Original Article

Cytokines as Immunological Markers for Follow up of Disease Activity During the Treatment of Pulmonary Tuberculosis

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Abstract

Background: Cytokines play a major role in protection against *Mycobacterium tuberculosis* infection and regulate the immune responses at a cellular level. Cytokine profile determines clinical outcome of the disease and responses to treatment as well. A T helper 1 (Th1) cytokine interferon gamma (IFN-γ) is one of the most important cytokines which activate the macrophages to produce tumor necrosis factor-alpha (TNF-α). Excessive production of TNF-α have been implicated in immunopathogenesis of tuberculosis. A T helper 2 (Th2) response leads to release of IL-4, and IL-10 promoting an anti-inflammatory macrophage response. Interleukin-4(IL-4), has been implicated to down-regulate IFN-γ, and thus has a harmful effect on TB patients. IL10 cytokine has the capacity to inhibit Th1 activation and thus terminates cell mediated immune responses.

Objective: The objective of the present study was to determine Th1 and Th2 cytokine profile in patients with tuberculosis to identify immunological marker for follow up of the disease activity, and to study the outcome of treatment.

Methods: To examine this, blood samples were collected from newly diagnosed HIV negative pulmonary tuberculosis patients and from apparently healthy individuals as controls following an informed consent. Blood samples were as well collected at several intervals during the treatment with anti-tuberculosis drugs. Levels of IFN-γ, TNF-α, IL-4 and IL-10 were measured pre and during treatment using commercial available enzyme-linked immune-sorbent assay (ELISA). Data were analyzed using SPSS 20. Receiver Operating Characteristic (ROC) Curve analysis has been carried out to assess their discriminative power and to determine cut-off values. Analysis has been carried out further by calculating other measures of diagnostic test accuracy.

Results: The median serum level of IL-4 was 20 and 35 pg/ml higher in new cases (untreated patients) and in patients under treatment with oral anti-tuberculosis, respectively, compared with that of controls (p=001). Levels of TNF-α were significantly increased in patients before and after the treatment than those in control (p=0.001). New cases had the highest median level (10pg/ml) followed by those under treatment group (6pg/ml). Levels for IFN-γ were not statistically different between patients and controls (p=0.351). Median levels of IL10 were similar in both controls and new cases groups (35pg/ml), but lower in patients under treatment group (20pg/ml). Increase in levels of IL-4 during treatment showed that Th2 immune responses still present and may indicate active disease and thus IL4 cytokine may be a possible marker for the disease activity.

Conclusion: serum levels of TNF-α in TB patients is useful in the evaluation of the disease activity during therapy, not replacing clinical parameters of disease activity in TB. Similar to TNF-α, IL-4 can also be used as marker for TB severity. On the other hand IL-4*test* can be used to diagnose TB in highly exposed suspects where a positive result is more likely to indicate TB.

Keywords: Tuberculosis; Cytokines; Immunological markers.

Tuberculosis (TB) remains a global health problem of enormous dimension[1] and continues to be one of the top infectious killers in sub Saharan Africa. The number of TB cases are steady increasing. In 2010, there were 350,000 tuberculosis-related deaths in HIV-infected people, most of them in developing countries[2]. It had been demonstrated that the protective immunity against *M. tuberculosis*

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is mediated by Th1 response and this mediated by Th1 cytokines like IL-12 , IFN-γ and TNF-α these mechanism may be opposing by Th2 cytokines like IL-4, for example, IL4 cytokine with anti-inflammatory properties has the capacity to down regulate INF-γ response by inhibiting Th0 to Th1 differentiation[3] to more appropriate levels during the course of the disease and during treatment. Also high levels of IL-4 may lead to tissue damage and cavity formation[4] and thus can be used as a marker for severity of the disease.

Clearance of resident bacteria by alveolar macrophages is dependent on the presence of lymphocytes as well as activation by IFN-γ, released by Th1 cells. Thus, it is clear that the Th1-type response plays an important role in immunity to *M. tuberculosis*. Down regulation of Th1-type response is achieved via the Production of IL-10 and other cytokines that deactivate macrophages. If down-regulation is improperly achieved, then either the disease may not be arrested or extensive tissue damage can accompany Latent infection[3].

