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Features of Cytokine Profile in Patients with Progressive TB-Induced Pulmonary Fibrosis Characterized by Various Intensities of Pulmonary Destructive Changes

Knoring BE[1], Davydova NI[2], Shulgina MV[1,4], Kravtsov VY[3*], Zilber EK[1] and Yablonskii PK[1,5]

[1]*Saint Petersburg Research Institute for Phthisiopulmonology, Saint Petersburg, Russia,*

[2]*A.M. Nikiforov Russian Centre for Emergency and Radiation Medicine EMERCOM of Russia, Saint Petersburg, Russia,*

[3]*Pushkin Leningrad State University, Saint Petersburg, Russia,*

[4]*Kashkin Research Institute of Medical Mycology of North-West State Medical University named after I.I. Mechnikov, Saint-Petersburg, Russia*

[5]*Saint Petersburg State University, Medical Faculty, Saint Petersburg, Russia*

[*]**Corresponding author:** Viacheslav Yu. Kravtsov, Pushkin Leningrad State University, 10 Peterburgskoe shosse, St.-Petersburg (Pusshkin), 196605, Russia, Tel: +79602571546; E-mail: kvyspb@mail.ru

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Abstract

Production of pro- and anti-inflammatory cytokines (IFN-γ, IL-2, TNF-α, IL-8, IL-4, IL-10) in patients with pulmonary TB infection with various spread and severity of the process were investigated in *in vitro* PPD- and PHA-stimulated peripheral blood mononuclear cells (PBMCs). It was demonstrated that each type of MDR-TB lung fibrosis is determined by specific traits of a multilevel cytokine network functioning according to activity of cytokine-producing cells.

The most prominent alterations in regulation of the immune response, which clearly correspond to the outcome of TB-process, are exhibited in cytokine profile PPD-induced cytokine profile displayed by *in vitro* stimulated PBMCs. It is known that PPD-stimulated PBMCs from patients with severe extensively MDR-TB lung fibrosis are distinguished by low production of pro-inflammatory cytokines IFN-γ, IL-2, and TNF-α, whereas a role for altered level of anti-inflammatory cytokine IL-10 remains controversial. Here, we found that most unfavorable course of TB-infection was observed in patients displaying low production both of pro- (IFN-γ etc.) and anti-inflammatory (IL-10) cytokines. In particular, high level of IL-10 produced by PPD-stimulated PBMCs from patients with progressive TB-lung fibrosis was associated with restricted inflammatory process and lung tissue damage. Insufficient production of IL-10 during severe course of TB-infection may underlie a pathogenesis of steadily progressing TB-lung fibrosis and is associated with an extensive spread of the disease.

By analyzing alterations in production of pro- and anti-inflammatory cytokines from PBMCs of patients with MDR-TB lung fibrosis, it may allow to predict spreading of destructive damage in the lungs, which may additionally serve as a rationale for correction of immune system.

A comparative analysis of *in vitro* PPD- and PHA-stimulated PBMCs together with evaluation of spontaneous cytokine production as well as their level in the blood serum provides additional information regarding functional state of immune cells in TB-patients.

Keywords: Tuberculosis; Multidrug-resistant tuberculosis; Phytohemagglutinin; Interleukin

Introduction

Prevalence of multidrug-resistant TB-infection (MDR-TB), which results in development of severe progressive course of TB-process, is characterized by rapid generalization, greater spreading, immunocompromised state and the lack of effect from the administered standard anti-TB therapy [1-11].

Currently, a promising approach is based on thorough investigation of the mechanisms underlying unfavorable outcome of TB-process. Introduction of immunomodulating agents into a standard therapeutic protocol of TB-patients represents one of the first-priority tasks in physiology [12-14].

It is known, that disturbed regulation of cytokine network underlies development of pathologies, which pattern depends on skewing cytokine balance [10,14-27]. At present, a great attention is paid to investigating pathogenesis of MDR-TB. In particular, peripheral blood mononuclear cells (PBMCs) from these patients are characterized by lowered capacity to recognize M. tuberculosis antigens [28], decreased NO production [29], down regulated phosphorylation of STAT1, STAT4 and consequently lowered production of cytokines [30]. In addition, there are numerous reports demonstrating that during severe TB-infection Th1-immune responses become down regulated, whereas Th2-response are up regulated [1-8,31-38].

An imbalance between pro- and anti-inflammatory cytokine profiles was observed in patients with MDR-TB [6,10,14-16,20,22,23,27,32,33,39]. In this regard, special attention was paid to production of TNF-α in response to M. tuberculosis antigens. In particular, it is known that TNF-α overproduction may result in profound lung injury, whereas its shortage may be associated with uncontrolled course of the infection [21]. It is emphasized that TNF-α and IFN-γ play a special role in controlling TB-dependent lung destruction [1,2,4-7,17,18,22-24,27]. However, a degree of the observed immunological disturbances as well as their skewing during pulmonary MDR-TB together with evaluation of their clinical relevance are ambiguous, which seem to be related to examining patients with progressive pulmonary TB-infection with differing severity of the disease, that was enrolled in different studies [10,15,17,20,29,30,33]. In particular, some investigations [9,19,23,24,30] demonstrated that during pulmonary TB-infection production of IFN-γ was down regulated. On the contrary, other studies showed that upon extensive TB-lung injury it was up regulated [24,38,10,20]; Eum et al. [25] however, found no changes in production of IFN-γ in patients with MDR-TB before the onset of therapy.

