JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Relevance of *TNF-α, IL-6* and *IRAK1* gene expression for assessing disease severity and therapy effects in tuberculosis patients

Ivana Buha[1], Vesna Škodrić-Trifunović[1,2], Tatjana Adžić-Vukičević[1,2], Aleksandra Ilić[1,2], Ana Blanka Protić[1], Mihailo Stjepanović[1,2], Marina Anđelković[3], Miša Vreća[3], Jelena Milin-Lazović[2,4], Vesna Spasovski[3], Sonja Pavlović[3]

[1] Clinic of Pulmonology, Clinical Centre of Serbia, Belgrade, Serbia
[2] School of Medicine, University of Belgrade, Belgrade, Serbia
[3] Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia
[4] Department for Medical Statistics and Informatics, Faculty of Medicine, University of Belgrade, Belgrade, Serbia

Abstract

Introduction: Tuberculosis (TBC) is a contagious chronic respiratory disease which despite the known cause, *Mycobacterium tuberculosis* (*Mtb*), and many decades of successful therapy, remains one of the leading global health problems. Immune responses against *Mtb* infection involve both of types of immunity, but cellular immunity, in which certain cytokines and Th1 cells play a key role, is crucial. A better understanding of the functions of the cytokine network involved in the state and progression of TBC could identify specific molecular markers for monitoring of disease activity as well as therapy outcomes in TBC patients.

Methodology: We investigated expression of *TNF-α, IL-6* and *IRAK1* genes using an RT-qPCR technique in peripheral blood mononuclear cells of 33 TBC patients and 10 healthy individuals.

Results: Comparison between TBC patients and healthy individuals revealed statistically significant differences for all analyzed genes. The levels of expression of TNF-α and IL-6 mRNA were higher, while the level of IRAK1 mRNA was lower in the TBC group compared to controls. Moreover, a strong positive correlation was observed between *TNF*-α and *IL-6* gene expression. When clinical parameters were analyzed, increased levels of TNF-α mRNA were detected in patients with a longer duration of therapy (>2 months) compared to those with a shorter therapy duration (< 2 months), and in patients without anemia.

Conclusions: Our results indicate that the inflammatory genes we examined play a crucial role in the pathogenesis of tuberculosis, and that the expression of the *TNF-α* gene could be a marker for monitoring the clinical effect of the ant-tuberculosis drugs during therapy.

Key words: gene expression; monitoring therapy; pulmonary tuberculosis; cytokines.

J Infect Dev Ctries 2019; 13(5):419-425. doi:10.3855/jidc.10949

(Received 13 October 2018 – Accepted 05 February 2019)

Introduction

Despite being one of the oldest human diseases, tuberculosis (TBC) is still a social and economic burden, especially in underdeveloped countries. One-third of humanity has been infected, according to the 2014 WHO Global Tuberculosis Report, resulting in nine million cases of active tuberculosis and 1.5 million deaths [1]. Both types of immunity appear in the host protective immune response against *Mtb* infection, but cellular immunity is crucial, in which certain cytokines and Th1 cells play a key role [2]. Better understanding of the mechanisms involved in the immunopathogenesis of TBC as well as the understanding of the functions of the cytokine network involved in these processes, is important to advance the control and prevention of this disease [3].

Tumor necrosis factor alpha (TNF-α) is a cytokine produced by macrophages, lymphocytes, neutrophils, and some endothelial cells. It participates in the development of systemic inflammation by stimulating the acute phase. The ability of *Mtb* to decrease the production of TNF-α in human peripheral blood mononucleated cells (PBMCs), leads to the chronicity of the TBC infection [4]. TNF-α influences the inflammatory response resulting in secretion of other cytokines (IL-1 and IL-6), and in migration of immune cells to sites of inflammation by promoting production of chemokines and adhesion molecules [5]. Also, TNF-α increases the capacity of macrophages for phagocytosis and killing of *Mtb* and stimulates apoptosis of macrophages [6]. It has a role in the immunopathology of TBC, apart from creation and

maintenance of granulomas and thus control of the progression of disease, preventing its spread. TNF-α acts as a major mediator in the destruction of lung tissue [7,8]. Therefore, elevated TNF-α levels are associated with cachexia and necrosis, which means excessive inflammation [9,10]. The human TNF- α gene maps to chromosome 6 p.

