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Changes in Serum Levels of TNF-α & IL-4 among New, Under-treatment & MDR TB Patients

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Authors' contributions

This work was carried out in collaboration between all authors. Author NF designed the study, wrote the protocol and wrote the first draft of manuscript. Author Nabeela performed the statistical analysis and managed the literature analysis. Authors MS and HMK managed and analyzed literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Tuberculosis (TB) remains a significant public health problem with an estimated one-third of the world's population being infected. Cytokines play a major role in protection against *Mycobacterium tuberculosis* infection and regulate the immune responses at cellular level. Most studies on cytokines during TB are from '*in vitro*'-stimulated lymphoid cells with few reports on *in vivo* plasma levels. This study was aimed to evaluate the levels of TNF-α & IL4 in new, under-

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treatment (UT) and multidrug resistant (MDR) pulmonary and extra-pulmonary cases.
Methodology: The study was conducted at the Department Of Microbiology, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, India. Levels of TNF-α and IL-4 were measured in 76 serum samples from TB patients by ELISA kit (Diaclone France) along with 10 BCG vaccinated control. A complete clinical, radiological & treatment data was collected on questionnaire forms of each patients.
Results: TNF-α levels were elevated in new (*P<0.05*) and MDR cases *(P<0.05*), but not significantly for UT cases (*P>0.05*). TNF-α and IL-4 levels showed no significant variations according to site of involvement in pulmonary vs. extra-pulmonary TB cases.
Conclusion: An understanding of the development of this response may lead to insight into pathogenesis and novel therapies for TB.

Keywords: Tuberculosis; IL-4; TNF-α; cytokines.

1. INTRODUCTION

Tuberculosis (TB) remains a major global health problem. In 2012, an estimated 86 million people developed TB and 1.3 million died from the disease [1]. Despite the implementation of TB control programs, case rates continue to soar. The situation is further complicated by a worldwide increase in multi-drug resistant (MDR) and the recent reports of extreme drug resistant (XDR) TB [2].

TB begins with the inhalation of *Mycobacterium tuberculosis (*Mtb*)* in the aerosols into the pulmonary alveoli. Mtb binds to the phagocytic receptors and enters resident alveolar macrophages, dendritic cells, and monocytes recruited from blood stream. Although TB has traditionally been linked to failed immunity, recent work has implicated excessive inflammation in increased TB susceptibility [3].

Cytokines play a major role in protection against Mtbinfection and regulate the immune responses at a cellular level. Upon stimulation by a pathogen, macrophages engulf the offending particle, and upon its destruction, they present smaller peptide antigens on their surface. These antigens are then recognized by Th1 cells, which in turn secrets various cytokines including IF-γ IL-12 and TNF-α. These cytokines in turn activate resting macrophages, which trigger the immune response. TNF-*α* is believed to play multiple roles in the immune and pathological responses in TB [4]. In order to suppress the immune response once an infection has been cleared, and to prevent autoimmune responses to self-antigens, other cytokines down regulate the immune system. IL-10 activates B and Th2 cells while inhibiting Th1 cytokine production. Activated Th2 cells secret IL-4, which also inhibit Th1 cell activity and inactivates macrophages [5].

Understanding of the mechanism involved in cell-mediated immune response against the Mtb, particularly the function of cytokine network involved, is of significant relevance to the development of effective control and prevention [6]. T cell response to an MDR-TB infection in human remains unclear [7]. Most studies on cytokines during TB development are from *in vitro* stimulated lymphoid cells with few reports on *in vivo* plasma levels [7,8,9]. There is a paucity of data regarding cytokine interplay in MDR-TB patients. With this background, the aim of the present study was to determine the correlation of serum levels of TNF-α and IL-4 in new, under-treatment and MDR TB cases and to compare these values in pulmonary and extra-Pulmonary TB.

2. METHODOLOGY

The present study was conducted at the Department Of Microbiology, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, India. A complete clinical and radiological data were collected. Informed consent was taken from all the subjects. The study was approved by the Institutional Bioethical Committee.