A lead role for TNF-α in damaging lung tissue has been justified in numerous studies [1,18,22-25,37], and during MDR-TB production of TNF-α was either unregulated [9,20,25] or down regulated [24,29]. However, amount of TNF-α correlated with bacteriologically proven M. tuberculosis but not with intensity of lung tissue damage [10]. In addition, ambiguous data were obtained on IL-10 levels in patients with MDR-TB vs. non-MDR-TB [9,10,15,25,35]. In particular, patients with MDR-TB were found to have either elevated IL-10 [10,19,20,25] or reduced [9], or even normal levels of IL-10 [35]. At the end of therapy, high amount of IL-10 found in sputum supernatant is considered as a risk factor for developing recurrences of TB-infection [21]. Thus, a role that cytokines might play in immunopathogenesis of TB-infection remains obscure. Perhaps, controversy of these results might be due to examining patients with MDR-TB differing in terms of clinical picture and chest X-ray data.

Based on this, it is very important to further investigate potential alterations in cytokine profile in patients with steadily progressing MDR-TB having different degree of spreading of destructive changes in the lungs, which would be useful to evaluate immune disturbances, justify feasibility of the proposed immunotherapy [14], and predict course and outcome of pathological process.

The goal of the current study was to seek for novel criteria for evaluating course of TB-process, its outcome and feasibility of immunotherapy in patients with progressive MDR-TB-induced pulmonary fibrosis with destruction of lung tissue and caverns based on profile of pro- and anti-inflammatory cytokines produced by *in vitro* PPD- and PHA-stimulated PBMCs.

Materials and Methods

Patients

For the study, there were enrolled 140 patients with pulmonary MDR-TB-infection with TB-induced pulmonary fibrosis, who were treated at the St. Petersburg Research Institute of Phthisiopulmonology within 2007-2012. 82 males and 58 females with TB-induced pulmonary fibrosis were recruited into the study, with age ranging from 19 to 63 лет (<20 y.o. – 10 persons, 20-50 y.o. – 110 persons, >50 y.o. – 20 persons). The diagnosis was confirmed according to clinical picture, chest X-ray and radiology (multi slice computed tomography) examination as well as confirmed by sputum microscopy and bacteriology testing.

In all cases, the disease had a chronic course lasting >2 years, characterized by lung tissue destruction, lack of positive clinical and X-ray dynamics or progression of the disease under a long-term extensive anti-TB polychemotherapy. Depending on severity and spreading of cavernous process, all patients were divided into four groups.

In group 1 (64 person), on admission to hospital patients with TB-induced pulmonary fibrosis had an extensive spreading of pulmonary process (affecting ≥6 lung segments), multifocal degradation of lung tissue with developing caverns, foci of bronchogenic TB-contamination, a bilateral process was found in 34 patients. Patients were found to have a marked intoxication (weakness, elevated body temperature, sweatiness, lowered appetite, weight loss), cough with sputum, blood spitting, chest pain, symptoms of cardiopulmonary insufficiency and were highly bacilli positive. Mycobacterium tuberculosis strains isolated from patients displayed secondary drug-resistance.

In group 2 (23 persons), patients had a tuberculosis empyema confirmed bacteriologically and histologically, with severe recurrent chronic course (e. pleurae chronicum), steady excreting MDR-M. Tuberculosis. Among them, bilateral pulmonary damage was found in 12 persons (52.2%), bronchopleural fistulas – in 9 persons (39.1%). All patients were debilitated or severely weakened.

In group 3 (53 persons), patients with TB-induced pulmonary fibrosis having relatively limited spreading of pneumosclerotic changes and pulmonary cavities (affecting <6 lung segments) were enrolled. The vast majority (79.2%) of patients had a unilateral TB-process in the lungs. Isolated M. tuberculosis strains also exhibited primary or secondary multiple-drug resistance. Intoxication process was less pronounced compared to patients from group 1 and 2.

In group 4 (21 person), oligosymptomatic patients with isolated pulmonary TB-lesions were also examined along with patients having TB-induced pulmonary fibrosis, who were characterized by dense round/oval formation (2-8 cm in diameter) representing a productive inflammatory focus with a large caseous core, fibrosis and well-developed fibrous capsule. Upon that, changes in the surrounding pulmonary tissue were slightly pronounced. Similar type of pulmonary pathology was denoted as lung tuberculoma.

Single lung tuberculoma (71.4%) was found in the majority of patients, with limited spreading (1-2 lung segments). In 14.3% cases (3 persons), small multiple lung tuberculomas were found, and in one case they were detected in both lungs. In 16 out of 21 patients (76.2%) TB-infection had a progressive course characterized by development of foci of destruction inside tuberculomas. Low bacilli positivity was registered only in 3 cases. Mild clinical manifestations were observed in the majority of patients, stable condition.

In group 5, there were enrolled apparently healthy persons (31 persons, volunteers), who never suffered from TB-infection, or had contact to persons discharging TB-bacteria, had no X-ray pulmonary changes, or autoimmune, allergic, acute and chronic inflammatory and infectious diseases, aged from 19 to 49 y.o. (average age 31 ± 0.9 y.o.), and included 17 males (57.1%) and 14 females (42.9%). All examined persons were HIV-negative.

Diagnostic Assays

Bacteriology assay

Samples (sputum, induced sputum, broncho-alveolar lavage or a lung biopsy, surgical material) from all patients were found culture positive (Loewenstein-Jensen culture and/or BACTEC MGIT960) and Ziehl-Neelsen (ZN) microscopy positive for M. tuberculosis, which were confirmed by IS6110-based real-time PCR analysis by using Amplitub-RV diagnostic kit (Syntol, Russia) and iCycleriQ5 apparatus (Bio-Rad, USA). Analysis of M. tuberculosis susceptibility to the first-line (isoniazid [INH], rifampin [RIF], pyrazinamide, ethambutol, and streptomycin) and second-line (ofloxacin, cycloserine, amikacin, kanamycin, capreomycin, and ethionamide) anti-TB drugs was done by using the method of absolute concentrations according to the guidelines of the Russian Ministry of Health (order no. 109 of 21 March 2003) and/or a BACTEC MGIT 960 system according to the manufacturer's recommendations (Bacteriology Laboratory at Research Institute of Phthisiopulmonology is externally quality assured by the Federal System for External Quality Assessment in Laboratory Medicine of Russian Federation).

