Dynamics of Th2 IL-4 and IL-10 Cytokines During the Treatment of Pulmonary Tuberculosis

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ABSTRACT

Tuberculosis is (TB), an infection caused by mycobacterium tuberculosis, responsible for high morbidity and mortality worldwide. It is one of the major causes of morbidity and mortality in Sudan. Cytokine profile determines clinical outcome of the disease and responses to treatment as well. A T helper 2 (Th2) responses lead to release of IL-4, and IL-10, promoting B lymphocyte activation leading to an antibody response and promoting an anti-inflammatory macrophage response. Interleukin-4 (IL-4), an anti-inflammatory cytokine 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. The aim of the present study was to determine Th2 cytokine profile in patients with tuberculosis to identify immunological marker for follow up of the disease activity and to study the outcome of anti-tuberculosis treatment as well. 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 also collected at several intervals during the treatment with anti-tuberculosis drugs. Levels of Th2 cytokines 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. The results showed that, 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=0.001). Median levels of IL10 were similar in both controls and new cases groups (35pg/ml), but lower in patients under treatment group (20pg/ml). Despite that, the difference in levels of IL-10 was not statistically different between patients and controls (p=0.243). 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. Result showed that, IL-4 has the potential to be used as marker for TB severity (specificity=91%) having the area under the curve AUC of 0.659.

Keywords: IL-4, IL-10, Pulmonary Tuberculosis
INTRODUCTION

It had been demonstrated that the protective immunity against M. tuberculosis 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 (Janis and Denise 2001) 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 (van Crevel R. et al., 2000) and thus can be used as a marker for severity of the disease.

IL-4 is a Th2-type cytokine which is antagonizes the action of Th1-type cytokines primarily interferon gamma (IFN-γ). IFN-γ is an important factor for the activation of the infected macrophages (van Crevel R. et al., 2000) which is the major effector mechanism of cell mediated immunity in the eradication of mycobacterium (Octavio M. and Rivero-Lezcano 2008; Bai Y. et al., 2008). IL-4 has modestly detrimental effects on the antibacterial efficacy of the Th1 response, and larger effects on the toxicity of TNF-α, and on fibrosis (Hernandez-Pando et al., 2004). IL-4 has also the capacity to Switches off signaling via Toll Like Receptor-2 and down regulate iIONS which may play crucial role in driving mycobacterium tuberculosis in to latency (Rook GA. et al., 2005).

The production of IL-4 is modulated by anti tuberculosis treatment (Octavio M. and Rivero-Lezcano 2008). It had been observed that there were elevated levels of mRNA for IL-4 and IL-4 antagonist (IL-4-δ2) from T-cell and non T-cell population in TB patients after anti tuberculosis chemotherapy and thus, appear to be important in pathogenesis of tuberculosis. (Wing W. and Chi Leung 2006)

Similar to IL-4, IL-10 is capable of inhibiting synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-3, TNFα and GM-CSF made by cells such as macrophages and regulatory T-cells. It also displays a potent ability to suppress the antigen-presentation capacity of antigen presenting cells. However, it is also stimulatory towards certain T cells and mast cells and stimulates B cell maturation and antibody production.

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 showed evidence of reactivation of tuberculosis with a highly significant increase in bacterial numbers within the lungs. This reactivation was shown to be associated with the formation of macrophage-dominated lesions, decreased mRNA production for TNF and IL-12p40, and a decrease in Ag-specific IFN-γ secretion. In addition, IL-10 plays a pivotal role during the chronic/latent stage of pulmonary tuberculosis, with increased production playing a potentially central role in promoting reactivation tuberculosis (Turner J. et al., 2002).

IL-10 plays a number of important roles in down-regulating an active immune response in TB, including deactivation of
macrophages, inhibition of T cell proliferation and suppression of cytokine production by Tlymphocytes. Study of gene polymorphisms had found that there were no association in IL-10 either with susceptibility or resistance to pulmonary TB (López-Maderuelo D. et al., 2003). It seems that higher IL-10 production appears to reflect IL-10-dependent suppression of adaptive immune responses and sustained long-term specific energy, in the PPD-negative individuals (Prabhu S. et al., 2007).

