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Original Article

Blood Cytokine Responses to Early Secreted Protein Antigen-6/Culture Filtrate Protein-10 Tuberculosis Antigens 2 Months after Antituberculosis Treatment among Patients with Drug-Susceptible Pulmonary Tuberculosis

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Abstract

Background: Human tuberculosis is a chronic inflammatory disease caused by *mycobacterium tuberculosis*. Pulmonary tuberculosis is the result of the failure of host immune system to control *mycobacterium tuberculosis*. The aim of the study was to asses the changes of the cytokines in active pulmonary tuberculosis patients before and after the use of anti-TB therapy. **Methods:** Multiple cytokine responses in active tuberculosis (TB) patients were investigated in this study following anti-TB drug therapy after 2 months. Ninety-six participants with pulmonary TB were engaged in the study between May 2018 and October 2018. Samples of blood were taken early before treatment at 0 and 2 months after using anti-TB therapy. The levels of interferon-gamma (IFN)-γ, interleukin-4 (IL-4), IL-6, IL-10, and tumor necrosis factor (TNF)-α in whole blood plasma collected from the QuantiFERON-TB Gold Plus were measured. **Results:** Compared with baseline levels, TNF-α, IL6 and IL10 were significantly lower following treatment whereas the IFN-γ and IL-4 increased significantly after treatment. The responses of five cytokines varied significantly after treatment ($P < 0.0001$) where IFN-γ was highest compared to other cytokines with 123.6%, AUC=0.757 and $P < 0001$, TNF-α AUC: 0.529 and $P = 0.743$, IL-4 AUC:0.557 and $P = 0.514$, IL-6 AUC:0.629 and $P = 0.047$, IL-10 AUC:0.549 and $P = 0.581$. **Conclusion:** It is concluded that changes of cytokines that observed during the treatment of TB patients play a very important role in monitoring pulmonary TB and can be suitable biomarkers to assess the effectiveness of anti-TB therapy in patients with TB.

Keywords: Antituberculosis therapy, biomarkers, cytokines, pulmonary tuberculosis, QuantiFERON-TB Gold Plus

Introduction

Tuberculosis (TB) infection remains a major challenge globally.[1] It is one of the leading infectious causes of death worldwide[1,2] responsible for an estimated 1.7 million deaths.[3,4] In 2017, TB caused an estimated 1.3 million deaths among HIV-negative people, and there were an additional 300,000 deaths from TB among HIV-positive people.[4] Tanzania is one of the highest burden countries with TB (among 30 countries) in the world.[5]

Mechanisms underlying host defense to *Mycobacterium* infection is poorly understood.[6,7] In response to infection, host immune cells secrete a number of cytokines and chemokine signals[7,8] which play active roles in initiation and regulation of the immune response at various stages of disease development.[7,9] Following anti-TB therapy, research has shown that the cytokine-mediated cell signaling is altered, which ultimately leads to recovery of the TB infection.[7] The knowledge of understanding different cytokines at different stages of TB infection is necessary in knowing the host response after treatment. TB is an old disease, but still, there is an urgent need of rapid and precise monitoring tools to assess

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the early response of anti-TB treatment. Sputum culture status is the only acceptable biomarker for TB treatment response,[10] but in resource-poor settings, the culture is unavailable. In additional waiting for the culture results for 6- 8 weeks to monitor the response of anti-TB is a challenge. The Ziehl–Neelsen sputum for smear is the available tool for diagnosis and the same time monitoring in resource-poor setting even though its limitations are well published.[11] Sputum for smear is not useful for monitoring anti-TB treatment response as it is unable to distinguish live from dead bacilli.[12] Using sputum has its limitation as for the patients with extrapulmonary TB and in children. Furthermore after 2 months of anti-TB medication to get quality sputum sample is difficult due to cough cessation or reduction, patients end up giving inadequate volume of sputum or excessive saliva. Given the limitations of using sputum for monitoring treatment, blood has been suggested as alternative.

Cell-mediated immunity plays an important role in human host defenses against TB.[13,14] The essential role of T cells in protection against TB infection has been well-documented.[13,14] In this research, we studied changes of different cytokines in pulmonary TB patients following anti-Tb treatment after 2 months.

Methods

Ethical approval

The study was approved by the National Institute of Medical Research in Tanzania. All participants were given written informed consent.