IL-4 is a Th2-type cytokines which is antagonized the action of Th1-type cytokines primarily interferon gamma which is an important factor for the activation of the infected macrophages[4].which is the major effecter mechanism of cell mediated immunity in the eradication of mycobacterium[5,6].

It had been determined that Immunity to *Mycobacterium tuberculosis* in humans requires IFN-γ for its effects on macrophages[7]. The plasma level of INF-γ were significantly higher in patient with mycobacterium tuberculosis, this could be ascribed to the role of T-helper1 (Th1) cells which are the major producer of type 1 cytokines like INF-γ in cell-mediated immunity against intracellular infection[8,9] to eliminate mycobacterial infections. IFN-γ might also improve or augment antigen presentation, leading to recruitment of CD4+ T-lymphocytes and/or cytotoxic T-lymphocytes, which might participate in mycobacterialkilling[10].TNF-α clearly plays an important but potentially complex role in the host response to *M. tuberculosis,* not only by synergizing with IFN-γ in activating macrophages but also by playing a role in the modulation of macrophages apoptosis and granuloma formation[11]and thus TNF-α appears to be crucial for the infection control and elimination of mycobacteria[12].During the early stages of TB there are elevated amount of TNF-α and this high level persists after the start of chemotherapy course. This clearly demonstrated when patients with latent TB infection rapidly progress to active disease when treated with TNF-α antagonists, such as Infliximab[13]. IL-10 has the capacity to inhibit synthesis of TNFα and IFN-γ as well. In tuberculosis, over expressed IL-10 showed no increase in susceptibility during the early stages of infection, but during the chronic phase of the infection, reactivation of tuberculosis with a highly significant increase in bacterial numbers within the lungs was demonstrated[14].Since, reliability of diagnostic or prognostic values of some markers for follow up patients with tuberculosis under treatment has not been well studied in Sudan, the aim of this study was to identify immunological marker for follow up tuberculosis patients, and to identify immunological marker associated with the outcome of anti-tuberculosis treatment. In addition Cytokines may be used as alternative predictive test for follow up of responses to anti-TB therapy together with conventional tests.

Materials and Methods

Study design:

This a cross sectional, descriptive longitudinal study.

Study area:

This study was conducted in Khartoum Hospital in the section of tuberculosis. It is one of tuberculosis Center for treatment and follow up in Khartoum. It belongs to tuberculosis national programme in Khartoum state.

Study population:

The study was conducted on 80 HIV negative tuberculosis patients who had a positive sputum for M. tuberculosis, matched with 57 healthy individuals in age and sex and ethnicity as controls. After the pretreatment specimens were collected patients received standard short-course chemotherapy.

Ethical Considerations:

Ethical approval for this study was obtained from the Ethical Committee of Review Board of Faculty of Medicine, Al Neelain University. The study protocol was scientifically reviewed by the ethical review board of AL Neelain University. The objective of the study was explained clearly to participating patients and controls. Written consents were obtained from participants.

Samples:

Venous blood samples (3-5 ml) were collected from patients before starting anti-tuberculosis treatment (ATT) and after 2,4,5 and 6 months of ATT. Blood samples were centrifuged at 5000 rpm for 10 minutes and then sera were collected and store at (-80) until use.

Cytokines Assays:

Sera from patients and control were screened for HIV using enzyme-linked immuno-sorbent assay Kit from Biorex and those whom were reactive for HIV were excluded from the study.

Cytokines levels in sera were assessed using commercial available ELISA Kits obtained from Komma biotech (Komma biotech INC.), Seoul were used to determine IFN-γ, TNF-α, IL-4 and IL-10 levels. Results were reported as pg/milliliter (pg/ml). The detection range of the assay is 32-2000pg/ml for IFN-γ, 16-2000pg/ml for TNF-α, 63-2000pg/ml for IL-4 and 47-3000pg/ml for IL-10 respectively.

