

Original Article

## Immunophenotyping of circulating mononuclear cells in active pulmonary tuberculosis

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### Abstract

**Introduction:** Interpreting the interactions between *M. tuberculosis* and the host innate and adaptive immune defense mechanisms is mandatory for understanding the pathogenesis of active pulmonary TB (APTb). The aim was to describe the distribution of mononuclear cells in APTb and their relation to disease severity.

**Methodology:** A case-control study of peripheral blood CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, B-lymphocytes, NK cells, T regulatory lymphocytes (Tregs) and monocytes by flow cytometry. The patients had clinical presentations of APTb, positive tuberculin skin tests, acid-fast bacilli smears and sputum cultures using BACTEC 960.

**Results:** There was a significant decrease in the haemoglobin level and the absolute lymphocytic count ( $p < 0.01$ ), while both the neutrophil count and erythrocyte sedimentation rate showed significant increase in the APTb patients compared to HC with  $p$ -values  $< 0.001$  and  $< 0.0001$  respectively. Both the CD4<sup>+</sup>/CD8<sup>+</sup> ratio and the percentages of CD3<sup>+</sup>–CD19<sup>+</sup> cells were significantly lower in APTb patients ( $p = 0.03$  and  $p = 0.005$  respectively). The percentages of CD4<sup>+</sup>, CD8<sup>+</sup>, CD3<sup>+</sup>–CD19<sup>+</sup>, CD14<sup>+</sup>, and CD3<sup>+</sup>–CD (16+56)<sup>+</sup> cells showed no significant differences, when comparing either disease severity groups, or cavitated and non-cavitated groups of APTb patients. There was significant increase in the CD4<sup>+</sup>25<sup>+</sup> lymphocytes in the advanced APTb patients than in the mild disease group ( $p < 0.05$ ).

**Conclusions:** B-lymphocytes and CD4/CD8 ratios were significantly lower in the APTb patients than controls with no association with disease severity. CD4<sup>+</sup> CD25<sup>+</sup>hi Tregs were significantly higher in the advanced versus mild groups.

**Key words:** Active pulmonary tuberculosis; flow cytometry; lymphocytes subsets; monocytes.

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### Introduction

Tuberculosis (TB) is the prime cause of death from a curable infectious disease and an important cause of morbidity in the world. The burden of TB is still high worldwide. In 2016, 6.3 million new cases of TB were reported (World Health organization global tuberculosis report, 2017) [1]. The majority of immunocompetent people infected with *M. tuberculosis* develop a latent infection with no clinical symptoms [2]. In some cases, mycobacteria can successfully infect host tissues, but extracellular mycobacteria are cleared rapidly by macrophages. Pathogenic mycobacteria persist for an extended period inside host tissues, likely within infected phagosome of host macrophage. Intracellular growth and persistence explain the ability of mycobacteria to produce chronic diseases. Tuberculosis may be reactivated in the setting of malnutrition, aging or acquired immunodeficiency [3]. The mechanisms of protective immunity against TB

have not been fully elucidated. Cell-mediated immunity is of particular importance in preventing clinically evident disease following infection. CD4<sup>+</sup> T cells have an essential role in this response, but other T-cell subsets such as CD8<sup>+</sup> are also supportive [4]. B lymphocytes, together with other antigen-presenting cells express the co-stimulatory molecules necessary for activating T cells. Primarily, CD4<sup>+</sup> T cells are involved in augmenting immune responses by secreting various cytokines, but some CD4<sup>+</sup> T cells also act as cytotoxic effector cells capable of lysing target cells directly [5]. The aim of this study was to evaluate a panel of lymphocyte subsets by flow cytometry in APTb patients compared to healthy controls to observe the difference in their distribution in relation to disease severity and complications of infection to identify possible immunological markers.

**Methodology**

This is a case control study on 50 patients and 30 age and sex matched healthy controls. The APTB patients presented to Fayoum University Hospital and Fayoum Chest Hospital from Fayoum Governorate in Egypt. The research was carried out in accordance with the Declaration of Helsinki, 2013. Patients were diagnosed on the basis of presence of recent clinical symptoms of tuberculosis, a positive sputum smear test for acid-fast bacilli confirmed by a positive culture of *M tuberculosis* within 8-16 days by BACTEC MGIT 960 System, and a characteristic chest radiograph. A written consent to the experimental laboratory procedure was obtained. Patients with any underlying, inherited or acquired, immunological disorder were excluded. The healthy subjects had no abnormal X-ray findings or history of TB and no other underlying disease and had a negative tuberculin test. They were not concurrently taking any medication and consented to the experimental laboratory procedure. APTB patients were divided into three groups of severity based on the X-ray findings according to the National Tuberculosis Association USA (1961) [6].