Immunological assays

Samples of peripheral blood were collected from patients with TB-infection and apparently healthy persons from ulnar vein into S-Monovette 7.5 ml, Lithium Heparin, test tubes (Sarstedt, Germany). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifuging in density gradient of Ficoll-Urografin (ρ=1.077 g/ml), according to the Manufacturer's instructions. Purified protein derivative of Mycobacterium tuberculosis (PPD) was purchased from the Research Institute of Vaccines and Sera (St. Petersburg, Russia), and applied for *in vitro* stimulation at final concentration 10 g/ml, phytohemagglutinin-M (PHA-M) (Sigma, Germany) – at concentration 30 g/ml. The cells (1.5×105/well) were cultured in 400 µl of RPMI-1640 medium (Sigma, Germany) added with gentamycin (50 g/ml) and 30%-lincomycin (50 g/ml) as well as 10%-heat inactivated serum from Group IV blood donors, in 24-well plates for immunological assays (Tissue Culture Plate, Sarstedt, USA). Cells were cultured in the incubator with humidified atmosphere, with 5% CO2, at 37ºC.

Concentration of interleukin (IL)-2, IL-4, IL-8, IL-10, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) was measured in supernatants harvested from *in vitro* PHA- and PPD-stimulated, unstimulated PBMCs as well as in samples of blood sera.

PHA as a stimulator was used to induce TNF-α production in the PBMCs based on experiments demonstrating that, although PHA was weaker than LPS, however, it skewed pattern of cytokine production similarly to LPS. Use of both stimulators provided demonstrated direct correlation in terms of affecting cytokine production by PBMCs. Thus, along with PPD as a standard TB-specific activator for PBMCs we also used PHA, which allowed assessing changes and dynamics in cytokine production and comparing them between the examined groups of patients.

After 24 hr-incubation, supernatants were harvested and tested with ELISA KIT (CJSC Vector-Brest, Novosibirsk, Russia) according to the Manufacturer's instructions (detection level: for INF-γ – 5 pg/ml, IL-2 – 2 pg/ml, IL-4 – 0.4 pg/ml, IL-10 – 1 pg/ml, IL-8–2 pg/ml, TNF-α – 2 pg/ml).

PPD- and PHA-induced proliferative activity of PBMCs was examined 96 hrs after *in vitro* cell culturing by estimating the rate of propidium iodide (PI)-positive cells at S and G2/M phase of the cell cycle by using FACS Calibur (BD, USA) flow cytometer and subsequent analysis by using CellQuest software. Proliferative response was positive: for PPD-stimulated cells at S-phase (diploid cells) >3%, for PHA-stimulated cells >17%.

Statistical analysis

Statistical analysis was performed by using Statistica 7.0 (StatSoft Inc., USA) software. Quantitative parameters did not have normal distribution, and were evaluated by using non-parametric Mann-Whitney U-test for independent samples. Relationship between the examined parameters was evaluated by performing a correlation analysis and calculating Spearman's rank correlation coefficient (r). Significance level was set at p<0.05.

Results

PPD- and PHA-stimulated cytokine profiles produced by PBMCs of all patients' groups and volunteers is are presented in Figures 1-6. Production of IFN-γ, IL-2, TNF-α was variously observed in PPD-stimulated PBMCs from all patients with pulmonary TB-infection (groups 1-4) (Figure 1). At the same time, PHA-stimulated PBMCs produced significantly higher amount of IL-4 (Figure 5). However, IL-10 production changed differently. In particular, PPD-stimulated PBMCs from patients with relatively limited MDR-TB lung fibrosis (group 3) unregulated IL-10 production, whereas during extensive spreading of pulmonary TB-process (group 1) it was decreased (Figure 6).

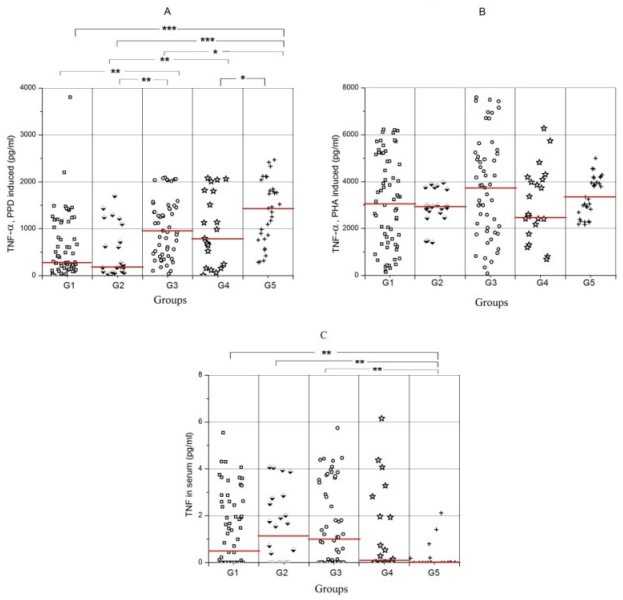

Figure 1: Production of IFN-*γ by in vitro* PPD (A) - and PHA (B)-stimulated PBMCs as well as in the blood serum (C) from patients with pulmonary TB-infection with various spreading of TB-process and from apparently healthy persons.

