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## Lymphocyte Sub-populations and Lymphocyte Activation Markers in Pulmonary TB Patients

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### ABSTRACT

**Background:** Tuberculosis is still a major health problem around the globe. Better knowledge of different aspects of the disease, including immunology and immuno-pathogenesis, promises better control measures. Studies regarding quantitative and qualitative changes of lymphocytes have developed our knowledge to a great extent. In this study, quantitative deficiencies of lymphocyte sub-populations and their activation markers have been evaluated.

**Materials and Methods:** Following documentation of smear positive pulmonary Tuberculosis in adult patients referred to Massih Daneshvari Hospital, 40 new cases without any other immunodeficiency conditions such as HIV, CRF, etc were randomly selected. Tuberculin skin test (TST) and chest radiography were performed for each case, and flow-cytometry was done from peripheral blood for lymphocyte sub-populations as CD3, CD4, CD8, CD4/CD8 ratio, CD19, CD16+56, and for Lymphocyte activation markers including CD25, CD69 and HLA-DR. Comparison was made with the historical control group in a study in same city, age as well as same flow -cytometry operator and same including & excluding criteria. Statistical analysis of data using Spearman's rho, Mann-Whitney U, and Asymp. Sig. (2-tailed) was done.

**Results:** 18 cases (45%) were male and 22 (55%) were female. Age distribution had a minimum of 17 and a maximum of 80 with a mean of 44.18 (19.65). Considering flow-cytometric marker correlation's, HLA-DR had significant relation to age ( $P=0.002$ ). The ratio of CD4+ cells also had significant relation to CD3, CD16+56 and HLA-DR markers. A significant difference was found only in the total of CD3+ lymphocytes by comparing PPD positive cases ( $\geq 10\text{mm}$ ) with PPD negative patients. The ratio of CD4+ was significantly lower than the normal population, and CD8+ was significantly higher comparing to the control population. The CD4/CD8 ratio was also significantly lower than the normal population.

**Conclusion:** Expression of lymphocyte sub-populations during the course of pulmonary Tuberculosis disease showed positive or negative correlation. By comparing lymphocyte sub-populations of TB patients with the control group, it is concluded that CD4+, CD8+, and CD4/CD8 ratio show significant differences that confirm significant quantitative defects in lymphocytes due to tuberculosis disease. (Tanaffos 2002; 1(4): 37-44)

**Key words:** Tuberculosis (TB), Lymphocyte, Marker, Lymphocyte activation marker.

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## INTRODUCTION

Tuberculosis remains a major health problem in the world today. It has expanded in the recent years with the emergence of AIDS pandemic (1). In addition to distinct therapeutic strategies, an extended knowledge of different aspects of the disease including its immunology and immuno-pathogenesis is the most effective tool for tuberculosis control.

Both controlling of tuberculosis in the body and conversion of infection to disease depend on the individual's immune status (2). During the immune response against mycobacterium tuberculosis, alveolar macrophages ingest the bacilli, and deliver their antigens to lymphocytes; this, in turn, leads to various reactions that activate the cell mediated immunity (CMI) (3,4).

Lymphocyte sub-populations proliferate following exposure to the mycobacterium antigen and start secreting specific cytokines (2,5). Cytokines are, in fact, messengers of the immune system, leading the system to a stronger defense against the disease.

Interferon (IFN- $\gamma$ ) and interleukin-12 (IL-12) are examples of those cytokines which are released from CD<sub>4</sub><sup>+</sup> T-helper cells and activate the Th1 system (6,7,8). Initial reactions together enhance cellular defense and cause a strong bactericidal activity of monocytes and macrophages (3,4). It has been shown that pulmonary TB patients have lower lymphocyte secretions against mycobacterial antigens compared to normal individual's cells. This difference is particularly noted in IL-2 secretion and expression of its specific receptor (IL-2R) in the course of the disease (9,10). Other studies have also shown functional defects in CD<sub>4</sub><sup>+</sup> cells during the course of tuberculosis (11,12).

There are also different variations regarding the quantity of lymphocytes. During the course of disseminated or progressive TB, circulating thymic lymphocytes especially CD<sub>4</sub><sup>+</sup> and CD<sub>8</sub><sup>+</sup> subsets undergo quantitative variations (13). The results of

the study pertaining to an increase or decrease in lymphocytes sub-populations are very much different and sometimes controversy (14). This study was performed to evaluate lymphocytes sub-populations and their activation markers in selected TB patients.

