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Original Research

CD4+ regulatory T cells and CD4+ activated T cells in new active and relapse tuberculosis

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Abstract: The aim of the present study was to examine characteristics of tuberculosis (TB) patients with different clinical forms and to study the frequency of Regulatory T cells (Treg cells) and Activated T cells in patients with new active and relapse TB. Forty-five pulmonary TB patients and a control group of 15 healthy individuals were enrolled in this study. Of the 45 TB patients, 15 were new cases with drug-susceptible active TB and 30 were relapsed cases (15 drug-susceptible and 15 multidrug resistant-TB). The age of study participants ranged from 21 to 68 years old. According to sex presentation, males were appreciably highly affected than females with a sex ratio of 2. The patients reported a mean recent weight loss of 8.9 kg. The Erythrocyte Sedimentation Rate was high in TB group, far exceeding the normal value. The results revealed that the number of CD3+ CD4+ T-cells significantly decreased whereas the level of blood Treg cells and expression of activation markers CD38 and HLA-DR on CD4+ T-cells significantly increased in TB group compared with the control group (p<0.05). The frequency of Treg cells was significantly higher in the TB group than the control group. Both the patients with new active TB and relapse TB demonstrated significantly higher levels of CD4+FoxP3+ Treg compared to healthy subjects (p<0.05). A high and significant percentage of Treg cells were found in patients with DS active TB than patients with MDR relapse TB. Interestingly, the frequency of CD4+FoxP3+ cells also differs according to the sputum smear microscopy status. The presence of high numbers of Treg cells and corresponding high immune activation may be an unfavourable factor that can predispose individuals to different clinical forms of TB, including relapse TB.

Key words: Tuberculosis; Regulatory T-cells; CD4+ T cell activation; FoxP3 protein; Relapse; Disease susceptibility; Multidrug-resistant tuberculosis.

Introduction

Worldwide, tuberculosis (TB) is one of the most prevalent infectious diseases and remains a major global health problem. It causes ill-health for approximately 10 million people each year and is one of the top ten causes of death, especially in developing countries (1). Recently, the number of retreatment, especially relapse cases and severe pulmonary tuberculosis with high resistance observation, has continued to increase despite the implementation of the Directly observed Therapies (DOTs) strategy for TB control (2).

Protective immunity against *Mycobacterium tuberculosis* (Mtb) is not completely understood but depends above all on T cell mediated immune responses where CD4 T cells play a central role (3). These immune responses are modulated by regulatory T cells (Treg cells), a subset of CD4+ T cells, through the mechanisms that depend on cell-cell contact mediated suppression and production of cytokines such as Interleukin-10 (IL-10) and Transforming Growth Factor beta (TGF-b) (4 - 6).

Following cell contact, Treg cells may kill responder T cells by a granzyme-dependent or perforin-dependent mechanism (5). T cell activation is also necessary for effector functions such as cytotoxicity, but the persistent immune activation associated with systemic inflammation is known to play a key role in disease progression (7). Previous studies have reported Treg cell expansion and immune activation in patients with active tuberculosis disease, suggesting that they are a susceptibility factor in the development of TB. Patients with Multidrug resistant (MDR) strains are also reported to perform more distinctly Treg population than patients with Drug susceptible TB patients (8 - 13). However, little is known about the frequency of Treg cells and activated T cells in the peripheral blood of patients with relapse TB.

Therefore, this study was planned to examine the characteristics of TB patients with different clinical forms and to evaluate the frequency of Treg cells and the expression of activation markers on CD4+ T cells from patients with new active and relapse TB.

Materials and Methods

Human study subjects and blood samples

Forty-five pulmonary TB patients were recruited at

Leon Daniello Pneumology Hospital, Cluj-Napoca, Romania. Of the 45 TB patients, 15 were new cases with drug-susceptible active TB and 30 were relapse cases (15 drug-susceptible and 15 multidrug resistant-TB). The diagnosis of pulmonary TB was based on clinical presentation and chest computed tomography examination, and confirmed by positive sputum culture (Solid Medium Lowenstein Jensen) for Mtb. Patient's age, sex and other information on clinical profile were obtained from TB registers at Leon Daniello Pneumology Hospital where all participants underwent chest X-rays and other routine investigations such as weight loss, white blood cell count, platelet count, hematocrit, hemoglobin and erythrocyte sedimentation rate. A control group of 15 healthy subjects with no history of TB and no significant past medical history was recruited from the Iuliu Hatieganu University of Medicine and Pharmacy.

A relapse was defined as a patient who has previously received full treatment and is declared cured or treatment completed, but returns and is found with bacteriologically positive (smear or culture) TB (1). Patients with failure or default during treatment were excluded. Other exclusion criteria were HIV infection, systemic autoimmune disorders and immune-suppressive therapy history. The study protocol was approved by the University of Medicine and Pharmacy Iuliu Hatieganu Ethical Committee on Human Research and written informed consent was obtained from each study subject.