Interleukin 6 (IL-6) is a multifunctional cytokine, whose gene is located in humans on chromosome 7. Increased production of IL-6 is an indicator of many chronic inflammatory diseases. Together with TNF-α and IL-1β it is found in early proinflammatory responses and is also known as an initiator of acute-phase protein production. The role of IL-6 in active TBC is predominantly negative, specifically, it accelerates the growth of *Mtb* in PBMCs and inhibits the production of TNF-α and IL-1β, which has a negative influence on the intracellular killing of microorganisms and development of granulomas [11], enhancing the spread of the disease. On the other hand, the role of IL-6 in the immune response to a subunit vaccine is positive [12].

Interleukin-1 receptor-associated kinase 1 (IRAK1) is an enzyme that functions in transmission of a signal crucial to the activation of nuclear factor kappa B (NF-kB). IRAK1 is a putative serine/threonine kinase, and NF-kB has an effect on the gene expression of cytokines (TNF-α, IL-6, IL-1β, IL-8). It also affects various micro-RNA (miRNA). Orchestrating the action of specific factors such as miR-146a inhibits the expression of IRAK1 and impairs NF-κB activity, which in turn suppresses expression of IL-6, IL-8, IL-1β, and TNF-α genes [13]. This is an example of negative feedback in cytokine regulation.

In this study, we investigated expression of *TNF-α, IRAK1* and *IL-6* genes and their correlation with clinical severity of TBC in a Serbian population. Analyses of changes in gene expression during *Mtb* infection as well during treatment could help in better understanding the mechanisms of host response to *Mtb*, and in identifying specific molecular markers of disease state and progression.

Methodology

Patients and controls

Patients were recruited at the Clinic for Pulmonary Diseases, Clinical Center of Serbia, between February 2013 and February 2014. The patient group consisted of 33 patients with active pulmonary TBC, with a mean age of 52.34 ± 17.61 years, 13 females and 20 males. Fourteen patients were smokers and two of them were ex-smokers. Family history of TBC was recorded in 18.2% of the patients. Massive changes in both lungs were found at X ray in 66.7% of patients; 15.1% were recurrent cases.

Laboratory examinations revealed positive inflammatory syndrome in 42,4% of patients, who had increased values of C-reactive protein, sedimentation and fibrinogen. Four patients had complications such as hemoptysis, and three patients had elevated levels of transaminases as a result of therapy.

The control group consisted of 10 healthy volunteers, who had no history of TBC and who were matched to the patient's group by ethnicity, age and gender. Expressions in control samples were homogeneous without deviation in terms of outliers. Because the differences were statistically significant, we did not increase the number of controls. We assume that the number of controls, one third of the number of test samples, is a good ratio for this type of analysis.

The exclusion criteria for patients with active TBC was presence of severe pathologies, including cancer, HIV infection, diabetes, cardiovascular diseases, atopy or autoimmune disease**s.** The exclusion criteria for the control group included the presence of active TBC or a pulmonary infection or other severe illness.

The study was performed in conformitye with the Declaration of Helsinki ethical guidelines. Informed consent was obtained from each patient and the protocol for the research project was approved by the Ethics Committee of the Clinical Centre of Serbia, permission number 2656/10. TBC diagnosis was as recommended by WHO: one positive result is required for a diagnosis of smear-positive pulmonary TBC. In our study we used sputum smear microscopy for all patients as well as culture. Twenty-three patients were direct smear positive, 10 were negative; all were culture positive, but while we were waiting for results of culture, we acquired positive Hains tests and MGIT identification for these patients. The Hain test has benefits when direct smears are negative. We followed the selection criteria from the national guidelines for microbiological diagnosis of tuberculosis, which was prepared and published within the project of the Ministry of Health of the Republic of Serbia "Control of Tuberculosis in Serbia".

All the TBC patients at the time of blood sampling were receiving anti-tuberculous therapy and were respondi ng to the first line treatment (isoniazid, rifampicin, ethambutol, pyrazinamide). Treatment takes place in two phases. In the first, the initial phase is aimed at achieving negative sputum smear microscopy and significant improvement of clinical findings. The second phase of treatment serves to

stabilize the process and prevent worsening of the disease. At the time of diagnosis, patients receive all four drugs for two months, followed by two drugs for four months (isoniazid and rifampicin). For those who have previously been treated for pulmonary tuberculosis, a longer course is applied. Our aim also was to measure any differences in expression of the genes between the two phases of TBC treatment.