## MATERIALS AND METHODS

40 patients were randomly selected from adults referring to the mycobacteriology clinic of Massih Daneshvari hospital with respiratory symptoms and radiological findings; they were hospitalized with sputum mycobacteriological test confirmation for tuberculosis. All the patients were new cases and had no history of anti-TB treatment. History or the presence of conditions leading to defective immune status such as AIDS, malignancies, organs transplantation, congenital immune deficiencies, cirrhosis, chronic renal failure, collagen vascular diseases, corticosteroid therapy, and chemotherapy were all defined as the excluding criteria. General information about the patients was gathered in a specified questionnaire. Following patients' consent to be enrolled in the study, tuberculin skin test (TST) was performed, and chest radiography along with venous blood samples for flow-cytometry was taken.

### Flow-cytometric evaluation

2 ml of peripheral blood sample was taken and placed in EDTA tubes. A panel of dual color conjugated antibodies was used with Fluorescein isothiocyanate (FITC) and phycoerythrin (PE) dyes from Becton-Dickinson company.

The antibodies included CD<sub>3</sub>/CD<sub>8</sub>, CD<sub>3</sub>/CD<sub>4</sub>, CD<sub>3</sub>/CD<sub>19</sub>, CD<sub>3</sub>/CD<sub>69</sub>, CD<sub>3</sub>/CD<sub>25</sub>, CD<sub>45</sub>/CD<sub>14</sub>, CD<sub>3</sub>/CD<sub>16+56</sub>, CD<sub>3</sub>/HLA-DR, and control IgG1/IgG2a. 100 $\mu$ L of each patient's blood sample were added to tubes containing 20 $\mu$ Ls of conjugated antibodies, then vertex was added too.

The tubes were placed in a dark area for 20-25 minutes at the room temperature. Next, 2 ml of lysing solution was added to each tube, and again

they were placed in a dark place for 10-12 minutes at room temperature. The tubes were centrifuged for 5 minutes and 2ml of PBS solution was added to each tube for washing. After washing for 5 minutes, the fluid standing on top was expelled, 0.5ml of PBS was added, and the cells prepared for analysis were counted by flow-cytometry technique using SimulSET software and FACS caliber (Becton-Dickinson Co.).

#### The control group

In order to compare our study results with the normal population, we used a study by Gasempour et al. (15) as control. The selected study was performed in adults (18-60 year old) and had the same excluding criteria as our study. It was performed by a university center in the same city (Tehran) last year. The operator of flow-cytometry was the same person in both studies. Selection of the control study was therefore, based on other experiences existing as a historical control in experimental studies (16).

#### Statistical Analysis

First, we evaluated the data using central indices. Next, statistical analysis was performed using Spearman's rho, Markers' correlation, Mann-Whitney U, and Asymp. Sig. (2-tailed) tests. Results were compared with the control study.

## RESULTS

18 cases (45%) were male, and 22 cases (55%) were female. The age range was 17 to 80 (mean:  $44.18 \pm 19.65$ ).

All cases had at least two positive smears for acid fast bacilli (AFB), with clinical symptoms and radiological findings consistent with pulmonary, TB diagnostic criteria (17). HIV serology was negative in all; only six cases (15%) had a history of diabetes mellitus type II.

Cavitary lesions were prominent in 22 cases (55%). TST showed indurations  $\geq 10$ mm in 26 cases (65%) which was considered as positive, and  $< 10$ mm in 14

cases (35%) which was considered as negative results. The mean induration size was  $12.3 \pm 6.6$  mm.

The ratios of lymphocyte sub-populations and activation markers are listed in table 1.

**Table 1.** Description of lymphocyte sub-populations and activation markers in case group.

Markers	Min	Max	Mean	Std. Deviation
CD <sub>3</sub>	38	88	69	11
CD <sub>19</sub>	1	27	13	7
CD <sub>4</sub>	18	59	38	11
CD <sub>8</sub>	14	61	33	11
CD <sub>4</sub> /CD <sub>8</sub>	0.47	3.74	1.27	0.67
CD <sub>16+56</sub>	5	46	18	9
CD <sub>25</sub>	3	16	9	3
CD <sub>69</sub>	2	20	8	3
HLA-DR	5	39	19	8

The age of patients did not show any significant correlation with various flow-cytometric marker counts except for HLA-DR. HLA – DR had a positive correlation with age advancing ( $p = 0.002$ ). Expressed ratios of CD<sub>4</sub><sup>+</sup> markers showed significant relation with CD<sub>3</sub>, CD<sub>16+56</sub>, and HLA-DR ratios. The correlation was positive with CD<sub>3</sub> (total T-cells) marker ratio ( $p < 0.0001$ ) and was negative with the ratios of expressed CD<sub>16+56</sub> and HLA-DR markers ( $p = 0.006, 0.011$ , respectively).

