Inverse correlation of Interferon-gamma and CD8+ T Lymphocytes in Tuberculosis Patients

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ABSTRACT

BACKGROUND & OBJECTIVES: Tuberculosis (TB) is an infectious disease caused by Mycobacterium tuberculosis. All over the world TB is one of the leading causes of mortality and morbidity. Interferon-gamma (IFN-γ), at the site of MTB infection, activates macrophages to kill intracellular mycobacteria. CD8+ T cells kill the cells infected by viruses and other intracellular microbes. Diagnostic significance of IFN-γ has been reviewed in latent and active TB. Aim of this study was to determine association between IFN-γ and CD8+ T-cells in the blood of pulmonary TB-patients. **METHODS:** Study consisted of 54-TB-patients and 38 healthy subjects. Level of IFN-γ was determined by ELISA and percentage of CD8+ T-cells by flowcytometry. **RESULTS:** Level of IFN-γ and percentage of CD8+ T-lymphocytes were high in the blood of TB patients compared to control. **CONCLUSION:** TB patients had high level of IFN-γ compared to healthy controls. IFN-γ and CD8+ T cells were negatively correlated with duration of treatment of tuberculosis. There was no difference in percentage of CD8+ T cells in TB patients after 3 months of chemotherapy compared to controls.

Key words: Interferon gamma, tuberculosis, CD8+ T lymphocytes

الملخص

المقدمة والاهداف: الدرن هو مرض معدي يسببه بكتريا عصيات الدرن. وهو احد الاسباب الرئيسية للمراضة والوفاة في كل العالم. الانترفيرون جاما ينشط كريات الدم البيضاء لقتل البكتريا داخل الخلية في حالات الاصابة بالدرن . خلايا تي المناعية من نوع سي دي 8 لها القدرة علي قتل الخلايا التي تحتوي علي فيروسات او جراثيم . تم مراجعة الاهمية التشخيصية للانترفيرون جاما في حالات الاصابة بالدرن الكامن او النشط. هدفت الدراسة لتحديد العلاقة بين الانترفيرون جاما و خلايا تي المناعية من نوع سي دي 8 في دم مرضي الدرن الرئوي. **المنهج:** شملت الدراسة علي 54 مريض بالدرن و 38 شخص سليم. تم تحديد مستوي الانترفيرون جاما بطريقة الاليسا و تحديد نسبة خلايا تي المناعية من نوع سي دي 8 بواسطة مقياس الخلايا . **النتائج:** وجد ان مستوي الانترفيرون جاما و نسبة خلايا تي المناعية من نوع سي دي 8 اعلي في دم مرضي الدرن بالمقارنة بالأشخاص السليمين. **الإستنتاج:** مستوي الانترفيرون جاما اعلي في دم مرضي الدرن بالمقارنة بالأشخاص السليمين. مستوي الانترفيرون جاما و نسبة خلايا تي المناعية من نوع سي دي 8 تتناسب تناسبا عكسيا مع مدة علاج الدرن. لا توجد فروق في نسبة خلايا تي المناعية من نوع سي دي 8 في مرضي الدرن بعد 3 شهور من العلاج بمضادات الدرن بالمقارنة بالأشخاص السليمين.

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by mycobacteria, mainly Mycobacterium tuberculosis [1]. All over the world TB is one of the leading causes of mortality and morbidity. In 2010, 8.8 million people had TB and 1.4 million died of TB [2]. Pakistan ranks eight among 22 high burden tuberculosis countries in the world [3] and in 2001, government of Pakistan declared TB as national emergency. The emergence of multi-drug resistant TB (MDR-TB) is another growing concern in the country [4].

Mycobacterium tuberculosis (Mtb) is large, non-motile, rod shaped intracellular bacterium, also known as acid-fast bacilli (AFB) [5]. Clinically tuberculosis may manifest as pulmonary or extra-pulmonary TB that may affect any body organ. Smear-positive pulmonary TB is the most infectious form and is diagnosed by microscopic examination or sputum culture. Smear-negative pulmonary TB is diagnosed by findings on chest x-ray or on treatment failure after standard antibiotics [6].

