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TNF-α and IL-10 Levels: Possible Risk Markers For Latent *M. tuberculosis* Infections Among Sudanese

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Abstract: Factors responsible for the control of *M. tuberculosis* infection includes T cells, macrophages and cytokines. The incidence and prevalence of latent tuberculosis in Sudan have not yet been reported and latent tuberculosis infection is regarded as a significant risk factor for active pulmonary tuberculosis. This study aimed to understand the role of immune responses particularly the cytokines profile (levels of TNF-α and IL-10) as risk factors of infection in patients with active and latent *M. tuberculosis* infections. A case control study was conducted in various hospitals in Greater Khartoum, a total of 48 patients and volunteers were enrolled (17 active pulmonary TB patients, 17 latent TB patients and 14 apparently healthy individuals). Whole blood culture with PPD, PHA and LPS stimulation and TNF-α and IL-10, measurement by ELISA were conducted. The results have shown TNF-α and IL-10 significantly higher in active TB patients compared to latent TB patients and healthy individuals ($p<0.001$). On the other hand, patients with latent TB infection had significantly higher levels of TNF-α and IL-10 compared to healthy individuals ($p<0.001$). Therefore, the differences in the TNF-α and IL-10 levels probably indicate pivotal roles in the pathogenesis of *M. tuberculosis* infection. Furthermore, TNF-α and IL-10 levels can provide a useful risk marker for the development of overt and latent TB infections.

Key words: TNF-α and IL-10, whole blood assay, pulmonary tuberculosis, latent tuberculosis, TB patients

INTRODUCTION

Tuberculosis (TB) remains the single largest infectious disease causing high mortality in humans, leading to 3 million deaths annually. Despite wide coverage of BCG vaccination through the EPI Program, Sudan ranks one of the top countries worldwide in terms of TB endemicity (WHO, 2008). The pathogenesis of TB is complex and manifested in various forms depending on the interactions between bacterial virulence factors and the host immune system (Dannenberg, 1994). *M. tuberculosis* has the ability to cause latent infection as other bacteria such as streptococcal infections, syphilis and *H. pylori* (Dermott, 1958; Rhen *et al*., 2003). Latent infections could reactivate when the host immune system is compromised by conditions such as HIV. In addition, latency increases emergence of Multidrug-Resistant TB (MDR-TB) and poses a significant threat to the control of TB (Dye, 1999). Latent Tuberculosis Infection (LTBI) is detected through skin testing (Mantoux test) or the quantiFERON blood test among persons with risk factors for TB such as co-patients and public health investigators of contacts of infectious TB cases (Jereb *et al*., 2003). For persons with untreated LTBI and intact immunity, the estimated risk of developing symptomatic TB disease is 5-10% over a lifetime (Verver *et al*., 2004) with about half of that risk occurring during the 1st year or 2 after infection. For persons who are immunocompromised by HIV co-infection, the estimated risk of developing disease increases to 5-10% per year (Selwyn *et al*., 1992).

The immunology of LTBI is complex and involves the interaction between CD4+, CD8+ T lymphocytes, macrophages and monocytes along with the production of cytokines such as Interferon-γ (IFN-γ), Tumor Necrosis Factor-α (TNF-α) and IL-10. Clinical studies have shown the use of TNF-α blockers and reactivation of LTBI

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(Keane *et al*., 2001). TNF-α plays a key role in controlling the primary challenge with *M. tuberculosis* (Bean *et al*., 1999). TNF-α is also essential for maintaining the state of latency (Wallis *et al*., 2004). TNF-α provides several functions relevant for protecting reactivation of TB, TNF-α is involved in the regulation of apoptosis of cells infected with *M. tuberculosis* (Balcewicz-Sablinska *et al*., 1998), TNF-α also supports the maturation program in DCs by inducing migration and the up regulation of the molecular mechanisms required for the activation of T cells (Moll, 2003). Furthermore, TNF-α induces antimicrobial activity of murine macrophages via the induction of reactive nitrogen intermediate (Chan *et al*., 1992). In addition, TNF-α recruits monocytes and lymphocytes to the site of infection via its action on the vascular endothelium. TNF-α has the capacity to establish gradients for chemokines and such as chemokine ligands CCL2 (Rothlein *et al*., 1988) and TNF influences the expression of Chemokine Receptors (CCRs) such as CCR5 (Patterson *et al*., 1999).