A negative correlation was also found for CD<sub>8</sub><sup>+</sup> population comparing with CD<sub>19</sub> (B-cells) ( $p=0.017$ ), and no significant relationship was found with other markers. The expression of CD<sub>16+56</sub> had a negative correlation with both CD<sub>3</sub> expression ( $p<0.001$ ) and CD<sub>4</sub> expression.

Evaluation of activation markers revealed only a negative correlation between HLA-DR and CD<sub>4</sub> expression. No significant difference was found between genders in the expression of flow-cytometric markers. There was a significant difference between PPD positive and PPD negative patients with regard to total CD<sub>3</sub> lymphocytes ratios ( $p=0.02$ ), but no

difference was found in other markers. There was no statistical significant difference in lymphocytes sub-populations and cell marker ratios between the cavitory and non-cavitory TB cases.

Table 2 demonstrates a comparison of lymphocyte sub-populations in smear positive TB patients with the historical control group. According to obtained results, the ratios of CD<sub>4</sub><sup>+</sup> and CD<sub>8</sub><sup>+</sup> expression in the two groups had significant differences; CD<sub>4</sub><sup>+</sup> population was significantly lower in the case group (p=0.001) and CD<sub>8</sub><sup>+</sup> population was significantly higher (p=0.009). Also, the CD<sub>4</sub> / CD<sub>8</sub> ratio was significantly lower than that of the normal population (p=0.001).

**Table 2.** Statistical comparison in lymphocyte sub-populations in the cases and control groups

	X1	X2	S1	S2	P-value
CD <sub>3</sub>	69	71	11	8	0.29
CD <sub>19</sub>	13	13	7	5	0.91
CD <sub>4</sub>	38	42	11	7	0.0003
CD <sub>8</sub>	33	28	11	8	0.003
CD <sub>4</sub> /CD <sub>8</sub>	1.29	0.6	0.67	0.6	0.002
CD <sub>16+56</sub>	18	16	9	7	0.13

1 = case    2 = control    X = Mean    S = std. deviation

## DISCUSSION

The immune response to mycobacterium tuberculosis is programmed and mediated by sensitized CD<sub>4</sub><sup>+</sup> and CD<sub>8</sub><sup>+</sup> lymphocytes (18,19). Interaction between these lymphocytes and macrophages results in the pathogenesis of tuberculosis (20). Therefore, an evaluation of lymphocyte cell counts can reflect the state of disease pathogenesis.

According to the study by Rodrigues et al., (21) which compared lymphocytes sub-population counts in three groups of healthy PPD positive, healthy PPD negative and pulmonary TB patients, the ratio of expressing CD<sub>4</sub> and CD<sub>8</sub> sub-populations was

significantly lower in the patient group (P<0.05). In this study, 18 healthy PPD positive subjects, 18 healthy PPD negative subjects, 20 active pulmonary tuberculosis patients, and 15 patients with TB at the end of a 6-month period of treatment were included. In addition to differences in the expression of lymphocyte sub-populations, the activation marker of CD<sub>38</sub><sup>+</sup> had a significant increase in the group with active disease (p=0.0055). The difference in lymphocytes sub-populations was not detected only in adults; in a study by Swaminathan et al. (22), it was shown very clearly that the CD<sub>3</sub><sup>+</sup> and CD<sub>4</sub><sup>+</sup> populations counts decrease in pediatric patients with active pulmonary tuberculosis. They compared Flow-cytometric markers of 17 healthy TST positive children with 22 pediatric tuberculosis patients without a prior history of treatment (new cases) and with 8 TB cases at the end of their treatment course. The differences of CD<sub>3</sub><sup>+</sup> and CD<sub>4</sub><sup>+</sup> cells between the healthy subjects and the patient group were significant. Comparison with the third group showed an increase in CD<sub>4</sub><sup>+</sup> cell counts at the end of anti-TB treatment course and a shift toward normal counts.