2.1 Inclusion Criteria

All the sputum positive patients by smear microscopy (new case, relapse, defaulter, treatment failure) with Tubercular effusion and pericardial effusion were enrolled.

- New case: A patient who has never taken anti-TB drugs for more than 1 month.
- Relapse: Previously received treatment, cured, and has once again positive for PTB.

- Failure: A TB patient who remains positive while on treatment, or becomes positive at 5th month or later, or was negative at start and becomes positive after 2 months of treatment.
- Treatment after default: A patient who returns to treatment following interruption of treatment for two months or more and is positive bacteriologically.
- MDR TB cases: History of at least one previous period of TB treatment under the centre direct observation (6 month documentation) 2 positive sputum smear, 1 positive sputum culture. Their susceptibility show resistant to isoniazid and rifampsin and their chest X-ray and clinical symptoms were compatible with pulmonary TB.

2.2 Exclusion Criteria

HIV & HCV antibodies positive, HBSAg positive any known concurrent infection, Allergy and Asthma.

2.3 Controls

Subjects who were tuberculin skin test (PPD) negative.

2.4 Sputum Culture and Drug Susceptibility Test

Sputum culture positive for Mtb was confirmed by inoculation of samples on Lowenstein Jensen (LJ) media. Briefly, sputum specimen were decontaminated with 4% NaOH and inoculated into LJ media and incubated for 6-8 weeks at 35-37ºC. When the growth was detected as positive, drug susceptibility test was carried out using absolute concentration method as previously described by Canetti et al. [10]. The reference strain H37Rv was used as control.

2.5 Blood Collection

76 serum samples were obtained from the patients with active TB before treatment. Patients of all age groups with both pulmonary & extra-pulmonary TB were included. Extra–pulmonary sites included were pleural, lymph nodes, soft tissues, meninges, gastrointestinal, bone and joints and disseminated disease. Sera were also obtained from patients with TB who had been treated for at least 2 weeks, but had not yet completed therapy at the time of blood sampling and from the patients who had completed anti-tuberculous therapy. Records of all the patients with active TB were reviewed for clinical data such as fever (rectal temperature > 38°C), anorexia, skin test, bacilli Calmette–Guérin (BCG) vaccination, direct microscopy, and culture results.

The 76 patients, covered 43.42% (33) new, 31.57% (24) UT and 25% (19) MDR TB cases, in which, 1.31% (1 case) had pneumothorax, 2.63% (2 cases) had pyopneumothorax, while 1.31% (1 case) had diabetes mellitus. None of the patients was HIV positive.

2.6 Cytokines Assay

For cytokine analysis, sandwich ELISA with monoclonal antibody sets (Diaclone SAS, Besancon Cedax, France), Streptavidin- Horse reddish peroxides conjugate and recombinant cytokines as standard were used. Briefly, 96 well plates were coated with cytokines (TNF-α & IL-4) according to the manufacturers protocols. Samples were added to all the wells. Diluted biotinylated anti-TNF-α were added and incubated at room temperature for three hours. After washing two times, streptavidin-HRP were added and incubated for 30 minutes. Tetramethylbenzidine (TMB) substrate was added to each well and incubated at room temperature for 12-15 minutes and the reaction was stopped by adding stop solution. Plate was read at 450 nm in an ELISA reader (Thermo Electron Corporation, Vantaa, Finland). The detection range of the assay was less than 8 pg/ml for TNF-α and 0.7 pg/ml for IL-4.

2.7 Statistical Analysis

All statistical analyses were performed using SPSS Statistics (version-20). Receiver operating characteristic (ROC) curve and other performance measures were performed using the statistical software Med Calc (version 10.2.0.0). Pair wise analysis using Chi square test was done to compare differences in cytokines levels between groups of patients. $P<0.05$ was considered significant.

