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ORIGINAL ARTICLE

Study of some T regulatory cell subsets in patients with multi-drug resistant pulmonary tuberculosis

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Abstract *Background:* Regulatory T-cells are $CD4^+$ cells involved in the regulation of suppression of immune response during infection. Many studies revealed that the number of these cells, increase in patients with active pulmonary TB (PTB. Few studies addressed this problem in MDR-PTB.

Objective: This work aimed at studying some T reg – cell subsets in patients with MDR-PTB, compared to those with active pulmonary TB who responded to treatment as well as to healthy control subjects.

Methods: Three groups were included in the study (20, in each group), group of healthy control and 2 groups as patients' groups (patients with MDR-PTB and patients' with PTB responding to treatment). Routine blood work and CXR were done for all subjects in addition to microbiological evaluation of sputum in patients' groups. T reg – cell subsets in peripheral blood were studied by flow cytometry, using monoclonal antibodies against the following markers, CD4, CD25 and FoxP3 for identification.

Results: Patients' groups, had higher frequency of T reg cell subsets, $CD4^+$ $CD25^+$ $FoxP3^+$ than the group of healthy subject, ($P < 0.01$) and treatment responders' group had non-significantly higher percentage of these cells than in patients with MDR-PTB ($P > 0.05$), but highly

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significant statistical difference for the percentage of total CD4$^+$ ($P < 0.01$). Patients' with more radiologically extensive disease, had higher level of these cells than, in other patients ($P < 0.05$), with significant positive correlation.

Conclusion: Although immune suppression characteristic of T reg – cells seems important in the pathogenesis of MDR-PTB, other mechanisms, immunologic, on non-immunologic are important as well.

Introduction

In 2010, there were 8.8 million (range, 8.5–9.2 million) new cases of TB, 1.1 million (range, 0.9–1.2 million) deaths from TB among HIV – negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB. According to the WHO report about global tuberculosis, five of six WHO regions (the exception being the African Region) are on track to achieve the stop TB partnership target of halving 1990 mortality rates by 2015. Yet, alongside this achievement, diagnosis and appropriate treatment of multidrug-resistant TB (MDR-TB) remain major challenges. In 2010, there were an estimated 650,000 cases of MDR-TB among the world's 12.0 million prevalent cases of TB [1].

T-cells, termed T regulatory (T reg) cells, which have immunosuppressive functions and cytokine profiles distinct from that of either Th1 or Th2 cells, have been intensely investigated during recent years [2]. TB induces a state of immune activation in the infected host, and an increased expression of activation markers on T cells in blood from patients with active TB has been described [2,3]. T regulatory cells (T reg) are CD4 + T cells involved in the regulation of self-tolerance, autoimmunity and suppression of immune responses during infections [4,5]. T reg – cells were first recognized as FoxP3$^+$ CD4 + CD25+ T cells, but expression of the intracellular marker forkhead box p3 (FoxP^3) and low cell-surface expression of the IL-7 receptor a-chain (CD127) have been suggested as more accurate markers. Many studies suggested a role of T reg in immune response to mycobacteal infection as it seems to be an increased level of immune activation and T reg in both latent and active TB infection that is only modestly influenced by preventive therapy. In Germany, latent infection with *Mycobacterium* tuberculosis is characterized by an increased frequency of T reg – cells in the bronchoalveolar lavage (BAL) [5]. Compared with controls, tuberculosis had the highest T reg cell frequency, but also the highest levels of CD4$^+$ T lymphocyte activation [3].

Heet et al. (2010) hypothesized that this susceptibility to *M. Tuberculosis* infection is linked to increased T regulatory (T reg) cells and Th2 cytokines in tuberculosis patients. CD4$^+$ FoxP3$^+$ regulatory. T-cell expansion in the lung suggests that T reg – cells may be related to the progression of *M. tuberculosis* infection and that the balance [6] between effector immune responses and suppression of immune responses is essential to control *M. tuberculosis* infection.

Many studies revealed that the number of T reg – cells increases in the blood and at the infection site in active TB patients [7–9] and in latent *M. tuberculosis* infection [5]. However, few studies had addressed this problem in MDR TB patients [10,11].