More than 90% of Mtb infected individuals do not develop disease and the organism remains dormant but in about 10% of individuals, there is reactivation of latent bacteria that proceeds to TB infection. Cell mediated immunity is considered vital in preventing

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spread of Mtb, whereas macrophages, dendritic cells, CD4+ T and CD8+ T cells are also essential in controlling TB [7].

Interferon-gamma (IFN-γ) is a soluble protein secreted by a number of cells which activates macrophages to kill intracellular mycobacteria and at the site of Mtb infection it is the major cytokine [8]. After disrupting IFN-γ gene, mice subjected to sub-lethal dose of intravenous Mtb progressed to disseminated TB [9]. IFN-γ knockout mice developed neither mature granulomas nor protective immunity after infection with a virulent Mtb strain [10].

Lymphocytes particularly CD4+ T cells secrete cytokines which can modify/amplify specific immune response. Based on cytokine secretion CD4+ cells are further divided into Th1 or Th2. Th1 cells produce IFN-γ, IL-2 whereas Th2 cells produce IL-4 [11]. Macrophages kill internalized Mtbs when activated by TNF-α and IFN-γ [12]. CD8+ T cells kill the cells that contain foreign antigens such as viruses and intracellular microbes [13].

There can be significant increase in CD8+ T cells with a notable reduction in CD4+ T cells during TB infection [14]. Number of CD4+ and CD8+ T cells varies during anti-tuberculosis treatment (ATT) but there is no significant change in the number of these cells between chronic TB patients and healthy controls [14].

Diagnostic significance of IFN-γ in latent and active TB has been reviewed [15]. Enzyme linked immunosorbent assay (ELISA) of IFN-γ provides information about the level of cytokine at a given point of time [16]. The present study was carried out to determine the level of IFN-γ and percentages of CD8+ T cells in the blood of pulmonary TB patients.

Materials and Methods

Study design: It was a cross sectional analytical study

Period & Place of Study: The study was performed during December 2011–November 2012, in the Department of Immunology, University of Health Sciences (UHS) Lahore after approval of Ethical Committee and Advanced Studies & Research Board of UHS and Shalimar Hospital Lahore. Written, informed consent was obtained from participants. According to the formula sample size was 385 but due to budget constraints, sample size was limited to 92; 54 patients and 38 healthy controls (Kukreja et al., 2001).

The subjects were from both genders between 15–70 years of age. Newly diagnosed TB patients not on anti-tuberculosis treatment (ATT) were labeled as Group-I and TB patients on ATT as Group-II. Each group consisted of suspected TB/diagnosed TB patients on clinical grounds and confirmed TB patients. Pregnancy, Hepatitis B, Hepatitis C, immunoproliferative disorder, malignancy, allergy, immunosuppressive therapy and TB patients on ATT for more than 3 months were excluded. Clinically suspected TB patients had clinical history, findings on chest x-ray, non-responsiveness to antibiotics and good response to ATT within two months. Sputum positive for ZN staining, sputum culture for AFB, tissue biopsy for granuloma/AFB or Mtb nucleic acids by molecular methods were considered as confirmed TB patients [5,6]. For controls subjects without history of chest infection in last three weeks were included.

Five ml of venous blood was drawn aseptically from anterior cubital vein and added into 3 vacutainers (BD); two containing ethylene diamine tetra acetic acid (EDTA) and one with gel. Blood samples were transported to the Department of Immunology, UHS. Complete blood count (CBC), total leukocyte count (TLC) and differential leukocyte count (DLC) was measured by Sysmex analyzer XT1800i.

Gel vial was centrifuged and plasma was separated and stored at -80°C for IFN-γ detection by ELISA technique (DIACLONE diagnostics, France). Assay was performed according to manufacturer's directions who claimed no cross reactivity with other autoantigens and detection limit was between 0.25-400pg/ml.

