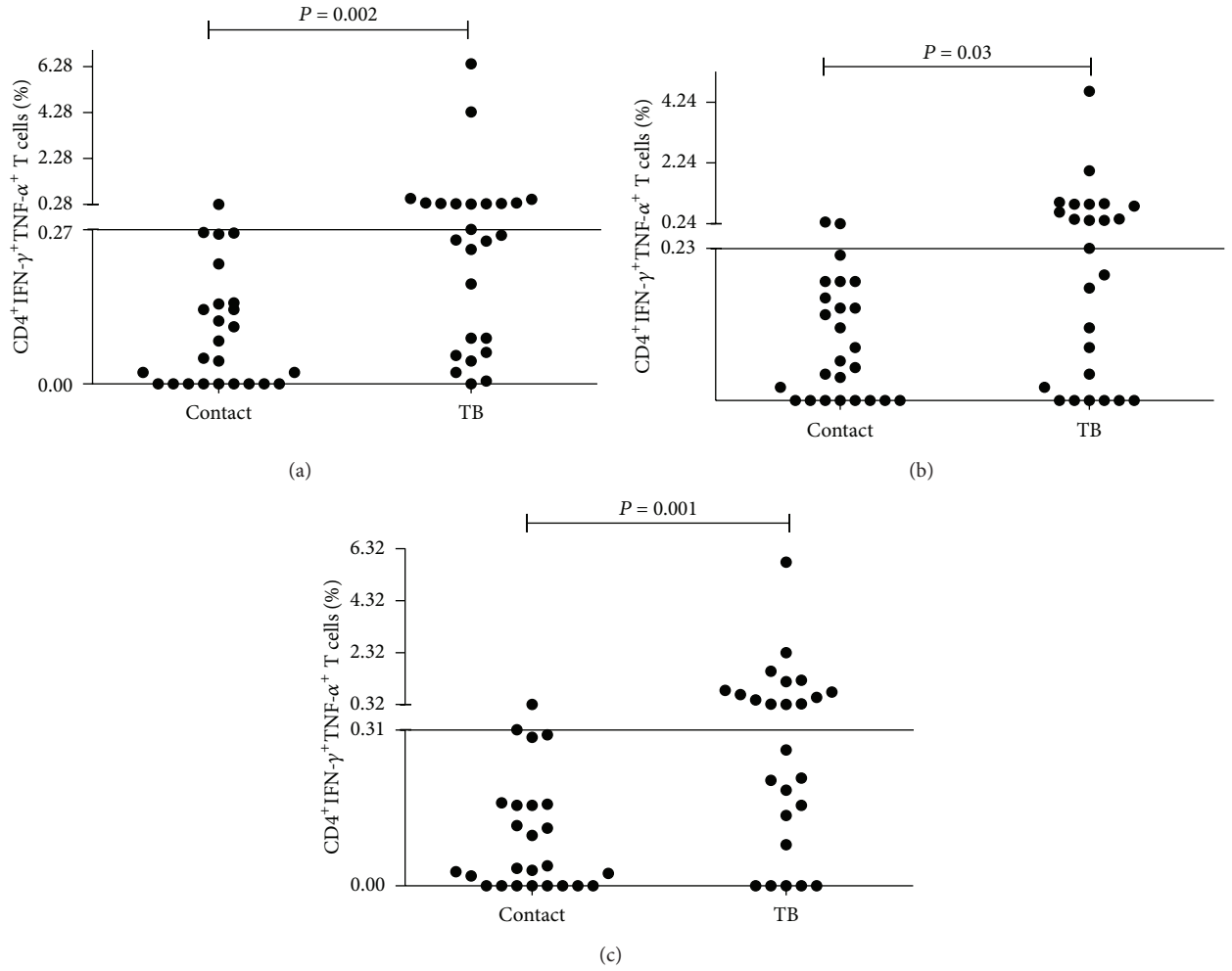

Figure 1: Enumeration of antigen-specific $CD4^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells in HHC and TB patients. Scatter plots show antigen-specific $CD4^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells in HHC and TB patients. The frequency of antigen-specific ((a) PPD, (b) ESAT-6, and (c) CFP-10) $CD4^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells was significantly higher in TB patients compared to HHC for all three antigens. The difference between HHC and TB was calculated by Mann-Whitney *U* test. TB: tuberculosis patients; HHCs: healthy household contacts.