Sample processing and mycobacterial assessment

Clinical samples were first decontaminated by the NaOH-N-acetyl-L-cystein-NaOH method and concentrated sediments were suspended in 1.0 ml of sterile phosphate buffer for Ziehl-Neelsen staining method. Aliquots of decontaminated specimens were cultured on L/J solid medium (14). Confirmed *Mtb* samples were subject to drug susceptibility testing using the proportional method on L/J medium. DS TB was defined as susceptible to isoniazid and rifampicin. MDR TB was defined as resistant to at least isoniazid and rifampicin.

Blood sampling and Immunophenotyping analysis

Peripheral Blood Mononuclear Cells (PBMC) were isolated from heparinized whole blood using Ficoll-Paque PLUS, cryopreserved in 10% dimethyl sulfoxide (DMSO) / 90% foetal bovine serum (FBS) and then stored in liquid nitrogen prior to subsequent analysis. Treg cells were defined as CD4+ FoxP3+ and CD4+ activated T cells as CD3+ CD4+ CD38+ HLA DR+. Frozen PBMCs were rapidly thawed and incubated for 30 min in the dark with PerCP anti-human CD4 (clone RPA-T4, Biolegend, San Diego, CA, USA) for surface phenotyping. Cells were then washed, fixed and permeabilized for intracellular staining with PE anti-human Foxp3 antibody (clone 206D, Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. To determine the activation status of CD4+ T cell populations, cells were stained with RPE-Cy5.5 conjugated CD3 (clone UCHT1), APC conjugated CD4 (clone RPA-T4), FITC conjugated CD38 (clone AT13/5) and RPE conjugated HLA-DR (clone YE2/36-HLK) (all from Bio-Rad Company, USA). Cells were finally analysed on a BD FACSCanto™ II Flow Cytometry.

Statistical analysis

To evaluate certainty of differences of the sample numerical characteristics, which are not subject to normal distribution, we used the Kruskal-Wallis test. The difference in indicators in the compared groups was considered to be statistically significant at the significant level $p<0.05$. Data were collected in Excel and statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Prism Software, Inc. CA, USA).

Results

A total of 45 TB cases and 15 healthy controls were included in this study. Clinico-demographic profile and

Table 1. Baseline characteristics of the study participants.

Subject groups	DS-Active TB (n=15)	DS-Relapse TB (n=15)	MDR-Relapse TB (n=15)	Control (n=15)
Age, mean (range)	43.4 (21 - 68)	43.7 (23 - 66)	43.9 (30 - 61)	42.3 (20-58)
Males /Females	8/7	10/5	12/3	9/6
Admission Temperature (^{O}C, Mean)	37.6	37.4	37.5	36.9*
BCG vaccinated (n)	14	11	12	12
Weight loss				
Yes (n, Mean in Kg)	11 (7.1)	10 (9.3)	11 (9.4)	-
Non	4	5	4	-
Sputum smear microscopy (n)				
Positive	10	10	9	-
Negative	5	5	6	-
Bilateral cavitation (n)				
Yes	6	8	10	-
Non	9	7	5	-
Laboratory findings (Mean ± SD)				
WBC count, x10^3 cells / µl	8.28 ± 0.6	7.32 ± 0.36	8.07 ± 0.24	9.2 ± 0.42*
Lymphocytes, x10^3 cells / µl	1.7 ± 0.5	2.03 ± 0.21	1.7 ± 0.33	2.9 ± 0.4*
Haemoglobin, g/dl	11.3 ± 1.25	11.5 ± 1.14	11.1 ± 1.08	11.7 ± 2.1
Haematocrit, %	31.9 ± 4.24	37.2 ± 6.21	33.4 ± 5.68	45.8 ± 7.3*
Platelet count, x10^3 cells / µl	387 ± 62.08	415 ± 55.36	328 ± 73.2	375 ± 54.13
ESR, mm/1h	53.6 ± 9.91	55.4 ± 8.92	73.06 ± 10.1	6.5 ± 1.2*
ESR, mm/2h	84.8 ± 14.3	76 ± 15.26	97.26 ± 12.3	14.6 ± 3.6*

DS: drug susceptible. WBC: white blood cell. * Statistically significant difference ($p< 0.05$) between all pulmonary TB patients and control subjects.

routine laboratory data are summarized in Table 1. The age of study participants ranged from 21 to 68 years old. According to sex presentation, males were appreciably highly affected than females with a sex ratio of 2 (30 males and 15 females). Overall, 64.4% of cases were sputum smear microscopy positive and 53.33 % had bilateral cavitation.

