

## Correlation of serum tumor necrosis factor- $\alpha$ , interleukin-4 and soluble interleukin-2 receptor levels with radiologic and clinical manifestations in active pulmonary tuberculosis

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THE precise clinical manifestations of tuberculosis are likely to result from a complex interaction between the host and the pathogen. We took serum samples from a group of patients with a variety of clinical and radiological stages of pulmonary tuberculosis in order to characterize tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-4 (IL-4) and soluble interleukin-2 receptor (sIL-2R) response. We further evaluated whether the levels of TNF- $\alpha$ , IL-4 and soluble IL-2R are related with each other, and also evaluated the levels of TNF- $\alpha$ , IL-4 and sIL-2R after anti-tuberculosis therapy and relation with radiologic scores. Forty-three inpatients with active pulmonary tuberculosis and 19 healthy controls participated in the study. Patients were divided into four categories radiologically on chest X-ray (minimal, moderate-advanced, far-advanced and with miliary infiltration). Concentrations of TNF- $\alpha$  ( $20.9 \pm 10/15.4 \pm 8$  pg/ml) and sIL-2R ( $2569 \pm 842/1444 \pm 514$  pg/ml) were statistically different between patients and controls ( $p = 0.02$  and  $p = 0.0001$ , respectively). Before chemotherapy there was a positive correlation between TNF- $\alpha$  and sIL-2R ( $r = 0.34$ ), but there was no correlation between IL-4 and TNF- $\alpha$ , and between IL-4 and sIL-2R ( $r = -0.23$  and  $r = -0.22$ ). The TNF- $\alpha$  level was not statistically different in four groups before and after chemotherapy. Results of this study provided some evidence confirming the previously reported role of TNF- $\alpha$ , IL-4 and sIL 2R in the control of tuberculosis, but these cytokines were not found related with disease severity.

**Key words:** Tuberculosis, Radiologic manifestation Cytokine

### Introduction

Cytokines are primarily involved in host responses to disease or infection and any involvement with homeostatic mechanism has been less than dramatic. Many cytokines are produced during tuberculosis (TB),<sup>1,2</sup> with a predominance of Th1 cytokines during the early stage<sup>3,4</sup> and Th2 cytokines in the later stages of the infection.<sup>5</sup> These cytokines exert important roles to limit or exacerbate the disease depending on their balance and combinations. An understanding of the basis of these associations and correlations during TB could be useful in elucidating protection/pathogenesis.

Some cytokines promote inflammation and are called proinflammatory cytokines [tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1, IL-6, IL-8], whereas other cytokines suppress the activity of proinflammatory cytokines and are called anti-inflammatory cytokines (IL-4, IL-10, IL-13).<sup>6</sup> TNF- $\alpha$  has harmful effects, such as acute-phase pathophysiological events including fever and tissue necrosis. It also plays a protective role against mycobacterial infec-

tion.<sup>7,8</sup> Soluble interleukin 2 receptor (sIL-2R) is a surrogate marker of T-lymphocyte activation and proliferation. A soluble fraction of the IL-2 receptor, released from the cell membrane, is detectable in serum and its concentration is known to be elevated in TB.<sup>9</sup>

In the literature, most reports on cytokines during TB are from studies on *in vitro*-stimulated lymphoid cells with few reports on *in vivo* plasma levels. We consider immunity of an individual to TB to sometimes be reflected in the plasma levels of some cytokines. In addition, the plasma is easily accessible, thus requiring simple procedures and equipment to process it. In the present study we therefore examined the levels of TNF- $\alpha$ , IL-4 and sIL-2R in the serum of pulmonary TB patients. To understand systemic T-cell response in pulmonary tuberculosis to some degree, we analyzed TNF- $\alpha$ , IL-4 and sIL-2R in serum. Also, to evaluate the hypothesis that different clinical and radiological manifestations of active pulmonary tuberculosis are associated with different patterns of cellular immune response systemically, we took serum samples from a group of patients with a

variety of clinical and radiological stages of pulmonary tuberculosis. We further evaluated whether the levels of TNF- $\alpha$ , IL-4 and sIL-2R are related with each other and also evaluated the levels of TNF $\alpha$ , IL-4 and sIL-2R before and after anti-TB therapy.