A thorough analysis of cytokine production revealed the following: in the majority of TB-patients, production of IFN-γ, IL-2 by PPD-stimulated PBMCs was lower compared to healthy volunteers (Figures 1 and 2). In particular, the lowest amounts of IFN-γ and IL-2 were produced in patients from group 1, which had multiple pulmonary cavities, and group 2, where patients had pleural empyema, (p<0.001, TB-patients vs. volunteers in all cases); median level for both cytokines was by 8-fold lower in TB-patients vs. volunteers.

When PBMCs from patients of group 1 and 2 were stimulated with PHA, production of IFN-γ and IL-2 was significantly decreased by 3- and 4-fold, respectively (p<0.001, Mann-Whitney test, TB-patients vs. volunteers in all cases) (Figure 2), which, however, was less pronounced in PHA- vs. PPD-stimulated PBMCs

PBMCs from patients with limited TB-induced pulmonary destructive changes (group 3 and 4) vs. volunteers had moderately down regulated production of IL-2 as well as IFN-γ from group 4 (Figure 1, p<0.05). Moreover, level of IL-2 was also decreased in group 3 and 4, whereas IFN-γ – only in group 4 (Figure 1, p<0.05). A clear-cut difference in magnitude of decreased amount of IL-2 and IFN-γ was observed depending on spreading of TB-process in the lungs (Figures 1 and 2). Down regulated production of IL-2 and IFN-γ turned out to be a typical sign of severity for MDR-TB-lung fibrosis.

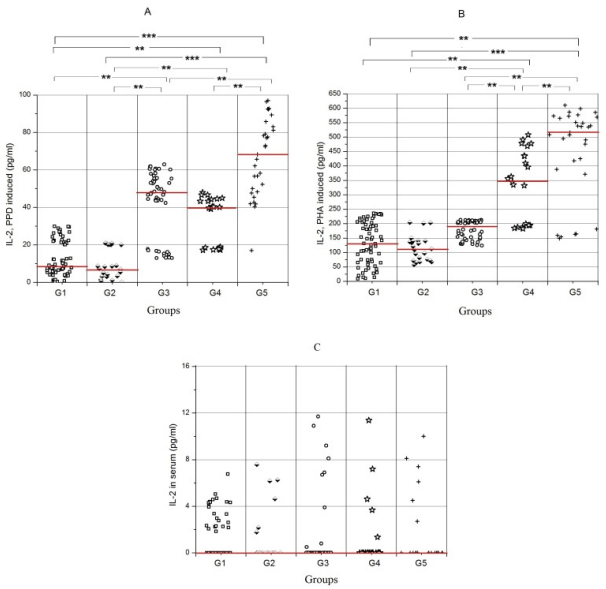

Figure 2: Production of IL-2 *by in vitro* PPD (A) - and PHA (B)-stimulated PBMCs as well as in the blood serum (C) from patients with pulmonary TB-infection with various spreading of TB-process and from apparently healthy persons.

More evident differences were found in the level of IL-2 produced by PBMCs from patients with generalized (group 1 and 2) vs. limited (group 3) TB-process in response to PPD-stimulation (by 5-7-fold vs. 1.5-fold after PHA-induced stimulation, Figure 2). However, when magnitude of lowered IFN-γ production (by 2-3-fold) was compared between group 1 and 3, virtually it was independent on the nature of the applied inducer (PPD or PHA). Thus, magnitude of down regulated PPD-induced IL-2 production was found to strongly match severity of progressing degradation of the lung tissue.

Previously, we demonstrated [7] that PPD- and PHA-stimulated PBMCs from patients with generalized MDR-TB-lung fibrosis (group 1 and 2) process were characterized with low proliferative response ($p<0.05$), which may be a sign of immunodeficiency. A markedly suppressed proliferative capacity of PBMCs during progressive MDR-TB lung fibrosis with multiple degradation cavities (group 1 and 2) is also confirmed by an inverse correlation with spreading of TB-process ($r=-0.54$; $p<0.05$) lacked in group 3.

Irrespective of spreading (group 1-3), all patients with progressive TB-induced pulmonary fibrosis were characterized by direct correlation between production of IL-2 and IFN-γ in response to PHA and PPD ($r=0.73$; $p<0.00001$). In addition, a direct correlation was found between proliferative capacity of PBMCs and IL-2 level ($r=0.68$; $p<0.02$), thus, indirectly evidencing that IL-2 might regulate their proliferation potential in patients with TB-induced pulmonary fibrosis.

The level of spontaneously produced IL-2 and its amount in the blood sera revealed no significant differences between all examined patients (group 1-4) and volunteers ($p > 0.05$, Mann-Whitney test, in all cases, Figure 2).

However, in group 1 and 2 level of spontaneously produced IFN-γ and his amount in blood serum were significantly higher compared to volunteers and patients with more limited TB-process (group 3 and 4, $p<0.05$, Figure 1).

Thus, long-lasting TB-process accompanied with extended degradation of the lung tissue is characterized by profoundly suppressed production of IL-2 and IFN-γ by PBMCs stimulated with PPD and PHA, which is paralleled with elevating baseline production of IFN-γ and its amount in the blood sera. Spreading of TB-process in the lungs is mostly associated with PPD-induced up regulated production of IL-2. Therefore, these changes in cytokine profile may reflect a degree of functional failure developed in immune system.

