Abstract

Background: Tuberculosis (TB) control is challenging due to poor drug compliance and emerging resistance. The need of the hour is to determine the prediction of disease cure and relapse. Patients’ immune response is crucial to the disease outcome. This study was designed to study the immune profile of TB patients during treatment and cure. Methods: The cross-sectional study included newly diagnosed pulmonary TB patients and healthy controls. Levels of serum cytokines/chemokines (Th1/Th2/Th17) were measured by BD cytometric bead array. The cell surface markers assessed in the study were CD3, CD4, CD8, CD16, CD56, and BD human regulatory T cell cocktail (CD4/CD25/CD127). Results: Data analysis observed statistically significant differences in CD3dim/CD56 + natural killer T cell cocktail (CD4/CD25/CD127). Results: The percentage of T regulatory was found to be high in patients when compared with healthy controls; the values were statistically significant (0.0002). Interleukin-6 was significantly associated with the disease (P < 0.0485). Discussion: A comprehensive understanding of role of CD3dim/CD56+ NKT in antimycobacterial immunity may enable new possibilities for NK cell-based prophylactic and/or therapeutic strategies against TB.

Keywords: Cytokines, immunity, natural killer cell, regulatory T cell

Introduction

Worldwide, the growing epidemic of tuberculosis (TB) is alarming. An estimated one-third of the world population is infected with Mycobacterium tuberculosis (MtB) and at risk of developing the disease. The lifetime risk of progressing to active TB when latently infected with TB is about 5%.[1] With more than eight million people progressing to active TB every year, and a death rate of about 25%, pulmonary TB (PTB) is one of the most life-threatening human diseases with more than 90% of global TB cases and deaths occurring in the developing world. Geographically, Southeast Asia and the Western Pacific Region accounted for 56% of the world’s TB cases. In India, 1.4 million TB cases were notified per year.[1] For immunologists, it has been of great interest that only an estimated 5%–10% of Mtb-infected, HIV-uninfected individuals develop active disease, the remainder being protected from illness by their immune system. The Bacillus Calmette–Guérin vaccine primarily protects young children from disseminated forms of the disease but is not effective in protecting adults from PTB.

Mechanism of immune response in immune-competent healthy individuals postexposure to tubercle bacilli may help to devise strategies for good and effective prophylactic regimens involving immunotherapeutic agents.[2] However, it is important to understand the mechanism of activation of effector cells in humans as different experimental models have different mechanisms, thereby limiting the extrapolation of data.[3–6] The major arm of host defense against TB is

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cell-mediated immunity. Classically, it has been elucidated that CD4 T cells play an important role in antimycobacterial immunity.\textsuperscript{[6]} To clarify the mechanisms of protective immunity against Mtb infection and disease in humans, many reports have addressed the potential immunological defect(s) by comparing immune phenotypes in actively diseased patients to those with latent infection. Most of these studies have demonstrated that CD4+ T lymphocyte subsets are generally low in peripheral blood of TB patients,\textsuperscript{[7]} whereas γδ T cell subsets have been shown to be elevated in some studies,\textsuperscript{[8]} and normal\textsuperscript{[9]} numbers have been observed in other studies. Some studies have demonstrated inconclusive reports on cell numbers of B-lymphocyte and natural killer (NK) cell in TB patients.\textsuperscript{[7,10‑12]} NKT cells have, to our knowledge, not been investigated in drug-naïve TB patients.

Factors contributing to TB susceptibility remain unclear, and follow-up data during therapy are scanty. However, Al‑Hajoj in a recent article commented on the various factors contributing toward the susceptibility of TB infection.\textsuperscript{[13]} The manipulation done by the tubercle bacilli to survive in the niche needs to be studied and evaluated. Various mechanisms have been elucidated in recent times with more emphasis on the molecular mechanisms such as microRNA (miRNA). Furci et al. reported that the miRNA is produced by the host in macrophages as a response to mycobacterial infection. The study provided new insights about regulation mechanisms in various host–pathogen interactions, at posttranscriptional levels.\textsuperscript{[14]} Such studies which shower light on host–pathogen interactions are the need of the hour. The discovery of the mechanisms of the natural protective resistance would benefit in the efforts to stop the spread of the disease and contribute to treatment shortening, possibly to disease prevention. The aim of the study was primarily to correlate immune parameters with fast and slow response to treatment in order to look for surrogate immunological biomarkers for disease and cure.