Despite the efforts to improve diagnosis and treatment there are many obstacles to TB control especially in developing countries, these including 1); Lack of concrete markers indicating success or failure of treatment during the early course of active disease, 2); Assessment of response to treatment using sputum culture conversion following 8 weeks of treatment require 6 weeks to perform. Reliability of diagnostic or prognostic values of some markers for follow up patients with tuberculosis under treatment has not been well studied in Sudan. Cytokines may be used as alternative predictive test for follow up of responses to anti-TB therapy together with conventional tests. Accordingly, the aim of the current study was to identify immunological marker for follow up tuberculosis patients, and to identify immunological marker associated with the outcome of anti-tuberculosis treatment.

MATERIALS AND METHODS

Study design:

This a cross sectional, descriptive longitudinal study. The 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 National Tuberculosis 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 pre-treatment specimens were collected patients received standard short-course chemotherapy.

Ethical Considerations:

Ethical approval for this study was obtained from the Institutional 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 in plain vacutainor 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 IL-4 and IL-10 levels. Results were reported as pg/milliliter (pg/ml). The detection range of the assay is 63-2000 pg/ml for IL-4 and 47-3000 pg/ml for IL-10.

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 (1 ml Tween-20 (50%) to 1 L PBS). 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 (1 ml Tween-20 (50%) to 1 L PBS). 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.

Statistical analysis:

All statistical analyses were performed using IBM SPSS Statistics (version 20) except for the analysis of diagnostic test accuracy (ROC curve and other performance measures) which was performed using the biomedical StatsDirect Statistical Software v2.7.9 (7/9/2012). Graphs, however, were all created using SPSS. The conventional 5% level of significance was used for all statistical tests.

RESULTS

1. Interleukin-4 (IL-4)

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 1 below shows the characteristics of the three groups.

Table 1: Characteristics of the three groups of subjects tested for IL-4

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

* (Male/Female)

From table 1 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. The interquartile range was 20 pg/ml in Controls, 120 pg/ml in the New cases group and 140 pg/ml in the Under treatment group. The interquartile range was 6 times as high in the New cases group and 7 times as high in the Under treatment group.
in the Under treatment group compared with that of Controls. This indicates that there was much more spread in the middle 50% of IL-4 levels of the New cases and the Under treatment groups than in that of the Controls group.

Table 2: Pairwise comparisons of the three groups

<table>
<thead>
<tr>
<th>Group1 vs Group2</th>
<th>Adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls vs Under treatment</td>
<td>0.005*</td>
</tr>
<tr>
<td>Controls vs New cases</td>
<td>0.017*</td>
</tr>
<tr>
<td>New cases vs Under Treatment</td>
<td>1</td>
</tr>
</tbody>
</table>

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

* Significant at 0.05 level.

The pairwise comparison table shows that there were statistically significant differences between the distributions within each of the two first pairs of table 2, namely Controls vs the Under treatment group and Controls vs the New cases group (adjusted p-values were 0.005 and 0.017, respectively). The Under treatment group and the New cases group, however, did not show a significant difference between their distributions as regards IL-4 level (adjusted p-value = 1).

**Diagnostic accuracy of Interleukin-4 test:**

To investigate the accuracy of IL-4 test, ROC curve analysis was first performed to assess its discriminative power. Data from only the New cases and the Controls groups were used for ROC curve analysis. A decision threshold (cut-off) and other performance measures corresponding to that threshold were calculated.

**ROC Curve analysis of interleukin-4 test**

ROC curve analysis was performed to evaluate the discriminative power of IL-4 as a marker for TB and to determine an optimum cut-off level. The ROC curve of IL-4 is shown in figure 1 below.
Figure 1: ROC Curve of interleukin-4

The area under the curve (AUC) of IL-4 was found to be 0.695 (CI: 0.56 to 0.83). AUC is significantly different from 0.5 (p-value = 0.006), indicating that IL-4 test has a discriminatory power that is significantly different from random allocation. The 95% CI for the AUC indicates that the test has low to moderate accuracy. The shape of the ROC curve, being parallel to the diagonal line, is not consistent with that of a good test.

The optimum cut-off level obtained for IL-4, assuming equal weights (importance) for sensitivity and specificity, was 110 pg/ml. Subjects with IL-4 levels ≥ 110 pg/ml were classified as test positives, while subjects with IL-4 levels < 110 pg/ml were classified as test negatives. The classification result is shown in table 3 below.