Conventional drug susceptibility testing was performed by the proportion method and showed that all new cases were susceptible to all first line drugs. Among the 30 relapse cases, 15 were drug susceptible and 15 were MDR, highlighting resistance to both rifampicin and isoniazid. The difference between TB cases and healthy controls was noted especially on subject temperature at recruitment, number of white blood cells, number of lymphocytes, and the erythrocytes sedimentation rates after 1h and 2h. Most subjects were already vaccinated with BCG and most patients have registered loss of weight.

CD4+ T cell activation increases both in active and relapse TB

Figure 1 illustrates the flow cytometric gaining strategy adopted to measure HLA-DR and CD38 expression on CD4+ T cells. Flow cytometric analyses showed a significant higher co-expression of CD38 and HLA-DR in TB patients than healthy subjects (p<0.05). This co-expression was higher both in active TB and relapse TB patients compared to controls, with no statistically significant difference between active and relapse cases (p<0.05). However, when the analyses were performed for each fraction separately, the percentage of CD4+CD38+ and CD4+HLA DR+ seems to be higher within patients with new active TB than relapse TB, although the difference was not statistically significant (Figure 2). No difference in T cell activation was found between patients with DS TB and MDR TB.

CD4+ Foxp3+ T cells increase both in patients with active and relapse TB

The frequency of Treg cells was significantly higher in the TB group than the control group. Both patients with new active TB and relapse TB demonstrated significantly higher levels of CD4+FoxP3+ Treg compared to healthy subjects (p<0.05). A high and significant percentage of Treg cells were found in patients with DS active TB than patients with relapsed cases with MDR-TB. Interestingly, the frequency of CD4+FoxP3+ cells also differs according to the sputum smear microscopy status, Treg cells being significantly more frequent in smear positive cases (Figure 3).

Discussion

According to the World Health Organization, tuberculosis cases are broadly classified into new or retreatment TB cases. Retreatment patients are further classified as relapse, treatment after failure and treatment after the loss to follow-up (15).

A total of 60 subjects were enrolled in this study: 15 cases with new DS active TB, 15 cases with DS relapse TB, 15 cases with MDR relapse TB and 15 healthy subjects without *M. tuberculosis* infection. The age range was 21-68 with no difference in median between

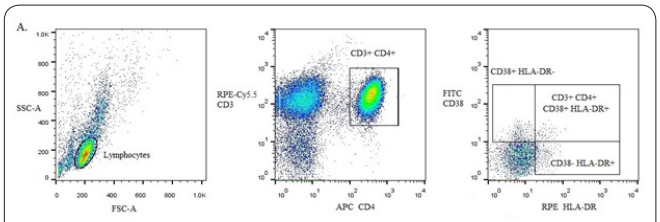

Figure 1. Flow cytometry gating strategy for measuring HLA-DR and CD38 expression on CD4+ T cells in healthy subjects, active and relapse TB patients.

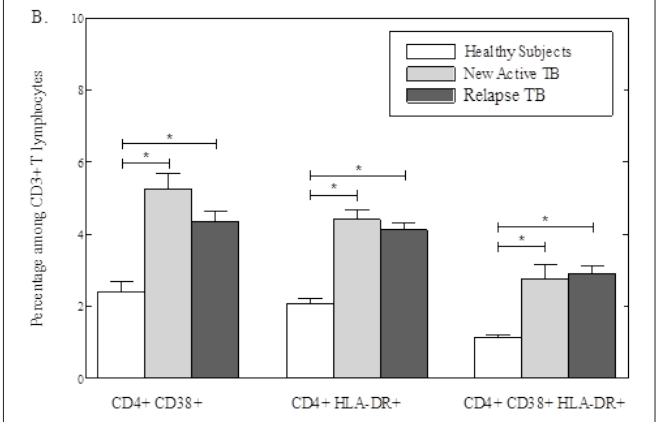

Figure 2. Distributions of CD4 activation markers on CD3+ T cells in patients with new active and relapse TB compared to healthy subjects (* p<0.05).