The IL-10 is an important regulatory cytokine in the immune response to mycobacterial infection that can inhibit the production of the proinflammatory cytokines resulting in macrophage deactivation and preventing the release of reactive nitrogen/oxygen intermediates by macrophages (Gong *et al*., 1996). The IL-10 is implicated at many levels of TB disease such as directing Th2-type cell expansion in the lung (Almeida *et al*., 2009). In addition, high level of IL-10 in the lung may impair the *M. tuberculosis* mediated alveolar macrophage apoptosis (Patel *et al*., 2009), IL-10 also regulates innate immune responses such as macrophage apoptosis after mycobacterial infection (Feng *et al*., 2002).

The measurement of cytokines released from stimulated monocytes and T-cells in response to mitogens, recall antigens and disease specific stimuli such as mycobacterial antigens (De Groote *et al*., 1992; Hussain *et al*., 1996) using simple whole blood assays have been used to understand the relationship between secreted host proteins and disease phenotypes and to realize prognosis or response markers. This study focuses on the differences in plasma levels between controls LTBI and TB patients in order to understand the role of TNF-α and IL-10 in immunopathogenesis of LTBI to use it as a risk factor to diagnose LTBI.

MATERIALS AND METHODS

Study groups: The study included 17 pulmonary TB patients attending the Abuanja Hospital, Alshap Hospital and Khartoum North Hospital, Khartoum, Sudan whose diagnosis was based on clinical and radiological data together with the identification of tubercle bacilli in sputum as described previously (Hussain *et al*., 1996). The TB patients (n = 17; consist of 6 female and 11 male with a mean age of 35±13). The LTBI patients (n = 17; consist of 10 females and 7 males with a mean age of 39±9 years) who were in contact with TB patients for >6 months but without showing symptoms and signs of active TB, tested with QuantiFERON®-TB test QFT manufactured by Cellestis Limited, Carnegie, Victoria, Australia (FDA, 2002). The third group is the healthy volunteers (n = 14; consist of 8 women and 6 men with a mean age of 42±11 years) not in contact with TB patients but living in the same area were included as controls with the negative tuberculin skin test. Clinical data were obtained from the patients medical records; all participants had negative HIV serology.

Ethical aspects: Written consent was obtained from all participants before sample collection. The study was approved by the Ethics Committee of the Institute of Endemic Diseaes, University of Khartoum, Khartoum, Sudan.

Mitogens and antigens: *M. tuberculosis* Purified Protein Derivative (PPD) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Endotoxin Lipo Ploy Saccharide (LPS) and Phytohemagglutinin (PHA) was purchased from Sigma (St. Louis, MO).

Whole blood cytokine assays

Stimulation of cells and collection of supernatants for cytokine analysis: The method used was based on that described by Weir *et al*. (1994). The blood (5 mL) was collected in 15 mL syringes by venipuncture from each donor and immediately mixed with sodium heparin (Leo Pharmaceuticals Ballerup, Denmark) at 20 U mL^{-1} in 50 mL plastic centrifuge tubes and further diluted 1/11 with sterile RPMI 1640 tissue culture medium containing 100 U mL^{-1} of penicillin/100 μg/mL streptomycin and 2 mM L-glutamine (Sigma). Diluted blood (900 μL $well^{-1}$) was dispensed in 24 well tissue culture plates (Nantong Hailun Biomedical Apparatus Manufacturing Co., Ltd. Nantong, China) within 2 h of collection. Cultures were stimulated with 100 μL $well^{-1}$ of mitogens (LPS at 25 μg mL^{-1}, PHA at 5 μg mL^{-1}) or antigen (PPD 10 μg mL^{-1}). Plates were incubated at 37°C in 5% CO_2. Supernatants were collected on day 2 from the wells and stored as 4×200 μL aliquots at -20°C.

Cytokines determination: Plasma TNF-α and IL-10 were measured using sandwich commercial ELISA kits (BD Biosciences, California, US) according to the

manufacturer's protocol. Samples were assayed in duplicate and results expressed as the average of the two readings in an ELISA reader (Atlas Medical, Cambridge, UK) at 450 nm. Concentrations of the respective analytes were determined using the Biotek KC 4 Version 3.4 Software.

Statistical analysis: The results were analyzed using Statistical Package of Social Sciences (SPSS) Version 16, the results are presented as the mean±SD Statistical significance of the difference between the level of cytokines was analyzed using ANOVA test. Data were considered statistically significant when $p \leq 0.05$.