Clinical data such as weight loss, anemia, sputum, diagnostic imaging such as thoracic X-Ray (XR), computed tomographic (CT) scan of the chest, and also, presence of hypoxia were analyzed for all patients. The diagnosis of TBC was made in those patients positive for acid-fast bacilli microscopy and/or culture. Patients were considered to have anemia when their hemoglobin was < 12 g/dL (women) or < 14g/dL (men). Diagnostic imaging was performed to analyze the existence or absence of pulmonary destruction. For assessment of severity of disease, we used the following scoring system: thoracic X-ray/chest CT finding of normal scored as 0, altered finding, without cavity = 1, with cavity = 2, without anemia = 0, anemia = 1, without hypoxia = 0, with hypoxia = 1, without weight loss = 0; with weight loss = 1 [14]. For the purpose of this study, the clinical severity of each patient was estimated with score obtained by summing these numerical values (1 would mean the lowest severity and 5, the highest).

RNA extraction and preparation of cDNA

Venous blood was collected with sodium citrate as anticoagulant, and peripheral blood mononuclear cells (PBMNC) were isolated by density-gradient centrifugation using Ficoll-Histopaque (GE Healthcare, Gothenburg, Sweden). Extraction of total RNA was performed using TRI Reagent Solution (Ambion, Foster, USA). Complementary DNA (cDNA) was synthesized starting from 1µg of total RNA. For cDNA synthesis RevertAid M-MuLV Reverse Transcriptase and random hexamer primers (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were used.

Relative quantification (RT-qPCR)

Expression level of *TNF*-α and *IL-6* genes was determined using following primers: *TNF*-α gene F: 5'-CCCAGGCAGTCAGATCATCTTC-3', R: 5'-AGCTGCCCCTCAGCTTGA-3'; *IL-6* gene: F: 5'-GCAAAGAGGCACTGGCAGAA-3', R: 5'-GGCAAGTCTCCTCATTGAATCC-3'. RT-qPCR was performed using SYBR Green Universal qPCR kit (KAPA Biosystems, Waltham, Massachusetts, USA).

GAPDH gene was used as endogenous control (primers: F: 5'-GTGAAGGTCGGAGTCAACG-3', R: 5'-TGAGGTCAATGAAGGGGTC-3'). For quantification of IRAK1 mRNA, standard gene expression assays (Hs01018347_m1; ThermoFisher scientific, Waltham, Massachusetts and KAPA PROBE qPCR kit (KAPA Biosystems, Waltham, Massachusetts USA) were used. *GAPDH* gene was used as endogenous control, with following primers: F: 5'-GAAGGTGAAGGTCGGAGT-3', R: 5'-GAAGATGGTGATGGGATTTC-3', probe: 5'-GGCTGAGAACGGGAAGCTTG-3'. Real-time PCR was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems, Waltham, Massachusetts USA). All experiments were performed in duplicate. Relative quantification analysis was performed by the comparative ddCT method, using median expression level of the healthy control group as calibrator for *TNF*-α and *IL-6* expression levels, and the HL-60 cell line as the calibrator for *IRAK1* gene expression (SDS 1.3.1.21, Applied Biosystems, Waltham, Massachusetts, USA). After initial denaturation which

Table 1. Baseline and clinical characteristics of patients with tuberculosis.

		TB patients (n = 33)	TB male (n = 20)	TB female (n = 13)	Control (n = 10)
Age		52.3 ± 17.6	51.4 ± 17.2	53.7 ± 18.8	53.6 ± 4.6
Gender	Male	20 (60.6%)	NA	NA	5 (50%)
	Female	13 (39.4%)			5 (50%)
TBC dg*	DM-	10(30.3%)	4 (20.0)	6 (46.2)	NA
	DM+	23 (69.7%)	16 (80.0)	7 (53.8)	
Therapy duration	< 2 month	28 (84.8%)	18 (90.0)	10 (76.9)	NA
	> 2 month	5 (15.2%)	2 (10.0)	3 (23.1)	
Presence of cavitation		21 (63.6%)	13 (65.0)	8 (61.5)	NA
Anemia		12 (36.4%)	10 (50.0)	2 (15.4)	NA
Hypoxia		4 (12.1%)	2 (10.0)	2 (15.4)	NA
Weight loss		21 (63.6%)	14 (70.0)	7 (53.8)	NA
Recurrence of TB	new	28 (84.9%)	16 (80.0)	12 (92.3)	NA
	recidivism	5 (15.1%)	4 (20.0)	1 (7.7)	
Complication		7 (21.2%)	4 (20.0)	3 (23.1)	NA

*DM-Direct sputum smear microscopy; TB-Tuberculosis; TBC dg-tuberculosis diagnosis; NA-non applicable.

was performed according to manufacturer's instructions, all reactions were done at 95°C/15s; 60°C/ 1min for 40 cycles. We did not measure serum concentrations of these cytokines.