3. RESULTS

Of the 76 patients 14 (34.1%) and 19 (54.2%) was new cases, 11 (26.8%) and 13 (37.1%) were under-treatment TB cases while 10 (24.3%) and 9 (25.71%) were MDR pulmonary and Extra-pulmonary TB cases respectively (Table 1). TNF-α levels was elevated in new and multi-drug

resistant TB patients compared to healthy controls (*P<0.05, P<0.05* respectively) but levels of TNF-α is less significantly in under- treatment cases (Fig. 1). TNF-α & IL-4 showed no significant variations according to the site of involvement in pulmonary vs. extra- pulmonary TB cases (*P>0.05*) (Fig. 2).

The levels of IL-4 showed no significant changes in new cases (*P>0.05*) and under-treatment cases (*P>0.05*) as compared to TNF-α; however, significantly increased levels were seen in MDR cases as compared to controls (*P<0.05*) (Figs. 3 and 4).

From the analysis of the diagnostic accuracy of TNF-α test, TNF-α can be used as a marker for TB in new cases having a good discriminatory power.

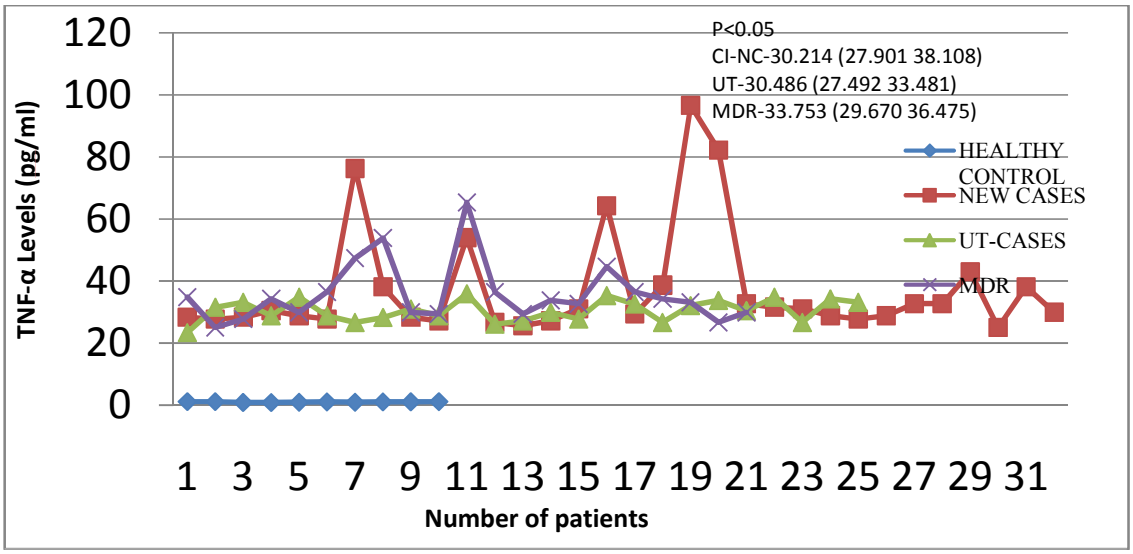

Fig. 1. Levels of TNF-α in NEW, UT & MDR TB cases
**UT-Under-treatment; *MDR- Multi-drug resistant*