**Methods**

**Ethics**

We confirm that all methods were carried out in accordance with relevant guidelines and regulations, and the study was ethically approved by the All India Institute of Medical Sciences (AIIMS), Ethics Committee approval number (IESC/T‑92/01.02.2013). The study was approved from ethical angle in the year 2013.

**Selection and description of participants**

The study was conducted at the TB Division, Department of Microbiology, AIIMS, New Delhi, India, which is a multispecialty tertiary care, teaching hospital with 2500-bed capacity. All patients provided written informed consent to be enrolled and to be the part of the study. Drug-naïve TB patients were enrolled before the initiation of antitubercular therapy and were formally followed for a period of 2 years.\textsuperscript{[15]} The patient participation was voluntary, and a written informed consent was obtained; demographic and clinical data including gender, age, origin, geography, microbiological details, and comorbidities including results of HIV testing, family TB history, diagnosis of previous TB, antimicrobial drug resistance, chest X-ray findings, and treatment prescribed were collected by treating physician and trained medical staff.

The study samples include a total of 12 patients with PTB and 11 healthy controls. Drug-naïve patients were enrolled before the initiation of antitubercular therapy and were formally followed till the completion of treatment.\textsuperscript{[15]} The patient participation was voluntary, and a written informed consent was obtained; demographic and clinical data including gender, age, origin, geography, microbiological details, and comorbidities including results of HIV testing, family TB history, diagnosis of previous TB, antimicrobial drug resistance, chest X-ray findings, and treatment prescribed were collected by treating physician and trained medical staff.

**Collection and processing of samples**

Twelve patients were enrolled and studied; they were followed formally throughout the treatment. Blood samples were taken at the time of diagnosis, before the initiation of treatment, and at 6-month follow-up after treatment.

**Smear preparation, staining, and culture**

Two sputum samples were collected (one spot and one early morning sample) and processed using N-acetyl-L-cysteine–sodium hydroxide–sodium citrate method.\textsuperscript{[16]} Samples were inoculated into liquid culture (MGIT, Becton Dickinson, Sparks, MD, USA) and solid culture (LJ, 7H11 agar).

**Acid-fast bacillus smears**

The processed specimens were used for making smears. All the smears were stained by the Ziehl–Neelsen method and examined with a light microscope.\textsuperscript{[17]} The results were quantified in accordance with the Revised National Tuberculosis Control Program standards.

**Culture**

Samples were inoculated into MGIT 960 nonradiometric automated isolation system (Becton Dickinson, Sparks, MD, USA); the MGIT tube containing 7 ml of 7H9 medium, supplemented with 0.8 ml of oleic acid-albumin-dextrose-catalase along with polymyxin B-ampicillin B-nalidixic acid-trimethoprim-azlocillin, was inoculated with 0.5 ml of decontaminated sample. Positive cultures were confirmed using TBe identification test (TBe ID, Becton Dickinson, Sparks, MD, USA) as Mtb.\textsuperscript{[18]} All specimens and/or cultures were stored at −70°C till further workup.

The phenotypic drug susceptibility testing (DST) was performed for all positive liquid cultures using MGIT 960. DST for rifampicin (RIF) and isoniazid (INH) was performed using the WHO recommended standard critical concentration of 1 μg/ml RIF and 0.1 μg/ml INH. A standard protocol was followed according to the manufacturer’s instructions. Strains resistant to INH and RIF were subjected to DST for second-line drugs, ofloxacin and kanamycin, by proportion method.\textsuperscript{[19,20]}
All inoculated drug-containing gas chromatography tubes were placed in the DST set carrier and entered into the MGIT 960 instrument as “unknown drugs” using the antimicrobial susceptibility testing entry feature.

**Immunophenotyping**

Venous blood was collected and processed for flow cytometry. The blood sample was centrifuged at 1500 g for 15 min to separate plasma; the plasma was cryopreserved at −70°C for cytometric bead array. Blood was processed within 4 h of phlebotomy. Peripheral blood mononuclear cell (PBMC) was isolated from ethylenediaminetetraacetic acid-treated blood on a Ficoll-Histopaque density gradient (Sigma, St Louis, MO, USA) and was cryopreserved using nine parts of fetal calf serum and one part of dimethyl sulfoxide at −196°C (liquid nitrogen), until use. Following PBMC extraction from venous blood, the cells were stained in the dark at room temperature for 30 min and were then acquired. Cells were surface stained with anti-CD3-FITC (BD Biosciences), anti-CD4-PE Cy7 (BD Biosciences), anti-CD8-Pac Blue (BD Biosciences), anti-CD16-APC H7 (BD Biosciences), and anti-CD56-PE (BD Biosciences) for 30 min at room temperature. The T regulatory cells were assessed using BD human regulatory T cell (Treg) cocktail (CD4/CD25/CD127). Following sample staining, testing was performed on a BD LSR11 flow cytometer (Becton Dickinson) fitted with four lasers, to allow the multicolor analysis of cells labeled with different fluorochromes. Acquisition data were analyzed using BD FACS Diva software, Becton Dickinson, Sparks, MD, USA following laser interrogation of the labeled cells. Cellular populations were discriminated on the basis of size and granularity using measurements of light forward and side scatter, respectively. Gating was performed according to protocols as illustrated in Figure 1. Using the same gates, cells stained with all antibodies for NK cells and Treg cocktail were then analyzed.

