

Diagnostic Accuracy of Cytokine Levels (TNF- α , IL-2 and IFN- γ) in Bronchoalveolar Lavage Fluid of Smear-Negative Pulmonary Tuberculosis Patients

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Key Words

Bronchoalveolar lavage fluid · IFN- γ · IL-2 · TNF- α · Tuberculosis

Abstract

Background: The determination of cytokine concentrations in serum and bronchoalveolar lavage fluid (BALF) may contribute to the diagnosis of tuberculosis (TB) since cytokines have been ascribed an important role in TB pathogenesis. **Objective:** To assess the diagnostic accuracy of TNF- α , IFN- γ and IL-2 levels in serum and BALF of smear-negative pulmonary TB patients. **Method:** BALF was obtained from the affected lobe in patients with smear-negative TB or other pulmonary diseases (OPD), and from the right middle lobe in healthy controls. ELISA and a nephelometric method were used to detect cytokine and albumin levels. **Results:** TNF- α levels in BALF were significantly elevated in the TB group (n = 15) compared with the OPD patients (n = 40) and controls (n = 17; p < 0.001). Although these three cytokines correlated well with each other in BALF (p < 0.0001, and r \geq 0.7, respectively), BALF IL-2 and IFN- γ levels were not significantly different among the groups (p > 0.05). BALF TNF- α or IFN- γ levels were significantly higher in patients with cavitory disease (n = 11) versus those without (n = 61; p < 0.05). However, no significant difference was found between cavitory (n = 7) and non-cavitory TB in cytokine levels (p > 0.05). Neither gender nor smoking status showed any statistical differ-

ences in cytokines in the groups (p > 0.05). Sensitivity and specificity of BALF TNF- α were found to be 73 and 76%, respectively. The positive and negative predictive values for BALF TNF- α were 44 and 91%, respectively. **Conclusion:** In cases of smear-negative TB, BALF TNF- α can be a useful tool to identify healthy subjects rather than smear-negative TB patients.

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Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, still represents a serious health problem in the world. Each year, approximately 8 million people develop active TB and 3 million die of this disease worldwide [1]. The pro-/anti-inflammatory cytokine balance has been shown to play an important role in the pathogenesis and activity of TB including granuloma formation, caseation necrosis and delayed-type hypersensitivity. Infection from *M. tuberculosis* leads to a strong cytokine response. The inflammatory response to this pathogen is a crucial step in the control of infection but may also contribute to long-term sequelae and associated pathology [2, 3].

Although *M. tuberculosis* isolation is the 'gold standard' for the diagnosis of TB [1], studies have shown that certain cytokine concentrations in serum as well as bronchoalveolar lavage fluid (BALF) may also contribute to

the diagnosis [4–7]. Interferon (IFN)- γ is a key cytokine in the control of *M. tuberculosis* infection. It is produced by both CD4- and CD8-type T cells and activates macrophages in TB. Interleukin (IL)-2 is a Th1 cytokine increasing the number of T lymphocytes as well as activating leukocyte chemotaxis. *M. tuberculosis* induces tumor necrosis factor (TNF)- α secretion by macrophages, dendritic cells and T cells, and is required for the control of *M. tuberculosis* infection when it is in its active stage [5]. Studies have shown that serum and BALF levels of IFN- γ , IL-2 and TNF- α are elevated in TB patients [5, 8, 9]. IL-4, -6, -10 and -12 and transforming growth factor (TGF)- β are further cytokines contributing to the pathogenesis of TB, and increased levels are supposed to inactivate macrophages and inhibit possible T-cell responses [2].

Assuming that ‘high cytokine levels are a hallmark in the pathogenesis of TB’, we think that some cytokines may be used for diagnostic purposes during the course of this disease. For this reason, we measured the levels of three cytokines, TNF- α , IFN- γ and IL-2, in our laboratory. BALF and serum cytokine levels were assessed in sputum samples of smear-negative, culture-positive active pulmonary TB patients before commencing anti-TB therapy, and levels were compared among groups.

Patients and Methods

Patients

All subjects were informed regarding the flexible bronchoscopy (FB) and BAL procedures before giving informed consent. The study was approved by the Ethics Committee of our institution. Healthy controls were offered financial support (\approx USD 100) to participate in the study. The patients studied demonstrated abnormal radiographic findings at the time of admission to the hospital. Three groups were studied: two patient groups, patients with TB and other pulmonary disease (OPD), and a control group.