The aim of this study was to assess some regulatory T-cell subsets in patients with MDR-TB in comparison to patients with active pulmonary TB who responded to anti-TB drugs as well as to healthy control subjects.

Patients and methods

This case control, cross-sectional study was conducted in chest unit, n medicine department, clinical pathology and microbiology departments, Suez Canal University in collaboration with Abbassia Chest and Ismailia Chest Hospitals.

The study included 3 groups of patients with pulmonary tuberculosis (P-TB), 20 patients in each group and a 3rd group of 20 healthy volunteers.

Group I: comprised re-treatment cases of P-TB (relapse, treatment failure and defaulters) whose sputa were positive for AFB with multidrug – resistance (MDR-TB). All patients in this group were negative for HIV.

Group II: comprised patients with newly-diagnosed smear positive active PTB who had received anti-TB drugs for at least 2 months and responded well, clinically, radiologically, and bacteriologically (i.e. sputum was negative for AFB at the end of the initiation period).

Group III: included 20 healthy volunteers with matched age, sex and all subjects had negative tuberculin skin testing.

All patients were subjected to the following

– Complete medical history and careful clinical examination.
– Chest X-ray, postero-anterior and lateral views (all subject) as a diagnostic tool and to assess the radiological extent as mild, moderate and far-advanced (or extensive). (National tuberculosis Association of USA, 1961) [12].
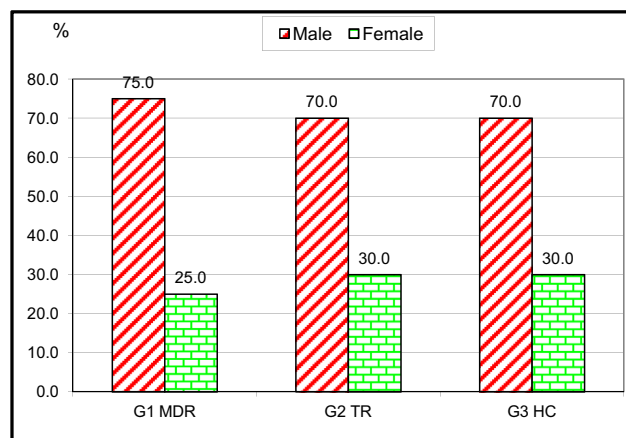– Sputum examination for AFB by Zeihl – Neelsen stain.

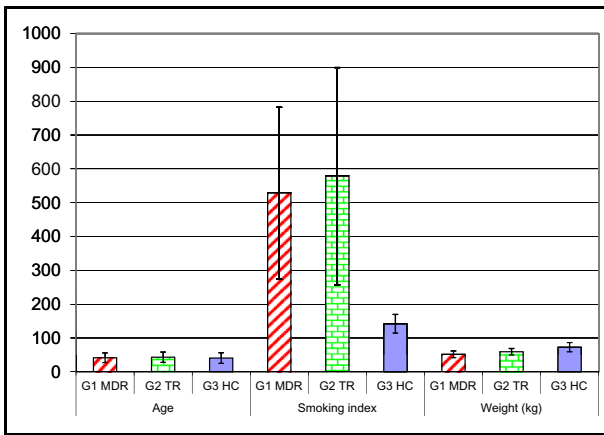**Fig. 1**   Comparison between three groups as regards sex.

Fig. 2 Comparison between three groups as regards age, smoking index and weight (kg).
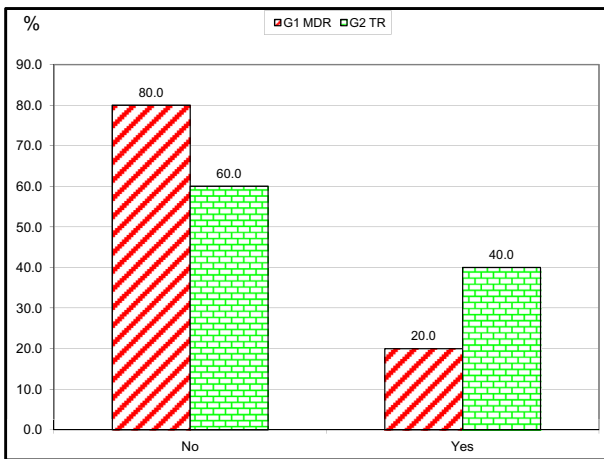

Fig. 3 Comparison between three groups as regards associated disease (DM).