**Cytometric bead array**

Human Th1/Th2/Th17 cytokine kit was used to measure the concentration of interleukin (IL)-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF), interferon-gamma (IFN-γ), and IL-17A in blood plasma obtained from patients and healthy controls. This array kit provides a mixture of seven capture beads with distinct fluorescent intensities that have been coated with capture antibodies specific for each cytokine. The tests were performed according to the manufacturer’s instructions. About 50 µl of assay beads, 50 µl of detection reagent, and 50 µl of the studied sample or standard were added consecutively to each sample tube and incubated for 3 h at room temperature, in the dark. Next, the samples were washed with 1 ml of wash buffer and centrifuged. The supernatant was discarded; the pellet was resuspended in 300 µl of buffer and acquired on the flow cytometer. Samples were measured on the BD LSR II flow cytometer.

Figure 1: Representative Flow cytometry (FACS) results of T cells in patients. Peripheral blood mononuclear cells were isolated, and the lymphocyte populations were gated, CD3, CD4, and CD8 were further used for the analysis. (a) To determine the phenotype and frequency of CD3dim/CD56+ natural killer T in the circulation, specific antibodies were used. (b) To determine the phenotype and frequency of T regulatory cells in the circulation, specific antibodies were used.
cytometer and analyzed by FCAP Array™ Software, Becton Dickinson, Sparks, MD, USA (BD Biosciences). Individual cytokine concentrations were indicated by their fluorescent intensities. Cytokine standards were diluted serially to construct calibration curves to determine the concentration of the analyte.

**Statistical analysis**

GraphPad Prism Software (San Diego, CA, USA) was used for statistical analyses. *P* < 0.05 was considered to be statistically significant. The results are expressed as the mean ± standard error.

**RESULTS**

A subset of 12 patients enrolled in the study was used for immunological analysis. Of the 12 patients, 10 were baseline culture positive and two patients were culture negative. After 2 months of treatment, four patients were culture positive and the remaining eight were culture negative. Negative culture after 2 months of treatment is used as an indicator of early treatment response, and these patients were termed as fast responders. Differences were seen in lymphocyte population of fast and slow responders. The percentages of CD4+ and CD8+ at diagnosis were seen to be depressed at diagnosis, but on comparing the counts at the end of treatment, it was observed to be similar to the cell counts of the controls. It has been reported that two subsets of NKT cell populations are present.

In our study, we too detected the different subset of NKT cells (CD3bright/CD56+ NKT cells and CD3dim/CD56+ NKT cells). The percentages of CD3bright/CD56+ NKT cells at diagnosis and at the end of treatment were not different from those of controls, whereas the CD3dim/CD56+ NKT cell percentages were higher in PTB patients when compared with healthy controls (57.9 ± 16.6 vs. 26.6 ± 12.5). The values were found to be statistically significant (P = 0.0001) [Table 1 and Figure 2]. The population of CD3dim/CD56+ NKT cells in PTB patients decreased following successful antitubercular therapy (6 months), and the difference was found to be statistically significant (*P* < 0.0001) (57.9 ± 16.6 vs. 24.8 ± 13.6) [Table 2 and Figure 3].

CD3dim/CD56+ NKT cells at diagnosis correlated with treatment response, i.e., they were significantly higher at diagnosis in fast responders [Table 3 and Figure 4]. The frequency of cells in fast responders (66.9 ± 10.7) versus slow responders (39.8 ± 9.6) was found to be statistically significant (0.0018). It was also observed that the CD3dim/CD56+ NKT cell count was lower in baseline and follow-up samples of patients who relapsed at the end of treatment.