Study design and population

This was a prospective cohort study involving two sites. Participants with pulmonary TB were enrolled between May 2018 and October 2018. Patients were considered eligible for inclusion if they were >18 years of age and had sputum smear-positive pulmonary TB detected by GeneXpert. Exclusion criteria includes participants who refused to provide informed consent, participants with extra pulmonary TB, and participants who were very sick i.e unconscious. The QuantiFERON-TB Gold Plus (QFT-Plus) was performed in each patient from the baseline (before starting treatment) and at 2 months of anti-TB medication. The levels of interferon-gamma (IFN-γ), interleukin-6 (IL-6), IL-10, IL-4, and tumor necrosis factor-α (TNF-α) were measured consecutively in the plasma harvested from the QFT-Plus tubes. All methods were carried out in accordance with the manufacturer's instructions.

Diagnosis and pulmonary tuberculosis treatment

The diagnosis of pulmonary TB was made based on clinical and microbiological status. Active pulmonary TB was confirmed using GeneXpert as derived from respiratory specimen (sputum). The standard regimen of fixed-dose combination of rifampicin, isoniazid, ethambutol, and pyrazinamide was used, and the dosage was given according to body weight of the patients. The duration of treatment was 6 months.

Plasma preparation using QuantiFERON-Tuberculosis Gold Plus

The preparation of plasma samples was performed using the QFT-TB Gold Plus tubes according to the manufacturer's instructions. For each patient, 1 ml of blood by venipuncture directly into each QFT-Plus blood collection (nil, TB1, TB2, and mitogen) where TB1 and TB2 tubes had specific TB antigen early secreted protein antigen-6 (ESAT-6), culture filtrate protein-10 (CFP-10), and the blood was incubated within 16 h at 37°C of blood collection. The plasma samples were harvested after centrifugation and stored below −20°C until use.

Human 5-Plex Magnetic Luminex Assay

The plasma which was stored below −20°C was removed from the freezer and kept to room temperature until all ice were completely melted. The assay of all cytokines, such as IFN-γ, IL-6, IL-4, IL-10, and TNF-α, were performed using Human Premixed Multi-Analyte Kit with the catalog number LXSAHM, and multiplexing analysis was done using Luminex™ 200 system. All the samples and reagents were prepared according to the manufacturer's protocol.

Statistical analysis

The analysis of data consisted of computing descriptive statistics for each study variable and then comparing them. Analysis of descriptive and inferential statistics was done using GraphPad Prism version 3.02. The baseline data were compared with final data using Wilcoxon matched-pair test. The responses of five cytokines were compared using Friedman test – a repetitive nonparametric one-way ANOVA alternative. In each case, a significant level was set at 5% with 95% confidence interval. The power of cytokines to discriminate infection was tested by doing receiver operating characteristic (ROC) curve, where area under the curve (AUC) of 0.5 was considered. ROC curve analysis was done using MedCalc version 18.10−© 1993–2018 MedCalc Software bvba.

Results

The study involved 96 people with pulmonary TB. A TB case was defined by scores calculated by combination of symptoms and smear results. The results from the study showed that all patients (equivalent to 100%) had symptoms of TB before medication was initiated. After 2 months of medication, patients with TB symptoms dropped significantly to 15.6%. Similarly, median case scores decreased from 3.99 to 0.18 ($P < 0.0001$) [Table 1 and Figure 1].

As indicated in Table 1 and Figure 2, the result showed significant changes of initial levels of each cytokine ($P < 0.0001$). Treatment of TB resulted into increased IFN-γ from 6.75 to 16.25 pg/ml and IL-4 from 2.5 to 2.8 pg/ml and the decreased TNF-α from 122 to 76 pg/ml, IL-6 from 88.25 to 56.5 pg/ml, and IL-10 from 89.7 to 61 pg/ml [Figure 2]. The percentage

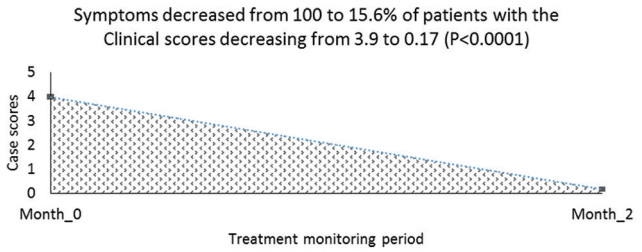

Figure 1: Graphical presentation of decrease of tuberculosis case scores before and after treatment within 2 months

