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Regulatory T lymphocytes in evaluation of the local protective cellular immune response to *Mycobacterium tuberculosis* in Romanian patients

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Abstract

*Active suppression by Regulatory T lymphocytes (Treg) might be important in controlling immune responses against **Mycobacterium tuberculosis** (Mtb). Our aim was to evaluate the local cellular immune response to Mtb, by evaluation of Treg cells in pleural fluid (PF) compared to peripheral blood (PB) from patients with active Mtb infection and healthy Romanian subjects.*

Tregs were isolated using MACS $CD4^+CD25^+CD127^{dim/-}$ (Miltenyi) and CD4 CD25 T cells and Foxp3 transcription factor were analyzed by flow cytometry. We found higher % of Tregs in PF compared to PB from patients or healthy Romanian subjects, which might explain the relatively effective local immune response against Mtb infection.

Keywords: RegulatoryTcells, CD4+CD25+FoxP3+, Mycobacterium tuberculosis, pulmonary tuberculosis

Introduction

Tuberculosis remains one of the most deadly diseases in the world affecting an astonishing number of the world's population. It is estimated that each year more than 9 million new cases of tuberculosis occur and approximately 2 million persons die from the disease [1]. Ninety-five percent of the tuberculosis cases occur in developing countries. Romania is ranked among the first in WHO Europe Region with an incidence of 115/100.000 inhabitants.

Approximately one-third of the world's population is latently infected with Mtb and 90% of these individuals will never develop active disease during their lifetime, indicating that the human immune system is capable, in most of the cases, of controlling Mtb infection effectively. However, it remains elusive why the immune system only restricts

microbial growth and fails to achieve sterile eradication of Mtb. Also, there is a wide spectrum of susceptibility to TB, even among an immunocompetent population.

Regulatory T cells (Tregs) are considered among the most informative in evaluation of current immune status, controlling homeostasis and immunopathology [2].

Effector CD4+T cells of the Th1 type dominate protective immunity and help to limit bacterial replication and dissemination *in vivo*, however it also causes significant immunopathology. Tregs, produced by the thymus, suppress the activation and expansion of naïve T cells and their differentiation to effector T cells, and appear to be critical in controlling immune homeostasis [3].

Immunologic reactivity against Mtb is compartmentalized in pleural space, and inflammatory cells and mediators are readily found at the site of disease. Active suppression by Tregs might play an important role in the down-regulation of T cell responses to foreign and self-antigens, including Mtb [4].

At this time, little is known about the profile of Treg cells in patients with TB from endemic settings as Romania is. If Treg cells can be expanded at the site of infection during active TB, those cells would be able to decrease the immune response against Mtb [5].

Our aim was to evaluate regulatory T cells (Tregs) at the site of infection with Mtb in Romanian patients. Tuberculosis (TB) pleurisy is accepted to be a suitable model for evaluating local protective cellular immune response to Mtb, since it can be spontaneously self-cured. Therefore, we evaluated Treg population in pleural fluid (PF) and time-matched peripheral blood (PB) samples from Romanian patients with tuberculous pleurisy (TP), compared to peripheral blood samples from healthy, Mtb uninfected individuals.

Results and Discussion

CD4+CD25+FoxP3+ T cells are increased in the blood and pleural fluids of TP patients To investigate whether Mtb infection in TP is associated with CD4+CD25+FoxP3+ Treg expansion, we monitored their proportion from total CD4+ lymphocytes.

The percentages of $CD4^+CD25^+Foxp3^+$ T cells in PF and PB from patients with TP and, PB from healthy control (Control-PB) subjects were determined by flow cytometry before (Fig.1) and after enrichment using MACS $CD4^+CD25^+CD127^{dim/-}$ (Miltenyi Biotec) (Table 1). The expression of forkhead transcription factor Foxp3 was examined both in $CD4^+CD25^+$ after first and second positive magnetic selection of the cells, using anti-CD4-FITC/anti-CD25- PE/anti-Foxp3-APC (Miltenyi Biotec).

The results depicted in Figure 1 indicate that the frequency of CD4+CD25+FoxP3+ T cells within the total CD4+ population was significantly increased in the PF compared with PB of the patients with tuberculosis ($p<0.05$) and in both type of samples from TP patients compared to healthy donors ($p<0.05$ for PB-control vs. PB-TP and $p< 0.001$ for PF-TP vs. PB-TP).