**T regulatory cocktail CD4+CD25high CD127low** live natural Treg population was detected using BD human Treg cocktail (CD4/CD25/CD127). The cell population was compared between patients and healthy controls at baseline, before initiation of treatment, and in the diseased condition. The percentage of Treg cells was higher compared to healthy controls (18.57 ± 6.58 vs. 7.15 ± 1.39) [Table 4 and Figure 5]. The percentages of Treg cells at diagnosis and at the end of treatment were found to be statistically significant (P = 0.0001) [Table 5 and Figure 6].

### Table 1: CD3dim/CD56 + NKT cells percent in peripheral blood of patients with pulmonary tuberculosis (PTB), and in healthy control cases

|              | PTB [n=12] | Healthy control [n=11] | P       |
|--------------|------------|------------------------|---------|
| CD3dim/CD56 + NKT cells | 57.9±16.6 | 26.6±12.5              | 0.0001  |

Data were presented as mean±SD, *P*<0.05 significance.

### Table 2: Comparison between CD3dim/CD56 + NKT cells in PBMCs in PTB patients before treatment and after 6 months of receiving anti-TB therapy

|              | PTB [n=12] | PTB [n=12] | P       |
|--------------|------------|------------|---------|
| CD3dim/CD56 + NKT cells | Before treatment | After anti-TB therapy | P<0.0001 |
|               | 57.9±16.6 | 24.8±13.6  |         |

Data were presented as mean±SD, *P*<0.05 significance.
of treatment were not different. This could be due to the chronic nature of the disease and a slow recovery after treatment [Table 5 and Figure 6].

We measured the concentrations of IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, and IL-17A secreted in the serum of TB patients, and these cytokine levels were compared to healthy controls. IFN-γ, TNF-α, IL-2, IL-4, IL-10, and IL-17A were not detected in the majority of the patients’ samples. Among the 12 patients, 9 patients showed IL-6 in the detectable limit in the baseline samples of the patient population. On comparing the baseline data with the follow-up data of the patients, it was found that 2 of the 12 patients showed elevated IL-6 levels, whereas the remaining 10 patients did not show elevated IL-6 levels. Two patients with an elevated level of IL-6 relapsed after completion of successful anti-TB therapy. IL-6 cytokine levels detected in the baseline sample decreased following treatment, and it was found that IL-6 levels were higher and in detectable limit in TB patients when compared with healthy controls.

IL-6 levels of patients when compared with that of healthy controls were found to be statistically significant; similarly, it was observed that absolute concentration of IL-6 in plasma of baseline samples of diseased patients and their 6-month follow-up was statistically significant [Figures 7 and 8].

The cytokines levels of the diseased patients when compared with the healthy controls showed detectable levels of IL-2 and IL-4. The majority of the studied cytokines were not detected in plasma of the patient population, or the other suggested reason for such result was the presence of very low concentration of cytokines, i.e., below 1 pg/ml. According to the suggestions by the manufacturer, the protocols were modified, and plasma samples of all patients with undetectable level of cytokines were diluted twofold before retesting. However, this modification did not alter the subsequent results and analysis.

### Table 3: Comparison between CD3dim/CD56 + NKT cells in PBMCs of fast responders and slow responders PTB patients

|                | PTB (n=8) fast responders | PTB (n=4) slow responders | P    |
|----------------|---------------------------|---------------------------|------|
| CD3dim/CD56 + NKT cells | 66.9±10.7               | 39.8±9.6                  | 0.0018 |

Data were presented as mean±SD, *P*<0.05 significance.

### Table 4: T regulatory cells percent in peripheral blood of patients with pulmonary tuberculosis [PTB], and in healthy control cases

|             | PTB [n=12] | HC [n=11] | P    |
|-------------|------------|-----------|------|
| T reg cells | 18.57±6.58 | 7.15±1.39 | 0.0002 |

Data were presented as mean±SD, *P*<0.05 significance.

### Table 5: T regulatory cells percent in peripheral blood of patients with PTB patients before treatment and after 6 months of receiving anti-TB therapy

|             | PTB [n=12] | PTB 6 months [n=12] | P    |
|-------------|------------|---------------------|------|
| T reg cells | 18.57±6.58 | 14.50±6.30          | 0.0637 |

Data were presented as mean±SD, *P*<0.05 significance.

### Discussion

The adaptive arm of immune response to Mtb infection is a subject for extensive research. The memory response has not been harnessed for the development of vaccines, and hence, it is important to elucidate the mechanisms of protective immunity against Mtb infection. Most of the studies have reported low levels of CD4+ T cells in PBMC of TB patients,[17,10] whereas elevated[8] or normal[19-21] levels have been reported for gamma-delta T cells.