While the proportion of PPD specific $CD4^+TNF\text{-}\alpha^+$ T cells was significantly higher in TB patients (median, 0.37%) compared to HHC (median, 0.57%) ($P = 0.036$), ESAT-6 or CFP-10 specific $CD4^+TNF\text{-}\alpha^+$ T cells did not differ between those two groups. The proportion of antigen specific $CD4^+IL\text{-}2^+$ or $CD8^+IL\text{-}2^+$ cells did not differ between HHC and TB patients (Table 1).

3.2. Frequency of Polyfunctional T Cells. There was an increase in the expression of $IFN\text{-}\gamma^+IL\text{-}2^+$ and $IFN\text{-}\gamma^+$ $TNF\text{-}\alpha^+$ (dual cytokine secreting) T cells, when PBMC from HHC and TB patients were stimulated with antigens for overnight. Interestingly, significantly higher frequency of $CD4^+$ $IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells and $CD8^+$ $IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells was observed in TB patients compared to HHC for all the three antigens (PPD, ESAT-6, and CFP-10) used in our study (Figures 1 and 2). Another subset $CD4^+IFN\text{-}\gamma^+IL\text{-}2^+$ T cells or $CD8^+IFN\text{-}\gamma^+IL\text{-}2^+$ T cells did not differ between HHC and TB patients (Figure 3).

The quantitative increase of $IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells in TB patients compared to HHC prompted us to calculate the sensitivity and specificity, when the numbers of $IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells were enumerated to differentiate HHC and TB patients. We derived cut-off points for each antigen specific of $IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cell subset using HHC as controls and TB patients as diseased.

With a cut-off value 0.255 (AUC, 0.672: 95% confidence interval (CI), 0.521–0.832) for PPD-specific $CD4^+$ $IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells, 12 out of 25 TB patients and 1 out of 25 HHCs were found to be positive. This yielded a sensitivity of 48% (95% CI, 27.8%–68.7%) with a specificity of 96% (95% CI, 74.7%–99.9%) (Table 2). The cut-off values 0.225 (AUC, 0.626 : 95% CI, 0.456–0.795) and 0.310 (AUC, 0.743 : 95% CI, 0.582–0.905) were derived for ESAT-6 and CFP-10 specific of $CD4^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells, respectively. ESAT-6- and CFP-10-specific $CD4^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells showed sensitivities of 48% (95% CI, 27.8%–68.7%) and 52% (95% CI, 31.3%–72.2%) with specificities of 100% and 96% (95% CI,

