ORIGINAL ARTICLE

PERCENTAGE OF $CD4^+$ AND $CD8^+$ T-LYMPHOCYTES IN BLOOD OF TUBERCULOSIS PATIENTS

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Background: Tuberculosis is a fatal infectious disease, mainly caused by *Mycobacterium tuberculosis*. Spread of TB is controlled by cell-mediated immunity. Purpose of this study was to determine $CD4^+$ and $CD8^+$ T cell percentages in TB patients. **Methods:** 77 subjects consisted of 39 patients of active tuberculosis and 37 normal healthy individuals were recruited for the study. Among patients, 27 were at different stages of anti-tuberculous therapy while rests of the patients were not taking treatment. Sixteen patients were sputum positive for AFB while other patients were sputum negative for AFB. T cells percentages were determined by flow cytometer. **Results:** In TB patients $CD4^+$ and $CD8^+$ T cells percentages were 34.4±9.8 and 32.0±9.8 while in controls these were 37.1±6.9 and 30.2±7.2 respectively but the difference was statistically insignificant. $CD4^+$ T cell percentage in newly diagnosed TB patients was 28.8±8.7 while it was 37.9±8.9 in TB patients who were on therapy and difference was statistically significant whereas difference in $CD8^+$ T-cell percentages was statistically insignificant. A negative correlation between $CD8^+$ T-cells percentage and the duration of ATT was found. **Conclusion:** $CD4^+$ and $CD8^+$ T-cells percentages may help to find out the immune status of TB patients before and after the completion of ATT.

Keywords: TB, $CD4^+$, $CD8^+$, ATT, AFB

INTRODUCTION

All over the world tuberculosis (TB) is one of the leading causes of mortality and morbidity. It is mainly acquired through airborne droplets. The incidence of TB is expected to increase substantially worldwide because of the interaction between TB and epidemics of human immunodeficiency virus.[1] According to WHO Global TB Report 2008, Pakistan ranks eight among 22 high-burden tuberculosis countries in the world.[2] TB was declared a national emergency by the government of Pakistan in 2001.[3] The emergence of multi-drug-resistant TB (MDR-TB) is another growing concern in the country.[4] *Mycobacterium tuberculosis* (Mtb); causative agent of TB, is a fairly large non-motile rod-shaped bacterium. It is an obligatory aerobe, intracellular pathogen which has predilection for lung tissue that is rich in oxygen supply. This is also called an acid-fast bacilli (AFB) because after staining, they resist de-staining by acid or alcohol.[5]

Clinically tuberculosis may manifest as pulmonary TB which affects lungs or extra-pulmonary TB that may affect any other organ of the body. There can be smear-positive pulmonary TB, which is the most infectious form and is diagnosed by microscopic examination or culture of sputum. Smear-negative pulmonary TB is diagnosed clinically or on chest x-ray findings or on treatment failure by standard antibiotics.[6,7] More than 90% of individuals infected with Mtb do not develop disease because their immune system is competent enough to get rid of this organism.[8] Macrophages, dendritic cells, $CD4^+$ T and $CD8^+$ T cells play important role in controlling TB infection.[9,10]

It has been found that there is considerable reduction in cell mediated immunity during TB infection.[11,12] Another study documented significant reduction in $CD4^+$ T cells but it was without notable reduction in $CD8^+$ T cells. However, reduction in $CD4^+$ T cells while increase in $CD8^+$ T cells has also been reported.[13] Less than 300 mm^3 $CD4^+$ T cells were found associated with poor prognosis.[14] Further, $CD4^+$ and $CD8^+$ T cells did not vary at different times of treatment with ATT and there was no significant change in $CD4^+$ and $CD8^+$ T cells between chronic TB patients and healthy controls .[16]

The present study was designed to determine the percentages of $CD4^+$ and $CD8^+$ T cells in TB patients regardless of their status of disease; whether active, inactive, acute or chronic and to compare these values with healthy subjects.

MATERIAL AND METHODS

The study was carried out in the Department of Immunology, University of Health Sciences Lahore. Study was approved by the Ethical Committees and Research Boards of University of Health Sciences (UHS) Lahore and Shalimar Hospital Lahore. It was a cross sectional analytical study. Study population consisted of 39 TB patients and 38 normal healthy subjects as controls. Written, informed consent was obtained from the study participants. Patients included in the study were from 15–70 years of age from both the

genders. TB patients were divided in two groups. Group-I had newly diagnosed TB patients who had not received ATT. Group-II had TB patients who had received ATT for not more than 3 months duration. Each group consisted of both suspected TB patients/ diagnosed on clinical grounds and confirmed TB patients. Pregnant, Hepatitis B, Hepatitis C, immuno-proliferative disorder, malignancy, allergy, on immunosuppressive therapy and TB patients on ATT for more than 3 months were excluded from the study.