RESULTS

Monocyte and T-cell cytokines are as effectively detected in stimulated Whole Blood cultures (WB): Latent TB patients (LTB = 17) were studied in parallel with active pulmonary TB patients (TB = 17) and Healthy community controls (EC = 14). Two potent mitogens were used LPS which stimulates cytokine secretion predominantly from monocytes (TNF-α) and PHA which induces cytokine secretion predominantly from T-cells (IL-10). In addition Mycobacterium specific antigen (PPD) was used to stimulate Whole Blood (WB) and Normal Saline (NS) was used as a control. For the measurement of the cytokines, supernatants were collected after 2 days of stimulation. As reported earlier both monocyte and T-cell cytokines were effectively detected in diluted WB cultures in response to mitogens (LPS and PHA).

Cytokine profiles in mitogen-stimulated WB cultures in active TB patients, latent TB patients and endemic controls TNF-α: Table 1 compares the differences in TNF-α secretion in latent TB patients, active pulmonary TB patients and healthy community controls to PPD, TNF-α and negative control (Fig. 1). A highly significant increase in production in active TB patients compared to latent TB patients and EC (ANOVA; p = 0.0003) indicating an overall as well as an antigen-specific activation of this anti-inflammatory cytokine in TB patients. Latent TB patients showed a significant highly plasma level of TNF-α in response to PPD compared to community controls (ANOVA; p = 0.001).

IL-10: IL-10 responses were similarly analyzed in the three groups (Table 1), IL-10 was significantly higher in response to PPD in active pulmonary TB patients compared to latent TB patients and community control (ANOVA; p = 0.005) (Fig. 2). On the other hand, latent TB patients showed significant higher levels of IL-10 compared to community controls in response to PPD (ANOVA; p = 0.001).

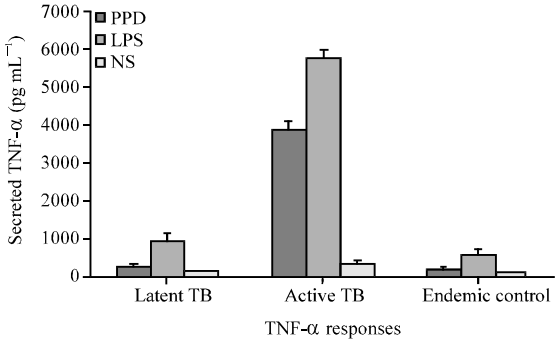

Fig. 1: Comparison of TNF-α released in response to PPD, LPS and normal saline in whole blood cultures from latent TB patients, active TB patients and healthy community controls. ANOVA test analysis was carried out to determine the significance of the differences between the three groups. TNF-α concentrations were higher in the active TB group compared to the latent TB and community control groups (p = 0.001). The latent TB group showed significantly higher concentrations of TNF-α compared to community control group (p = 0.001)

Table 1: TNF-α and IL-10 cytokines profiles in stimulated whole-blood culture of patients with pulmonary tuberculosis, latent TB patients and healthy community controls

	Latent TB (n = 17)			Active TB (n = 17)			Endemic control (n = 14)			
Cytokine	X	SD	SEM	X	SD	SEM	X	SD	SEM	p-values*
PPD										
TNF-α	296.4	179.4	51.8	3900.0	653.1	206.5	234.8	199.2	41.6	0.0003
IL-10	183.7	42.1	12.2	410.8	163.2	051.6	101.7	67.2	14.0	0.0050
LPS										
TNF-α	956.6	691.2	199.5	5756.2	756.1	239.1	598.3	576.4	120.2	0.0010
PHA										
IL-10	389.1	111.9	32.3	577.0	247.4	78.2	351.7	202.2	42.2	0.0001
NS										
TNF-α	88.0	45.4	13.1	349.3	203.5	64.3	23.0	11.7	2.4	0.0100
IL-10	66.2	25.1	7.3	97.3	39.7	12.5	39.6	23.8	11.7	0.0200

Results are expressed as means (X)±SD and±SEM around the mean (pg mL^{-1}). *ANOVA test was used to determine the significance of differences

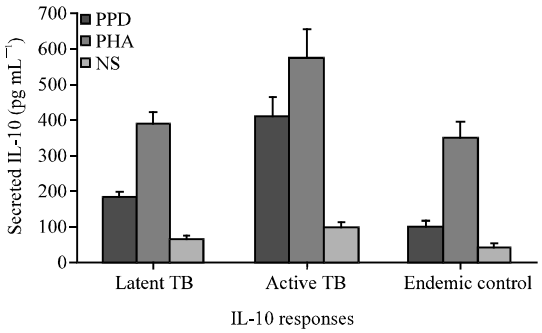

Fig. 2: Comparison of IL-10 released in response to PPD, PHA and normal saline in whole blood cultures from latent TB patients, active TB patients and healthy community controls. ANOVA test analysis was carried out to determine the significance of the differences between the three groups. IL-10 concentrations were higher in the active TB group compared to the latent TB and community control groups ($p = 0.001$). The latent TB group showed significantly higher concentrations of IL-10 in response to PPD compared to community control group ($p = 0.001$)