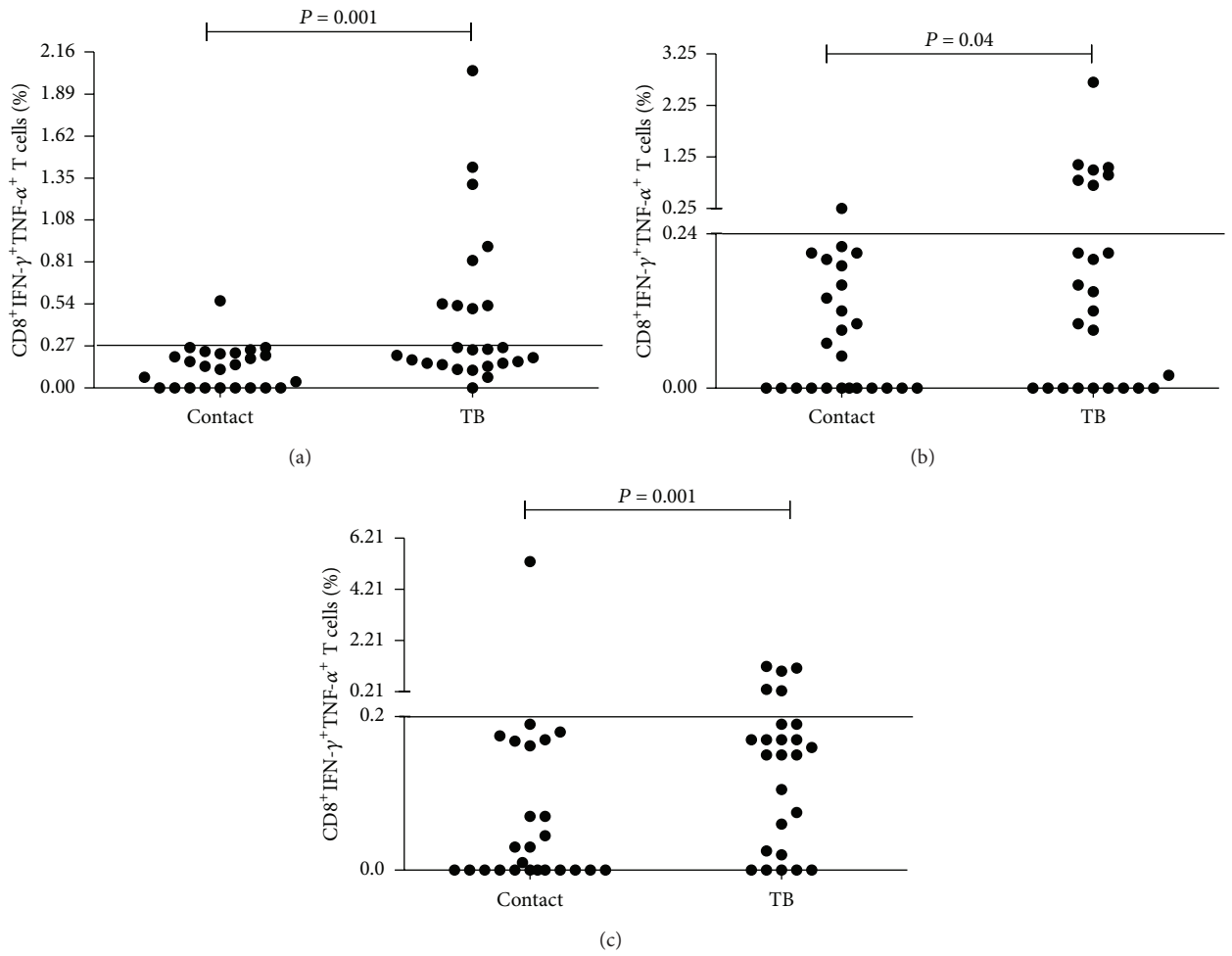

Figure 2: Enumeration of antigen-specific $CD8^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells in HHC and TB patients. Scatter plots show antigen-specific $CD8^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells in HHC and TB patients. The frequency of antigen ((a) PPD, (b) ESAT-6, and (c) CFP-10) $CD8^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells was significantly higher in TB patients compared to HHC for all three antigens. The difference between HHC and TB was calculated by Mann-Whitney *U* test. T: tuberculosis patients; HHCs: healthy household contacts.