The *TB group* included sputum smear-negative patients who were strongly suspected to have active TB on the basis of clinical and radiological findings. The diagnosis of TB was eventually confirmed by positive sputum culture for TB bacilli or BALF smear and/or culture positivity.

The *OPD group* included subjects with pulmonary infiltrates suspicious for TB, but who were eventually diagnosed with other diseases. Preliminarily, this group was combined with the TB group (*pulmonary disease group*). Once the exact diagnoses were established, these two groups were separated and renamed. In the OPD group, three sputum specimens and BAL smear as well as culture had been negative. All patients underwent FB to elucidate the nature of the pulmonary infiltrates. Patients with complete or partial resolution of radiographic findings were included in the OPD group. In the absence of resolution, these cases were either excluded from the study or if appropriate included in the TB group.

The *healthy control group* included volunteers without any pulmonary symptoms or active disease. Active smoking was not an exclusion criterion in this group as long as there was no evidence of obstructive lung disease on spirometry. These subjects were free of any pulmonary disease during the 1-year follow-up.

None of the study subjects had any evidence of human immunodeficiency virus infection and none were receiving corticosteroids or other immunosuppressive drugs.

Methods

Sputum Smear. The early morning sputum was collected and immediately sent to the in-house laboratory for processing. Sputum was first treated with sodium hydroxide and shaken well for 20 min, followed by centrifugation for 20 min at 3,000 rpm. The supernatant was discarded and part of the resultant sediment was used to prepare a smear on the glass slides and evaluated by the Ziehl-Nielsen method. The rest of the sediment was cultured on Lowenstein-Jensen medium.

FB and BALF. All patients underwent FB, and BALF was obtained. Indications for FB were suspicion of TB based on clinical and radiological findings and suspicion of lung cancer/obstructive pneumonia or atelectasis in sputum smear-negative patients. Following determination of the lesion site based on radiographic findings, BALF was obtained from the affected bronchi of the patients. In the control group, BALF was obtained from the right middle lobe bronchus by wedging an Olympus BF type P20D bronchoscope into a forth- or fifth-generation bronchus under local anesthesia. For BAL, 50-ml aliquots of normal saline were instilled using a syringe attached to the suction port at room temperature. A total of 200 ml of saline were sequentially instilled and immediately retrieved manually. The resultant fluid was filtered through four layers of sterile gauze, pooled and immediately stored at 4°C for future processing. The pooled fluid was centrifuged at 400 g for 10 min. The supernatant was then divided into 200- μ l aliquots and rapidly frozen at -70°C. Serum was also obtained from all subjects by centrifugation of whole blood using standard procedures on the day BALF was collected and rapidly frozen at -70°C until analysis.

Determination of Cytokine Concentrations. A sandwich enzyme-linked immunosorbent assay (ELISA) was used to detect the concentrations of TNF- α , IFN- γ and IL-2 in BALF (bTNF- α , bIFN- γ and bIL-2) and serum (sTNF- α , sIFN- γ and sIL-2). The kits for TNF- α and IL-2 assays were purchased from Biosource International (Nivelle, Belgium) and for IFN- γ from CytElisa (CytImmune Sciences, Rockville, Md., USA). The frozen aliquots of BALF and serum were thawed at room temperature for each assay. The minimal detectable level was 1.7 pg/ml for TNF- α , <5.1 pg/ml for IL-2 and 11.9 pg/ml for IFN- γ .

Standardization of Cytokine Concentrations in BALF with Albumin. We also presented our data as ratios of the concentration of cytokines to the concentration of albumin in BALF, i.e. the crude BALF cytokine levels directly measured by ELISA were divided by the BALF albumin levels, and data were given as ratios of the amount of cytokine per milligram of albumin. Albumin concentration was measured by a nephelometric method (Image, Beckman Coulter, Fullerton, Calif., USA) using reactive anti-serums. This standardization method removes the variable of dilution and allows comparison between data from different subjects and investigators.

Statistical Analysis

SPSS 11.0 was used for statistical analyses. Values were expressed as means \pm SD, medians and ranges. The Kruskal-Wallis test was used to compare cytokine levels among groups. A multiple comparison test was employed to detect statistically significant differences among groups [10]. In the TB group, cytokine levels were compared between patients with and without cavitory disease using the Mann-Whitney U test. Cytokine levels of patients with cavitory disease were also compared to those of other groups using the same test. Numbers and percentages of smokers/nonsmokers and males/females were investigated by cross-tabulation using the χ^2 test. The null hypothesis was rejected at $p < 0.05$ in all statistical tests.