DISCUSSION

M. tuberculosis infection phenotypes are multi-factorial where cytokines play a pivotal role in the modulation of disease type/severity. TNF-α is a potent modulator of early inflammatory responses to *M. tuberculosis*. It is critical for prevention of establishment of mycobacterial infection and the maintenance of latent TB. This study showed that the TNF-α levels are significantly higher in patients with a proven encounter with Mycobacteria (LTBI and pulmonary TB) compared to community controls. The results showed the TNF-α level are higher in active TB patients compared to apparently healthy community controls. This is in agreement with previous reports that have concluded that there is an elevation in TNF-α level in active TB when compared to healthy individuals (Arriaga *et al*., 2002; Sahiratmadja *et al*., 2007). Active TB patients showed higher TNF-α levels compared to the latent TB patient group, the findings are concordant with the results obtained by previous study (Caccamo *et al*., 2010) where they reported that the level of TNF-α is higher in 90% of patients with active TB and in only 10% of patients with latent TB. This difference in TNF-α level between Latent TB patients, active pulmonary TB patients and healthy individuals can be used as a diagnostic test in differentiating latent TB from active TB and healthy state.

Also, TNF-α could also be a useful prognostic marker, reports by Dlugovitzky *et al*. (2000) suggested that levels of TNF-α in the early phase of the disease is significantly higher than the level in the late phase of the disease. In the same line, another study concluded that clinical improvement of patients with pulmonary TB is associated with reduction in TNF-α level (Junior *et al*., 2008).

It is clear that TNF-α plays a major role in maintaining the latency of Mycobacterial infection and prevent the progression of the latent infection to the active form of the disease. This was confirmed by the fact that neutralization of TNF-α in latent TB infection leads directly to reactivation of the microorganism and development of active TB when anti-TNF-α antibodies were used therapeutically in inflammatory diseases such as Psoriasis and rheumatoid arthritis (Wallis, 2007; Yun *et al*., 2007; Miller and Ernst, 2009).

This study also has shown that the IL-10 level is significantly increased in active TB compared to the latent TB form and healthy individuals. In addition, the latent TB form has significantly increased IL-10 levels compared to healthy individuals. The increase in IL-10 level in active form is probably due to the fact that IL-10 induced down-regulation of Th-1 response and hence shifts the Th-1/Th-2 balance to a pure Th-2. Therefore, IL-10 plays a key role in the progression of early TB infection or reactivation of LTBI. While in the latent form, the Th-1 responses play a major role in controlling IL-10 levels. However, a polish study reported that it is not the diminished production of Th1 cytokines but rather the parallel overproduction of Th2 cytokines which are essential in the development of active TB (Winek *et al*., 2009).

Many studies reported results that are concordant with results with regard to the significant high of IL-10 levels in active TB compared to the latent form. However, when comparing with other intracellular pathogens. Some studies on Post Kala-azar Dermal Leishmaniasis (PKDL) patients in Sudan it reported that the pathogenesis of PKDL is largely immunologically mediated with high concentrations of interleukin 10 in the peripheral blood of Visceral Leishmaniasis (VL) patients predicting the development of PKDL. During VL, interferon-γ is not produced by Peripheral Blood Mononuclear Cells (PBMCs). It is known that the regulatory T cells (T reg) have an arsenal of suppressor mechanisms including IL-10 production. Neutralization of IL-10 in cultures stimulated with CFP-10 and PPD showed no association between IL-10 and the inhibition of IFN-γ and IL-17 production, suggesting that Tregs use another suppressive mechanism different from IL-10 production to

inhibit effector T cell responses (Marin *et al*., 2010). As an interpretation of the results, researchers may conclude that IL-10 level is higher in active compared to latent forms and hence provide an evidence of Th-2 in response to active TB disease and re-activation of LTBI.

CONCLUSION

Differences in the TNF-α and IL-10 levels probably indicate they are critical for the prevention of establishment of *M. tuberculosis* infection and the maintenance of latent tuberculosis. Furthermore, they can provide a useful risk marker for the development of overt and latent TB infections.

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