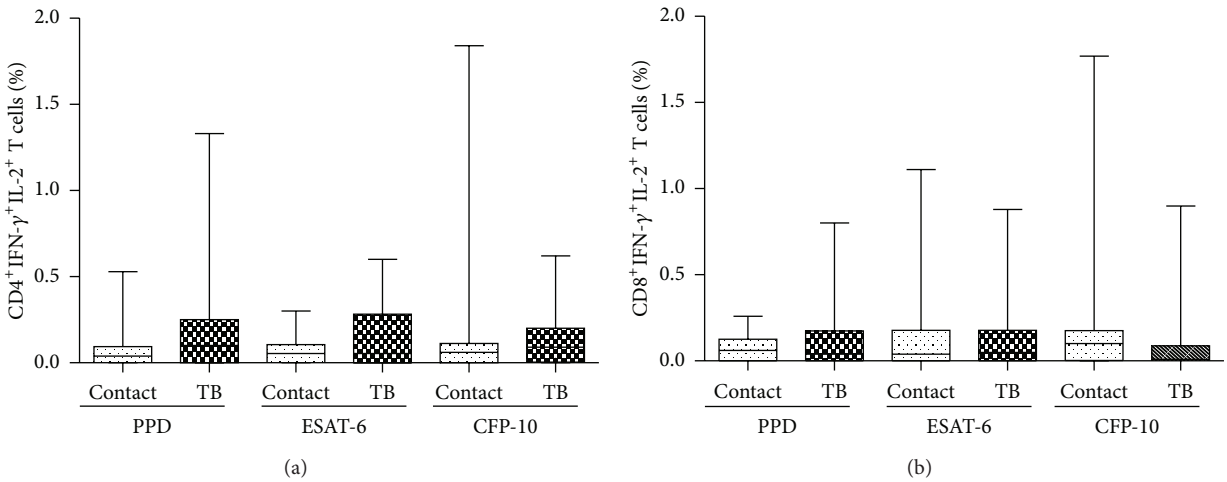

Figure 3: Enumeration of antigen-specific dual cytokine-secreting T cells in HHC and TB patients. Box and Whisker plots show range, interquartile range, and median of antigen-specific $CD4^+IFN\text{-}\gamma^+IL\text{-}2^+$ T cell (a) and $CD8^+IFN\text{-}\gamma^+IL\text{-}2^+$ T cell (b) in HHC and TB patients. The frequency of $IFN\text{-}\gamma^+IL\text{-}2^+$ T cell subset did not differ between HHC and TB patients. The difference between HHC and TB was calculated by Mann-Whitney *U* test. TB: tuberculosis patients; HHCs: healthy household contacts.