In TB and non-TB groups, receiver-operating characteristic curve (ROC) analyses were performed for sIFN- γ and bTNF- α to determine cutoff levels increasing the true-positive (TP) rate.

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic efficiency of bTNF- α was calculated as described in our previous study [11].

$$\text{Sensitivity} = \text{TP} / (\text{TP} + \text{FN})$$

$$\text{Specificity} = \text{TN} / (\text{TN} + \text{FP})$$

$$\text{PPV} = \text{TP} / (\text{TP} + \text{FP})$$

$$\text{NPV} = \text{TN} / (\text{TN} + \text{FN})$$

$$\text{Diagnostic efficiency} = \text{TP} + \text{TN} / (\text{TP} + \text{TN} + \text{FP} + \text{FN}),$$

where FN = false negative, FP = false positive and TN = true negative. The correlation analyses were done using the Spearman test.

Results

The TB group included 15 patients (11 males and 4 females), with a mean age of 48.5 ± 18.3 years (range: 18–75). Eight (53.3%) of them were smokers. Constitutional symptoms (fever, weight and appetite loss, and sweating) were noted in all TB patients. On chest X-ray, cavitory lesions were seen in 7 patients (46.6%); heterogeneous and homogenous opacities were seen in 6 (40%) and 2 patients (13.3%), respectively. Sputum culture was positive in 10 patients (66.6%), BALF smear was positive in 3 (20%), and BALF culture was positive in 8 (53.3%) patients. The OPD group comprised 40 patients (23 males and 17 females), including 16 (40%) smokers, with a mean age of 51.7 ± 13.8 years (17–76); 3 of them had asthma, 18 pneumonia, 4 lung cancer, 4 chronic obstructive pulmonary disease, 9 bronchiectasis and the other 2 had pulmonary embolism, which were diagnosed after a follow-up of ≥ 6 months. The healthy control group included 17 individuals (10 males and 7 females), with a mean age of 51.2 ± 9.2 years (range: 42–77 years). Six (35.5%) of them were smokers at the time of analysis and all of them had normal pulmonary function.

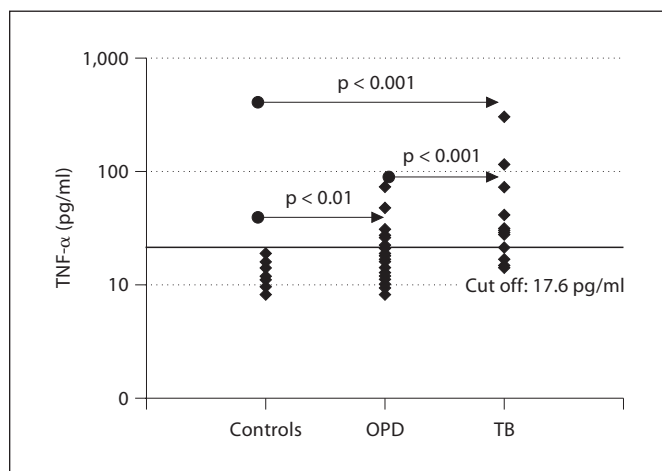


Fig. 1. bTNF- α levels of the groups: 11 of 15 TB patients were above its calculated cutoff of 17.6 pg/ml. All healthy controls were below this cutoff value. The y-axis is shown in logarithmic scale.

All subjects tolerated the FB procedure and BAL collection well, with $>60\%$ of the total volume infused being collected in all cases.

bTNF- α levels were significantly increased in the TB group compared with both non-TB groups ($p < 0.01$ and $p < 0.001$). None of the healthy controls had bTNF- α levels over the cutoff value. Although being lower than in the TB group, bTNF- α levels were significantly higher in the OPD group than in the healthy controls ($p < 0.05$; fig. 1). On the contrary, no statistical differences were found among the groups in bIL-2 and bIFN- γ levels ($p > 0.05$), although bIFN- γ levels were higher in the TB patients (table 1).

Serum cytokine levels were not statistically different between the groups ($p > 0.05$; table 1). Cytokine levels in BALF and serum did not differ between males or females and smokers or nonsmokers ($p > 0.05$).