*Group 1* (mild cases, *n* = 23). Involving a single lobe with no cavities.

*Group 2* (moderate cases, *n* = 18). Unilateral disease affecting two or more lobes with cavities, reaching a total diameter no more than 4 cm.

*Group 3* (advanced cases, *n* = 9). Bilateral disease with massive involvement and multiple cavities.

*Sputum specimen processing*

All steps were performed in biological safety cabinet level II. Morning sputum samples were collected under sterile conditions. Sodium hydroxide decontamination of the sputum sample was carried out by the previously described procedure [7-9]. The decontaminated pellet was resuspended with 1 mL PBS, and 500 µL were added to the MGIT tube previously inoculated with 800 µL of the PANTA & growth supplement solution (Becton- Dickenson, New Jersey, USA). MGIT tubes were then incubated into BACTEC

MGIT 960 System (Becton- Dickenson, New Jersey, USA). Positive MGIT tubes were removed from the instrument for preparation of smears and staining by Ziehl Neelson and Gram stains using biosafety level (BSL) II practices and containment facilities. Reporting of preliminary results was done after acid-fast smear evaluation. Acid fast bacilli (AFB) positive samples were subcultured on solid media and reported as: AFB-positive.

*Flow cytometry analysis*

Fresh EDTA peripheral blood samples for patients and controls were used for analysis. Absolute lymphocyte counts (ALCs) were analyzed using Sysmex automated cell counter XS-800i (Sysmex corporation , Kobe , Japan). Lymphocyte subsets analysis was performed by flow cytometry using a Coulter EPICS XL-MCL (Beckman Coulter, Fullerton, Cal, USA) and fluorescently labeled monoclonal antibodies with an isotope control from Beckman Coulter. Each lymphocyte subpopulation count was expressed as a percentage of the total of CD3<sup>+</sup> lymphocytes. For the detection of lymphocytes by flow cytometry, an initial region gate was set at the region of lymphocytes for detection of the surface markers: CD4 FITC/CD8 PE, CD3 FITC/CD19 PE, CD3 FITC/CD (16+56) PE and CD4 PE/CD25 FITC. Gating was set at the region of the monocytes for the surface marker CD14 Per Cp.

*Statistical methods*

Data were statistically described in terms of mean ± standard deviation (± SD), median and range, or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using Mann Whitney *U* test for independent samples when comparing 2 groups and Kruskal Wallis H test (one-way ANOVA) when comparing more than 2 groups. *p* values less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs SPSS (Statistical Package for the

**Table 1.** Haematological parameters of patients and controls.

Parameter	TB patients mean ± SD	Controls mean ± SD	p value
Hb (g/dL)	Male	12.4 ± 2.1	0.001
	Female	11.2 ± 1.8	0.011
TLC ×10 <sup>9</sup> /L	8.0 ± 4.3	7.5 ± 2.1	1.02
Neutrophils %	65.8 ± 9.2	57.1 ± 6.9	0.001
Lymphocytes %	23.6 ± 9.0	31.9 ± 2.1	0.01
Monocytes %	9.8 ± 4.2	8.7 ± 2.2	1.05
ESR mm/1 <sup>st</sup> hour	66 ± 29.6	5 ± 3.8	0.0001

**Table 2.** Comparison between the mean absolute lymphocytes count, mean percentages of lymphocytes subpopulations and monocytes in the APTB and HC groups.