– Routine blood work (all study subjects), including CBC, ESR, LFTs, S-creatinine, fasting and 2-h post prandial sugar and HIV screening (in group I only).
– Patients in group I were also subjected to sputum culture on Lowenstein- ensen medium and drug – susceptibility testing for first line anti-TB drugs.
– Flow cytometric study of monocyte – depleted peripheral blood mono-nuclear cells (PBMC) and dis included (DH) CD25 and Fox P_3 +ve regulatory T-cells (T regs).

T reg subset study by flowcytometer

Anticoagulated blood was collected in EDTA vacutainer tubes. T reg subsets regarding their CD25 and foxp3 were identified using monoclonal antibodies for the following markers, CD4 antibodies labeled by FITC fluorochrome, anti FOXP3 labeled by PE fluorochrome and, anti CD25 labeled by APC fluorochrome. Surface staining was done first for both CD4 and CD25 according to the regular procedures, then it was followed by cellular permeabilization and intracellular staining for FOXP3. After staining, data acquisition by FACS calibur using cell quest software and paint gate software for data analysis was done. (BD Bio-sciences, San José, CD, USA).

Statistical analysis

Data were analyzed using Statistical Program for Social Science (SPSS) version 18.0. Quantitative data were expressed as mean ± standard deviation (SD). Qualitative data were expressed as frequency and percentage.

The following tests were done

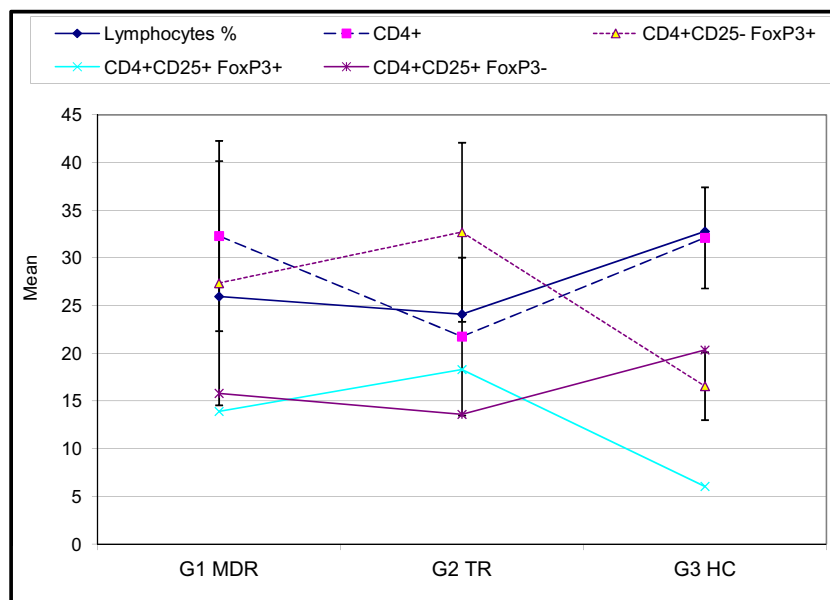• Independent-sample t-test of significance was used when comparing between two means.