Some authors have reported B-lymphocyte and NK cells in TB patients,[17-10,21-23] Although the role of NK cells in
Some authors have reported that activated NK cells have been found in the spleen and liver in patients with TB. Paidipally et al. reported that through IL-21 production, activated T cells enhance the response of NK cell to lyse Mtb-infected human monocytes and restrict its growth in monocytes. It has been shown that a significantly high number of NKT cells have been seen in TB patients. It should be noted that NKT cells are recruited into circulation following a TB infection as these cells recognize glycolipid antigens. In the current study, NKT cells were found to be significantly higher in “fast responders.” The cellular immune response upon activation by Mycobacterium may lead to an increase in array of various lymphocyte populations, particularly the T lymphocytes.

Studies on NK cells against Mtb infection report that NKT cell numbers are lower in PTB patients, whereas some studies have demonstrated significantly higher CD56dimCD16+ NK cells in active TB patients when compared with healthy controls. Huang et al. reported increased Tim-3-expressing CD56dimCD16+ NK cells in disease population. This subset of NK cells has decreased cytotoxicity and reduced production of IFN-γ. Junqueira-Kipnis et al. reported that in response to an infection with Mtb, the NK cell population in the lung expands and expresses some cell surface markers which are associated with activation and can produce IFN and perforin, in turn activating NK cells, but it is important to note that despite the increased activity in NK cell population, the bacterial load within the lungs remained unaltered in diseased patients. Chackerian et al., 2002, reported the protection of susceptible mice from TB and reduction in bacterial burden following stimulation of NKT cells in mice by CD1d ligand alpha-galactosylceramide. NKT cells are said to act by producing IL-21 and bridging the innate and adaptive immune response. A complete understanding of their role in antimycobacterial immunity is important as it may open new possibilities for NKT cell-based prophylactic and/or therapeutic strategies against TB.

T regulatory cells (Tregs) modulate the immune system. It is observed that Treg cells express CD3, CD4, high levels of CD25, low levels of CD127, and the intracellular marker forkhead box p3 (FOXP3) receptor. The Treg activity in TB is not clear; few studies have evaluated their role in the disease. Sahmoudi et al. reported that Tregs in lymph node mononuclear cells show a strong correlation with Th1-type immune response post-Mtb antigenic stimulation. Few studies reported increased numbers in adults with TB infection when compared to healthy controls, particularly in patients with extrapulmonary TB (EPTB). It is also noted that Treg cells persist even in patients who have been cured of EPTB. Wergeland et al. reported the highest frequency of CD4+ CD25+ CD127− Treg in active TB (P = 0.001), followed by latent TB infection (LTBI) group (P = 0.006) when compared with controls. Zewdie et al. demonstrated an association of Treg frequency with TB disease, but they...
also reported that association with the disease depends on the phenotypic markers used. The frequency of CD4 + CD25+/hi T cells was higher in TB patients when compared with LTBI individuals; however, no difference was seen in the frequency of CD4+ CD25+ FOXP3+ CD127lo Treg among different groups.[36]

Parida and Kaufmann et al. defined a biomarker as a “characteristic that can be objectively measured and evaluated as an indicator of a physiological or pathological process or pharmacological response (s) to a therapeutic intervention.”[36] However, no biomarkers have been identified which can be used effectively for TB diagnosis. Therefore, the urgent need of the hour is to develop a suitable biomarker that could be related to the clinical outcome of the disease. As previously described, it was established that cytokines can function as biomarkers, and several authors have suggested that IFN-γ and TNF-α can be used as excellent biomarkers for the prognosis of TB. One of the most important outcomes of our study was elevated level of IL-6 cytokine in patients at baseline and in patients who relapsed. Our observation with respect to production of IL-6 cytokine following TB infection is in consonance with earlier published reports. Previously, it has been reported that IL-6 is the only cytokine significantly increased following interaction of cells with mycobacteria.[37] Doherty et al. reported that C-reactive protein, Acute phase proteins (APP), can be used as a biomarker of TB.[38] Singh and Goyal reported increased levels of serum amyloid P-component and APP in mice and IL-6 in Mtb-infected mice.[39] Our results are seen to be concordant with Singh and Goyal reports which also demonstrated the enhanced levels of only IL-6; some other reports have also reported this finding.[39] Thus, IL-6 can be developed as a potential immunological biomarker of TB.

CONCLUSION
The results demonstrated in this study, during active TB, revealed elevated levels of Treg cells and NKT cells in the peripheral blood mononuclear cells. These cells may prove to be useful for targeted immune interventions for disease detection and control.

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Conflicts of interest
There are no conflicts of interest.

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