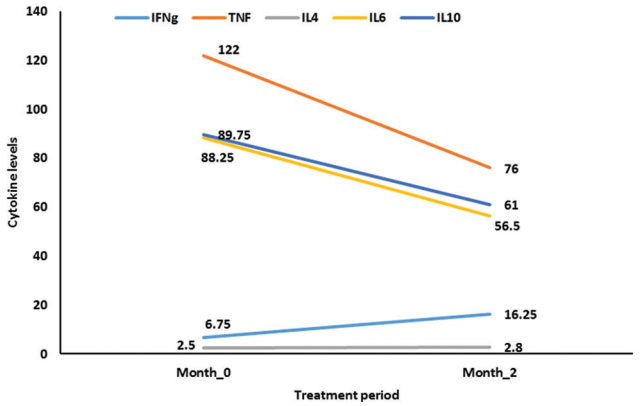

Figure 2: Changes of cytokine levels after medication (from month 0 to month 2). IFN-γ: Interferon-gamma, TNF: Tumor necrosis factor, IL-4: Interleukin-4, IL-6: Interleukin-6, IL-10: Interleukin-10

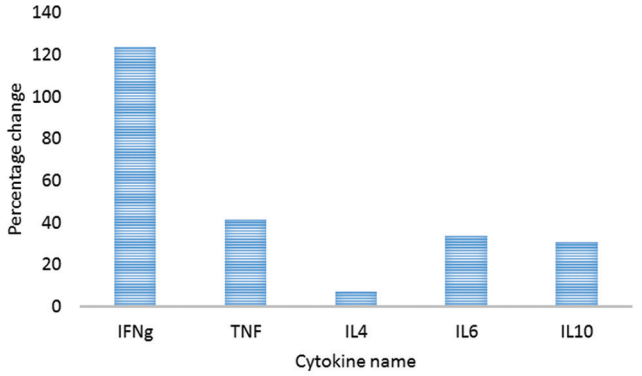

Figure 3: Comparative percentage changes of the levels of cytokines recorded after 2 months of tuberculosis treatment. IFN-γ: Interferon-gamma, TNF: Tumor necrosis factor, IL-4: Interleukin-4, IL-6: Interleukin-6, IL-10: Interleukin-10

response for each cytokine is presented in Table 2 and Figure 3. As indicated in Table 2, the responses for five cytokines varied significantly after treatment ($P < 0.0001$), where IFN-γ was highest compared to other cytokines. The percentage responses are summarized in Figure 3, indicating that IFN-γ had the highest response of 123.6%, followed by TNF-α (41.5%), IL-6 (33.7%), IL-10 (30.68%), and IL-4 (6.9%). The ROC curves were used to analyze the discriminating power of the cytokines against the health status of study patients after medication. We saw that IFN-γ (AUC = 0.757, $P < 0001$) and IL-6 (AUC = 0.629, $P = 0.047$) had the ability to distinguish sick and healthy individuals. The corresponding sensitivity and specificity of each cytokine is shown in Table 3 and Figures 4-9.

Discussion

The profile of cytokine secretion in response to mycobacteria infection has been studied to identify immunological changes in active TB. Changes in cytokines could be designed as important biomarkers for monitoring progress of the patients with TB.

There have been several studies that explained about the importance of cytokines as a biomarker for monitoring the progress of the patients using anti-TB. Previously, the most studied cytokine was TNF-α. One of the largest studies found that TNF-α increased after the use of anti-TB treatment.[15] On Other studies where Tumor necrosis factor alpha was stimulated with TB- specific antigens reported to decrease after the use of anti-TB medication.[16-19] In our study, we found that after the stimulation of the cells with TB-specific antigens ESAT-6/CFP-10, TNF-α decreased following anti-TB therapy.