Parameters	APTB (n = 50)	HC (n = 30)	p value
ALC (cell/mm <sup>3</sup> )	1,950 ± 327	2,208 ± 629	0.08
CD3 <sup>+</sup> (%)	66.23 ± 8.53	62.38 ± 19.45	0.96
CD3 <sup>+</sup> CD4 <sup>+</sup> (%)	37.7 ± 12.9	38.6 ± 11.3	0.83
CD3 <sup>+</sup> CD8 <sup>+</sup> (%)	28.01 ± 6.9	23.34 ± 6.7	0.53
CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio	1.46 ± 0.47	1.66 ± 0.28	0.03
CD3 <sup>-</sup> CD19 <sup>+</sup> (%)	7.46 ± 5.4	12.1 ± 4.71	0.005
CD3 <sup>-</sup> CD (16+56) <sup>+</sup> (%)	7.65 ± 5.44	6.36 ± 4.93	0.52
CD14 <sup>+</sup> (%)	0.61 ± 0.51	0.78 ± 0.92	0.52
CD4 <sup>+</sup> CD25 <sup>+</sup> (%)	4.17 ± 2.81	2.87 ± 1.89	0.67

APTB: Active pulmonary tuberculosis; HC: Healthy Controls.

Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

**Results**

The APTB patients were 35 males and 15 females with age range 15- 65 years and mean ± SD (40 ± 25). Both age and gender showed no significant difference between patients and controls  $p > 0.05$ .

The mean neutrophil counts and ESR results were significantly higher in patients than controls ( $p = 0.001$ ) and ( $p = 0.0001$ ) respectively while lymphocyte counts were significantly lower in the APTB group (0.01) (Table 1).

The means ± SD of the absolute lymphocytes counts (ALC), the percentages of T-cell subsets (T helper cells and T cytotoxic cells), B cells, NK cells, CD4<sup>+</sup>CD25<sup>+</sup> (T regulatory cells) and CD14<sup>+</sup> (monocytes) were compared between patients and controls and showed a significantly lower CD4<sup>+</sup>/CD8<sup>+</sup> ( $p = 0.03$ ) ratios and CD3<sup>-</sup>CD19<sup>+</sup> ( $p = 0.005$ ) in the patient group (Table 2).

*Correlation with severity and complications*

The absolute lymphocytic count was lowest in the APTB group with advanced disease severity, followed by the group with moderate severity and highest in the group with mild severity but these differences were not

statistically significant. The CD4<sup>+</sup>CD25<sup>+</sup> cells (T regulatory lymphocytes) percentages were highest in the APTB group with advanced disease severity and lowest in the group with mild severity ( $p < 0.05$ ). No significant difference was found in CD3<sup>+</sup> (total T lymphocytes), T lymphocyte subsets (CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>), CD3<sup>-</sup>CD19<sup>+</sup> cells (B lymphocytes), CD3<sup>-</sup>CD (16+56)<sup>+</sup> cells (natural killer cells) and CD14<sup>+</sup> (monocytes) percentages in the three groups (Table 3).

The patients were once more grouped into non-cavitated (group I 23) and cavitated (group II and III = 27) and statistically re-analyzed, however no statistically significant difference was found in any of the lymphocyte subsets (Table 4).

**Discussion**

The significantly higher neutrophil percentage in APTB patients compared to HC ( $p$ - value: 0.001), confirm the earlier reports [10,11]. This finding suggests the important role of neutrophils in tuberculosis infection as they have been shown to be the prevalent infected phagocytic cells in the airways of patients with active pulmonary TB. They drive the activation [12] and regulation of both the innate and adaptive immune responses [13,14]. A study by Pokkali *et al.* showed that neutrophils infiltrate the inflammatory sites and phagocytose mycobacteria

**Table 3.** The mean absolute lymphocyte counts, mean percentages of lymphocytes subpopulations and monocytes in the different groups of severity disease.

Parameter	Advanced	Moderate	Mild	P-value
ALC (cell/mm <sup>3</sup> )	1,300 ± 224	1,609 ± 454	2,620 ± 187	0.63
CD3 <sup>+</sup> (%)	62.10 ± 9.48	68.20 ± 2.84	67.80 ± 9.38	0.573
CD3 <sup>+</sup> CD4 <sup>+</sup> (%)	49.0 ± 13.29	36.18 ± 14.9	41.84 ± 11.34	0.733
CD3 <sup>+</sup> CD8 <sup>+</sup> (%)	23.75 ± 1.77	27.45 ± 11.09	28.44 ± 5.91	0.622
CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio	1.78 ± 0.73	1.35 ± 0.21	1.51 ± 0.57	0.649
CD3 <sup>-</sup> CD19 <sup>+</sup> (%)	10.6 ± 2.1	3.9 ± 3.6	9.9 ± 6.3	0.058
CD3 <sup>-</sup> CD (16+56) <sup>+</sup> (%)	14.95 ± 5.3	5.4 ± 5.6	9 ± 3.29	0.203
CD14 <sup>+</sup> (%)	0.18 ± 0.35	1.55 ± 0.6	0.35 ± 0.2	0.766
CD4 <sup>+</sup> CD25 <sup>+</sup> (%)	5.87 ± 2.3	5.3 ± 1.6	3.3 ± 1.8	0.766

thereby inhibiting the bacillary spread initially until the accumulated macrophages get activated and that neutrophils are very competent in amplifying the innate immune response and conferring protection at the early phase of infection [15].