## Materials and methods

### Subjects

Forty-three inpatients (20 male and 23 female) with active pulmonary TB and 19 healthy controls (eight male and 11 female) participated in the study. All patients were recruited from the Tuberculosis Hospital and Erciyes University Medical Faculty, Clinic of Pulmonology in Kayseri, which is located in the central region of Turkey. Their mean age was  $36.4 \pm 15.5$  years (range 16–67 years). On entry, all patients had positive smear for acid-fast bacilli in sputum or bronchial lavage and subsequent cultures of these specimens yielded tubercle bacilli. None of the patients had any evidence of concomitant bacterial or viral infections as indicated by sputum and blood cultures and viral serologic study including HIV. Five patients had diabetes mellitus. All patients were administered anti-TB therapy in which isoniazid, rifampicin, pyrazinamide and streptomycin or ethambutol were used.

We evaluated patients with physical examinations, chest radiographs and routine laboratory tests. All patients had pulmonary TB symptoms such as cough, fever, and hemoptysis. To assess the presence, form, spread and size of the TB cavities and infiltrations in the lungs, all 43 patients received plain postero-anterior and lateral chest radiographs. To avoid observer bias, three pulmonary physicians initially assessed the radiographs independently before the laboratory studies. Patients were divided radiologically into four categories; minimal, moderate-advanced, far-advanced, and with miliary infiltration. In minimal TB, minimal lesions include those of slight to moderate density but that do not contain demonstrable cavitations. They may involve a small part of one or both lungs, but the total extent, regardless of distribution, should not exceed the volume of the lung on one side that occupies the space above the second condrosteral junction and the spine of the fourth thoracic vertebra or the body of the fifth thoracic vertebra. Moderate-advanced TB lesions include disseminated lesions of slight to moderate density that may extend throughout the total volume of one lung or the equivalent in both lungs; dense or confluent lesions limited in extent to one-third of the volume of one lung; and total diameter of cavitations, if present, must be less than 4 cm. In far-advanced TB, total cavity diameters are more than 4 cm and

lesions are more extensive than moderately advanced.<sup>10,11</sup>

Additionally, appetite and weakness were asked and assessed as present or not. Weight loss was measured as a difference between weight of patients before disease and on the time of applying to our clinic. Body mass index, erythrocyte sedimentation rate, and body temperature were measured initially and after chemotherapy. Body mass index was measured as body weight (kg)/square of height ( $m^2$ ). Tuberculin skin tests with PPD (purified protein derivative) were conducted in 13 patients, reactions were calculated 72 h after by the same technician (mean  $14.6 \pm 6.6$  mm). A control group of 19 healthy volunteer subjects (eight women and 11 men) with a mean age of  $34.9 \pm 12.7$  years (range 22–65 years) was also studied. We also evaluated normal volunteers with chest radiographs, routine laboratory tests and physical examinations. None of the control groups had any evidence of infections and systemic disorders. All patients and volunteers gave informed consent and the Erciyes University Medical Faculty, Department of Pulmonology, approved the protocol.

### Immunoassays

All serum samples were determined from the patients and the control group of peripheral venous blood samples prior to the initiation and the sixth month of anti-TB chemotherapy, and samples were preserved at  $-70^\circ\text{C}$ . All samples were assigned code numbers and processed by an investigator. For the assay, frozen serum samples were transferred by cold chain rules (with  $\text{CO}_2$  ice) to the Zonguldak Karaelmas University Medical Faculty Laboratory of Immunology on the same day. All measurement was performed in 24 h. IL-4, sIL-2R and TNF- $\alpha$  enzyme-linked immunosorbent assay (ELISA) kits were purchased by Biosource International Inc. (Camarillo, California, USA) and used according to the recommendations of the manufacturer. The minimum detectable dose of TNF- $\alpha$  is 1.7 pg/ml, of sIL-2R is 12 pg/ml and that of IL-4 is 0.1 pg/ml, and there is no cross-reactivity with other cytokines. All samples were assayed in duplicate. These parameters were measured in patients after 6 months of anti-TB treatment.