IL-10 is an important anti-inflammatory cytokine reported to affect multiple cell types, such as macrophages, monocytes, dendritic cells, CD4+ T-cells, and CD8+ T-cells.[13,20] It inhibits CD4+ T-cell responses by inhibiting the antigen-presenting cell function of TB-infected cells.[13,21] Recently, the secretion of *Mycobacterium tuberculosis* (MTB)-enhanced intracellular survival protein from MTB cells was reported, which possibly increases IL-10 expression.[13,22] Findings from different studies that have reported the role of IL-10 in TB infection are inconsistent.[15,23-25] One study reported that IL-10 increased with treatment.[15] On the other hand, another longitudinal study reported the reduction in IL-10 levels after being in treatment.[23] In this study, we found that IL-10 after being stimulated with TB-specific antigens decreased after anti-TB treatment. This decrease may be due to modified cytokine expression in infected individuals after treatment.[13]

IL-6 appears to be the major cytokine elaborated by mycobacteria-infected peritoneal macrophages(PMs) *in vitro* and thus may function as a potential biomarker of mycobacterial infection, either stand-alone or along with other cytokines.[26] Several studies have investigated the responses of IL-6 during anti-TB therapy.[13] Some studies reported decreased IL-6 during the intensive phase of anti-TB therapy.[1,18,27] Other studies (two of which predominantly included HIV-positive patients) found no change of IL-6.[1,28,29] In this study, IL-6 tends to decrease following anti-TB treatment. The increased levels of these three cytokines, such as IL-6, IL-10, and TNF-α, in active TB patients and their decrease following anti-TB treatment might be due to temporary stimulation of regulatory immune mechanisms that can lead to subsequent repair of immune homeostasis.

Table 1: Descriptive summary of cytokine's levels before and after medication

Statistics	Case scores		IFN-λ		TNF-α		IL4		IL6		IL10	
	Baseline	Final	Baseline	Final	Baseline	Final	Baseline	Final	Baseline	Final	Baseline	Final
Sample size	96	96	96	96	96	96	96	96				
Symptomatic patients	96	15										
Minimum	3	0	0.5	3.5	87	34.5	0.2	0.5	40	30	44	36
25% percentile	4	0	3	10.25	105	63.5	1.6	1.7	75.75	46.5	75	50.75
Median	4	0	6.75	16.25	122	76	2.5	2.8	88.25	56.5	89.75	61
75% percentile	4	0	38.25	50	192.5	85.5	5.2	5.2	105.5	69.75	101	70
Maximum	5	2	63	80	12265	10988	55	58	162	90	411	275
Mean	3.99	0.18	16.65	28.49	409.5	289.2	4.87	5.13	91.61	57.92	92.26	63.09
Std. Deviation	0.55	0.41	18.21	22.75	1654	1487	7.9	8.17	24.29	15	37.8	24.99
Std. Error	0.06	0.04	1.86	2.32	168.8	151.8	0.81	0.83	2.48	1.53	3.86	2.55
Lower 95% ci	3.878	0.09396	12.96	23.88	74.38	12.06	3.27	3.475	86.69	54.88	84.6	58.03
Upper 95% ci	4.102	0.2602	20.34	33.1	744.5	590.6	6.472	6.784	96.54	60.95	99.92	68.15
P	*P*<0.0001		*P*<0.0001		*P*<0.0001		*P*<0.0001		*P*<0.0001		*P*<0.0001	

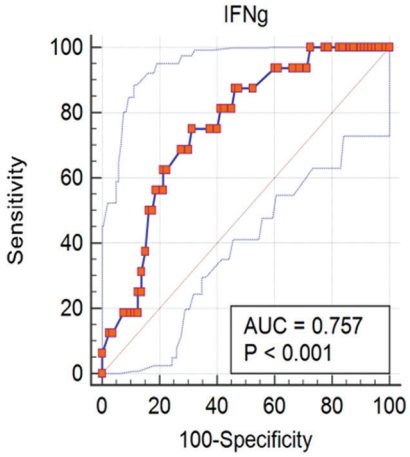

Figure 4: Sensitivity and specificity for interferon gamma which also show area under the curve in receiver operating characteristics with *P* value. IFNg: Interferon gamma, AUC: Area under the curve, *P*: *P* value

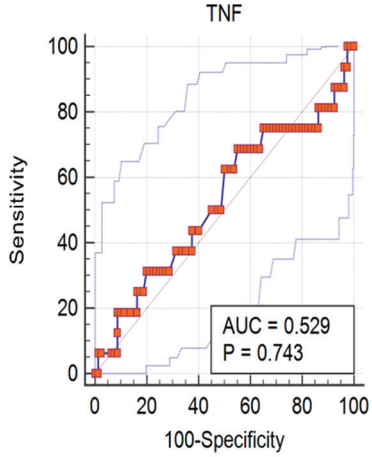

Figure 5: Sensitivity and specificity for tumor necrosis factor which also show area under the curve in receiver operating characteristics with *P* value. TNF: tumor necrosis factor alfa, AUC: Area under the curve, *P*: *P* value