Sandwich Enzyme Immuno-sorbent assay (ELISA) for the quantitative measurement of Human IFN-Gamma levels:

Briefly, 100 µl of each sample were added to each well in duplicate into 96 well plates pre-coated with antigen-affinity purified Rabbit anti-Human IFN Gamma along with controls. The plates were covered with the Plate Sealer and Incubated at room temperature for at least 2 hours. Washing was undertaken in 4 times by using washing buffer (PBS, Tween-20 (50%). Then 100 µl of the reconstituted detection antibody (1 µg/ml) of biotinylated antigen-affinity purified anti- Human IFN Gamma were then added to each well. Plates were incubated at room temperature for 2 hours and then were washed 4 times. Then 100 µl of the diluted color development enzyme ((Streptavidin-HRP conjugate) (60 ul) (1/200 dilute)) were added per well, covered with the plate sealer provided and incubated for 30 minutes at room temperature (or 37°C for 30 minutes). Following washing 4 times, 100 µl of color development solution were added to each well (A mixture of 1 volume of color development reagent A (TMB solution) and 2 volumes of color development reagent B (Substrate (H_2O_2) Solution). Then plates were incubated at room temperature for a proper color development for (3-15 minutes) and to stop the color reaction, 100 µl of the stop solution (2M H_2SO_4) were added to each well. Plates were read at 450 nm wavelength.

Sandwich Enzyme Immuno-sorbent assay (ELISA) for the quantitative measurement of Human IL-10 levels:

Briefly, 100 µl of each sample were added to each well in duplicate into 96 well plates pre-coated with Antigen-affinity purified Rabbit anti-Human IL-10 along with controls. The plates were covered with the Plate Sealer and Incubated at room temperature for at least 2 hours. Washing was undertaken in 4 times by using washing buffer (PBS, Tween-20 (50%). Then 100 µl of the reconstituted detection antibody (1 µg/ml) of Biotinylated antigen-affinity purified Rabbit anti-Human IL-10 were then added to each well. Plates were incubated at room temperature for 2 hours and then were washed 4 times. Then 100 µl of the diluted color development enzyme ((Streptavidin-HRP conjugate (60 ul)) (1/200 dilute)) were added per well, plates were covered with the plate sealer provided and incubated for 30 minutes at room temperature (or 37°C for 30 minutes). Following washing 4 times, 100 µl of color development solution were added to each well (A mixture of 1

volume of color development reagent A (TMB solution) and 2 volumes of color development reagent B (Substrate (H2O2) Solution). Then plates were incubated at room temperature for a proper color development for (3-15 minutes) and to stop the color reaction, 100 µl of the stop solution (2M H2SO4) were added to each well. Plates were read at 450 nm wavelength.

Sandwich Enzyme Immuno-sorbent assay (ELISA) for the quantitative measurement of Human IL-4 levels:

Briefly, 100 µl of each sample were added to each well in duplicate into 96 well plates pre-coated with antigen-affinity purified Rabbit anti-Human IL-4 along with controls. The plates were covered with the Plate Sealer and Incubated at room temperature for at least 2 hours. Washing was undertaken in 4 times by using washing buffer (PBS, Tween-20 (50%). Then 100 µl of the reconstituted detection antibody (1 µg/ml) of Biotinylated antigen-affinity purified Rabbit anti-Human IL-4 were then added to each well. Plates were incubated at room temperature for 2 hours and then were washed 4 times. Then 100 µl of the diluted color development enzyme ((Streptavidin-HRP conjugate (60 ul)) (1/200 dilute)) were added per well, plates were covered with the plate sealer provided and incubated for 30 minutes at room temperature (or 37°C for 30 minutes). Following washing 4 times, 100 µl of color development solution were added to each well (A mixture of 1 volume of color development reagent A (TMB solution) and 2 volumes of color development reagent B (Substrate (H2O2) Solution). Then plates were incubated at room temperature for a proper color development for (3-15 minutes) and to stop the color reaction, 100 µl of the stop solution (2M H2SO4) were added to each well. Plates were read at 450 nm wavelength.