Out of 54 TB patients, 39 subjects were selected randomly for immunophenotyping. Fluorescein isothiocyanate (FITC) tagged MoA CD8, phycoerythrin (PE) tagged MoA CD3, allophycocyanin (APC) tagged MoA CD4, and peridinin-chlorophyll-protien (PerCP) tagged MoA CD45 (DIACLONE, France) was used. Lyse-wash method using whole blood was performed. CD4/CD8 cells ratio was analysed by FACS Calibur

4-color analyser (BD, USA). 100µl of whole blood was added to 2 FACS tubes. 10µl each of CD3, CD4, CD8, and CD45 was added to one tube and isotype control to other. Tubes were mixed and incubated in dark at room temperature for 15 minutes. Two ml of BD FACSLyse was added to each tube. Tubes incubated in dark for 12 minutes, centrifuged at 250g for 10 minutes and supernatant was discarded. Pellet was re-suspended and cells were washed twice by adding 2ml of sheath fluid, mixed, centrifuged and supernatant was discarded. Cells were re-suspended in 0.5ml of sheath fluid with 2% paraformaldehyde. Fluorescence attributable to FITC, PE, APC and PerCP labeled monoclonal antibodies were determined using excitation by 15mW, 488ηM, argon-ion blue laser. Machine was calibrated and fluorescent signal compensation was performed using Cell Quest Pro software (BD) and Calibrite beads (BD). Data was acquired, templates were designed for two parameter dot-plot representing forward angle light scatter-side scatter (FALS-SS), and SS-CD45. Former dot-plot was used as indicators of satisfactory sample preparation while the latter to identify lymphocyte population. Lymphocytes (CD45 brightest population with lowest side scatter) in SS-CD45 dot-plot were gated and data for CD3+CD4+ and CD3+CD8+ cells was acquired. CD4+, CD8+ cells were analysed using two parameters dot-plot with log FITC fluorescence (CD3) on X-axis and log PE or APC fluorescence (CD4 or CD8) on Y-axis. Isotype control was run for each sample and fluorescence channel boundary was selected above which no more than 1% of control cells were detected and below this all stained cells were considered negative.

Statistical Analysis

Data was analyzed using SPSS-16.0, mean ± SD for quantitative variables, frequencies and percentages for qualitative variables and two-independent sample t test was applied to observe group mean differences of T cell percentages and IFN-γ levels. Pearson correlation was applied to observe correlation between IFN-γ levels, mean T cell percentages and duration of ATT, and p≤0.05 was considered as statistically significant.

Results

Among 54 TB patients, 25 (46.30%) were males and 29 (53.70%) were females, while in control group, 18 (47.30%) were males and 20 (52.63 %) were females. Mean age of TB patients and controls was 27.8 and 26.6 years respectively (Table 1). 27 (50%) patients had confirmed TB that included 24 (44.44%) ZN positive sputum and 3 (5.56 %) biopsy/granuloma positive while 27 (50%) were clinically diagnosed TB patients. 11 (20.37%) TB patients were on ATT while 43 (79.62%) were newly diagnosed. The results of CBC and ESR (erythrocyte sedimentation rate), CD4+cells and their comparison between TB patients and controls were made and described previously Hussain et al [17] and Afzal et al [18].

The mean±SD of IFN-γ was high in TB patients (48.69 ±4.20) pg/ml compared to controls (12.99±5.7) pg/ml and there was significant difference (p=0.002). Percentages of CD8+ cell were also high in TB patients but not statistically significant (Table-3).

The mean ± SD of IFN-γ was high in newly diagnosed TB patients (59.68±28.78) pg/ml compared to TB patients on ATT (36.85±24.76) pg/ml and there was significant difference (p=0.001). Mean ± SD of CD8 cells was not significantly different between newly diagnosed TB patients and TB patients on ATT (Table 4).