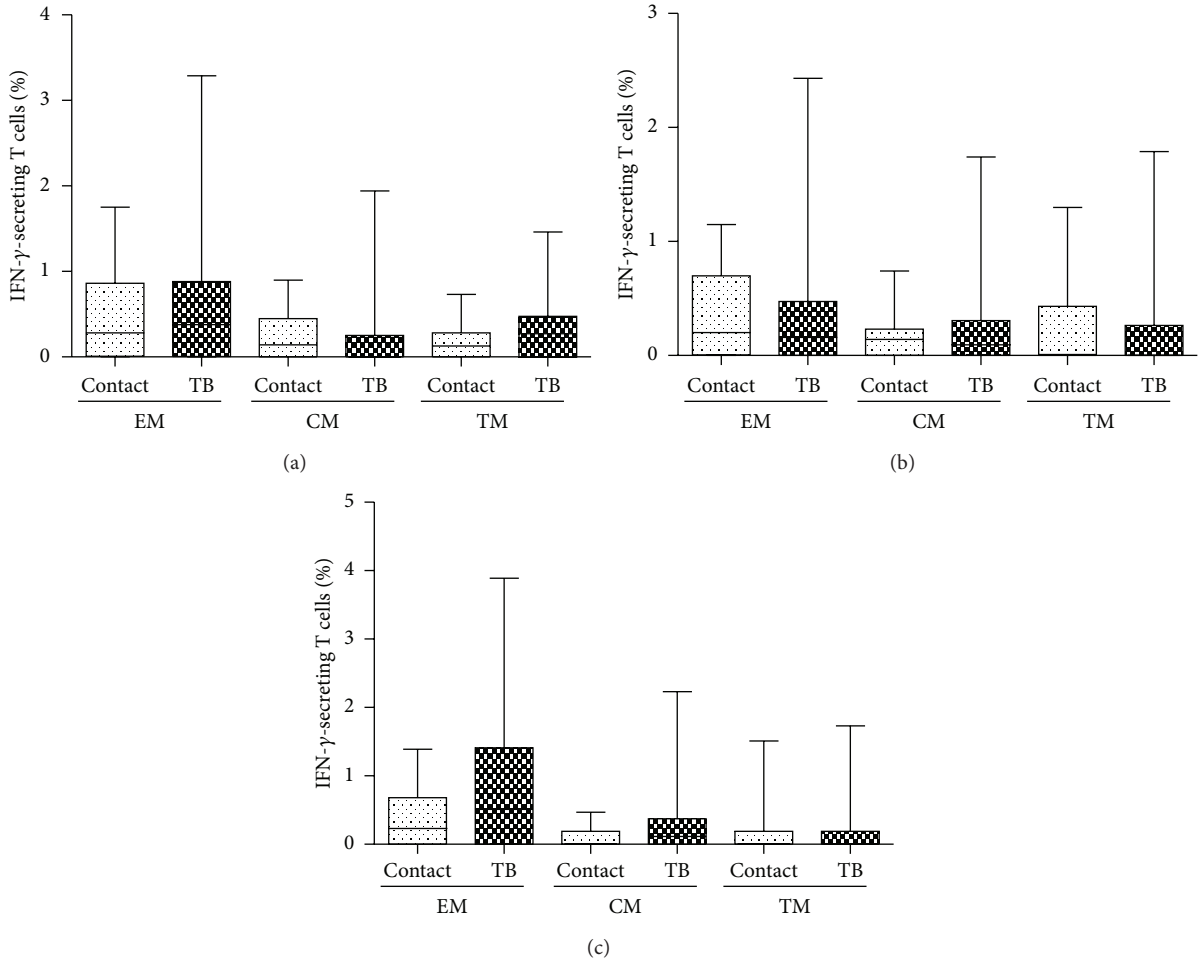

Figure 4: Memory phenotype of antigen-specific $CD4^+IFN\text{-}\gamma^+$ T cells in HHC and TB patients. Box and Whisker plots show range, interquartile range, and median of antigen-specific IFN-γ-secreting memory T cell subsets in HHC and TB patients. Effector memory subset secretes more IFN-γ than other memory subsets in HHC and TB patients. The difference between HHC and TB was calculated by Mann-Whitney test. EM-Effector memory T cell; CM: Central Memory T cell; TM: terminally differentiated memory T cell; TB: tuberculosis patients; HHCs: healthy household contacts.

74.7%–99.9%), respectively, for differentiating TB patients and HHC. When the results of ESAT-6 and CFP-10 were combined, it showed a sensitivity of 68% with a specificity of 96%.

Similarly, PPD-, ESAT-6-, and CFP-10-specific $CD8^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells showed sensitivities of 36%, 20%, and 32% and specificities of 96% for diagnosing TB patients. The combination of ESAT-6 and CFP-10 results did not improve the sensitivity (Table 2).

3.3. Memory T Cell Subtypes. Based on the expression of CD27 and CD45RA, memory subsets can be classified as central memory ($CD27^+CD45RA^-$), effector memory ($CD27^-CD45RA^-$), and terminally differentiated memory ($CD27^-CD45RA^+$) subsets (49). Frequency of IFN-γ^+ T cells in all the CD4 and CD8 memory subsets were enumerated (Figures 4 and 5).

Upon overnight stimulation with antigens (PPD, ESAT-6, and CFP-10), effector memory subset expressed a significantly higher level of IFN-γ, compared to terminally differentiated memory and central memory subset. However, the frequency of the IFN-γ-secreting T cells from any of these T cell subsets did not differ significantly between HHC and TB patients.

4. Discussion

This study enumerated the number of polyfunctional T cells in QFT-IT positive HHC and TB patients. In addition, this study also assessed the memory subsets, which were responsible for the secretion of IFN-γ in QFT-IT positive HHC and TB patients during the short-term culture with *M. tuberculosis* antigens. We used ESAT-6 and CFP-10 antigens to identify *M. tuberculosis*-specific T cells, since these antigens are used in commercial QFT-IT assays. In addition, we also used PPD as it is suggested that it induces IFN-γ secretion in HHC, as well as in TB patients.

We found that the numbers of $IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells were significantly higher in TB patients compared to HHC. This is the first study which compared the $IFN\text{-}\gamma^+TNF\text{-}\alpha^+$