Sandwich Enzyme Immuno-sorbent assay (ELISA) for the quantitative measurement of Human TNF-alpha levels:

Briefly, 100 µl of each sample were added to each well in duplicate into 96 well plates pre-coated with Antigen-affinity purified Rabbit anti-Human TNF-alpha along with controls. The plates were covered with the Plate Sealer and Incubated at room temperature for at least 2 hours. Washing was undertaken in 4 times by using washing buffer (PBS, Tween-20 (50%). Then 100 µl of the reconstituted detection antibody (1 µg/ml) of Biotinylated antigen-affinity purified Rabbit anti- Human TNF-alpha were then added to each well. Plates were incubated at room temperature for 2 hours and then were washed 4 times. Then 100 µl of the diluted color development enzyme ((Streptavidin-HRP conjugate (60 ul)) (1/200 dilute)) were added per well, plates were covered with the plate sealer provided and incubated for 30 minutes at room temperature (or 37°C for 30 minutes). Following washing 4 times, 100 µl of color development solution were added to each well (A mixture of 1 volume of color development reagent A (TMB solution) and 2 volumes of color development reagent B (Substrate (H2O2) Solution). Then plates were incubated at room temperature for a proper color development for (3-15 minutes) and to stop the color reaction, 100 µl of the stop solution (2M H2SO4) were added to each well. Plates were read at 450 nm wavelength.

Statistical analysis

Initial data analysis was carried out for both **age** and ***test results*** (cytokine levels). Statistical tests of normality and visual inspection showed that age could be regarded normally distributed and was therefore treated parametrically. ***Test results***, however, were not normally distributed; they had different shapes of distributions in the three groups and highly extreme values. Attempts to transform ***test results*** (to induce normality) failed due to the presence and pattern of the extreme values. Therefore, ***test results*** were treated non-parametrically.

One-way analysis of variance (ANOVA) was performed to test for differences between mean ages of the three groups.

Results:

To assess the potential of four cytokines; Interferon-Gamma (IFN-γ), Tumor necrosis factor-alpha (TNF-α), Interleukin-4 (IL-4) and Interleukin-10 (IL-10), as markers for

Tuberculosis (TB) during treatment, data were collected on subjects drawn from three groups. The groups were: (1) newly diagnosed cases of TB (the New cases group); (2) patients undergoing or already completed treatment of TB (the Under treatment group); and (3) apparently healthy individuals as controls (the Controls group). Four *tests* were administered to determine the serum levels of the four cytokines. Each subject underwent one or more of the four *tests* and the serum level determined by each *test* was recorded together with the subject's age and sex. The number of subjects in each group differed with regard to the four *tests*.

Table 1 below shows the number of individuals subjected to each of the four *tests* within each group.

Interferon-gamma levels were measured in 40 subjects in the three groups. The mean age of the subjects was 32.2 (CI: 29.5 to 37) years. Table 1.1 below shows the characteristics of the three groups.

As can be seen by table 1.1, in the New cases group, the median level of IFN-γ was about twice as much as that of the controls group (31.5 compared to 16 pg/ml).Tumor Necrosis Factor-Alpha levels were measured in 136 subjects in the three groups. The mean age of the subjects was 30.3 (CI: 28.3 to 32.3) years. Table 2 below shows the characteristics of the three groups.

From table 2, the new cases group had the highest median TNF-α level; two and a half times that of controls and about one and a half times that of the under treatment group. While the highest level of TNF-α was observed in the under treatment group (125 pg/ml) followed by that of the new cases group (80 pg/ml), the highest level in the controls group was only 28 pg/ml.

From the pairwise comparisons, it was found that significant differences existed within two of the three pairs, namely Controls vs the

Table 1: Study subjects classified by group and *test*

Group	*Test*			
	IFN-γ	TNF- α	IL-4	IL-10
Controls	9 (5/4)	57 (25/32)	56 (25/31)	15 (8/7)
New cases	14 (9/5)	26 (19/7)	17 (14/3)	9 (5/4)
Under Treatment	17 (10/7)	53 (31/22)	46 (28/18)	16 (9/7)
Total	40 (24/16)	136 (75/61)	119 (67/52)	40 (22/18)

Numbers shown in parentheses are (males/females)