Fig. 4 Comparison between three groups as regards T reg – cell subsets.
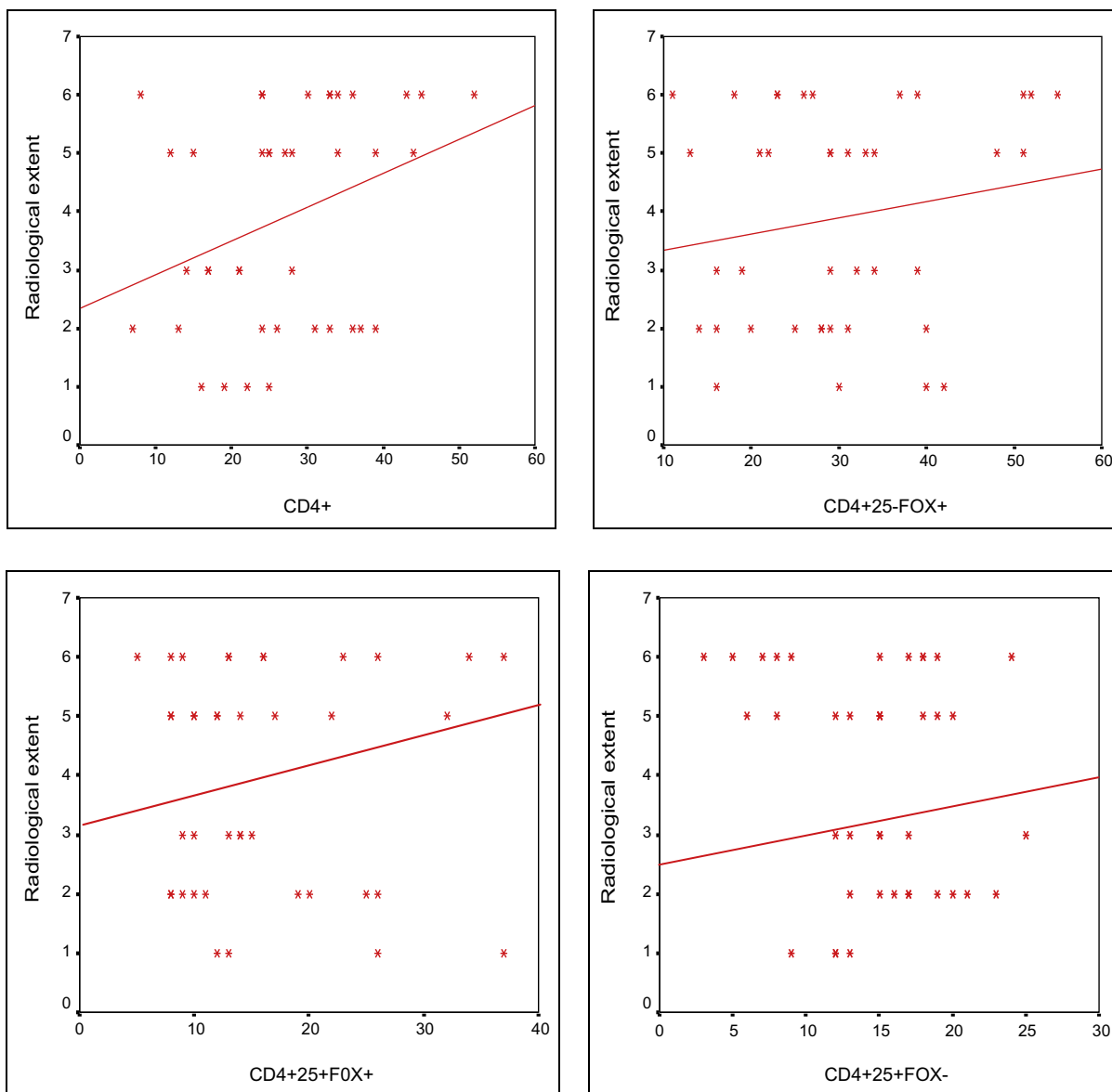
Fig. 5   significantly positive Correlation between radiological extent and T reg – cell subsets.

Table 1   Comparison between three groups as regards sex.

Sex	Groups						Total	
	G1 MDR		G2 TR		G3 HC			
	No.	%	No.	%	No.	%	No.	%
Male	15	75.00	14	70.00	14	70.00	27	45.00
Female	5	25.00	6	30.00	6	30.00	33	55.00
Total	20	100.00	20	100.00	20	100.00	60	100.00
X2	10.909							
P-value	0.052							

MDR: multidrug resistance; TR: treatment responders; HC: healthy control; X^2: chi-square test.
There is no significant difference among the study groups regarding sex, P-value > 0.05.