The level of TNF-α, which is involved in development of TB-granuloma, is closely linked to the features of the course of TB-process [10,18,22-24,26]. According to our data (Figure 3), amount of TNF-α tended to decrease only in PPD-stimulated PBMCs from all TB-patients compared to healthy volunteers. In addition, the most pronounced decline was noted in patients with extensive TB-induced pulmonary fibrosis (group 1) and pleural empyema (group 2), which were strongly significant compared to volunteers ($p <0.001$, in both cases, Figure 3). In particular, median TNF-α level in group 1 and 2 was 5-fold lower than in volunteers. Importantly, the extensive TB-process (group 3) but not limited TB-induced pulmonary fibrosis (group 1 and 2) was characterized by 3-4-fold lower level of *in vitro* induced TNF-α (Figure 3; $p<0.001$). In contrast, the level of PHA-induced production of TNF-α did not significantly differ between patients with progressive TB-induced pulmonary fibrosis (group 1-2) having a marked degradation of the lung tissue and volunteers ($p>0.05$; Figure 3).

The vast majority of patients with MDR-TB-induced pulmonary fibrosis (group 1-3) were found to have significantly decreased baseline production of TNF-α ($p<0.05$, Mann-Whitney test) by PBMCs, whereas its level in the blood serum was significantly higher ($p<0.05$) compared to volunteers. A strong inverse correlation between both parameters was revealed ($r=-0.67$; $p=0.003$).

Thus, progression of MDR-TB-induced pulmonary fibrosis with extensive degradation of the lung tissue is characterized by the low level of TNF-α produced by PPD-stimulated PBMCs, whereas PHA-induced production TNF-α did not differ from volunteers.

When PPD-stimulated PBMCs were examined for IL-8 production, it was found that both TB-patients and volunteers did not differ by medians of the cytokine (Figure 4). However, one third of patients with TB-induced pulmonary fibrosis, and none of volunteers, were noted to have a substantially up regulated production of this cytokine (>150000 pg/ml).

When PBMCs were stimulated *in vitro* with PHA, no significant differences in IL-8 production were observed. Altogether, it suggests that the capacity of PBMCs from TB-patients with pleural empyema (group 2) to up regulate PHA- induced IL-8 production and in some patients with TB-induced pulmonary fibrosis PPD-induced production of IL-8 reflects an intensity of ongoing inflammatory process (Figure 4). A significant decrease of the baseline production of IL-8 was observed in patients with TB-induced pulmonary fibrosis ($p<0.05$). However, no similar decline was found in patients with TB-granuloma (group 4; $p>0.05$). The blood levels of IL-8 in TB-patients (group 2-4) were similar to those found in volunteers. Moreover, in group 2 and 3 IL-8 level tended to increase, whereas it was significantly up regulated only in group 1 (Figure 4).

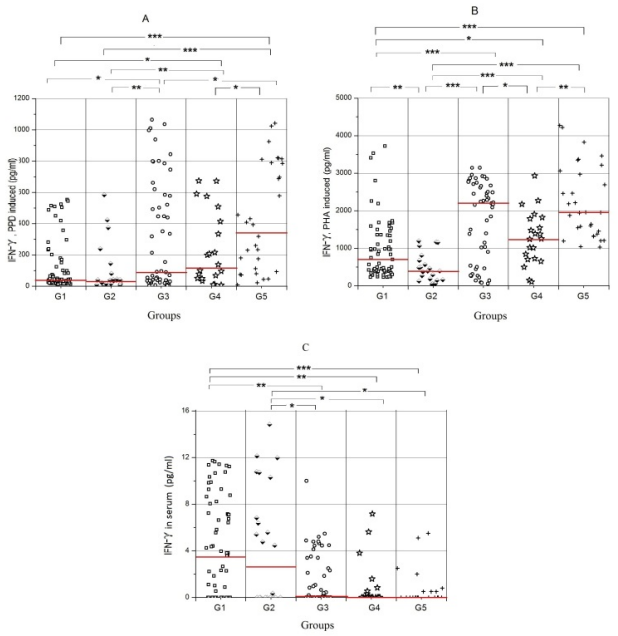

Figure 3: Production of TNF-α *by in vitro* PPD (A) - and PHA (B)-stimulated PBMCs as well as in the blood serum (C) from patients with pulmonary TB-infection with various spreading of TB-process and from apparently healthy persons.

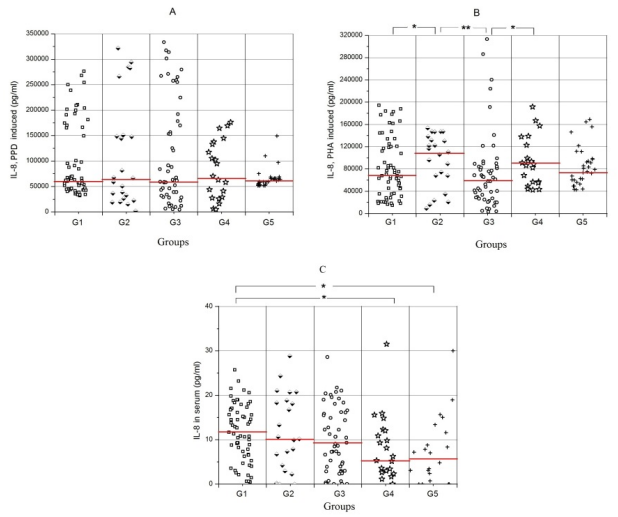

Figure 4: Production of IL-8 *by in vitro* PPD (A)- and PHA (B)-stimulated PBMCs as well as in the blood serum (C) from patients with pulmonary TB-infection with various spreading of TB-process and from apparently healthy persons.

The data obtained evidence that patients with TB-induced pulmonary fibrosis were characterized by suppressed IL-8production.

Production of IL-4 by PHA-stimulated PBMCs was significantly up regulated in all TB-patients, most markedly during extensive TB-induced pulmonary fibrosis from (group 1; $p<0.001$; Figure 5). However, PPD-induced PBMCs only tended to clearly elevate production of IL-4 in case of the most severe types of TB-pathology (group 1 and 2) compared to patients with limited TB-process and volunteers (group 3-5; $p>0.05$, Figure 5).