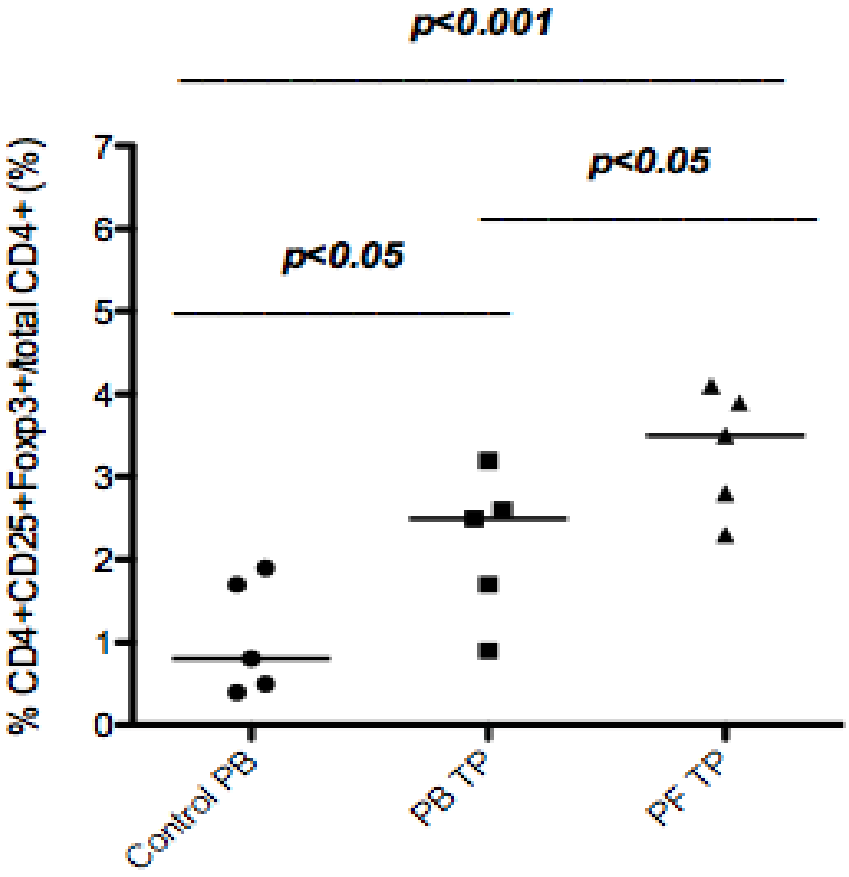


Figure 1. CD4+CD25+FoxP3+ T cells were increased in the peripheral blood (PB) of patients with pulmonary tuberculosis (PT). PBMCs isolated from the blood of healthy donors (PB control, n=5, mean age (range)= 34.8 (26-43)), PBMCs and cells from pleural fluid from pulmonary tuberculosis patients (n=5, mean age (range) = 36.8 (21-66)) were analyzed for surface expression of CD4 and CD25 and intracellular expression of FoxP3. Samples were acquired after written informed consent obtained from the study subjects. The research project was approved by the institutional Ethics Committee. All patients were HIV negative. All samples were examined for the presence of M. tuberculosis, fungi, and malignant cells. Samples were analyzed by EPICS XL Beckman Coulter or FACSCalibur Becton Dickinson.

Flow Cytometers. Each dot represents the value for each individual patient. Difference among groups was analyzed using Student's T Test and p values were indicated on the figure. The proportion of CD4+CD25+FoxP3+ T cells within total CD4+ T cells was significantly higher in PB-TP and PF-TP compared to PB control ($p<0.05$).

% $CD4^+CD25^+$ T cells in PF *versus* PB from patients and normal subjects after MACS separation

In PF from patients infected with MTB, a purity of 85.7±4.2% CD4+CD25+ was obtained even after first positive selection using MACS $CD4^+CD25^+CD127^{dim/-}$ strategy, compared to 58.2+/-18.4 for the cells from peripheral blood of patients and 55.7 ± 27.1% for the cells from peripheral blood from control subjects (Table I). After

second positive selection (III) purities higher than 90% were obtained for all investigated samples and high percentage Foxp3+ in these populations confirmed the fact that most of them were Tregs (Table1, Fig.2).