### Statistical analysis

The Kolmogorov–Smirnov test was used for confirmation of parameters. All figures are presented as mean  $\pm$  SD unless otherwise stated. Non-parametric Mann–Witney U, Wilcoxon and Kruskal–Wallis tests were used for comparisons between the groups of subjects. Spearman rank correlation was used to examine the relationship between the expression individual cytokines and clinical parameters. All  $p < 0.05$  were considered significant.

**Table 1.** Demographics and clinical characteristics of TB patients

Total patients	43
Sex	20 men; 23 women
Age, mean $\pm$ SD	36 $\pm$ 15 (range 16–67)
Chest X-ray	
Minimal (%)	9 (20.9%)
Moderate-advanced (%)	19 (44.2%)
Far-advanced (%)	11 (25.6%)
Milier (%)	4 (9.3%)
Cavity (%)	12(27.9%)
Symptoms and findings	
Weight loss	28 (65.1%)
Weight loss, mean $\pm$ SD (kg)	3.24 $\pm$ 3
Boy mass index $\pm$ SD (kg/m <sup>2</sup> )	19.6 $\pm$ 3
Appetite	25 (58.1%)
Weakness	23 (53.4%)
Fever > 37°C	25 (58.1%)
Body temperature, mean $\pm$ SD (°C)	37.9 $\pm$ 0.9

## Results

### Patient demographics

Demographic data of the 43 patients recruited from Erciyes University and Tuberculosis Hospital are presented in Table 1.

### Immunoassay findings

We studied the total amount of IL-4, sIL-2R and TNF- $\alpha$  in serum. The mean concentration of IL-4, TNF- $\alpha$  and sIL-2R levels are presented in Table 2. Concentrations of TNF- $\alpha$  and sIL-2R were statistically different ( $p = 0.02$  and  $p = 0.0001$ , respectively), but IL-4 levels were not significantly different between controls and patients ( $p = 0.40$ ) (Table 2). Before chemotherapy there was a positive correlation between TNF- $\alpha$  and sIL-2R ( $r = 0.34$ ,  $p = 0.02$ ), but there was no correlation between IL-4 and TNF- $\alpha$ , and between IL-4 and sIL-2R ( $r = -0.23$  and  $r = -0.22$ ). After chemotherapy, IL-4, TNF- $\alpha$  and sIL-2R levels were not correlated with each other ( $r = 0.16$ ,  $r = 0.10$ , and  $r = -0.01$ ). Also, TNF- $\alpha$ , IL-4 and sIL-2R levels were not correlated with the PPD reaction in 13 tuberculosis patients.

**Table 2.** Age, weight, body mass index (BMI), TNF- $\alpha$  and s IL 2R in controls and patients before and after chemotherapy

Parameter	Case X $\pm$ SD (n = 43)	Case $\pm$ SD* (n = 43)	Control $\pm$ SD (n = 19)	$p_1$	$p_2$
Age	36 $\pm$ 15	–	34 $\pm$ 12	0.95	–
BMI	19 $\pm$ 2	23 $\pm$ 3	24 $\pm$ 3	0.00	> 0.05
Weight	57 $\pm$ 8	66 $\pm$ 11	68 $\pm$ 12	0.01	> 0.05
TNF- $\alpha$ (pg/ml)	21 $\pm$ 10	22 $\pm$ 26	15 $\pm$ 8	0.02	0.26
sIL-2R (pg/ml)	2569 $\pm$ 842	1457 $\pm$ 457	1445 $\pm$ 515	0.00	0.81
IL-4 (pg/ml)	0.5 $\pm$ 1	0.5 $\pm$ 0.6	0.3 $\pm$ 0.3	0.40	0.15

Data presented as mean  $\pm$ SD.  $p_1$ , Comparison of case and control groups before chemotherapy;  $p_2$ , comparison of case and control groups after chemotherapy.

\*Levels of cytokine after anti-tuberculosis chemotherapy.