Patients included in the study on clinical suspicion of tuberculosis had clinical history, chest x-ray findings, history of non-responsiveness to standard course of antibiotics and history of good response to anti-tuberculosis treatment within two months of treatment. Positive sputum ZN smear, positive sputum culture for AFB, positive tissue biopsy for granuloma or AFB or positive Mtb nucleic acids by molecular methods was considered as confirmed TB patients.

Three (3) ml of venous blood was drawn aseptically from anterior cubital vein and added into 2 vacutainers BD, each containing ethylene diamine tetra acetic acid (EDTA). Blood samples were transported to the Department of Immunology, UHS in icebox. One tube was utilized for complete blood count (CBC), while other tube was used for immunophenotyping. All samples were processed within four hours and analyzed on the same day.

Sysmex automated haemanalyser (Sysmex 1000X-i) was used for obtaining haemoglobin, total leukocyte count (TLC) and differential leukocyte counts (DLC). Fluorescein isothiocyanate (FITC) tagged MoA against CD4, phycoerythrin (PE) tagged MoA against CD8 and peridinin-chlorophyll-protien (PerCP) tagged MoA against CD45 were used for immunophenotyping. All these antibodies were purchased from Diaclone (DIACLONE SAS, Besancon France). Lyse-wash sample preparation method using whole blood was performed. 100 µl of whole blood of each subject was placed into 2 FACS tubes. 10 µl of each of CD4-FITC, CD8-PE, and CD45-PerCP monoclonal antibodies were added to one tube and isotype control to the other. Tubes were mixed and incubated in dark at room temperature for 15 minutes. Two ml of BD FACSLyse (<15% formaldehyde and <50% diethylene glycol, was diluted 1:10 in deionised water immediately before use) was added to each tube. Tubes were re-incubated in dark for 12 minutes. Centrifuged at 250 g for 10 minutes and supernatant was discarded. Pellet was broken and cells were washed twice by adding 2 ml of sheath fluid, mixed, centrifuged and supernatant was discarded. Cells were resuspended in 0.5 ml of sheath fluid with 2% paraformaldehyde.

Cells were analysed with a FACS Calibur 4-color analyser (BD Biosciences, California USA). Fluorescence attributable to FITC, PE and PerCP labelled monoclonal antibodies were determined using excitation by 15 mW, 488 ηM, argon-ion blue laser. Before running the samples, machine was calibrated and fluorescent signal compensation was performed using Cell Quest Pro software (BD) and Calibrite beads (BD). Subsequently data was acquired using the same settings.

Templates were designed for two parameter-dot-plot representing forward angle light scatter-side scatter (FALS-SS), and SS-CD45. The former dot-plot was used as an indicator of satisfactory sample preparation while the latter was used to identify lymphocyte population, avoiding contamination from debris. The lymphocytes (CD45 brightest population with lowest side scatter) in SS-CD45 dot-plot were gated and data for $CD4^+CD45^+$ and $CD8^+CD45^+$ T cells was acquired.

The $CD4^+$ and $CD8^+$ T cells were analysed using two parameters dot-plot with log FITC fluorescence (CD4) on X-axis and log PE fluorescence (CD8) on Y-axis. It was carried out using Cell Quest Pro software. As $CD4^+$ and $CD8^+$ single positive cells were clearly distinguishable from $CD4^+CD8^+$, double positive cells so for differentiation of true positive cells (high $CD4^+$ or $CD8^+$ positive) from low positive cells (non-specific binding) isotype control were run for each test sample (whether patient or control population) and a fluorescence channel boundary was selected above which no more than 1% of control cells were detected. This channel was set as a limit below which all stained cells were considered negative and quadrants were set and these quadrants were overlaid on that for test samples.

The data was analysed using SPSS-16.0. Mean±SD was given for quantitative variables such as blood $CD4^+$ and $CD8^+$ T cell percentages, haemoglobin concentration, white cell count (TLC), neutrophil percentages, lymphocyte percentages, monocyte percentages and ESR. Frequencies and percentages were given for qualitative variables like gender, ATT and sputum status. Two-independent sample *t* tests were applied to observe group mean differences of $CD4^+$ and $CD8^+$ T cell percentages. Pearson correlation was applied to observe correlation between mean $CD4^+$ and $CD8^+$ T cell percentages and duration of ATT, and $p<0.05$ was considered as statistically significant.