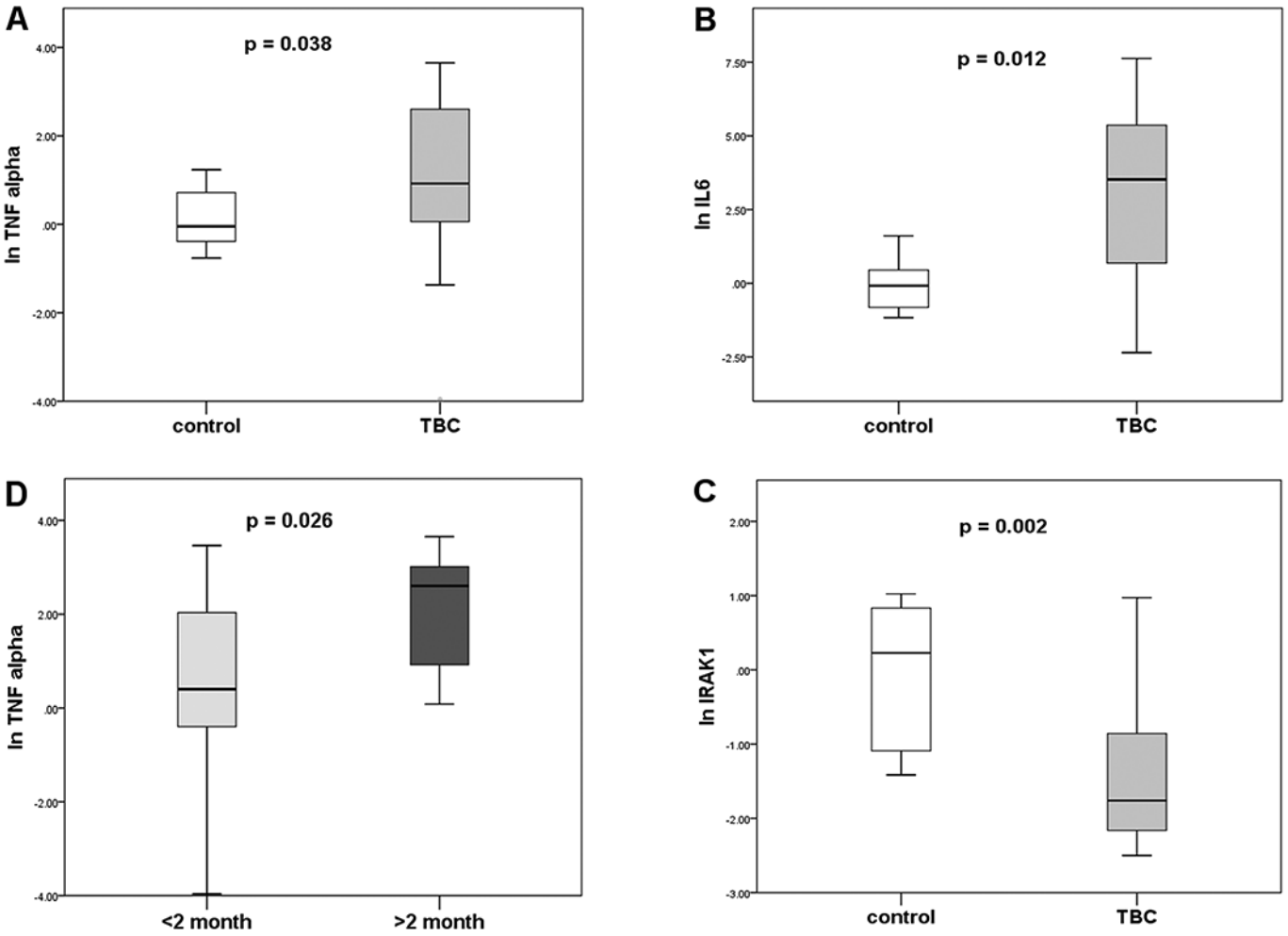
Statistical analysis

Data are analyzed by descriptive and analytic statistics. Testing between groups was performed using Mann-Whitney U test. Spearman`s rank-order correlation coefficient (r) was used to test relationships between variables. Differences were considered to be statistically significant when the p-value was less than 0.05. Nominal data are presented as n (%). Numerical data were tested for normality of distribution (graphic and mathematical methods). According to normality of distribution, data were presented as means ± sd (for normally distributed data) or medians (25^{th}-75^{th} percentile) for skewed data. For graphic presentation, skewed data were log transformed. The figure parameters shown, like relative expression data, were analyzed using SPSS for Windows 20.0 software (SPSS, Inc, Chicago, IL, USA).

Results

Baseline and clinical characteristics of the patients are presented in Table 1. There was no difference regarding age and gender between the TBC and control groups (p = 0.717; p = 0.551). Most of our patients had been on anti-tuberculosis therapy for less than two months. More than 2/3 of patients had cavitation and weight loss. When we compared clinical characteristic between men and women, there was no difference with regard to age (p = 0.726), TBC diagnosis (p = 0.110), presence of cavitation (p = 0.840), days of therapy (p = 0.306), hypoxia (p = 0.643), weight loss (p = 0.346), or recidivism (p = 0.335). There was a significantly higher frequency of anemia in men (p = 0.043).

Figure 1. Relative expression levels of TNF-α, IL-6 and IRAK1 genes in TBC patients and healthy individuals. A) TNF-α; B) IL-6; C) IRAK1. Data are shown as box plots. The line inside the box represents the median. Each box represents the 25th to 75th percentiles of data. P values were calculated by Mann–Whitney U test. A p value of < 0.05 is taken to indicate statistical significance.