• A one-way analysis of variance (ANOVA) when comparing between more than two means.

• Chi-square (X^2) test of significance was used in order to compare proportions between two qualitative parameters.

Table 2 Comparison between three groups as regards age, smoking index and weight (kg).

Parameters	G1 MDR		G2 TR		G3 HC		ANOVA		LSD		
	Mean	±SD	Mean	±SD	Mean	±SD	F	P-Value	I & II	I & III	II & III
Age	41.60	14.28	43.30	15.26	40.75	15.61	33.71	0.862	0.722	0.859	0.594
Smoking index	528.44	253.93	578.24	321.25	142.00	27.75	3754.13	0.016*	0.668	0.019*	0.005**
Weight (kg)	51.68	9.91	59.40	9.59	72.80	13.68	2285.01	<0.001	0.034*	<0.001**	<0.001**

MDR: multidrug resistance; TR: treatment responders; HC: healthy control. F: ANOVA test; LSD: least significant difference.
* P-value <0.05 S.
** P-value <0.01 HS. There is a highly statistically significant difference between patients' groups and healthy control group regarding weight, P-value <0.001 and significant statistical difference regarding smoking index, P-value <0.05.

Table 3 Comparison between three groups as regards associated disease (DM).

Associated disease (DM)	Groups				x^2/t^*	P-Value
	G1 MDR		G2 TR			
	No.	%	No.	%		
No	16	80.00	12	60.00	1.905	0.168
Yes	4	20.00	8	40.00		
Total	20	100.00	20	100.00		
Duration in year (DM)						
Mean ± SD	23.0 ± 10.37		20.0 ± 7.07		0.491*	0.638

MDR: multidrug resistance; TR: TREATMENT responders; HC: Healthy control; X^2: Chi-square test; t: Independent sample t-test.
There is no statistically significant difference among the patients' groups regarding the incidence and duration of diabetes mellitus (DM), P-value >0.05.

Table 4 Comparison between three groups as regards T reg – cell subsets.

Parameters	G1 MDR		G2 TR		G3 HC		ANOVA		LSD		
	Mean	±SD	Mean	±SD	Mean	±SD	F	P-Value	I & II	I & III	II & III
Lymphocytes%	25.95	8.43	24.10	6.32	32.78	4.41	4154.046	<0.001**	0.437	0.002**	<0.001**
CD4$^+$	32.30	9.97	21.75	8.28	32.10	5.30	728.217	<0.001**	<0.001**	0.938	<0.001**
CD4$^+$CD25$^-$FoxP3$^+$	27.35	12.81	32.70	9.39	16.55	3.55	1353.617	<0.001**	0.077	<0.001**	<0.001**
CD4$^+$CD25$^+$ FoxP3$^+$	13.90	6.57	18.30	9.72	6.04	1.22	771.491	<0.001**	0.056	0.003**	<0.001**
CD4$^+$CD2$^+$ FoxP3$^-$	15.80	5.81	13.60	4.24	20.35	3.82	237.017	<0.001**	0.144	0.003**	<0.001**

MDR: multidrug resistance; TR: treatment responders; HC: healthy control; F: ANOVA test; LSD: Least significant difference.
** P-value <0.01 HS. There are highly statistically significant differences between patients' groups and healthy control group regarding T reg – cell subsets. There is highly significant statistical difference between both patients' groups regarding CD4$^+$ cells.

Table 5 Correlation between age, smoking, weight, radiological extent, duration of DM and T reg – cell subsets.

Parameters	CD4+		CD4 + 25-FOX+		CD4 + 25 + F0X+		CD4+25+FOX-	
	r	P-Value	r	P-Value	r	P-Value	r	P-Value
Age	−0.278	0.082	0.046	0.778	0.015	0.925	−0.248	0.122
Smoking index	−0.137	0.503	−0.040	0.846	−0.027	0.897	−0.121	0.557
Weight (kg)	−0.270	0.092	−0.042	0.798	0.124	0.447	−0.028	0.863
Radiological extent	0.531	0.037*	0.871	0.045*	0.633	0.037*	0.793	0.032*
Duration in years (DM)	0.080	0.838	−0.098	0.802	−0.295	0.440	0.189	0.626

r: pearson correlation co-efficient test.
* P-Value <0.05. There is significant positive correlation between the radiological extent and studied T reg – cell subsets; other parameters show non-significant correlation.