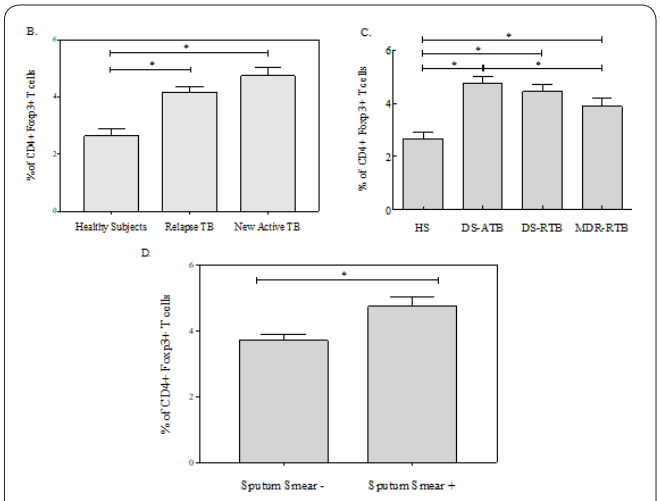

Figure 3. Frequencies of regulatory T cells among study groups. (A) Flow cytometry gating strategy for Treg cells in healthy subjects, active and relapse TB patients. Treg cells were defined as CD4+FoxP3+ cells. (B) The percentage of Treg cells in healthy subjects, relapse TB and new active TB. (C) The percentage of Treg cells in healthy subjects (HS), DS active TB (DS-ATB), DS relapse TB (DS-RTB) and MDR relapse TB (MDR-TB). (D) The frequency of Treg cells according to sputum smear results. * p<0.05.

groups. The patients reported a mean recent weight loss of 8.9 kg. The Erythrocyte Sedimentation Rate (ESR) in TB group far exceeded the normal value. ESR indicate inflammatory changes in the whole body induced by TB infection and has been empirically a classic laboratory index for diagnosis and follow-up of patients with tuberculosis (16, 17). There was not much difference in basic investigations between different groups among TB patients, except in ESR values. Most patients with

active TB had a much higher ESR value compared to the patients with relapse TB. In agreement with previous studies, patients with TB had significantly lower number of CD3+ and CD3+CD4+ cells (18). Patients with TB also displayed significant higher expression of peripheral CD38+HLA-DR+ on CD4+ T-cells than the healthy subjects ($p<0.05$). These results are in line with previous findings, suggesting that infection with TB is associated with immune activation (19). In the present study, an increased CD4+ T cell activation was also predominately found in the active TB patients and relapse patients compared with healthy subjects ($p<0.05$). A high but not statistically significant activation markers was observed in MDR-relapse than DS-relapse ($p>0.05$).

CD4+ cells are critical in immunity against Mtb infection. They play two important roles: first, to produce cytokines that govern the cell mediated immune responses; and secondly, to eliminate the infected macrophages via apoptosis (20, 21). The results of this study suggested a relative immune activation in TB patients which could possibly be a response to dampen the immune response directed against Mtb. The high immune activation could reflect immune dysregulation which is probably a feature of individuals at risk for relapse tuberculosis. Understanding the mechanism of relapse is key to guiding TB control measures.

The results also showed that the level of blood Treg cells, identified as CD4+Foxp3+ T cells, was higher in TB patients compared to controls. High levels of circulating Treg cells have also previously been found in patients with active and latent TB (22 - 26). However, to our best knowledge, no such studies have been performed in patients with relapse TB. The main strength of the study is the evaluation of the level of Treg cells at the same time in new active and relapse TB. Results clearly showed that Treg cells are elevated both in patients with active and relapse TB compared to controls ($p<0.05$) and no statistically significant difference was found between DS and MDR TB cases. This is consistent with a recent study which has shown that there was no difference in Treg cell frequency between DS TB and active MDR TB patients (27). Interestingly, patients with positive sputum smears showed a higher percentage of Treg cells comparatively to patients with negative sputum smears.

In this study, Treg cells were defined as CD4+Foxp3+. There is still controversy about identification markers of human Treg cells (27 , 28); however, a recent study by Lim *et al*. has shown that CD4+ Foxp3+ can be used to identify both natural (CD4+ CD25+ FoxP3+) and induced (CD4+ CD25- FoxP3+) T cells (27). Furthermore, Churina *et al*. showed that CD4+ CD25+ FoxP3+ Treg (natural regulatory T cells) and CD4+ CD25- FoxP3+ Treg (induced regulatory T cells) increased in TB (29). Other several previous studies also used CD4+FoxP3+ as the criterion for Treg cell identification (30, 31). In spite of all these findings, the exact role of Treg cells in TB is not certain and there is still continuing research interest in that domain for a better understanding (27).

This study is very informative but has also some limitations. First, the study groups used were relatively small, which limited the ability to perform detailed subgroup analyses. Second, the authors have studied cells from peripheral blood rather than from the site of infection. A larger study that assesses Treg cell frequency and activation according to the disease compartment itself is warranted because immune activation in the periphery may differ from the immune response at the site of disease. Third, other activation markers were not studied here, including those expressed early after activation, such as CD69 and CD25, and those expressed later, such as CD71 and CD40.

The results of this study show significant differences in the levels of Treg cells and CD4+ activated T cells among patients with active and relapse TB. The presence of high immune activation and corresponding high number of regulatory T cells may reflect immune dysregulation that can predispose patients to different clinical forms of tuberculosis, especially relapse TB. Further investigations on Treg cells, activated T cells and Treg specific cytokines such as IL-10 and TGF-b functional parameters will improve our understanding of the exact role of these determinants in the development of relapse TB.

Competing interests

The authors have no conflict of interest to declare.

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