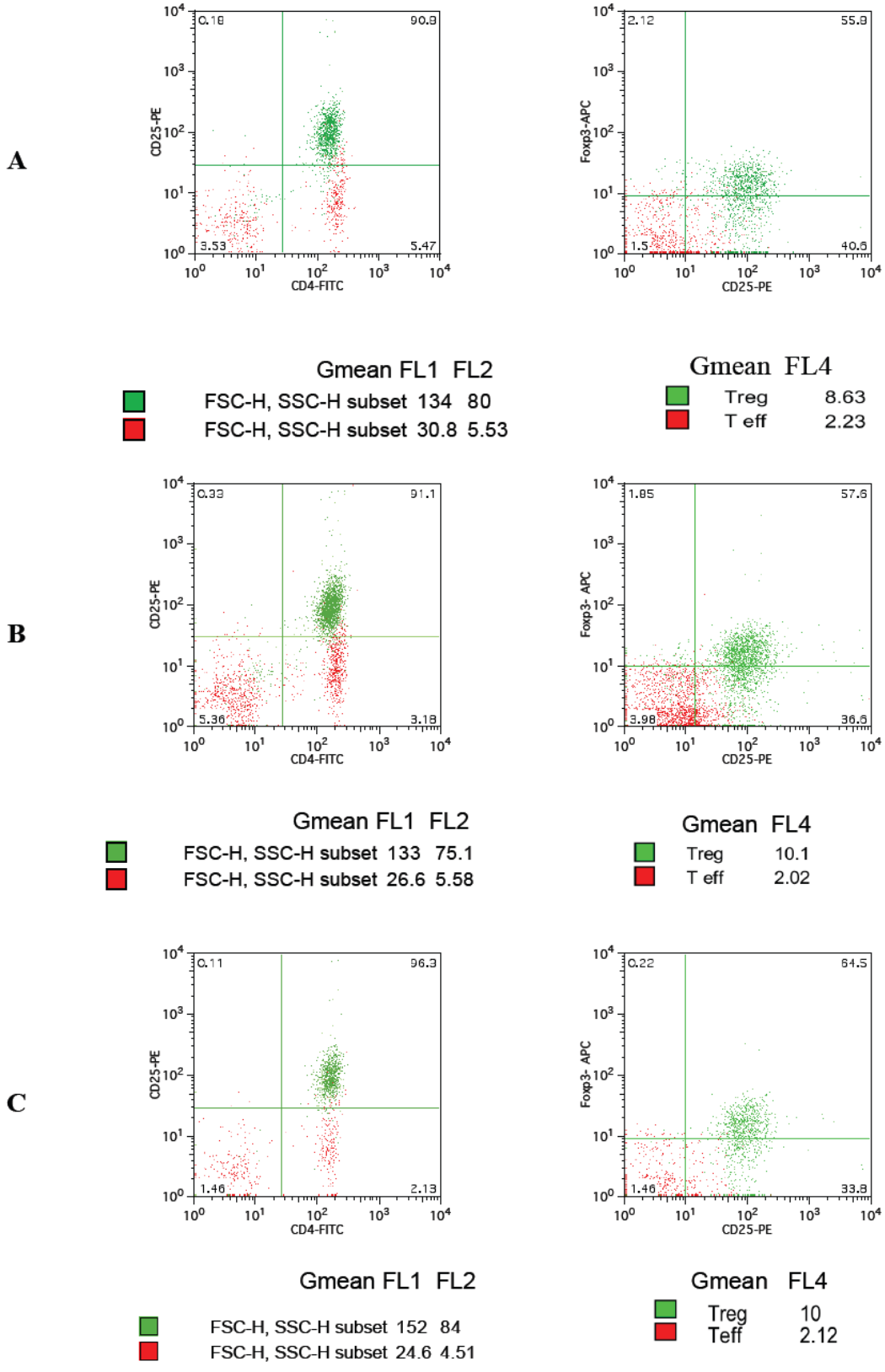
Percentages CD4 $CD25^{+}Foxp3^{+}$ T cell in PF were higher than those in PB from patients with TP and healthy subjects in this study. We also found that CD4 CD2 T cells infiltrating the pleural space were regulatory T cells since they expressed a high level of Foxp3 transcription factor.