The concentrations of TNF- $\alpha$ , IL-4 and sIL-2R are presented in Table 3 according to chest X-ray scores in patients. To analyze the differences between cytokine concentrations of the affected lungs, the Wilcoxon rank-paired test was employed, as well as for the X-ray scores. Patients with TB showed correlation between sIL-2R and TNF- $\alpha$  levels according to chest X-ray lesions in moderate-advanced ( $r = 0.33$ ), far-advanced ( $r = 0.27$ ) and miliary ( $r = 0.91$ ) TB. Nevertheless, IL-4 and sIL-2R levels were not correlated in minimal, moderate-advanced and far-advanced cases. In minimal and far advanced cases, IL-4 and TNF- $\alpha$  levels showed negative correlation ( $r = -0.57$  and  $r = -0.34$ ). There is no correlation between the concentrations of IL-4, sIL-2R and TNF- $\alpha$  in moderate-advanced and miliary TB. After chemotherapy, the concentration of sIL-2R and TNF- $\alpha$  levels was correlated in far-advanced cases ( $r = 0.32$ ). TNF- $\alpha$ , IL-4 and sIL-2R levels of four groups were not different statistically before and after chemotherapy as well ( $p > 0.05$ ). In four groups of patients there were statistically differences for serum sIL-2R levels before and after chemotherapy, but TNF- $\alpha$  and IL-4 levels were not statistically different before and after chemotherapy. In patients with TB, there was no correlation for TNF- $\alpha$ , IL-4 and sIL-2R levels with body mass index, age, weight loss, fever and erythrocyte sedimentation rate ( $p > 0.05$ ). There was no statistically difference between widespread of lesions and age ( $p = 0.11$ ). Considering TNF- $\alpha$ , IL-4 and sIL-2R levels, there were no statistical differences between male and female, with and without cavity, diabetic and non-diabetic, with and without sequelae ( $p > 0.05$ ) (Table 4).

## Discussion

The pleiotropic cytokine TNF- $\alpha$  has been shown to be associated with both protection and pathogenesis in mycobacterial infections.<sup>12</sup> In mice experimentally infected with *Micobacterium bovis* bacillus Calmette-Guérin (BCG), *in vivo* administration of anti-TNF- $\alpha$  antibodies inhibited the development of granulomas in host organs resulting in the dissemination of the

**Table 3.** Concentrations of TNF- $\alpha$ , SIL-2R and IL-4 according to chest X-ray findings before and after chemotherapy

	TNF- $\alpha$ (pg/ml)		SIL-2R (pg/ml)		IL-4 (pg/ml)		p
	p		p		p		
	Before chemotherapy	After chemotherapy	Before chemotherapy	After chemotherapy	Before chemotherapy	After chemotherapy	
Total (n = 43)	21 $\pm$ 10	22 $\pm$ 27	2570 $\pm$ 842	1457 $\pm$ 457	0.5 $\pm$ 1	0.5 $\pm$ 0.6	0.86
Minimal (n = 9)	21 $\pm$ 8	19 $\pm$ 10	2475 $\pm$ 1063	1329 $\pm$ 395	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.34
Moderate-advanced (n = 19)	19 $\pm$ 8	28 $\pm$ 39	2446 $\pm$ 639	1587 $\pm$ 402	0.3 $\pm$ 0.5	0.7 $\pm$ 0.7	0.10
Far-advanced (n = 11)	23 $\pm$ 15	16 $\pm$ 6	2635 $\pm$ 705	1282 $\pm$ 424	1.2 $\pm$ 2.0	0.6 $\pm$ 0.7	0.39
Miliary (n = 4)	23 $\pm$ 9	22 $\pm$ 10	3185 $\pm$ 1465	1603 $\pm$ 800	0.1 $\pm$ 0.0	0.5 $\pm$ 0.7	0.39
p*	0.76	0.79	0.68	0.32	0.28	0.08	

Data presented as mean  $\pm$  SD.

\*Comparison between four groups of cytokine levels.

bacilli.<sup>13,14</sup> An increased production of TNF- $\alpha$  was reported following stimulation of monocytes by lipopolysaccharides or BCG in newly diagnosed TB patients versus monocytes from chronic cases.<sup>15</sup> Our findings agree with the aforementioned reports that elevated amounts of TNF- $\alpha$  are detectable during the early stages of TB, and extended it to plasma after chemotherapy of pulmonary TB patients.