Expression level of TNF-α, IL-6 and IRAK1 genes

When expression levels of *TNF-α, IL-6* and *IRAK1* and genes of TBC patients were compared to those of the control group, statistically significant differences were observed for all the analyzed genes. The expression levels of *TNF*-α were median 2.51 (1.06-13.47 *vs* median 0.96 (0.68-2.05); p = 0.038. For *IL-6, the* median was 33.91 (1.98-212.77) *vs* median 0.92 (0.44-1.57); p = 0.012. (Figure 1 A, B). These two were thus higher in the TBC group. The levels of mRNA for the *IRAK*1 gene were median 0.17 (0.12-0.42) *vs* median 1.26 (0.34-2.30); p = 0.002, thus significantly lower in the TBC group compared to the control group (Figure 1 C).

Comparison of expression levels of TNF-α, IL-6 and IRAK1 genes according to clinical characteristics of TBC patients

We further analyzed the *TNF*-α, *IL-6* and *IRAK1* gene expression in relation to to clinical characteristics. Expression of *TNF*-α gene was similar between men and women (median 1.0 (0.7-2.1) *vs* 2.5 (1.1-13.5), p = 0.912). Analysis of *TNF*-α gene expression showed increased levels of *TNF*-α mRNA in patients with longer duration of therapy (>2 months) (median 14.79 (13.47-34.59) compared to those with shorter therapy duration (< 2 months) (median 1.89 (0.96-9.19); this difference was statistically significant (p = 0.026). When different clinical parameters were analyzed, there was a significant difference between the groups of patients with and without anemia (median 14.79 (13.47-34.59) *vs* median 1.89 (0.96-9.19); p = 0.026). (Figure 1 D).