The significantly lower absolute lymphocyte counts in the APTB patients confirm the association between active infection and lymphopenia [16,17]. This lymphopenia was found in a previous report to be transient and was corrected after treatment [18]. The erythrocyte sedimentation rate (ESR) was significantly higher due to the presence of acute phase reactants in the APTB patients. However, Ukpe *et al.* stated that active TB is associated mostly with higher ESR values than our study ( $\geq 100$  mm/h) [19].

The significant hematological abnormalities observed in APTB patients suggest that the differential diagnosis of tuberculosis should be considered in patients with these abnormalities and to use these hematological tests to monitor response to treatment.

The CD4<sup>+</sup>/CD8<sup>+</sup> ratio was significantly lower in APTB patients ( $p = 0.03$ ). The diminished CD4/CD8 ratio in this study is due to the increase in the CD8<sup>+</sup> T cell population rather than the depletion of the CD4<sup>+</sup> T cells compartment. This is evident from the the CD4<sup>+</sup> and CD8<sup>+</sup> percentages, ( $37.7 \pm 12.9$ ) and ( $28.01 \pm 6.7$ ) respectively, in the patients compared to the healthy controls, ( $38.6 \pm 11.3$ ) and ( $23.34 \pm 6.7$ ) respectively. Although the role of T-helper lymphocytes is predominantly recognized to limit progression of *M. Tuberculosis* infection, the CD8<sup>+</sup> T cells have been demonstrated to support other innate immunity compartments in the defense against this intracellular pathogen. TB specific CD8<sup>+</sup> T cells, can recognize infected cells, produce cytokines such as TNF and IFN  $\gamma$ , lyse infected cells and kill *M. tuberculosis*. CD8<sup>+</sup> cells are class I restricted, and to recognize other cells require antigen presentation via endogenous antigen processing pathways. Therefore *M. tuberculosis*

specific CD8<sup>+</sup> cells recognize infected cells while CD4<sup>+</sup> cells recognize infected cells, or cells that have phagocytosed dead bacteria and their antigens via class II MHC presentation [20].

The main finding in this study was that the CD3<sup>-</sup>CD19<sup>+</sup> cells (the B lymphocytes) were significantly lower in our APTB patients ( $p = 0.005$ ). Our results agreed with Winthrop *et al.* and Gea-Banacloche who reported the occurrence of mycobacterial infections upon rituximab-mediated depletion of B-cells [21,22]. In contrast, Wu *et al.* reported that CD19<sup>+</sup> were significantly increased compared with healthy controls [23]. However, the contribution of humoral immunity, including antibodies and specific functions of B cells, to *M. tuberculosis* infection in humans remains largely unknown [24]. B-cells are antigen presenting cells with various inflammatory and immune regulatory functions. In tuberculosis, they contribute to the granulomatous reaction in both humans and mice [25]. A recent study on an animal model, revealed that anti CD20 induced B-cell depleted granulomas, had no impact on disease progression or clinical outcome. It rather resulted in granuloma immunomodulation, with altered local T cell and cytokine responses, increased bacterial burden, and lower levels of inflammation [26]. Therefore, other studies need to be carried out to establish cause or effect in case of reduced B cell counts in APTB patients. B cells could be consumed in the granulomatous reactions rather than circulating which may also explain the lowered counts in this study. On the other hand, the depleted B-cells may remove the protection provided by the humoral immune compartment against TB.

Regulatory T cells (Tregs) initially described as CD4<sup>+</sup> CD25<sup>+</sup> cells, now more widely as CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> cells, represent 5-10% of the circulating T cell population with only 1-2% being CD4<sup>+</sup>CD25<sup>+</sup>hi. They inhibit autoimmunity and have an anti-inflammatory function that reduces tissue injury but delays bacterial eradication resulting in a lowered recovery rate [27].