Virulent and avirulent *Micobacterium avium* strains were shown to induce TNF- $\alpha$  in murine macrophages, but the virulent strain induced TNF- $\alpha$  later than the avirulent one.<sup>16</sup> These studies suggest that TNF- $\alpha$  may be required early to limit mycobacterial multiplication. It is therefore apparent that the rapid induction of TNF- $\alpha$  is a crucial factor in the elimination of TB in the host. However, the level of TNF- $\alpha$  in our study was variable from patient to patient. The variation could be owing to many factors including host and bacterial factors, but not disease severity and other accompanied diseases such as diabetes mellitus (Table 4).

Previous reports have suggested that an association between TNF- $\alpha$  with pathology in TB accompanied by fever, weight loss, shock and tissue necrosis.<sup>17,18</sup> Human infection with *Micobacterium tuberculosis* can lead to a range of clinical outcomes, from asymptomatic lifelong infection to progressive primary TB to what has been traditionally regarded as typical reactivation disease with pulmonary infiltrates and often parenchymal lung destruction.<sup>19</sup> Cellular immune response phenotypic CD4<sup>+</sup> T-cell differences seem to be related to different clinical presentations of illness. A preponderance of CD4<sup>+</sup> T cells at the site of infection is associated with recovery following anti-TB therapy.<sup>20</sup>

The macrophage-activating molecule, TNF, participates in the development of microbicidal granulomas<sup>13</sup> and may be critical to the immune response directed at *M. tuberculosis in situ*.<sup>21</sup> The activation and recruitment of cells into an area of inflammation is a crucial step in the development of delayed-type hypersensitivity (DTH) responses. DTH is immunologically a process similar to cell-mediated immunity, involving T cells and cytokines. However, DTH leads to pathologic responses, such as granulomatous inflammation, calcification, caseation necrosis, and cavity formation. Granulomas usually form as a result of the persistence of a non-degradable product or as the result of DTH responses.<sup>22</sup> The formation of tuberculous granulomas in humans is associated with the expression of characteristic cytokine profiles and indicates that the expression of certain cytokines is associated with the development of specific pathologic features resulting in granulomas.<sup>23</sup> In our study, TNF- $\alpha$  levels were no different before and after chemotherapy as high level. This indicates that TNF- $\alpha$  may play a significant role in DTH and granulomatous reaction in TB. However, TNF- $\alpha$

**Table 4.** Concentrations of TNF- $\alpha$ , sIL-2R and IL-4 according to gender and clinical conditions before and after chemotherapy

	N	TNF- $\alpha$ (pg/ml)		sIL-2R (pg/ml)		IL-4 (pg/ml)	
		Before chemotherapy	After chemotherapy	Before chemotherapy	After chemotherapy	Before chemotherapy	After chemotherapy
Male/female	20/23	19/22	29/16	2540/2594	1498/1421	0.4/0.6	0.7/0.4
Sequela, +/-	26/17	21/21	19/27	2743/2305	1467/1442	0.6/0.4	0.4/0.9
Cavitary, +/-	12/31	20/21	14/25	2410/2631	1391/1483	1.2/0.2*	0.9/0.4
Diabetes, +/-	5/38	18/21	16/23	2945/2520	1540/1446	0.1/0.5*	0.5/0.5

\* $p < 0.05$ .

levels were not correlated to sequela and cavity formation in our cases.

Th1-type cells secrete high levels of IL-2, TNF- $\alpha$  and interferon- $\gamma$ . This activates macrophages and promotes cell-mediated immune responses against invasive intracellular pathogens. IL-2 receptor may exist on the cell membrane as a signal transducing unit or in a soluble form in the extra cellular fluid. Similar to the literature, our results showed that sIL-2R levels were higher in TB patients in the early stages of disease with correlating TNF- $\alpha$  levels. sIL-2R levels were statistically reduced in four groups after chemotherapy independently of TNF- $\alpha$  level. In a recent study, TNF- $\alpha$  was readily detected in sputum from TB patients at baseline and responded to anti-TB therapy.<sup>24</sup> The elevated TNF- $\alpha$  level even after chemotherapy may be due to the systemic response being different to the local response.