Intragroup comparisons for bTNF- α , bIL-2 and bIFN- γ levels were also performed between patients with and without cavitory lesions. Although these cytokines were increased in patients with cavitory disease ($n = 7$), the differences were not statistically significant in the TB group ($p > 0.05$). However, the differences in bTNF- α and bIFN- γ were significant in 11 patients with (7 TB and 4 OPD patients) and those without cavitory disease ($n = 61$; $p < 0.02$ and $p < 0.03$; table 2).

bTNF- α levels were raised in symptomatic patients compared with symptom-free TB patients (63.7 ± 98 and 38.9 ± 35.3 pg/ml, respectively) but nonsignificant ($p > 0.05$).

Table 1. Cytokine levels (pg/ml) in BALF and serum

Groups	TNF- α	IL-2	IFN- γ
<i>BALF levels</i>			
TB			
Mean \pm SD	52.2 \pm 74.2 ^{a, b}	30.8 \pm 7.7	13 \pm 16
Median	28.3	29.8	8.5
Range	14.4–302	17.3–44.1	0–64.7
OPD			
Mean \pm SD	18.9 \pm 14.7 ^b	30.8 \pm 8.8	7.1 \pm 4.7
Median	13.9	33.9	6.2
Range	8.1–74.1	10.2–46.2	0.2–20.7
Controls			
Mean \pm SD	11.8 \pm 2.9	32.6 \pm 4	7.2 \pm 2.4
Median	11.3	33.1	6.8
Range	8.1–18.5	24.4–42.4	4–11.6
<i>Serum levels</i>			
TB			
Mean \pm SD	35.3 \pm 14.0	2.8 \pm 3.8	11.5 \pm 13.2
Median	30.8	1.3	7.7
Range	23–81.5	0–11.9	0–44.9
OPD			
Mean \pm SD	35.3 \pm 25.4	2.5 \pm 3.2	21.9 \pm 28.1
Median	28.8	1.7	14.9
Range	16–178.9	0–14.8	0–140.6
Controls			
Mean \pm SD	30.2 \pm 10.4	4.4 \pm 7	9.7 \pm 28.2
Median	29.0	0.8	0
Range	18.2–59.3	0–24.2	0–115.3

^a $p < 0.01$ vs. bTNF- α of OPD patients; ^b $p < 0.001$ vs. bTNF- α of controls.

ROC analysis was performed to determine the exact cutoff level for bTNF- α . Using the cutoff of bTNF- α , but not sIFN- γ ($p > 0.05$), we were able to differentiate TB from non-TB patients ($p < 0.001$). Sensitivity and specificity were 73 and 76%, respectively, using a cutoff value of 17.6 pg/ml for bTNF- α , leading to a diagnostic efficiency of 75%. The PPV and NPV for bTNF- α were 44 and 91%, respectively. Therefore, the cutoff level chosen was 17.6 pg/ml. As ROC curve analysis was not significant for other cytokines ($p > 0.05$), cutoff levels were not determined.

BALF cytokine levels correlated positively with standardized BALF cytokine levels (for TNF- α $r = 0.322$, $p < 0.01$; for IFN- γ $r = 0.865$, $p < 0.001$, and for IL-2 $r = 0.336$, $p < 0.05$). Moreover, these standardized cytokine levels were strongly mutually correlated: bTNF- α and bIFN- γ $p < 0.0001$, $r = 0.79$; bTNF- α and bIL-2 $p < 0.0001$, $r = 0.78$, and bIL-2 and bIFN- γ $p < 0.0001$, $r = 0.86$.

Table 2. BALF cytokine levels (pg/ml) in patients with cavitory disease (n = 11; 7 TB and 4 OPD patients) and those without (n = 61)

Groups	bTNF- α	bIFN- γ
Cavitory disease		
Mean \pm SD	34.1 \pm 32.6 ^a	15.8 \pm 17.7 ^b
Median	17.3	10.4
Range	13–116.3	0.9–64.7
No cavitory disease		
Mean \pm SD	22.3 \pm 38.5	7.0 \pm 4.3
Median	13.9	6.3
Range	8.1–302	0–20.7

^a $p < 0.02$, ^b $p < 0.03$, vs. no cavitory disease.

Discussion

Many studies have shown that cytokines are involved in the pathogenesis of TB. Cytokine levels in BALF have frequently been studied in patients with active TB [4, 6, 12–16]. We aimed to determine the diagnostic accuracy of three cytokines, TNF- α , IFN- γ and IL-2, in BALF and serum of sputum smear-negative active pulmonary TB patients.