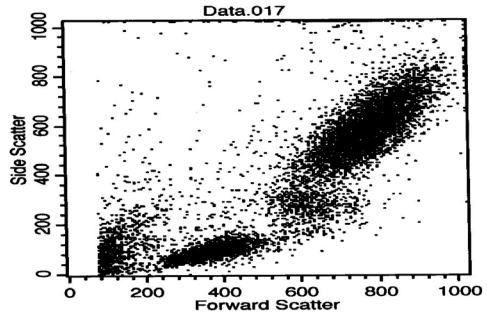


Figure 1: Distribution of different blood cells

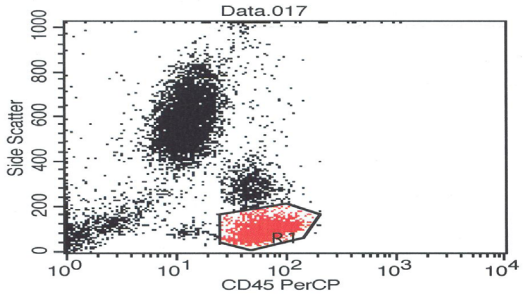


Figure 1: Lymphocytes selected from different cells of the blood

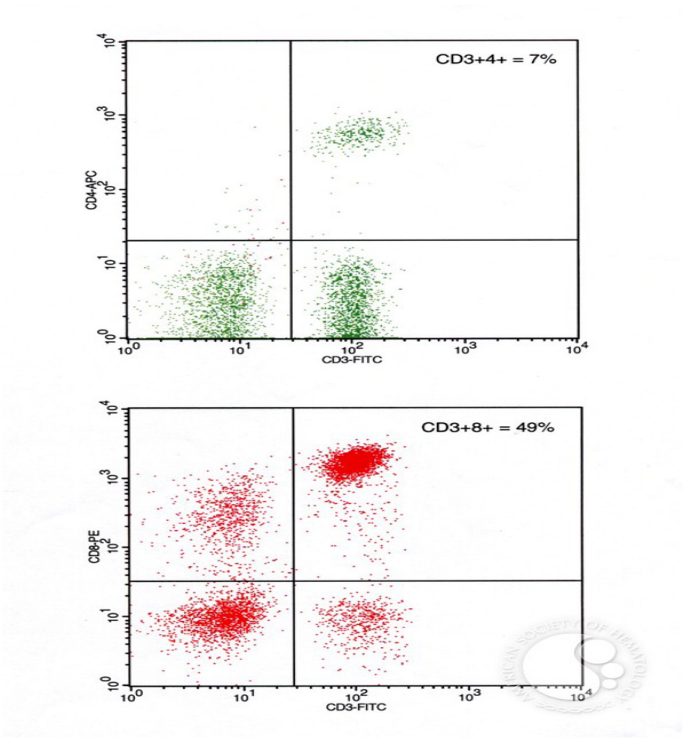


Figure 1: Upper: T lymphocytes i.e. CD3 and CD4 cells = 7% Lower: T lymphocytes i.e. CD3CD8 cells = 49%

The level of IFN-γ and percentage of CD8+ T cells decreased with the duration of treatment. By applying Pearson's correlation test, an inverse correlation was observed between mean percentage of CD8+ T cells and mean level of IFN-γ with the duration of ATT (r=-0.433, p=0.021) (Figure-1).

Discussion

Hematological parameters, percentages of CD4+ T, CD8+ T cells and level of IFN-γ in TB patients and controls have been described and discussed elsewhere [17, 18]. There is inverse correlation between the level of IFN-γ and the percentage of CD8+ T cells with the duration of ATT. It probably indicates that CD8+ T cells are good source of IFN-γ [19-21]. It has been documented that IFN-γ and tumor necrosis factor-α play role in the induction of inducible nitric oxide synthase and nitric oxide [22, 23]. In an earlier work, specific mycobacterial antigens like ESAT6 and CFP-10 were used to stimulate IFN-γ production [24]. Although the current study did not use such specific antigens, but significantly high level of IFN-γ has been observed in TB patients compared to controls.