Table 1.1: Characteristics of the three groups of subjects *tested* for IFN-γ

Group	Number Of Subjects*	Age (years) Mean (95% CI)	Interferon-Gamma (pg/ml)				
			Minimum	Maximum	Median	Percentile 25th	75th
Controls	9 (5/4)	28.3(22.4 to 34.3)	8	31.5	16	16	19
New cases	14 (9/5)	36.7 (28.8 to44.6)	5	275	31.5	16	31.5
Under Treatment	17(10/7)	33.2 (27.3 to39.1)	5	450	16	15	16
All groups	40(24/16)	32.2(29.5 to 37)	5	450	16	15	31.5

* (Male/Female)

under treatment group and Controls vs the New cases group (adjusted *p*-values < 0.001).
Serum levels of IL-4 were measured in 119 subjects. The mean age of the subjects was 29.3 (CI: 27.3 to 31.3) years. Table 3 below shows the characteristics of the three groups.
From table 3 above, it can be seen that the median level of IL-4 was 20 and 35 pg/ml higher in the New cases group and the Under treatment group, respectively, compared with the median level of Controls.
Serum levels of Interleukin-10 were measured in 40 subjects in the three groups. The mean age of the subjects was 32.7 years (CI: 28.8 to 36.6). Table 4 below shows the characteristics of the three groups.
From table 4 above, it is can be seen that the median level of IL-10 was the same in both Controls and New cases (35 pg/ml) but lower in the Under treatment group (20 pg/ml).

Table 2: Characteristics of the three groups of subjects *tested* for TNF-α

Group	Number Of Subjects*	Age (years) Mean (95% CI)	Tumor Necrosis Factor-Alpha (pg/ml) Minimum	Maximum	Median	Percentile 25th	75th
Controls	57(25/32)	28.8(25.9 to31.7)	2	28	4	2.5	6
New cases	26 (19/7)	37.2(31.0 to 43.4)	4	80	10	6	15
Under Treatment	53(31/22)	28.7(26.0 to 31.4)	1.5	125	6	4.5	14
All groups	136(75/61)	30.3(28.3 to 32.3)	1.5	125	5	4	10

* (Male/Female)

Table 2.1: Pairwise comparisons of the three groups

Group1 vs Group2	Adjusted[1] *p*-value
Controls vs Under treatment	< 0.001*
Controls vs New cases	< 0.001*
New cases vs Under Treatment	0.308

Each row tests the null hypothesis that Group1 and Group2 are the sameas regards TNF-α levels.
[1]P-values adjusted for multiple comparisons.
*Significant at 0.05 level.

Discussion:

In this study it is evident that the highest level of IL-4 related to the under treatment group. Despite that, the under treatment group and the new cases group, however, did not show a significant difference between their distributions as regards IL-4 level. This finding by the current study agrees with previous studies[8,9,15] provide evidence that, the Th2 response is unlikely to be an inflammatory epiphenomenon, indicating that, all patients improved clinically and radiologically during anti-TB therapy, but the IL-4 response did not decline substantially after anti-tuberculosis chemotherapy[16]. On the other hand, high level of IL-4 occur in TB most often in developing countries and this could be due to influence of high exposure to environmental mycobacteria, followed by high-dose challenge with M.tuberculosis[17]. In order to achieve the balance between the inflammatory and the protective immune response, the production of the anti-inflammatory cytokines like IL-4, IL-10 in response to M. tuberculosis antigens down regulate the immune response and limit tissue injury by inhibiting excessive inflammatory response. The opposing of this action will be achieved by increasing the interferon-γ and

decreasing TNF-α. Some studies demonstrated that high TNF-α initial levels in TB patients decreased significantly during the treatment, while the inflammatory process decreased at the same time[18]. High levels of TNF-α from PBMCS in patients with active pulmonary tuberculosis was demonstrated, however, a significant difference was observed in the level of TNF-α after the administration of TB treatment[19], accordingly, this could be predicative marker indicate the activity of the disease.

In the present study, TNF-α level in the new cases group was two and a half times that of controls and about one and a half times that of the under treatment group i.e., lower in control group than in patients before treatment and after treatment. However, the elevation of TNF-α levels in patients under treatment could be due to spontaneous or induced apoptosis of mononuclear cells and found to be increased among PBMC from patients with newly diagnosed TB, compared with that of healthy control subjects[20].