**Table 4.** Comparison between mean absolute lymphocytes count, mean percentages of lymphocytes subpopulations and monocytes in the cavitated and non-cavitated patient groups.

Parameter	Cavitated	Non cavitated	P value
ALC (cell/mm <sup>3</sup> )	1,531 ± 709	2,620 ± 1,984	0.452
CD3 <sup>+</sup> (%)	65.76 ± 6.13	67.8 ± 9.38	0.530
CD3 <sup>+</sup> CD4 <sup>+</sup> (%)	40.45 ± 14.58	41.84 ± 11.34	0.714
CD3 <sup>+</sup> CD8 <sup>+</sup> (%)	26.22 ± 8.83	28.44 ± 5.91	0.855
CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio	1.49 ± 0.49	1.51 ± 0.57	0.906
CD3 <sup>-</sup> CD19 <sup>+</sup> (%)	6.16 ± 4.90	10.60 ± 6.61	0.195
CD3 <sup>-</sup> CD (16+56) <sup>+</sup> (%)	7.78 ± 6.82	9.00 ± 3.29	0.660
CD14 <sup>+</sup> (%)	1.21 ± 1.44	0.35 ± 0.20	1.00
CD4 <sup>+</sup> CD25 <sup>+</sup> (%)	5.60 ± 1.95	3.3 ± 1.8	0.480

Our results showed a non-significant increase of CD4<sup>+</sup>CD25<sup>+</sup>hi T cell subset in the APTB group compared to healthy controls. There was also no significant difference either between the disease severity groups or among the cavitated and non-cavitated groups except that they were significantly higher in the advanced group when compared with the mild disease group ( $p < 0.05$ ). This confirms expansion and activation of Tregs in APTB. Other studies reported higher frequencies of Tregs obtained in APTB with much higher recovery using CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>CD127<sup>+</sup> low markers. Also introducing CD45RO<sup>+</sup>Ki-67<sup>+</sup> to the previous Tregs markers delineates active primed Tregs which were found to be significantly increased in TB patients compared to latent TB and healthy controls [27]. Singh *et al.* observed a tight relation between the bacillary load and Treg cells frequency in the peripheral blood of APTB patients and stated that high pathogen load generates Treg cells both by inducing inflammation as well as directly, thereby suppressing the host effector immune response. They also reported tight correlation between T regs and disease severity and decline after successful therapy [28]. Sharma *et al.* also observed that activated T cells (CD4<sup>+</sup>CD25<sup>+</sup>) were overrepresented in APTB patients with tubercular pleural effusion and patients with Miliary TB [29]. In *M. tuberculosis* infection there is activation and expansion of regulatory T-cell (Treg) populations [30]. T regulatory cells have multiple inhibitory effects. They can directly suppress CD4<sup>+</sup> cell activity or induce the expression of IL-10 and TGF  $\beta$  (inhibitory cytokines) [31] and thus prevent the eradication of tubercle bacilli [32]. However, failure in Treg cells decline during therapy was tightly associated with multidrug resistances (MDR) tuberculosis. Abrogating Treg cells could significantly rescue the MTB specific Th-1 response. In an experimental study by Scott-Brown *et al.*, T reg cells were depleted in mice before aerosol infection with MTB, they observed 1 log less of colony-forming units of MTB in the lungs of the infected mice suggesting an immunotherapeutic potential of T reg cells [32]. Previous data has demonstrated that attenuation of Treg cells along with BCG vaccination has a positive impact on the protective immunity against tuberculosis. Therefore, while designing a vaccine for boosting the immunity against MTB repairing of the immune deficit by inhibiting the Treg cells may be critical in rescuing protective immunity [33].

## Conclusion

The main finding in this study is the statistically significant reduction in CD3-CD19<sup>+</sup> cells in the APTB patients compared to control with no association with disease severity. More studies are still required to elucidate cause or effect of B cell reduction in APTB patients. Also CD4/CD8 ratio was significantly decreased but with no association with disease severity. CD4<sup>+</sup> CD25<sup>+</sup>hi T regs were significantly higher in the advanced group compared to the mild disease APTB patients so they can be a marker for disease severity and can be used for monitoring anti-tuberculous drugs. T cell subset immunophenotyping in APTB patients to study the active primed T regulatory cells, can describe more clearly the role of T regs in APTB patients' severity.

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