RESULTS

Thirty-nine TB patients (mean age 27.87 years) and 38 normal controls (mean age 26.68 years) were selected for study. Gender distribution of the subjects is given in Table-1. Sputum and ATT status is given in Table-2. Percentages of samples from pulmonary and extra-pulmonary sites are given in Table-3. Different laboratory parameters of TB patients and controls are summarised in Table-4.

It was found that TB patients had low percentages of $CD4^+$ T lymphocytes (34.4±9.8) and high percentages of $CD8^+$ T lymphocytes (32.0±9.8) as compared to controls (mean $CD4^+$ 37.1±6.9%, mean $CD8^+$ 30.2±7.2%) but this difference was not statistically significant for both of these parameters, *p*-values 0.171 and 0.356 respectively (Table-5). Mean percentage of $CD4^+$ T lymphocytes in TB patients who were not taking ATT was significantly decreased as compared to those TB patients who were taking ATT but there was no statistically significant difference between mean percentages of $CD8^+$ T lymphocytes in TB patients who were not taking ATT and TB patients who were on ATT (Table-6).

When mean percentage of $CD4^+$ T lymphocytes in TB patients who were not taking ATT was compared with controls significant reduction was noted but there was no statistically significant difference between mean percentages of $CD8^+$ T lymphocytes in TB patients not taking ATT and controls (Table-6).

No significant correlation was observed in mean percentage of $CD4^+$ T cells and duration of ATT (r=0.110, *p*=0.586). Interestingly significant inverse correlation was observed between mean $CD8^+$ T cell percentage and duration of ATT (r=-0.433, *p*=0.021) i.e., with increasing duration of ATT there was decrease in $CD8^+$ T cell percentages (Figure-1).

Table-1: Number of subjects by gender (n=77)

	Male	Female	Total
Patients	15	24	39
Control	18	20	38

Table-2: Sputum AFB and ATT status of TB patients

Study Population	Number of Sputum AFB +	Number of Sputum AFB -	Patients on ATT	Patients not on ATT
Males	7	8	10	5
Females	9	15	11	13
Total	16	23	21	18

Table-3: Distribution of samples from various sites

Site	Number	%
GIT	2	5.13
Lymph Node	4	10.26
Pulmonary	29	74.36
Bone	3	7.69
TBM	1	2.56

Table-4: Laboratory parameters of patients and controls

Parameter	TB Patients Mean±SD	Controls Mean±SD	*P*
Hb (g/dl)			
Male	12.5±2.0	14.9±1.2	0.000*
Female	11.3±1.9	12.6±1.2	0.011*
TLC $\times 10^9$/L	8.1±3.3	7.4±2.0	1.02
Neutrophil %	66.3±10.2	57.1±6.9	0.000*
Lymphocyte %	23.7±9.0	32.3±2.1	0.000*
Monocyte %	9.9±4.1	8.8±2.1	1.05
ESR mm/1st hour	66±29.6	5±3.8	0.000*

*Significant

Table-5: Mean $CD4^+$ and Mean $CD8^+$ T lymphocyte Percentages in TB patients and Controls

Parameter	Controls (n = 38)	TB Patients (n = 39)	*p* value
$CD4^+$ T cell%	37.1±6.9	34.4±9.8	0.171
$CD8^+$ T cell%	30.2±7.2	32.0±9.8	0.356

TB: Tuberculosis, **CD4+ T cell:** CD4+ T lymphocyte, **CD8+ T cell:** CD8+ T lymphocyte

Table-6: Mean percentage of $CD4^+$ and $CD8^+$ T lymphocyte in TB patients on ATT and without ATT

Parameter	Patients on ATT (n=21)	Patients without ATT (n=18)	*p*	Controls (n=38)	Patients without ATT (n=18)	*p*
$CD4^+$ T cell %	37.9±8.9	28.8±8.7	0.004*	37.1±6.9	28.8±8.7	0.001*
$CD8^+$ T cell %	30.8±10.0	34.01±9.3	0.319	30.2±7.2	34.01±9.3	0.0484

*Significant

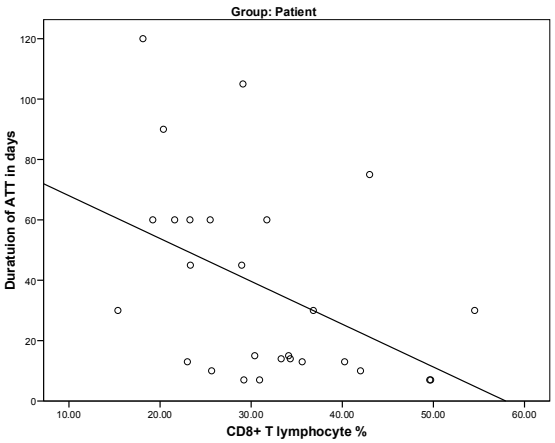

Figure-1: Relationship between $CD8^+$ T lymphocytes and duration of ATT

DISCUSSION

The TB patients had significantly decreased Hb concentration, i.e., they were anaemic and total leukocyte count of TB patients was with in normal range. Differential leukocyte count revealed that TB patients had significantly increased percentage of neutrophils, lymphocyte percentage was significantly decreased and there was no significant difference in monocyte percentages compared to controls. ESR value was also characteristically and significantly high in TB patients than healthy controls which is in accordance with previous studies.