- Pearson's correlation coefficient (r) test was used for correlating data.
- Probability (P-value)
  - P-value < 0.05 was considered significant.

– *P*-value 0.01 was considered as highly significant.
– *P*-value > 0.05 was considered non-significant.

Results

Figs. 1–5 and Tables 1–5.

Discussion

Multidrug – resistant TB (MDR-TB) and the recently defined cases of extensively drug-resistant (XDR-TB) and even totally drug resistant TB constitute major health problems [13,14].

In patients with drug – resistance, an impaired Th_1 immune response was described [15,16]. However, the mechanisms of this deficiency remain elusive.

In our study, the frequency of T reg-Cells was increased in the blood of patients with multidrug – resistant pulmonary tuberculosis (MDR-PTB) as well as in patients who responded well to anti-tuberculosis treatment when compared to healthy control, tuberculin skin test-negative subjects with significant statistical difference ($P < 0.05$). These results are consistent with previous studies by He et al. [3] Chen et al. [17] and Semple et al. [18] who found that the levels of these cells were higher in patients with active pulmonary tuberculosis than in healthy control subjects. Pinheiro et al. [11] showed that the PBMC from patients with MDR-PTB had significantly higher percentage of $CD4^+$ $CD25^+$ $FoxP3^+$ and $CD4^+$ $CD25^+$ $CD127^-$ cells than in healthy control subjects. In the present study, we found that the level of $CD4^+$ $CD25^+$ $Fox P3^+$ was higher in responders to treatment than in patients with MDR-PTB with non-significant statistical difference ($P < 0.05$). These results are not in agreement with the study done by Pinheiro et al. [8], who found that the level of these cells was higher in patients MDR-TB. Our results could be a explained by the T reg – cell dynamics (or re-distribution) between the blood and the sites of infection/inflammation as suggested by Semple et al. [18], which is augmented overtime with persistence of the drug-resistant TB bacilli. These cells modulate the immune response to decrease tissue damage in cases of chronic infection like MDR-PTB.

Another explanation is the fact that we did not study other T reg-cell subsets like, $CD4^+$, $CD25^+$, $CD39^+$ [19], $CD4^+$ $CD25^+$ $CD127^-$ [11,20] and $CD8^+ CD45$ RA^+ $CCR7^+$ $FoxP3^+$ [21] which could have increased levels in the blood of patients with MDR-PTB. Our results are in agreement with the studies of Ribeiro et al. [22] and Semple et al. [18], which showed an increase in T reg – cell frequency in the disease-involved lung, compared to PBMCs of PTB patients.

Herzmunn et al. [5] found an increase in the level of T reg – cells in subjects with latent TB infection when compared to those with negative tuberculin skin testing. In our study all the healthy control subjects had negative testing.

The radiological extent of lesions with more extensive ones in the present study in either group of patients (groups I and II) showing significant positive correlation with the number of T reg – cells and this result is consistent with those of Pinheiro et al. [11] and Dheda et al. [23] On the other hand, the age, smoking index, the duration of associated DM showed non-significant correlation with the number of T reg – cells.

In summary, the study disclosed that the frequency of $CD4^+$, $CD25^+$, $FoxP3^+$, cells was higher in the blood of both patients with MDR-PTB and responders to treatment, compared to healthy subjects and there was non-significant statistical difference between both patients' groups regarding the level of these cells. It is concluded that, although immune suppression characteristic of T reg – cells seems important in the pathogenesis of MDR-PTB, other mechanisms, immunologic or non-immunologic are important, as well. More studies with larger number of patients are needed to study different T reg. cell subsets in both the blood and the lungs of patients with MDR-PTB.

Conflict of interest

None.

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