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

Sayed Labib a,*, Nermine El-Maraghi b, Soha Younes b, Lobna Metwally b, Omar Dessouki b, Nader Nemr c

a Chest, Int. Medicine Department, Suez Canal University, Egypt
b Microbiology and Clinical Pathology Departments, Suez Canal University, Egypt
c Infectious & Endemic Diseases Units, Int. Medicine Department, Suez Canal University, Egypt

Received 3 July 2013; accepted 22 July 2013
Available online 4 September 2013

KEYWORDS
Multidrug resistant pulmonary TB (MDR-PTB); Regulatory T-cells (T reg); CD4; CD25; FoxP3

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
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 (FoxP3) 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 mycobacterial 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.
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 P3 +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.
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 1** Comparison between three groups as regards sex.

| Sex   | Groups | G1 MDR |  | %     | G2 TR | No. | %     | G3 HC | No. | %     | Total | 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.

Fig. 5 significantly positive Correlation between radiological extent and T reg – cell subsets.

- 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.
 Pearson’s correlation coefficient ($r$) test was used for correlating data. • Probability ($P$-value) – $P$-value < 0.05 was considered significant.
of T reg–cells and this result is consistent with those of II) showing significant positive correlation with the number of T reg–cells. The age, smoking index, the duration of associated DM showed non-significant correlation with the number of T reg–cells. 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 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 Dhe da 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.

References

[1] Global Tuberculosis Control WHO, 2011.
[2] J. Wergeland, J. Abmus, A.M. Dyhrhol-Riise, T regulatory cells and immune activation in Mycobacterium tuberculosis infection and the effect of preventive therapy, Scand. J. Immunol. 73 (2011) 234–242.
[3] X.Y. He, L. Xiao, H.B. Chen, et al, T regulatory cells and Th1/Th2 cytokines in peripheral blood from tuberculosis patients, J. Clin. Microbiol. Infect. Dis. 29 (6) (2010 Jun) 643–650.
[4] A.S. De Almeida, C.T. Fiske, T.R. Sterling, et al, Increased frequency of regulatory T cells and T lymphocyte activation in persons with previously treated extra pulmonary tuberculosis, Clin. Vaccine Immunol. 19 (2) (2012 Jan) 45–52.
[5] C. Herzmann, M. Ernst, S. Ehlers, et al., Increased frequencies of pulmonary T reg – cells in latent M. tuberculosis infection, Eur. Respir. J., 2012 http://dx.doi.org/10.1183/09031936.00214611.
[6] Shangs, Gibbs S, Henao-Tamayo M. Increased virulence of an epidemic strain of Mycobacterium massiliense in mice. PLOS One 6 (2011) e24726 http://dx.doi.org/10.1371/Journal.Pone.0024726.
[7] V. Guyot-Revol, J.A. Innes, et al, Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis, Am. J. Respir. Crit. Care Med. 173 (2006) 803–810.
[8] P.R. Antas, E.P. Sampoio, Another round for the CD4+ CD25− regulatory T-cells in patients with tuberculosis, Am. J. Respir. Crit. Care Med. 176 (2007) 214–215.
[9] P.K. Sharma, P.K. Suha, A. Singh, et al, Fox P3+ regulatory T cells suppress effector T-Cell function at pathogenic site in miliary tuberculosis, Am. J. Respir. Crit. Care Med. 179 (2009) 1061–1070.
[10] L. Geffen, N. Yokobori, L.E. Basi, et al, Patients with multidrug-resistant tuberculosis display impaired Th1 response and enhanced regulatory T-cell levels in response to an outbreak of multidrug-resistant M. tuberculosis M and Ra strain, Infect. Immun. 77 (2009) 5025–5034.
[11] R.O. Pinheiro, E.B. de Oliveira, G. dos Santos, et al, Different immunosuppressive mechanisms in multidrug – resistant tuberculosis and non tuberculosis mycobacteria patients, Clin. Exp. Immunol. 171 (2013) 210–219.
[12] National Tuberculosis Association of the USA, Diagnostic standards and classification of tuberculosis, New York, National tuberculosis Association, 1961.
[13] J.A. Caminero, Multidrug-resistant tuberculosis epidemiology, risk factors and case finding, Int. J. Tuber.c Lung Dis. 4 (2010) 382–390.
Study of some T regulatory cell subsets in patients with multi-drug resistant pulmonary tuberculosis

[14] Multidrug and extensively drug-resistant TB (M, XDR-TB), Global report on surveillance and response, WHO Global Rep. 2010 (2010) 71c.

[15] E.K. Jo, J.K. Park, H.M. Dockrell, Dynamics of cytokine generation in patients with active pulmonary tuberculosis, Curr. Opin. Infect. Dis. 16 (3) (2003) 205–210.

[16] C. Lienhardt et al, Active tuberculosis in Africa is associated with reduced Th1 and increased Th2 activity in vivo, Eur. J. Immunol. 32 (6) (2002) 1605–1613.

[17] X. Chen, B. Zhou, M. Li, et al, CD4+ CD25+ Fox P3+ regulatory T-cells suppress mycobacterium tuberculosis immunity in patients with active disease, Clin. Immunol. 123 (2007) 50–59.

[18] P.L. Semple, A.B. Blinder, M. Davids, et al., Regulatory T-cells attenuate mycobacterial stasis in alveolar and blood-derived macrophages from patients with TB. Am. J. Respir. Crit. Care Med. 2013, as DOI. 10.1164/recen.201210–1934oc.

[19] T. Chiacchio, R. Casetti, O. Butera, et al, Characterization of regulatory T-cells indentified as CD4+ CD25high CD39+ in patients with active, Tuberculosis 156 (2009) 463–470.

[20] N. Yu, X. Li, W. Sony, et al., CD4+ CD25+ CD127 (low/−) T-cells: a more specific T reg population in human peripheral blood inflammation, 2012.

[21] M. Suzuk, A.L. Jugger, C. Konya, et al, CD8+ CD45 RA− CCR7− FoxP3+ T-cells with immunosuppressive properties: a novel subset of inducible human regulatory T-cells, Immunology 189 (5) (2012) 2118–2130.

[22] R. Ribeiro-Rodrigues et al, A role for CD4+ CD25+ T-cells in regulation of the immune response during human tuberculosis, Clin. Exp. Immunol. 144 (1) (2006) 25–34.

[23] K. Dheda, I.T. Booth, J.F. Huggett, et al., Lung remodeling in pulmonary tuberculosis. J. Infect. Dis. 192, 1201–1209.