There is compelling evidence that CD8+ T cells are critical in different ways for protective immunity against TB: first, the adoptive transfer and cell depletion studies in vivo have demonstrated that CD8+ T cells are involved in controlling Mtb infection. Second, CD8+ T cell lines and clones can recognize Mtb antigens in vitro, lyse Mtb-infected macrophages in an antigen specific manner, and restrict the growth of Mtb in macrophages [25,26], and third, beta-2 macroglobulin deficient mice as well as TAP-deficient mice failed to express MHC class I molecules and succumbed to Mtb infection [27,28]. Therefore, CD8+ T cells can contribute to the immune response against Mtb infection by at least three possible pathways: the release of IFN-γ, lysis of infected targets, and a direct antimicrobial activity. The present study provides indirect evidence that human CD8+ T cells release IFN-γ.

Table 1: Age and Gender Distribution

Subjects	Mean Age (years)	Male	Female	Total
TB Patients	27.8	25 (46.30%)	29 (53.70%)	54
Controls	26.6	18 (47.30%)	20 (52.63%)	38

The in vitro study of Cho 2000 et al [29] also demonstrated that human MHC class I restricted CD8+ T cells specific for Mtb protein epitopes contribute to host defense by the release of IFN-γ, by lysing infected target cells, and by directly killing the intracellular bacteria. Claudia 2006 et al [30] also documented that CD8+ T cells control Mtb infection in healthy house hold contacts of TB patients.

Table 2: Mean CD8+ T lymphocyte Percentages and mean IFN-γ level in TB patients and Controls

Parameter	TB patients Mean ± SD	Controls Mean ± SD	p Value
IFN-γ (pg/ml)	48.69 + 4.20	12.99 ± 5.7	0.002*
CD8+ T cell%	32.0±9.8	30.2±7.2	0.356
* Statistically significant p value ≤0.05			

Gariby et al 2008 [31] documented Mtb specific CD8+ T cells secreted IFN-γ and decreased percentage of CD8+ T cells in TB patients compared to latent TB

infection subjects which returned to normal after 4-month therapy. It is different from the current study because they included active and latent TB patients while in the current study TB patients were compared with controls.

The study of Nadia 2009 et al [32] showed that majority of Mtb specific CD8+ T cells secreted IFN-γ when stimulated by Mtb antigens but they reported ex-vivo percentages of CD8+ T cells were significantly lower in TB patients as compare to the subjects with latent TB infection which return to normal after 4-month therapy. This discrepancy is probably due to fact that they made TB patients into two groups; active and latent TB patients while in the current study this categorization was not made and TB patients had been compared with controls. Iukary T et al (2012) [33] also suggested levels of interferon-gamma increases after treatment of latent tuberculosis infection in high-transmission setting. Further WHO (2011) [34] made recommendations on the use of commercial TB interferon-gamma release assays in low and middle income countries.

Table 3: Mean CD8+ T lymphocyte percentages and Mean IFN-γ in newly-diagnosed TB patients and TB Patients receiving ATT

Parameter	Newly Diagnosed TB patients Mean ± SD	TB patients on ATT Mean ± SD	p value
CD8+ T cell%	34.01 ± 9.3	30.8 ± 10.0	0.319
IFN-γ (pg/ml)	59.68 ± 28.78	36.85 ± 24.76	0.001*
* Statistically significant p value ≤0.05			

The results of current study are in partial agreement with study of Virginie et al [35]. They documented that patients of active pulmonary TB had increased frequency of Mtb-specific IFN-γ producing CD8+ T cells as compared to patients suffering from latent TB. In the current study, categorization of TB patients into active and latent was not done and TB patients were compared with healthy controls.

Conclusion

The level of IFN-γ and CD8+ T cells decreases with the advancement of duration of TB treatment.

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Conflict of Interest

There is no conflict of interest of any author.

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