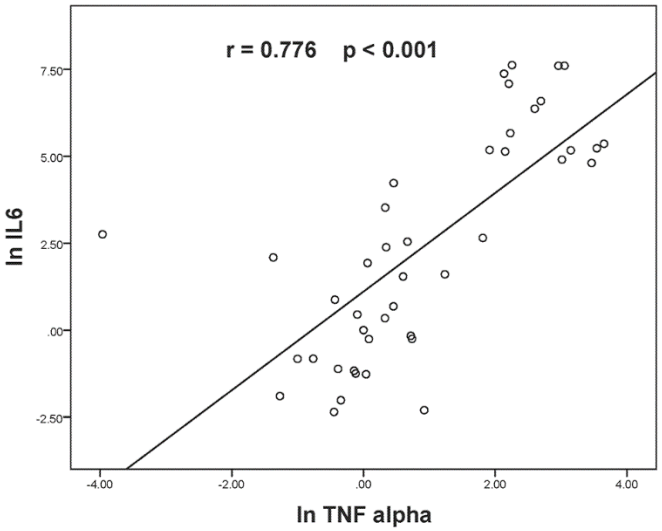
When the levels of *IL-6* gene were analyzed, we found a trend of increased expression in women as compared to men (median 33.9 (2.0-212.8) *vs* 0.9 (0.4-1.6), p = 0.071), in patients with complications (median 176.15 (68.74-583.45) *vs* 11.79 (0.44-187.35); p = 0.074) and in patients without anemia (median 135.07 (12.73 *vs* 0.61 (0.22-606.35); p = 0.056), but these differences did not reach statistical significance.

Expression of the *IRAK1* gene was similar between men and women (median 0.2 (0.1-0.9) *v*s 0.2 (0.1-0.4), p = 0.839). Expression levels of the *IRAK1* gene were similar between patients with different days of therapy, anemia, hypoxia; no statistical significant difference was observed between these groups.

Correlation of expression level of TNF-α, IL-6 and IRAK1 genes

When we evaluated the expression of *TNF-α, IL-6* and *IRAK1* genes in TBC patients, statistically significant positive correlation was observed between expression of the *TNF*-α and *IL-6* genes (r = 0.776, p < 0,001) (Figure 2). There was no correlation between expression of *TNF*-α and *IRAK1* genes (r = 0.163, p = 0.364), and *IRAK1* and *IL-6* (r = 0.027, p = 0.880).

Figure 2. Correlation between expression of TNF-α and IL-6 genes. A strong positive correlation was observed between mRNA level of TNF-α and IL-6. Each symbol represents an individual TBC patient. The correlation



Discussion

The respiratory tract is the main target of *Mtb* infection. Macrophages are responsible for intracellular killing of *Mtb*, but they also process and display antigen to lymphocytes and are therefore included in the effector phase of the specific immune response. At the same time, they are the principal target cells. After macrophages phagocytose *Mtb*, that leads to secretion of various cytokines, particularly proinflammatory cytokines, from the same macrophages and indirectly, of other immune cells. These cytokines are responsible for both the initial and the delayed expression of inflammation. Thus, an immune response against *Mtb* infection is mediated primarily by macrophages and T lymphocytes (delayed type hypersensitivity, or IV-T cell-mediated type) which plays a key role in the process of gaseous necrosis and formation of granulomas. The response causes tissue damage but at the same time, limits and localizes inflammation. Therefore, a better understanding of the interaction between *Mtb* and macrophages may contribute to the better control of TBC [15]. Despite this response, in early stages of the innate immune response, *Mtb* can avoid the macrophage defense functions and actively grow and replicate in the host [16]. In addition to

successfully avoiding phago-lysosomal degradation, *Mtb* can also inhibit the production of proinflammatory cytokines (TNF-α and IL-1), which can reduce the responses by infected cells facilitate survival within the cells [17]. The regulation of early immune responses by affecting the induction of proinflammatory cytokines is important for the outcome of disease [12,18].

It is well known that TNF-α has an important role in maintaining granulomas and protection of reactivation of latent TBC.

Our results showed increased levels of TNF-α in PBMNs of TBC patients compared to healthy individuals. Moreover, there were increased levels of TNF-α in patients with longer therapy duration (> 2 months) compared to those treated for less than two months. These results point to the significance of TNF-α in the defense against mycobacteria. Previous studies have reported controversial results concerning the levels of TNF-α during antitubercular therapy. Tang *et al*., [19] and Portales-Pérez *et al*. [20] found decreased TNF-α levels in TBC patients after therapy. However, Moura *et al*. [21] did not observe significant differences in TNF-α levels after treatment. These studies in a certain way confirm the role of TNF-α in both the pathophysiology and the protective immunity against TBC.

Both the anti-inflammatory and the proinflammatory roles of IL-6 could contribute to the pathogenesis of TBC [22]. Our results showed increased levels of IL-6 mRNA in the patient group compared to healthy subjects. Levels of IL-6 were found to be somewhat increased in women compared to men, as well as in patients with complications and in patients without anemia, but these differences did not reach statistical significance.