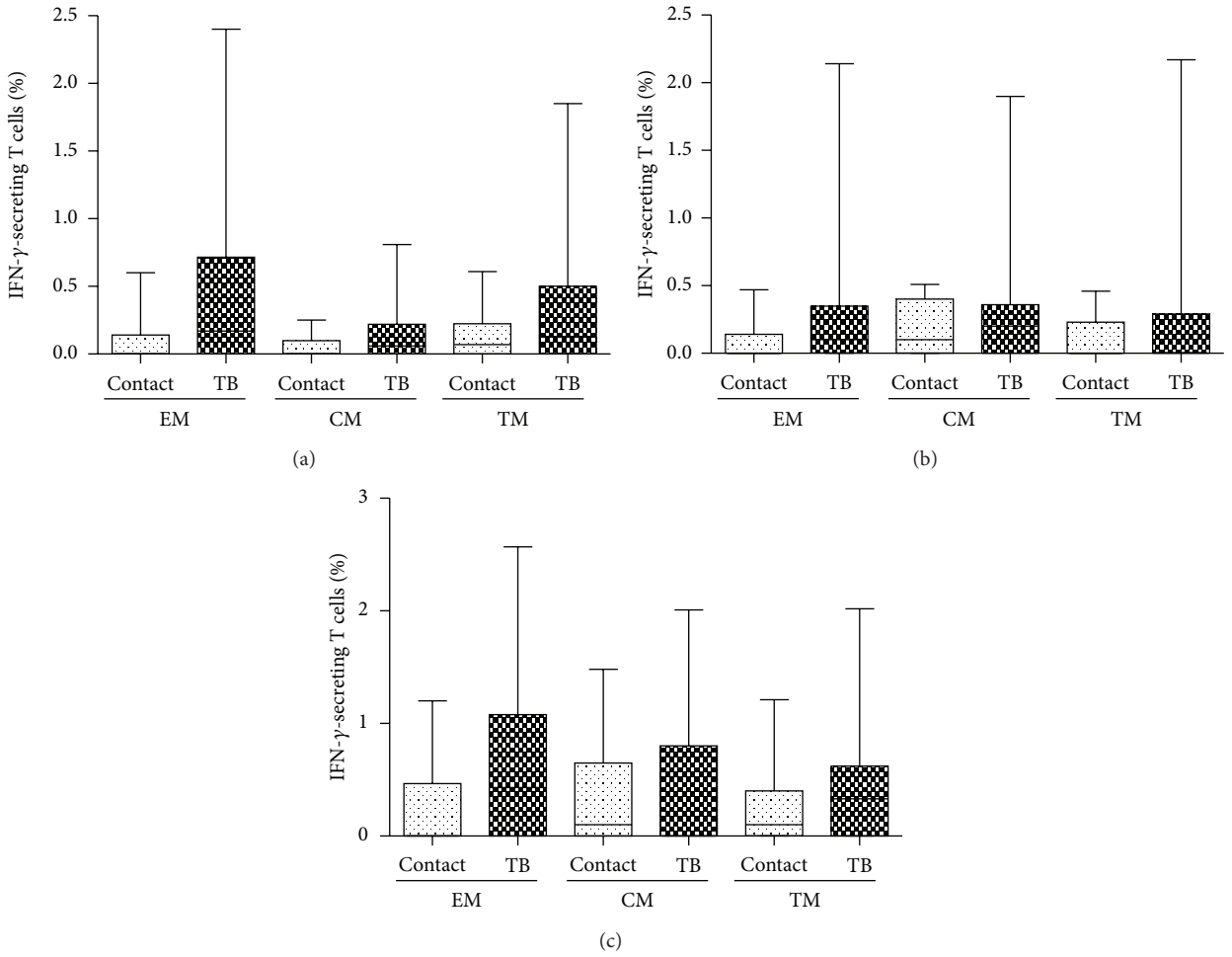

Figure 5: Memory phenotype of antigen-specific $CD8^+IFN\text{-}\gamma^+$ T cells in HHC and TB patients. Box and Whisker plots show range, interquartile range, and median of antigen-specific IFN-γ-secreting memory T cell subsets in HHC and TB patients. Effector memory subset secretes more IFN-γ than other memory subsets in HHC and TB patients. The difference between HHC and TB was calculated by Mann-Whitney test. EM: effector memory T cell; CM: central Memory T cell; TM: terminally differentiated memory T cell; TB: tuberculosis patients; HHCs: healthy household contacts.

Table 2: Sensitivity and specificity for $CD4^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ and $CD8^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells in discriminating HHC and TB patients.

Phenotype	Antigen	Cut-off value	% Sensitivity (95% CI)	% Specificity (95% CI)
CD4	PPD	0.27	48 (27.8–68.7)	96 (79.7–99.9)
	ESAT-6	0.23	48 (27.8–68.7)	100
	CFP-10	0.31	52 (31.3–72.2)	96 (74.7–99.9)
	ESAT-6 + CFP-10		68 (49.7–86.3)	96 (79.7–99.9)
CD8	PPD	0.27	36 (18.0–57.5)	96 (79.7–99.9)
	ESAT-6	0.24	20 (6.8–40.7)	96 (79.7–99.9)
	CFP-10	0.20	32 (15–53.5)	96 (79.7–99.9)
	ESAT-6 + CFP-10		32 (15–53.5)	96 (79.7–99.9)

T cells between HHC and TB patients. An earlier study, which was conducted in HIV positive latent TB subjects, also reported higher frequency of $IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ CD4 cells [20]. However, this study did not compare the number of $IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T cells between latent and active TB patients.