TNF- α is a cytokine with a long history in TB research and is believed to play multiple roles in immune and pathological responses of TB patients. Pleural fluid TNF- α levels were used to prognosticate tuberculous pleurisy with high sensitivity and specificity rates [17]. In the present study, bTNF- α levels were found to be significantly increased in the TB group compared with both OPD patients and healthy controls. Several studies have reported that TNF- α causes tissue necrosis and is responsible for systemic symptoms of TB; high BALF concentrations were noted in patients with active TB indicating active bronchoalveolitis [8, 14–19]. Increased BALF concentrations were also detected in other pulmonary diseases such as chronic obstructive pulmonary disease and lung cancer [20–25]. Consistent with these results, bTNF- α was also higher in the OPD group than in healthy controls in our study. In the TB group, the bTNF- α level was higher than in the OPD group suggesting that it may be more specific to TB than to other diseases. In our study, using 17.6 pg/ml as cutoff point, sensitivity and specificity of bTNF- α were high (73 and 76%, respectively), but lower than expected. This may be due to the fact that all TB patients were in an early stage and smear negative, and therefore probably had a low bacillus load. In this re-

gard, one of the most striking findings of the present study was that all the control subjects were below this cutoff level of bTNF- α , and therefore NPV and diagnostic efficiency were found to be fairly high (91 and 75%, respectively), indicating that it could reliably detect healthy subjects. Thus, bTNF- α seems to be a good diagnostic tool in smear-negative patients with active TB.

Animal studies have shown that IFN- γ plays a pivotal and essential role in protective cellular immunity in TB infection [26, 27]. There are many *ex vivo* studies showing decreased concentrations of IFN- γ in TB patients [28–31]. Conversely, in previous studies, sIFN- γ levels were found to be significantly increased in active TB patients [4, 5, 9, 32–34]. However, there was no significant difference in the serum levels of these cytokines among groups. Consequently, there was no need to establish cut-off levels for these cytokines, indicating that serum cytokines were not reliable diagnostic tools to distinguish TB from other inflammatory diseases or controls.

As expected, a positive correlation was observed between BALF cytokine levels and standardized BALF cytokine levels, in agreement with a previous study [35]. Even though IL-2 and IFN- γ levels were not statistically elevated in our TB group, it is easy to accept that high levels of these cytokines, including bTNF- α , mirror local inflammation and further support the suspicion of TB.

The weakness of our study is a small number of patients with active tuberculosis. However, it was difficult to find patients with a strong clinical suspicion for active TB and three negative sputum smears for the organism. Second, the OPD group neither represents the entire spectrum of pulmonary diseases nor a single specific pathology and hence the normal cytokine levels are of little relevance. Third, due to technical limitations, only three of the cytokines known to be involved in TB could be evaluated. Interestingly, the positive results of the study increase its applicability for institutions where measurements of all cytokine levels may not be possible. Lastly, almost 35% of our healthy controls were smokers. It has been shown that cigarette smoking induces many proinflammatory mechanisms in the lung and may also alter cytokine levels [36]. However, apparently stratification by smoking did not affect cytokine concentrations in BALF. Since both patients and controls were smokers, results may not have been adversely affected.

Efficient and affordable diagnostic tools to aid in the identification of TB disease in acid-fast bacillus smear-negative subjects are needed and would help to initiate targeted therapy in affected patients earlier, increase cost-effectiveness, decrease the duration of hospitalization

and also prevent transmission of the disease. The data presented indicate that a TNF- α value >17.6 pg/ml helps in the selection of treatment options in smear-negative patients suspected to have TB clinically and/or radiologically. Therefore, bTNF- α levels above the cut-off value may help to initiate anti-TB therapy in smear-negative TB patients before culture results are obtained. However, trials in larger patient cohorts are warranted. It would also be interesting to study the effect of anti-TB treatment on TNF- α levels, similar to previous studies on IFN- γ and TGF- β in sera and BALF [37]. In the future, the significance of BALF cytokines will also need to be evaluated against emerging new diagnostic tools, e.g. the detection of mycobacterial DNA by PCR, and the identification/enumeration of ESAT-6 and CFP-10 induced IFN- γ producing BAL cells using the ELISPOT assay [38].

Conclusion

Cytokines play important roles in the pathogenesis of TB. Many authors have investigated cytokines in smear-negative and TB-positive subjects. This is the first study to show that bTNF- α was significantly elevated in smear-negative active TB patients, and interestingly, bTNF- α may help to identify healthy controls rather than smear-negative TB patients, especially for values if <17.6 pg/ml.

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