Table 3: IL-4 test results of 18 new TB cases and 55 TB-free controls

<table>
<thead>
<tr>
<th>Test Result</th>
<th>Disease Status</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TB +ve (New cases)</td>
<td>TB –ve (Controls)</td>
</tr>
<tr>
<td>IL-4 +ve</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>IL-4 –ve</td>
<td>11</td>
<td>50</td>
</tr>
</tbody>
</table>

Diagonal segments are produced by ties.
Figure 2: Proportionate Reduction in Uncertainty for IL-4 test

Positive IL-4 test results provide more clarification of the disease status of subjects as their likelihood of having TB approaches one. A positive test will at most clarify 77% of the diagnostic uncertainty. Negative results provide more clarification of the disease status of subjects as their likelihood of having TB approaches zero. But the maximum PRU is only 33% meaning that a negative test result leaves out at least twice as much uncertainty as it can clarify.

2. Interleukin-10

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.

Table 4: Characteristics of the three groups of subjects tested for IL-10

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of subjects*</th>
<th>Age (years)</th>
<th>Interleukin-10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (95% CI)</td>
<td>Minimum</td>
</tr>
<tr>
<td>Controls</td>
<td>15 (8/7)</td>
<td>30.7(23.7 to 37.7)</td>
<td>15</td>
</tr>
<tr>
<td>New cases</td>
<td>9</td>
<td>40.5(31.4 to 31)</td>
<td>14</td>
</tr>
</tbody>
</table>
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). Controls had a single value for both the 25th and the 75th percentiles, (interquartile range = 0) because ten out of the 15 subjects had the same IL-10 level (35 pg/ml). The interquartile range of the Under treatment group was more than double that of the New cases group (65 and 30 pg/ml), indicating that the middle 50% levels of the Under treatment group were more spread out than those of the New cases group. The interquartile ranges are depicted in figure 4 below as a mere horizontal line for Controls, a box for New cases and another box that is about twice as high for the Under treatment group.

**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 did not show a significant difference between their distributions as regards IL-4 level. IL-4 level from patients was also found to be higher than that in controls. This finding agrees with previous studies (Poveda F. et al., 1999; Thillai M. et al., 2012) provide evidence that, the Th2 response is unlikely to be an inflammatory epiphenomenon. On the other hand, it indicates that all patients improved clinically and radiologically during anti-TB therapy, but the IL-4 response did not decline substantially. This argues against the IL-4 being a nonspecific, inflammation-driven, bystander response. Rather, if IL-4 antagonizes mycobactericidal macrophage activity, it may explain why, even after several months of treatment, when bacterial load is very low, the immune response still cannot contain the disease, and treatment must continue or may indicate that there is switch from Th1 to Th2 i.e., indicating active disease. It seems that after anti-tuberculosis chemotherapy, IL-4 mRNA level remained unchanged, where IL-4 antagonist (IL-4δ2) level increased in parallel with IFN-γ (Wing Yew and Chi Leung 2005). Thillai M. and others (2012) correlated high levels of IL-4 with TNF-α and accordingly, the current study agrees with previous studies that TB patients under treatment compared to healthy normal control had increased IL-4 level. In addition to that, 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 (Rogelio Hernández-Pando et al., 2008).

The current study suggests that IL-4 can be used to confirm rather than to rule out severity of TB or poor response to medical treatment. Our approach in this respect is that the likelihood ratios indicate that LR (+) of 4.28 is greater than the inverse of LR(-), which is 1.49 indicates that a positive result is more informative than a negative result.

IL-10 cytokine has been considered as Th2 cytokine with an anti-inflammatory effect, acting in the inactivation of macrophages through inhibiting the production of IL-12 and consequently reducing the production of IFNγ by T lymphocytes (Flynn JL and Chan J 2001). Levels of IL-10 is higher in cases of tuberculosis. In the present study the median level of IL-10 was similar in both controls and new cases (35 pg/ml) but it was lower in the under anti-tuberculosis treatment group (20 pg/ml) however the difference was not statistically significant. The decrease in IL-10 levels following therapy, may suggest the transient induction of regulatory immune mechanisms leading to subsequent restoration of immune homeostasis. On the other hand level of IL-10 in patients under treatment may suggest increased macrophages activation.

Taken together, IL-4 has the potential to be used as a marker for TB, having had a moderate discriminatory power (AUC = 0.659). IL-4 test can also be used to diagnose TB in highly exposed suspects where a positive result is more likely to indicate TB. Levels of IL-10 were not statistically significantly differ in the three groups. The distributions of IL-4 levels of the Under treatment group were significantly different from Controls; but was not significantly different from the New cases group.

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