Mycobacterial components activate the NF-kB signaling pathway, leading to miRNA expression. The activity of various miRNAs, resulting in the production of a number of cytokines, depends on level of transcription. Expression of negative control factors may be initiated by activation of the IL-1 receptor (IL-1R), working on the principle of negative feedback. There are regulatory mechanisms working to inhibit IL-1R at all levels of the transmission signal. Thus, control by miRNA146a,targeting its downstream molecules such as IRAK1, can limit the extent of the inflammatory reaction [15] and may be switched off when there is no infection. This system could, on the one hand, avoid the additional damage caused by excessive immune responses; on the other hand, it could also be utilized by mycobacteria to facilitate their better replication [23]. The lower levels of IRAK1 found in our study support the strategy of *Mtb* to downregulate inflammatory processes in the granuloma, which is in concordance with other studies in which IRAK1 down-regulation robustly reduced inflammatory responses [24,25].

It has long been assumed that the central role of Th1 cells in the defense against TBC is due to their ability to activate macrophages and to stimulate phagocytosis, the maturation of the phagosome, production of reactive nitrogen intermediates, and antigen presentation. The differentiation of Th17 and regulatory T cells (Treg) depends on the balance of IL-6, IL-1β and TGF-β. It is possible that there is involvement of IL-6 and IL-1β in Th17 and Treg cell differentiation. Therefore, further studies of IL-6 and IL-1β expression and activities may be needed to explain the interaction among these factors in TBC infection, pathogenesis and treatment [26,27].

Conclusion

The results of our study suggests that TNF-α gene expression is an important indicator to evaluate both the severity of TBC and the clinical response to antitubercular drugs (DOTS) over a period of prescribed drug therapy.

Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development, Republic of Serbia (Grant No. III41004).

The study was approved by the Ethics Committee of Clinical Center of Serbia. Written informed consent was obtained for all patients.

References

1. World Health Organization ((2014 Global tuberculosis report 2014. Available: http://www.who.int/tb/publications/global_report/en/. Accessed 25 April 2019.
2. Kinjo Y, Kawakami K, Uezu K, Yara S, Miyagi K, Koguchi Y, Hoshino T, Okamoto M, Kawase Y, Yokota K, Yoshino K, Takeda K, Akira S, Saito A (2002) Contribution of IL-18 to Th1 response and host defense against infection by *Mycobacterium tuberculosis*: a comparative study with IL-12p40. J Immunol 169: 323–329.
3. Wang CH, Lin HC, Liu CY, Huang KH, Huang TT, Yu CT, Kuo HP (2001) Upregulation of inducible nitric oxide synthase and cytokine secretion in peripheral blood monocytes from pulmonary tuberculosis patients. Int J Tuberc Lung D5:283-291.
4. Jönsson B, Ridell M, Wold AE (2013). Phagocytosis and cytokine response to rough and smooth colony variants of *mycobacterium abscessus* by human peripheral blood mononuclear cells. APMIS 121: 45-55.
5. Cavalcanti YVN, Brelaz MCA, Neves JK, Ferraz JC, Pereira VR (2012). Role of TNF-alpha, IFN-gamma, and IL-10 in the development of pulmonary tuberculosis. Pulm Med: 745483