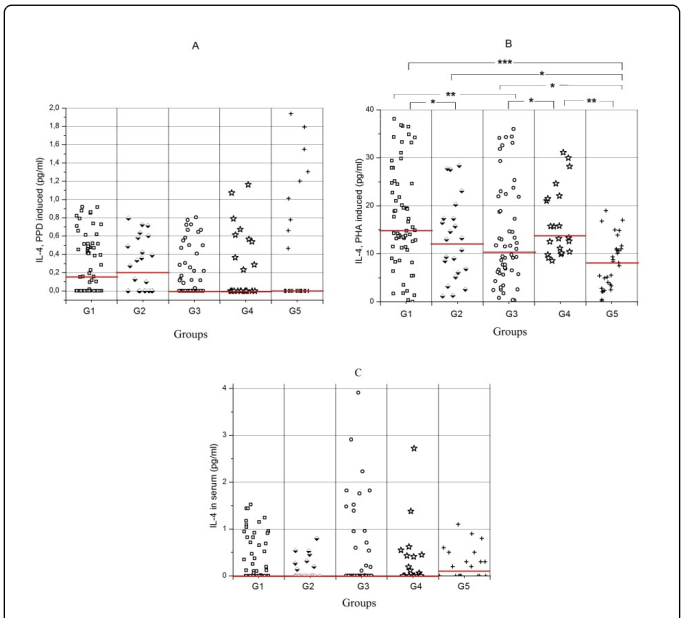

Figure 5: Production of IL-4 *by in vitro* PPD- (A) and PHA (B)-stimulated PBMCs as well as in the blood serum (C) from patients with pulmonary TB-infection with various spreading of TB-process and from apparently healthy persons.

No significant differences in IL-4 level between TB-patients and volunteers were observed during spontaneous production and its blood level ($p> 0.05$, Figure 5).

At the same time, a correlation analysis revealed that in patients with TB-induced pulmonary fibrosis up regulated PPD-induced production of IL-4 and its blood level was associated both with significantly extended spreading of TB-process ($r=0.6$; $p=0.03$) as well as down regulated PPD-induced production of TNF-α ($r=-0.64$; $p=0.017$). Thus, these relationships may point at development of Th2-skewed non-protective immune response in such patients.

Analysis of production of anti-inflammatory cytokine IL-10 in TB-patients revealed, that PPD-stimulated PBMCs in group 3 (limited TB-induced pulmonary fibrosis) was up regulated by 2-fold (Figure 6), and in contrast, was down regulated by 2-fold in patients from group 1 with severe course of pulmonary TB-process ($p<0.05$; in both cases). Furthermore, patients with extensive TB-induced pulmonary fibrosis were also noted to down regulate IL-10 production after being stimulated with PHA ($p>0.05$; Figure 6).

Thus in patients with advanced process (group 1), there is a significantly lower production of IL- 10 in response to PPD (2-fold) compared to group 3 patients with reduced degradation in the lung ($p<0.001$; Figure 6).

In addition, patients with extensive vs. limited TB-induced lung destruction (group 1 vs. group 3) were noted to significantly decrease PPD-induced production of IL-10 by PBMCs (2-fold; $p<0.001$). In

addition, a significantly decreased spontaneous IL-10 production and its elevated level in the blood serum were detected in entire group of patients with progressive TB-induced pulmonary fibrosis ($p<0.05$, Figure 6).

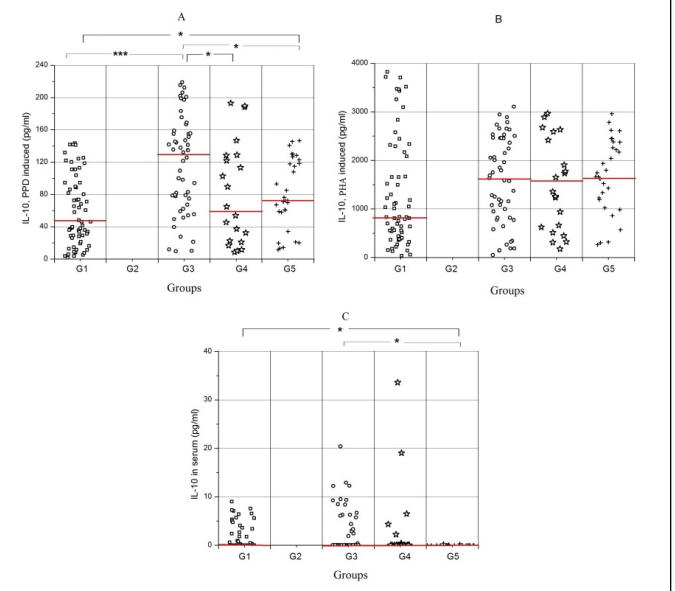

Figure 6: Production of IL-10 *by in vitro* PPD (A) - and PHA (B)-stimulated PBMCs as well as in the blood serum (C) from patients with pulmonary TB-infection with various spreading of TB-process and from apparently healthy persons.

Interestingly, patients with limited MDR-TB-induced pulmonary fibrosis (group 3) were shown to have an inverse correlation between spreading of TB-induced pulmonary destruction and baseline IL-10 production ($r=-0.69$; $p=0.01$). It may imply that moderate endogenous and adequate production of IL-10 triggered in the PBMCs agrees with a more favorable course of the disease. In the same group of patients with limited TB-process in the lungs a direct correlation between the level of PPD-stimulated production of IL-10 and PPD-induced production of IL-8 ($r=0.85$; $p=0.001$), TNF-α ($r=0.67$; $p=0.003$) and IL-2 ($r=0.54$; $p=0.02$) was revealed. These associations apparently indicate the development of relatively balanced production of both pro- and anti-inflammatory cytokines, thereby contributing to limiting inflammation and intensity of pulmonary destruction [21].