Young et al. also compared the number of single cytokine-secreting T cells and polyfunctional T cells in TB patients before and after chemotherapy and found that the number of polyfunctional T cells was decreased after successful TB therapy [21]. During the chronic infection with *M. tuberculosis*, the host slowly loses the important characteristics of the immune cells such as cytokine secretion and cytolytic property [22]. We speculate that the exhaustion of T cells in the TB infected individuals endows T cells with polyfunctions

such as the secretion of more than a single cytokine. This might be the reason for the higher number of IFN-γ^+TNF-α^+ polyfunctional T cells in TB patients compared to HHC in our study. Due to the small number of study subjects in each group, we could not find any association between clinical outcome and changes in the number of polyfunctional T cells. Therefore, further studies are needed to confirm the role of polyfunctional T cells in protective mechanism against TB.

On the other hand, we found that the number of single (either IFN-γ or TNF-α) cytokine-secreting T cells did not differ between these two groups, except the PPD-specific TNF-α. This study results corroborates with our earlier observations [23, 24]. PPD-specific TNF-α was significantly higher in TB patients compared to HHC. The recent study conducted by Harari et al. also found that TB patients were found to have higher frequency of antigen-pecific TNF-α compared to subjects with latent TB [25]. However, the number of ESAT-6- and CFP-10-specific TNF-α did not differ between TB patients and HHC in our study.

When we analyzed the sensitivity and specificity of antigen-specific CD4$^+$IFN-γ^+TNF-α^+, in differentiating HHC and TB patients, the combination of ESAT-6 and CFP-10 CD4$^+$IFN-γ^+TNF-α^+ showed a moderate sensitivity of 68% with a specificity of 96%. CD8$^+$IFN-γ^+ TNF-α^+ T cells could produce only 36% of sensitivity with a specificity of 96%. There was no difference in demographic details or clinical and bacteriological features between the TB patients which were dual cytokine positive and those who were negative. Therefore, further studies with larger number of study subjects are needed to answer why few of the TB patients in this study had low number of IFN-γ^+TNF-α^+ T cells, similar to HHC. Further studies have to also test the sensitivity of IFN-γ^+ TNF-α^+ T cells in smear negative, clinically diagnosed TB cases, where confirmation of TB is difficult. Furthermore, the cut-off points calculated in the study for CD4$^+$IFN-γ^+ TNF-α^+ T cells also have to be reconfirmed with large number of study subjects.

The profile of IFN-γ^+IL-2$^+$ T cells was studied in various viral infections and TB patients [26–31]. In many of the earlier studies, IFN-γ^+IL-2$^+$ T cells were suggested as positive correlates to the protection. Antigen clearance of past influenza infection was correlated with CD4 T cells secreting IFN-γ^+IL-2$^+$ [27]. While high viral load in cytomegalovirus was associated with high number of IFN-γ alone positive T cells, the subjects with low viral load had high number of IFN-γ^+IL-2$^+$ dual cytokine-secreting T cells [28]. Similar trend was observed for other viral infections such as hepatitis C virus, HSV, Epstein-Barr Virus, and HIV [29–31]. The TB patients, who have completed successful anti TB treatment, were found to have higher frequency of IFN-γ^+IL-2$^+$ or IFN-γ^+IL-2$^+$TNF-α^+T cells [32, 33].

Based on the above observations, we expected that number of IFN-γ^+IL-2$^+$ T cells would be higher in HHC compared to TB patients. However, this trend was not found in our study. There was less number of IFN-γ^+IL-2$^+$ T cells, similar to TB, in HHC. An earlier study conducted in subjects with latent TB and HIV-1 infection also reported a fewer number of IFN-γ^+IL-2$^+$ T cells [20].

Based on the expression of phenotypic markers CD45RA and CD27, the antigenic memory cells are classified as effector memory T cells, central memory T cells, terminally differentiated T memory cells, and naïve cells. The present study results indicate that the effector memory T cells secrete IFN-γ in response to *M. tuberculosis*-specific antigens during the short-term incubation. This study results corroborate with the earlier study results conducted by Goletti et al. [34]. But some of the antigen specific cells were having CD45RA$^+$ and CD27$^-$ phenotype. This indicates that the terminally differentiated effector T cells also secrete IFN-γ, to some extent, in response to *M. tuberculosis* antigens during the short-term incubation. However, number and phenotype of IFN-γ-secreting T cell were similar in HHC and TB patients.