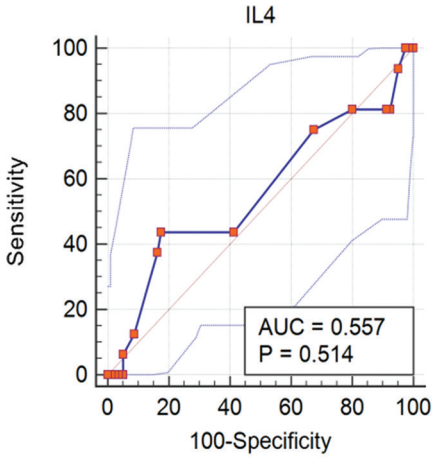

Figure 6: Sensitivity and specificity for IL-4 which also show area under the curve in receiver operating characteristics with *P* value. IL-4: Interleukin 4, AUC: Area under the curve, *P*: *P* value

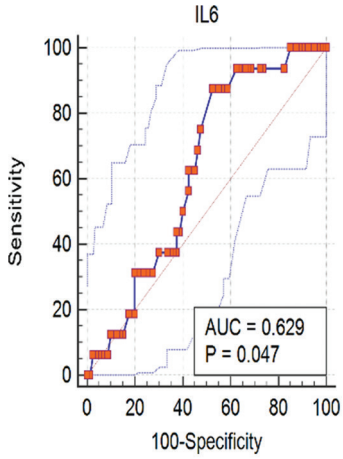

Figure 7: Sensitivity and specificity for IL-6 which also show area under the curve in receiver operating characteristics with *P* value. IL-6: Interleukin 6, AUC: Area under the curve, *P*: *P* value

Table 2: Friedman test (non-parametric Repeated One-way ANOVA) results to compare median percentage responses across the five cytokines

Descriptive statistics of % changes	IFN-λ	TNF-α	IL4	IL6	IL10
Number of values	96	96	96	96	96
Minimum	11.29	10.37	-13.04	10	7.143
Median	123.6	41.49	6.905	33.69	30.68
Maximum	3100	94.68	166.7	65.91	56.12
Mean	215.2	44.92	14.77	35.58	30.8
Std. Deviation	355.7	18.52	27.23	11.99	8.509
Std. Error	36.3	1.89	2.779	1.224	0.8685
Lower 95% CI	143.1	41.17	9.251	33.15	29.08
Upper 95% CI	287.3	48.67	20.29	38.01	32.53
Friedman test					
P	*P*<0.0001				
Friedman statistic	204.9				

Dunn's Multiple Comparison Test	Difference in rank sum	*P*	Summary
IFNg %Change vs TNF% Change	113	*P*<0.001	***

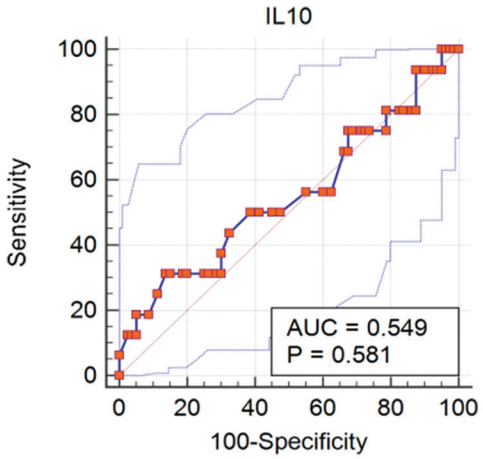

Figure 8: Sensitivity and specificity for IL-10 which show area under the curve in receiver operating characteristics with *P* value. IL-10: Interleukin 10, AUC: Area under the curve, *P*: *P* value