In addition to that, high levels of TNF-α in the current study may indicate that the enrolled patients may have necrotic granuloma with high number of of TNF-α producing cells. Furthermore, a virulent strains of mycobacterium tuberculosis bacterium have been found to be more potent inducers of TNF-α-dependent apoptosis than their virulent counterparts[21].

The current study had also demonstrated that cytokine directing a Th1 response (IFN-γ) was not significantly elevated in serum of patients with TB during treatment with anti-TB drugs. This finding is in agreement with results presented by Hirsch CS. and others (1999)[22] that IFN-γ immunoreactivity in PPD-stimulated culture supernatants from HIV-uninfected patients remained depressed for at least one year after initiation of chemotherapy, suggesting that the immunosuppression of TB is not only immediate and apparently dependent (at least in part) on immunosuppressive cytokines early during the course of Mycobacterium TB infection but is also long lasting, presumably relating to a primary abnormality in T-cell function. But however, an increased production of IFN-γ after the anti-tuberculosis treatment was assumed to be due to antigens released after death of mycobacteria caused by chemotherapy[19].

High levels of IL-10 cytokine has the capacity to reduce the production of IFN-γ by T lymphocytes[23]. In the present study IL-10 was lower in the patients under anti-tuberclosis treatment

Table 3: Characteristics of the three groups of subjects *tested* for IL-4

Group	Number Of Subjects*	Age (years)	Interleukin-4 (pg/ml)				
		Mean(95% CI)	Minimum	Maximum	Median	Percentile 25th	75th
Controls	56 (25/31)	28.8 (25.9 to 31.7)	5	590	20	10	40
New cases	17 (14/3)	34.2 (26.0 to 42.5)	15	370	40	20	140
Under Treatment	46 (28/18)	28.0 (25.5 to 30.6)	10	800	55	20	160
All groups	119(67/52)	29.3(27.3 to 31.3)	5	800	20	15	100

* (Male/Female)

group, however, the difference was not statistically significant. Low level of IL-10 in patients under treatment may suggest increased macrophages activation. This assumption comes from that, levels of TNF-α detected in sera from patients under treatment

had TNF-α level about one and a half times that of the of the control group i.e., the reduction is not drastic.

Taken together, serum levels of TNF-α in TB patients is useful in the evaluation of the disease activity of TB during therapy, not replacing clinical parameters of disease activity in TB, such as symptoms, chest X-rays, and culture and smear results, but used in addition to these conventional parameters. TNF-α can be used as marker for TB severity, having had a good discriminatory power (AUC = 0.824). For the chosen cut-off level of 5 pg/ml, TNF-α test had a sensitivity of 85% and a lower specificity of 70%. A negative result would indicate absence of TB more than would a positive result indicate presence of TB.

Table 3.1: Pairwise comparisons of the three groups

Group1 vs Group2	Adjusted *p*-value
Controls vs Under treatment	0.005*
Controls vs New cases	0.017*
New cases vs Under Treatment	1

Each row tests the null hypothesis that Group1 and Group2 are the sameas regards IL-4 levels.

* Significant at 0.05 level.

IL-4 also has the potential to be used as a marker for TB severity, having had a moderate discriminatory power (AUC = 0.659). For the chosen cut-off level of 110 pg/ml, had a low sensitivity (39%) but a much higher specificity (91%). It was less sensitive but more specific than TNF-α test. The PRU graph shows that a positive result would indicate TB in highly suspected subjects, but a negative result would not rule out TB even is minimally suspected subjects.

IL-4 *test* can be used to diagnose TB in highly exposed suspects where a positive result is more likely to indicate TB. However, it is of little value in screening for TB in the general population because only 39% (which is the sensitivity of IL-4 *test*) of the diseased will then be picked out and 61% of them will be left undetected. Accoringly, the current study recommends determination of treatment response phenotypes before the start of treatment, and reliable predictive models, using combinations of host markers would allow targeted interventions for patients at risk for slow treatment response to standard tuberculosis therapy.

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