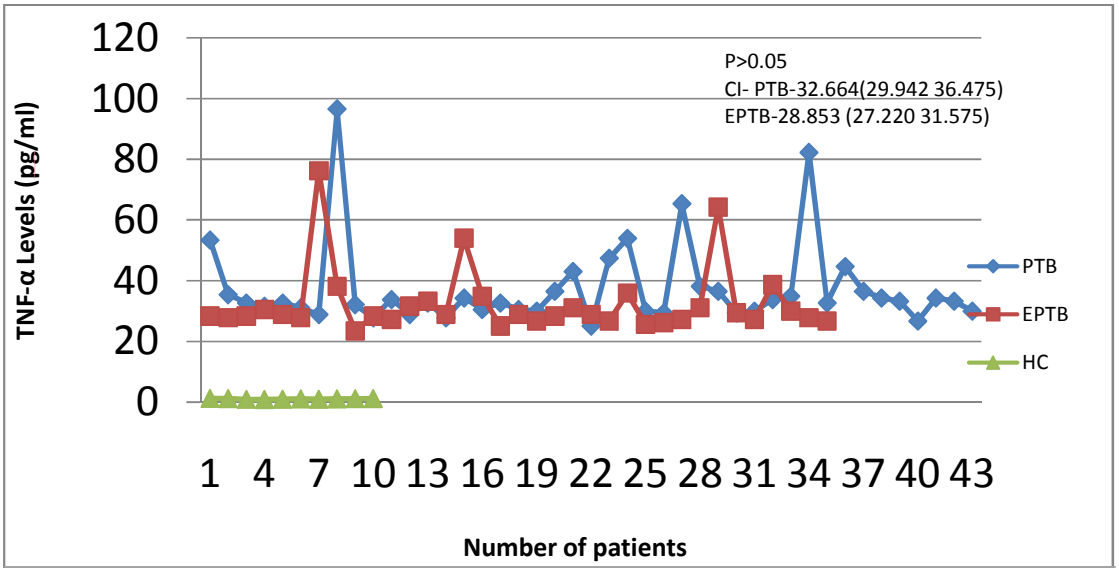

Fig. 2. Levels of TNF-α in PTB and EPTB
**PTB- Pulmonary tuberculosis; *EPTB- extra-pulmonary tuberculosis; *HC- Healthy control*

Table 1. Distribution of study subject in relation to site of TB

Patient group	EPTB (n=35)	PTB (n=41)	P -value	Significant P-value for Chi square
New	14 (34.1%)	19 (54.2%)	*P=0.104* (NS)*	*P<0.05*
Under-treatment	11 (26.8%)	13 (37.1%)	*P=0.458* (NS)*	*P<0.05*
MDR	10 (24.3%)	9 (25.71%)	*P=1.000* (NS)*	*P<0.05*
Total	35 (85.2%)	41(117.01%)		