Patients from group 1 with extensive degradation of lung tissue lacked significant correlations between the level of the produced IL-10 and levels of other cytokines. Overall, patients from these patients differed by a significantly lower production of IL-10 compared to patients with limited TB-induced pulmonary fibrosis (Figure 6).

Thus, irrespective of spreading of TB-induced pulmonary destruction, patients with MDR-TB lung fibrosis were found to have:

- Decreased production of IL-2 and IFN-γ by PPD- and PHA-stimulated PBMCs, TNF-α - in response to PPD, increased amount of IL-4 in response to PHA as well as elevated amount of blood serum TNF-α.

With this regard, depending on the course of the diseases certain differences in cytokine production were observed.

Patients with extensive pulmonary TB-process those with pleural empyema were characterized by:

- Sharply decreased production of IFN-γ and IL-2 by PPD- and PHA-stimulated PBMCs,

- Decreased PPD-induced production of IL-10,

- Elevated PHA-induced production of IL-4,

- Elevated level of IFN-γ in the blood serum,

- Suppressed proliferative response of PBMCs in response to PPD and PHA.

Patients with limited TB-induced pulmonary destruction (group 3) were characterized by up regulated PPD-induced production of IL-10 by PBMCs.

Discussion

Changes in cytokine production and degree of proliferative capacity of PBMCs found in patients with TB-induced pulmonary fibrosis evidence about a profound suppression of different arms of immunity, primarily cellular immunity. The most pronounced imbalance in cytokine production and degree of proliferative capacity of PBMCs were documented in patients with extensive TB-induced pulmonary fibrosis. It was found that upon extensive and complicated TB-process associated with extended destruction caverns in lungs and intensive bacilli excretion (group 1 and 2) down regulated/lacked IFN-γ and IL-2 as well as suppressed proliferation of PPD- and PHA-stimulated PBMCs occurred. It seemed to be related to a transitory or stable unresponsiveness of the cells to antigenic stimuli, which, overall, may point at Th2-skewed immune response.

Unregulated spontaneous production of the cytokines as well as elevated level of IFN- γ in the blood sera may be related to the strong in vivo pre-activation of immune cells due to a long-lasting antigenic load. Moreover, recently it was found that $Foxp^3+CD^{4+}$ regulatory T cells might also be able to produce IFN-γ, which, however, seems to inhibit activation of Th1 cells, inhibit proliferation and differentiation of naïve T cells, and induces their early death, by apoptosis [40]. Thus, it may be assumed that the amount of IFN-γ produced *in vitro* by PBMCs from TB-patients may not always correspond to its functional impact to immune response. Altered IFN-γ and IL-2 production in patients with extensive TB-induced pulmonary fibrosis may evidence about improper defense mechanisms and a markedly suppressed T-cell arm [7,27 32,34,36,38].

At the same time, proliferative capacity of PPD- and PHA-stimulated PBMCs was found to directly correlate with the amount of the produced IL-2, indirectly evidencing that IL-2 appropriately regulates proliferation of PBMCs in patients with TB-induced pulmonary fibrosis. Magnitude of decreased IL-2 production by PPD-stimulated PBMCs along with their suppressed proliferation was found to most strongly correspond to degree of spreading of TB-induced destruction process.

A significant role in TB-pathogenesis is played by TNF-α, not only participating in defense reactions and triggering inflammation, but also in destruction and reparation contributing to inflammation [6,17,18,26]. High level of TNF-α in the blood serum is associated with inflammation and cell elimination via direct cytotoxic effect or apoptosis [41] as well as suggest its inability to bind to specific receptors on target cells. As a result, T cell response and phagocyte

function and granuloma development may be impaired including extension of the inflammatory process [22,26]. We found that low level of PPD- along with barely altered PHA-induced production of TNF-α by PBMCs from patients with extensive pulmonary destruction primarily may be due to suppression of TNF-α-producing cells caused by long-term in vivo antigenic overload. Magnitude of TNF-α production was interconnected to spreading of TB-process. Lack of full-featured immune response in terms of produced TNF-α and IFN-γ associated with their high levels in the blood serum, may result in excessive activation of macrophages and cytotoxic T cells followed by damage to lung tissue and impaired development of granuloma.

In addition, spontaneous production of TNF-α and IFN-γ was found to inversely correlate between each other (r=-0.69; p=0.008) that may evidence dysregulated immune response, usually being accompanied by severe course of TB-infection [2,6].

Importantly, we found that along with decreased production of IFN-γ, IL-2, TNF-α by PPD-stimulated PBMCs from TB-patients they also up regulated production of IL-4, which may correspond to Th2-skewed immune response and neutrophil activation observed in some TB patients [4,16]. Progression of pulmonary TB-process considered to be related to Th1/Th2 imbalance with dominant Th2-mediated immune reactions [1,2,4,6,7,9,39].

Production of anti-inflammatory cytokine IL-10 by *in vitro* PPD-stimulated PBMCs was found to be significantly down regulated in patients with steadily progressing extensive TB-process in the lungs compared to patients with limited TB-induced pulmonary fibrosis [9,15]. Low amount of PBMC-produced IL-10 in the course of extensive TB-induced pulmonary fibrosis did not result in restricting of inflammatory process. In contrast, PBMCs from patients with limited MDR-TB-induced pulmonary fibrosis were found did not demonstrate the decrease in production of IL-10. Upon that, up regulated production of IL-10 by PPD-stimulated PBMCs was associated with elevated production of pro-inflammatory cytokines such as IL-8 and TNF-α, which resulted in pronounced inflammatory response, apparently having a favorable role for resolution of the process [22]. In our study moderate baseline production of endogenous IL-10 also correlated with favorable outcome of TB-infection.