In TB patients, clinical and radiological manifestation varieties may be related to anti-inflammatory cytokine response variability rather than proinflammatory cytokines. In active pulmonary TB, certain cytokines have been postulated to relate to cavity formation, although the detailed mechanism of cavity formation is not yet known. Abe and coworkers showed that increased serum vascular endothelial growth factor levels subdue cavity formation in active pulmonary TB. In their study, serum levels of IL-8 and TNF- $\alpha$  were not related to cavity formation.<sup>25</sup> To parallel this study, we observed that sIL-2R, IL-4 and TNF- $\alpha$  levels were not significantly different between groups with and without cavities.

Cytokines such as IL-1 $\beta$ , IL-6, IL-8, IL-12, interferon- $\gamma$  and TNF- $\alpha$  have been detected at higher concentrations in more affected lungs,<sup>26</sup> suggesting that these mediators are associated with the degree of TB disease and may be related to the intensity of inflammation. In contrast to this knowledge, our results showed that TNF- $\alpha$  and sIL-2R levels were not related to chest X-ray scores of tuberculosis. Radiological abnormalities were quantified by means of four different scores evaluated by three physicians in reading the conventional chest radiographs images. Significant differences between the four groups were not observed for sIL-2R, IL-4 and TNF- $\alpha$ , suggesting that these mediators were not asso-

ciated with the severity of tuberculous disease. In contrast to our findings, Casarini *et al.* found that significant difference was observed for TNF- $\alpha$ , suggesting that this mediator was associated with the severity of tuberculous disease as evaluated by high resolution computerized tomography.<sup>27</sup> In a different study, IL-2 concentrations were also inversely correlated with radiological score, unlike our results.<sup>28</sup>

Several of the immune theories focus on the central role of Th1/Th2 cross-regulation. In particular, it has been hypothesized that there is a switch from a Th1 to a Th2-dominant cell-mediated immune response leading to active disease, as is seen in other infections. However, attempts at isolating Th2 cells and Th2-type cytokines from the infection has not always been successful. IL 4 expression in samples from infected individuals was shown to be lower than in those from uninfected controls.<sup>29</sup> This would seem to indicate that the role played by Th1/Th2 immunity might not be a simple one. A second hypothesis is that a true switch from a Th1 to a Th2 response does not necessarily occur, but instead that the relative strength of the Th1 response determines latency or active disease in TB.<sup>30</sup> Presence of the correlation between TNF- $\alpha$  and sIL-2R levels in our study showed the cross-regulation of cytokines in active pulmonary TB. These findings are in concordance with the literature. When the severity of the disease is increasing, the correlation of these two cytokines seems more significant. But IL-4 response was not correlated with TNF- $\alpha$  and sIL-2R levels, similar to literature.

Evidence from the murine model of TB has linked major histocompatibility complex class I restricted CD8<sup>+</sup> T cells with protective immunity against *M. tuberculosis* infection.<sup>31</sup> Further evidence suggests that these CD8<sup>+</sup> cells are more important in controlling pulmonary infection during the chronic phase of disease rather than in the early stages of infection.<sup>32</sup> CD8<sup>+</sup> cells are capable of producing macrophage-activating cytokines and display cytolytic activity against infected macrophages.<sup>33</sup> Previous studies have shown increased IL-4 cytokine production in TB patients compared with healthy normal subjects or contacts,<sup>34,35</sup> although others have demonstrated reduced type 1 cytokine production in the absence of

increased IL-4.<sup>36,37</sup> An earlier study using ELISA also demonstrated increased numbers of IL-4 secreting cells in the peripheral blood of TB patients.<sup>38</sup> In our study, IL-4 response was not different after chemotherapy. We cannot clarify the BCG vaccination effects on Th2 response in control subjects because it is routinely done in our country.

In conclusion, understanding the biology of cytokine production and responsiveness among TB patients is important, because this cytokine may have a predominant protective role in *M. tuberculosis* infection. Results of this study have provided some evidence confirming the previously reported role of TNF- $\alpha$ , IL-4 and sIL 2R in the control of TB. However, these cytokines are not related with disease severity and outcomes. Types of cellular immune response may affect presentation and outcome in pulmonary TB, and an understanding of the development of this response may lead to insights into pathogenesis and novel therapies for TB. Therefore, further studies are required to explain the mechanism of outcomes and cytokines in pulmonary TB.

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Received 23 October 2002

Accepted 11 November 2002