Table 1. % $CD4^{+}CD25^{+}$ T cells in PF and PB from patients and normal subjects after MACS separation, and analysis of % Foxp3+ in the $CD4^{+}CD25^{+}$ isolated cells

	CD4+CD25+ purity (% of separated cells)	
	Pre-enrichment (I)	**Enrichment (II)**
controls PB	55.7+/-27.1	79.2+/-5.4
PB from TP patient	58.2+/-18.4	80.6+/-7.5
PF from TP patients	85.7+/-4.2	90.1+/-4.5
	Foxp3+ purity (% of CD4+CD25+ cells)	
	Pre-enrichment (I)	**Enrichment (II)**
controls PB	56.62+/-4.9	58.62+/-5.2
PB from TP patient	57.6+/-3.7	59.06+/-3.5
PF from TP patients	62.64+/-7.0	63.98+/-7.4

Separation of the T cell populations was achieved using **MACS $CD4^{+}CD25^{+}CD127^{dim/-}$ (ON130-094-775 Miltenyi Biotec)**. Non-target cells were eliminated after a magnetic labeling performed using anti-Biotin MicroBeads and biotin-conjugated mAb against CD8, CD19, CD123, CD127mAb (pre-enrichment). After magnetic separation, CD4+CD127dim/- unlabeled cells were pre-enriched in the effluent (I). Further, was performed an enrichment (II and III) using MicroBeads conjugated with anti-CD25 mAb. CD4+CD25+CD127dim/- cells were enriched in the effluent and analyzed for CD4, CD25 and Foxp3 the expression.

Figure 2. % $CD4^{+}CD25^{+}$ and %Foxp3+ in samples from control (A) or PB (B) and PF (C) from TB patients after MACS separation (second positive selection - III).



Dot plot representation are obtained from overlaying T $CD4^+CD127^-CD25^+$ cells (here called Treg - red) on T $CD4^+CD25^-$ (here called Teffector =Teff - green) obtained as non-target cells during last positive selection. Teff population was used for gating Foxp3+ cells as Low *et al.* [6] recently suggested. $CD4^+$were gated for analysis of Foxp3 expression. Representative dot-plots for each type of sample.

Higher percentage of $CD4^+CD25^+CD127^{dim/-}$ obtained after first positive selection might be explained by the higher level of $CD25^+$, which is a characteristic of Tregs cells and was confirmed by the higher percentage of $Foxp3^+$ cells in this population. Analysis of the $CD4^+/CD25^+$ purities of the cells obtained after first positive selection (enrichment II) might be a better modality to assess the percentage of Tregs in a biological sample.

Analysis of $Foxp3^+$ level in selected populations was not discriminative enough for a rapid evaluation of Tregs in initial samples and is more difficult to evaluate, Foxp3 being an intracellular antigen. Since usually the separated Treg cells are necessary in subsequent functional experiments, it is important to have a fast reliable method to evaluate the number of these cells in the purified samples.

At present expansion of Treg cells in TB is still under intense debate.

Guyot-Revol and colleagues [7], and others [8, 9] found that regulatory lymphocytes (Treg cells) were expanded in patients with active tuberculosis (TB) from a non-endemic setting and may play a role in suppression of type 1 immune responses. On the contrary, Gazzola and colleagues [10] showed no increase in CD4_CD25high T cells in patients with tuberculosis compared with healthy control subjects and they highlighted the significance of accurate approaches to evaluate the frequency and level of FoxP3 expression at the single cell level. Our results, obtained on samples from a setting with high endemicity, showed a significant increase of the frequency of the CD4+CD25+FoxP3+ T cells in TP compared to control.

Further studies are required to delineate the role of $CD4^+CD25^+$ $Foxp3^+$ T cells in TP and should be focused on identifying the mechanisms involved in the immunoregulatory properties of pleural Treg cells.

We presume that an increased percentage of $CD4^+CD25^+Foxp3^+$ T cells in PF might be due to active recruitment or local differentiation. Recent studies showed that both these hypothesis are possible [11]. Therefore, further studies on the mechanisms by which $CD4^+CD25^+$ $Foxp3^+$ T cells were recruited into PE will be very useful.

Conclusions

Analysis of the $CD4^+/CD25^+$ purities of the cells obtained after first positive selection might be a better modality of assessment of the percentage of Tregs in a biological sample than $Foxp3^+$ level determination, which will take longer to be performed.

The increased level of Treg cells in tuberculous pleural fluid explains the relatively effective local immune response against Mtb infection.

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