However, numerous studies demonstrated that the amount of suppressive cytokines IL-10 and TGF-β produced by induced regulatory T cells (iTregs), Tr1 and Th3 was up regulated in the peripheral blood from patients with progressive TB-infection [42]. Mainly, functions of iTregs and Tr1-cells are related to production of IL-10, whereas Th3-cells secrete TGF-β, primarily acting to restrain immune response [43,44]. Upon that, a steady tendency towards up regulated production of IL-10 and TGF-β going in parallel with deterioration of the TB-process was found [45,46], with subsequent suppression of T cell-response and development of energy in TB-patients [47,48]. A pool of antigen-specific Tregs is known to increase in parallel with increasing number of effector T cells for providing proper control over extending immune response [49]. Down regulated *in vitro* PPD-stimulated PBMCs production of both pro- (TNF-α, IFN-γ), but not anti-inflammatory (IL-10), cytokines evidences about dominant suppressive effects of regulatory T cells and/or insufficient activation of pro-inflammatory T cells required to override inhibitory signals and develop a full-fledged protective immune response.

On the other hand, there are some data evidencing about unambiguous role IL-10 during extensive inflammatory processes. For instance, it was reported that *in vitro* production of IL-10 by BCG-stimulated PMBCs significantly differed in patients with TB-induced pulmonary fibrosis having negative or positive Monteux test [34]. In particular, patients with allergic reaction to PPD skin test were found to have significantly lower production of IL-10 (in supernatants from BCG-stimulated PBMCs) compared to patients with positive Monteux test [34].

Likewise, it was demonstrated that patients with severe unstable asthma were noted to down regulate *in vitro* production of IL-10 compared to patients with mild and stable asthma [50].

Similar results regarding lowered concentration of TGF-β in the blood sera of children and adolescents with destructive vs. limited forms of TB-infection were also reported [51].

It may be assumed that upon chronic inflammation long-lasting antigenic stimulation may affect all arms of adaptive cellular immunity. Upon that, lowered production of pro-inflammatory cytokines and preserved secretion of anti-inflammatory cytokines may indicate at less severe course of the disease due to retained capacity of immune system to control magnitude of tissue damage triggered by innate immune cells [52].

On the other hand, simultaneous suppression of both Th1-cells and Tregs may evidence about exhaustion, energy and depletion of antigen-specific T cells or potential aggravation of immune reactions due to lowered control over activity of innate immune cells.

Differences found by others and us evidence about developed exhaustion of functional reserves of immune cells including Tregs (and other suppressor T cells) in patients with long-lasting persistent extensively MDR-TB lung fibrosis.

Given the fact that IL-10 is not the only antagonist of pro-inflammatory cytokines contributing to restraining magnitude of the immune response, the discrepancy found by us may also be explained by other cytokines involved in regulating Th1/Th2-balance during severe TB-infection such as excessive production of TGFβ [53,54], which role remains to be further investigated in TB-patients. It is plausible to assume that a pronounced decrease in production of IL-10 together with pro-inflammatory cytokines in the blood sera from patients with extensive TB-induced pulmonary fibrosis may result from profoundly suppressed immune response inevitably leading to further aggravated course of TB-infection.

Proper production of the suppressive cytokine IL-10 evidences about a possibility during TB-infection to control inflammatory response and able to cause tissue damage during TB-induced pulmonary fibrosis [39].

Lowered baseline production of IL-2, IL-8, TNF-α, IL-10 was most pronounced during severe course of TB-infection, which might indicate at suppression of immune cells due to toxic action of Mycobacterium-derived products on the host cells and/or up regulated use of these cytokines. Thus, the cytokine imbalance clearly reflects impairing of the immune system functioning [6,10,14,19,20,21,23,28,33].

The data obtained revealed that the most profound alterations in regulation of immune response clearly agree with outcome of TB-process manifested in produced cytokines as well as magnitude of proliferative response of PBMCs mainly to PPD. It justifies the need of using PPD as a cytokine inducer for assessing functional activity of immune cells in TB-patients.

Additional information regarding features of regulation and adequacy of immune response in patients with TB-induced pulmonary fibrosis of various severity may be obtained by conducting a comparative analysis of cytokines profile produced by *in vitro* PPD- and PHA-stimulated PMBCs as well as their levels in blood sera.

PPD-induced IL-10 production by PBMCs may serve as an indicator of disease severity in patients with severe progressive TB-lung process with profound secondary immunodeficiency and low production of other cytokines, particularly IL-2. During progressive TB-lung fibrosis, IL-10 was shown to have a positive effect by restricting inflammation and lung tissue damage. During severe course of the disease, its insufficient production may underlie pathogenesis of steady progression of TB-lung fibrosis and is associated with marked spread of TB-process [21].

A marked immunosuppression observed in patients with TB-induced pulmonary fibrosis may result in low efficacy of anti-TB therapy. Surgical interventions often used in these cases deteriorate immunodeficiency, thereby further increasing a risk of developing post-surgical pleuropulmonary complications. Upon that, it seems very important to predict disease course, outcome of pathological process and opportunity of developing post-operative complications. Failure of immune system characterized with low production of IL-2, TNF-α together with elevated level of anti-inflammatory cytokines is typical to the majority of patients with progressive TB-infection. However, reduced production of IL-10, suppressed lymphocyte proliferation together with lowered production of pro-inflammatory cytokines might be the most threatening sign evidencing about unfavorable course of pathology. A combination of such changes might serve as a prerequisite for applying immunomodulatory agents in patients while performing pre-surgical immune system correction.

Altogether, patients with a marked imbalance of immune regulatory mechanisms are recommended for pre-surgical immune system correction.

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