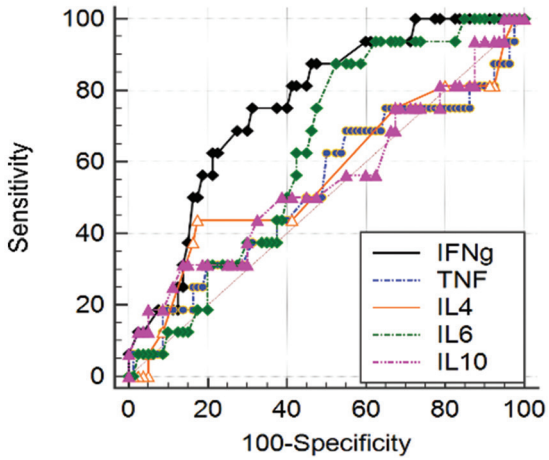

Figure 9: Combined for all cytokines. Sensitivity and specificity of 5 cytokines which also show area under the curve in receiver operating characteristics with *P* value of each cytokine. IFN-γ: Interferon-gamma, TNF: Tumor necrosis factor, IL-4: Interleukin-4, IL-6: Interleukin 6, IL-10: Interleukin 10, AUC: Area under the curve, *P*: *P* value

In our study, the level of IFN-γ found from the participants with active pulmonary TB increased significantly after 2 months of anti-TB therapy. Several studies reported that the pleural fluids of patients with TB have a high number of IFN-γ-induced cells;[30,31] this suggest these cells move to the pleural tissue and lungs during the active phase of the disease, and hence, they may be low temporarily in the blood. This explains the reason of lower level of IFN-γ obtained before the use of medication (anti-TB) in our study. To support our results there are other studies reported the increase of Interferon gamma after the anti-TB therapy.[10,25,32,33]

In this study, the increase of IFN-γ after 2 months could be due to the use of anti-TB therapy that cleared out the *Mycobacterium,* and this suggests the important role of IFN-γ in the response of immunity against MTB.

We found a slight increase of IL-4 in our study. Some studies reported an increased level of IL-4 in patients with pulmonary TB, especially those who had cavities in their lungs.[9,34,35] Still, it is not well explainable if IL-4 mirrors disease activity in human.[36,37] Many studies reported that no IL-4 level was detected in patients with TB, and also, there is no significant difference in IL-4 levels between patients with TB and healthy individuals.[7,38-42]

Our study had some limitation such as small sample size (only 96 participants) and this is due to limited cases that met inclusion criteria and also few cytokines tested (5 cytokines). We could not continue with the study further up to 5 months of anti-TB where another blood samples could be taken which would allow us to observe the changes in cytokines up to the end of anti-TB therapy may be it would help to describe the better immunological changes up to the end of treatment to our participants.

Table 3: Sensitivity and Specificity of each Cytokine

	IFN-λ	TNF-α	IL4	IL6	IL10
Sample size	96	96	96	96	96
Positive group[a]	16 (16.67%)	16 (16.67%)	16 (16.67%)	16 (16.67%)	16 (16.67%)
Negative group[b]	80 (83.33%)	80 (83.33%)	80 (83.33%)	80 (83.33%)	80 (83.33%)
Area under the ROC curve (AUC)	0.757	0.529	0.557	0.629	0.549
Standard Error[a]	0.0594	0.087	0.0879	0.0649	0.0884
95% Confidence interval[b]	0.659 to 0.839	0.424 to 0.631	0.452 to 0.659	0.524 to 0.725	0.444 to 0.651
Z statistic	4.336	0.328	0.653	1.986	0.552
Significance level *P* (Area=0.5)	<0.0001	0.7431	0.5137	0.047	0.5807
Youden index J	0.4375	0.1375	0.2625	0.35	0.175
Associated criterion	≤8.5	≤58	>0.3	>27.5	≤16
Sensitivity	75	68.75	43.75	87.5	31.25
Specificity	68.75	45	82.5	47.5	86.25

Conclusion

From the results of this research work, cytokine has a major role in immunological response during the pathogenesis of pulmonary TB. The use of anti-TB therapy effectively can cause immunological response in TB patients after 2 months of treatment which can be categorized by increase and decrease in secretion of cytokines these are IFN-γ, IL-4, and TNF-α, IL-6, IL-10 respectively. The studied markers herein can still be used to predict/determine early anti-TB response, which is crucial in determining whether a given treatment is effective or not, which may then help reduce unnecessary treatment cost that may be incurred in nonresponsive/resistant treatments.

Future studies should include a large number of participants, and if possible, all forms of TB (pulmonary and extrapulmonary TB) and cytokine levels measured the entire course of anti-TB treatment. Finally, the cytokine concentration can be measured as early as 2 weeks after starting anti-TB.

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Conflicts of interest

There are no conflicts of interest.

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