Altogether, this study concludes that TB patients were found to have higher number of IFN-γ^+TNF-α^+ T cells, compared to HHC. The effector memory subset, with a phenotype of CD45RA-CD27-T cells, plays an important role in the secretion of IFN-γ compared to other subsets during short term incubation with TB antigens.

Conflict of Interests

The authors declare that they have no conflict of interests, including specific financial interests or relationships or affiliations to the subject matter or materials discussed in the paper.

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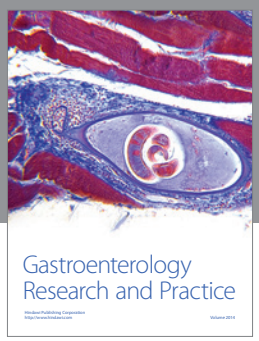

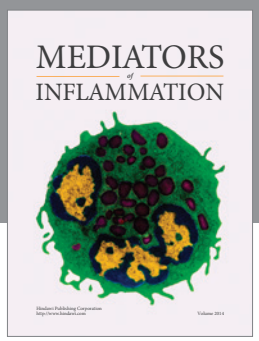

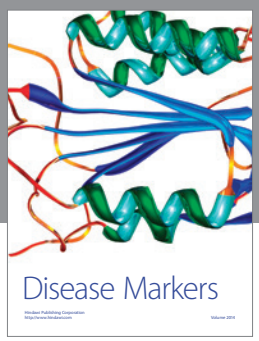

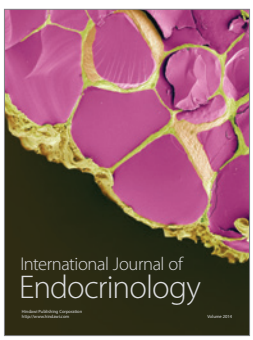

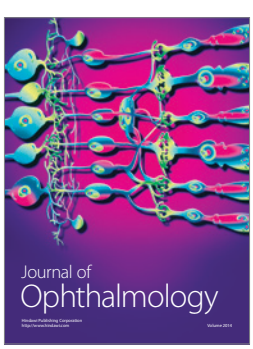

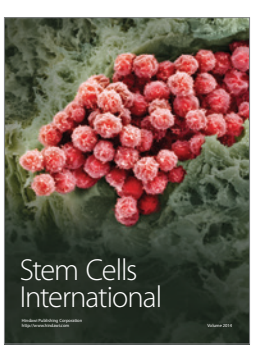

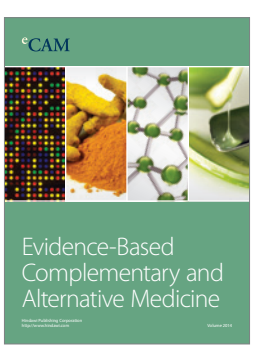

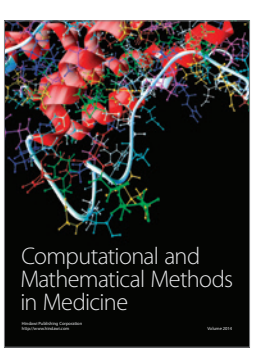

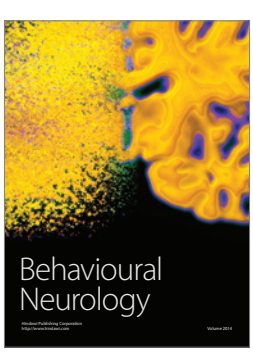

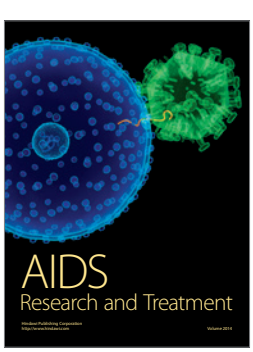

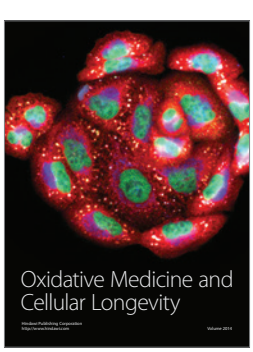

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