6. Keane J, Remold HG, Kornfeld H (2000). Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. J Immunol 164: 2016–2020.
7. Dambuza I, Allie N, Fick L, Johnston N, Fremond C, Mitchell J, Quesniaux VF, Ryffel B, Jacobs M (2008) Efficacy of membrane TNF mediated host resistance is dependent on mycobacterial virulence. Tuberculosis 88: 221–234.
8. Flynn JL, Chan J, Lin PL (2011) Macrophages and control of granulomatous inflammation in tuberculosis. Mucosal Immunol 4: 271–278.
9. Bekker LG, Moreira AL, Bergtold A, Freeman S, Ryffel B, Kaplan G (2000) Immunopathologic effects of tumor necrosis factor alpha in murine mycobacterial infection are dose dependent. Infect Immun 68: 6954–6961.
10. Ehlers S, Benini J, Held HD, Roeck C, Alber G, Uhlig S (2001) αβ T cell receptor-positive cells and interferon-γ, but not inducible nitric oxide synthase, are critical for granuloma necrosis in a mouse model of mycobacteria-induced pulmonary immunopathology. J Exp Med 194: 1847–1859.
11. Zhang Y, Broser M, Rom WN (1994) Activation of the interleukin 6 gene by *Mycobacterium tuberculosis* or lipopolysaccharide is mediated by nuclear factors NF-IL6and NF-kB. Proc Natl Acad Sci USA 91: 2225-2229.
12. Martinez AN, Mehra S, Kaushal D (2013). Role of interleukin 6 in innate immunity to *Mycobacterium tuberculosis* infection. J Infect Dis 207: 1253-1261.
13. Lu LF, Liston A (2009) MicroRNA in the immune system, microRNA as an immune system. Immunology 127: 291–298.
14. Andrade Junior DR, Santos SA, Castro ID, Andrade DR (2008) Correlation between serum tumor necrosis factor alpha levels and clinical severity of tuberculosis. Braz J Infect Dis 12: 226-233.
15. Li S, Yue Y, Xu W, Xiong S (2013) MicroRNA-146a represses Mycobacteria-induced inflammatory response and facilitates bacterial replication via targeting IRAK-1 and TRAF-6. PLoS ONE. 8: e81438.
16. Flynn JL, Chan J (2003) Immune evasion by *Mycobacterium tuberculosis*: living with the enemy. CurrOpinImmunol15:450–455.
17. Singh Y, Kaul V, Mehra A, Chatterjee S, Tousif S, Dwivedi VP, Suar M, Van Kaer L, Bishai WR, Das G (2013) *Mycobacterium tuberculosis* controls MicroRNA-99b(miR-99b) expression in infected murine dendritic cells to modulate host immunity. J Biol Chem. 288: 5056–5061.
18. Cooper AM (2009). Cell-mediated immune responses in tuberculosis. Annu Rev Immunol 27: 393–422.
19. Tang S, Xiao H, Fan Y, Wu F, Zhang Z, Li H, Yang Y (2002) Changes of proinflammatory cytokines and their receptors in serum from patients with pulmonary tuberculosis. Zhonghua Jie He He Hu Xi Za Zhi. 6: 325-329. [Article in Chinese]
20. Portales-Pérez DP, Baranda L, Layseca E, Fierro NA, de la Fuente H, Rosenstein, González-Amaro R (2002) Comparative and prospective study of different immune parameters in healthy subjects at risk for tuberculosis and in tuberculosis patients. Clin Diagn Lab Immunol 9:299-307.
21. Moura EP, Toledo VPCP, Oliveira MHP, Spíndola-de-Miranda S, Andrade HM, Guimarães TMPD (2004) Pulmonary tuberculosis: evaluation of interferon-gamma levels as an immunological healing marker based on the response to the Bacillus Calmette-Guerin. Mem Inst Oswaldo Cruz 99: 283-287.
22. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S (2011) The pro-and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 5: 878-888.
23. Means TK, Wang S, Lien E, Yoshimura A, Golenbock DT, Fenton MJ (1999) Human toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. J Immunol163: 3920–3927.
24. Nahid MA, Pauley KM, Satoh M, Chan EKL (2009) miR-146a is critical for endotoxin-induced tolerance: implication in innate immunity. J BiolChem284:34590-34599.
25. Zeng Z, Gong H, Li Y, Jie K, Ding C, Shao Q, Liu F, Zhan Y, Nie C, Zhu W, Qian K (2013) Upregulation of miR-146a contributes to the suppression of inflammatory responses in LPS-induced acute lung injury. Exp Lung Res 39:275-282.
26. Saini C, Kumar P, Tarique M, Sharma A, Ramesh V (2018) Regulatory T cells antagonize proinflammatory response of IL-17 during cutaneous tuberculosis. J Inflamm Res 11: 377-388.
27. Shu CC, Wu MF, Wang JY, Lai HC, Lee LN, Chiang BL, Yu CJ (2017) Decreased T helper 17 cells in tuberculosis is associated with increased percentages of programmed death ligand 1, T helper 2 and regulatory T cells. Respir Res 18: 128.

Corresponding author

Sonja Pavlovic, PhD
Laboratory for Molecular Biomedicine
Institute of Molecular Genetics and genetic Engineering,
University of Belgrade
Vojvode Stepe 444a,
11010 Belgrade, Serbia
Phone: +381637708372
Fax: +381 11 3975 808
E-mail: sonya@sezampro.rs

Conflict of interests: No conflict of interests is declared.