On comparing $CD8^+$ T cell percentages between newly diagnosed TB patients and TB patients on ATT, it was found that $CD8^+$ T cell percentages were high but it was not statistically significant. This finding is in accordance with the studies of Piheu *et al*[14], Deveci *et al*[12], and Escobar *et al*[16]. Our results disagree with the results of Singhal *et al*[13], Thomas *et al*[15] and Shijubo *et al*[17]. Singhal *et al*[13] reported significant increase in $CD8^+$

T cell values in TB patients. Probably discrepancy could be due to the difference of study population since they included pulmonary tuberculosis patients only while in our study some of the patients (n=10) were suffering from extra-pulmonary tuberculosis as well. Additionally, in their study none of the patients were on ATT, while 21 out of 39 patients in our study were at different stages of ATT. Thomas *et al*[15] included only pulmonary TB patients and according to clinical grading they divided them in three groups (Grade-1: minimal lesions, Grade-2: moderately advanced lesions, Grade-3: far advanced lesions). They reported significantly increased $CD8^+$ T cell values only in Grade-3 patients. In our study, categorisation of TB patients according to clinical grading was not done and we selected TB patients randomly, i.e., some of them were newly diagnosed and others were at different stages of ATT. Shijubo *et al* [17] reported significantly decreased $CD8^+$ T cell values in TB patients compared to controls while we observed rather increased $CD8^+$ T cell values although that was not statistically significant. To the best of our knowledge, it was the only study and it was published about eighteen years ago, which concluded decreased number of $CD8^+$ T cells in TB patients.

We also found decreased number of $CD4^+$ T lymphocytes in newly diagnosed TB patients as compared to TB patients on ATT and healthy controls and the difference was statistically significant. Thomas *et al*[15] found significantly reduced $CD4^+$ T cell values in TB patients and similarly Pilheu *et al* [14] found reduced number of $CD4^+$ T cells values in severe pulmonary TB patients. Our results disagreed with the results of Gariby *et al* [16] who reported non-significant difference in $CD4^+$ T cells values in TB patients. Probably the discrepancy was due to the fact that Gariby *et al* selected only chronic pulmonary TB patients.

The comparison of $CD4^+$ and $CD8^+$ T cells percentages between TB patients and healthy volunteers showed low values of $CD4^+$ T cells and high values of $CD8^+$ T cells in TB patients but the difference was statistically non significant. These results were in agreement with the studies of Vieria *et al* [18], Onwubilili *et al*[19], and Pilheu *et al*[14]. They also reported non significant reduction in the levels of $CD4^+$ T cells and similarly there was non significant increase in the levels of $CD8^+$ T cells in TB patients. The studies of Vieria *et al*[18] and Onwubilili *et al*[19] comprised of small number of patients, which was 10 and 13 respectively. Additionally they performed immuno-phenotypic analysis of T lymphocytes by immuno-fluorescence method whereas we used flow cytometry which was more accurate than immuno-fluorescence technique. Our results disagreed with the findings of Singhal *et al*[13] and Thomas *et al*[15]. This might be due to the fact that in the above two studies, they only included newly diagnosed pulmonary TB patients but in our study TB patients were randomly selected, i.e., both newly diagnosed and TB patients at different stages of ATT and now it has also been documented that immunological changes caused by Mtb could be reverted to normal with ATT.[13,19,20]

Since in this study TB patients at different stages of ATT were included, therefore to find the correlation of $CD4^+$ and $CD8^+$ T cells with duration of ATT, a comparison was made between the mean values of these parameters. No significant correlation was observed between mean $CD4^+$ T cell percentages and duration of ATT but a significant inverse correlation was observed between mean $CD8^+$ T cell percentages and duration of ATT, i.e., with increasing duration of ATT, $CD8^+$ values were decreased.

CONCLUSION

It would have been of value to measure $CD4^+$ and $CD8^+$ T cells in newly diagnosed TB patients. $CD4^+$ T cell percentages were profoundly decreased in newly diagnosed TB patients and there was negative correlation of $CD8^+$ T cell percentages with duration of ATT.

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