In a study by Singal et al. (23), consistent with previous studies mentioned, a difference in lymphocytes sub-populations in the TB patient group was shown. 30 TB patients were compared with 11 healthy subjects. A significant decrease in the ratios of CD<sub>4</sub><sup>+</sup> and CD<sub>3</sub><sup>+</sup> cells, and an increase in the ratio of CD<sub>8</sub><sup>+</sup> cells were shown in TB patients. Researchers in Japan also demonstrated a significant decrease in CD<sub>4</sub><sup>+</sup> cells ratios and a significant increase in CD<sub>8</sub><sup>+</sup> cells ratios of pulmonary TB patients relative to normal population (24).

In all aforementioned studies, quantitative disorders of lymphocytes sub-populations was evident, which confirmed the results of our study. The difference in CD<sub>8</sub><sup>+</sup> cells counts (increase or decrease) can be due to either different stages of disease in which the subjects were enrolled in the

study, cellular changes following the initiation of anti-tuberculosis treatment, or a difference in the immune status of smear positive and smear negative pulmonary tuberculosis. These situations were different in the mentioned studies; nevertheless, a decrease in the  $CD_4^+$  population was common to all of them. The reason for these quantitative changes might be due to the secretion of various cytokines in the trend of immune response during the course of tuberculosis. This hypothesis persuaded Garcia et al. (25) to evaluate the rate of cytokine secretion from lymphocytes of patients. They demonstrated the fact that peripheral blood lymphocytes in TB patients secrete significant amounts of interleukin-10 (IL-10) following exposure to TB bacilli before anti-TB drug treatment. However, the secretion of Interferon Gamma (IFN- $\gamma$ ) was shown to be insignificant. As a consequence,  $Th_2$  cells are active, but they do not secrete IL-10 at the end of 6 months anti-TB treatment anymore; instead they secrete IFN- $\gamma$ . The reason for this phenomenon is the effect of mycobacterium on the immune system.

We didn't find any significant difference between the TB group and the normal population with regard to other lymphocytes sub-populations. Other studies also have not found any quantitative relation between natural killer cells (NKC) and pulmonary tuberculosis although a qualitative difference was present. Studies by Bermudez (26) and Yaneda (27) revealed that NKCs of TB patients were activated by lymphokines and had apoptotic activities by killing the cells containing mycobacterium tuberculosis. In other words, they had a role in defense against this intracellular pathogen.

There was no significant difference in the ratios of lymphocytes sub-populations between sexes or in different age groups. Among activation markers, only HLA-DR had a significant increase concordant with age advancing.

In the study by Gasempour et al. (15),  $CD_4^+$  and  $CD_{19}^+$  lymphocyte counts had significant relationship with age. Kawiak et al. (28) also noted a decrease in monocytes, total  $CD_3^+$ , and  $CD_8^+$  lymphocytes as well as an increase in  $CD_4^+$  cells and  $CD_4^+ / CD_8^+$  ratio with increase in age. Moreover, Lee et al (29), proved increases in NKC,  $CD_3^+$ , and  $CD_8^+$  cells from birth to adulthood. It is worth consideration that the scheme of lymphocytes sub-population changes pertains to healthy subjects in the aforementioned studies and it may show variations in the patients.

These changes were not observed in active tuberculosis patients in our study. Finally, a thorough discussion can not be made about the results obtained in correlation between the ratios of expression of lymphocytes and pre-inflammatory markers.

For example, the reverse correlation of  $CD_{16+56}$  marker expression with  $CD_4^+$  cell expression is a complex finding that may be due to various cytokines secreted during the course of the disease. A better understanding of these associations requires further molecular investigations.

## CONCLUSION

Based on the conducted study, there are significant correlation, either positive or negative, between some of the lymphocytes sub-populations. In smear positive pulmonary TB patients, there is no significant relationship between age and the ratio of lymphocytes expression although the relationship is significant for HLA-DR. Comparing PPD positive with PPD negative, smear positive TB patients, only the ratios of  $CD_3^+$  cells had significant differences, and other lymphocytes sub-populations and activation markers were not significantly different. We also conclude that variations in lymphocytes sub-populations has no influence on chest radiographic changes. Comparing the results of our study with the control group, it became evident that in smear positive TB group, the  $CD_4^+$  cell counts and the

CD<sub>4</sub><sup>+</sup>/CD<sub>8</sub><sup>+</sup> ratio decreased, and CD<sub>8</sub><sup>+</sup> cell counts increased significantly.

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