** For chi square test P<0.05 consider as significant*

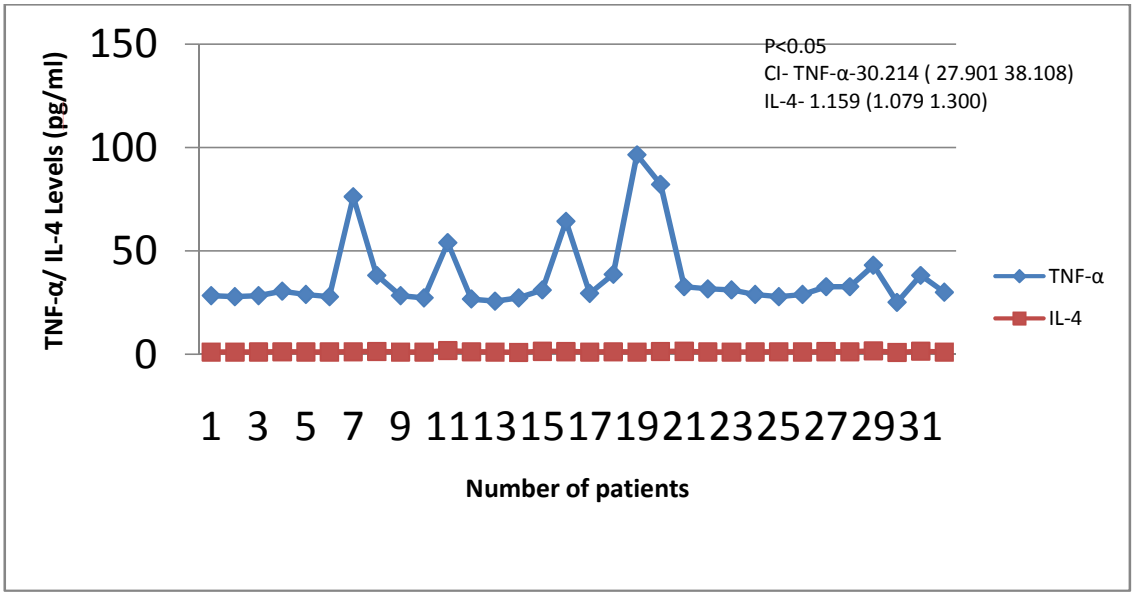

Fig. 3. Levels of TNF-α and IL-4 in TB cases

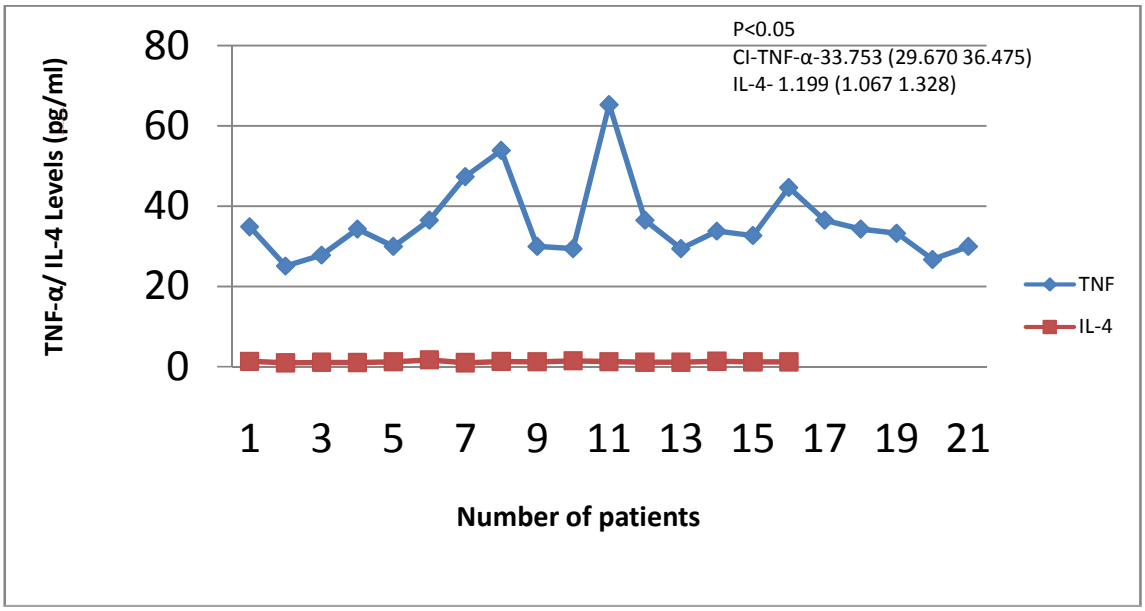

Fig. 4. Levels of TNF- α and IL-4 in MDR TB cases

4. DISCUSSION

Immunological studies conducted on cytokine production have in the past focused on *ex-vivo* cytokine production capacity of isolated peripheral blood mononuclear cells (PBMC) or $CD4^+$ T cells with inconsistent results [11-13]. Additionally *ex-vivo* stimulated production of cytokines did not provide insight into the exact interplay of various cytokines *in-vivo.*

We found significantly higher serum levels of TNF-α in new TB cases than healthy controls. Previous studies have shown higher serum levels of TNF-*α* in active TB patients than control subjects [14-17]. In particular, patients with PTB accompanied by systemic manifestations (persistent fever, weight loss) showed increased TNF-*α* compared with controls [18].

Also we found that serum TNF-α levels declined significantly in UT cases ($P<0.05$). Similarly, Tang et al. [19], Portales- Perez et al. [20] and Kawagnahi et al. [21] found decreased TNF-α levels in TB patients after therapy. However, Dejoba Siawaya et al. [22] did not observe significant differences in TNF-α level after treatment. These studies reinforced the belief that TNF-α has a role in both the physiopathology and protective immunity against TB and that reduction of inflammatory process is associated with disease improvement [23].

TNF-*α* is produced at the site of disease in TB patients [20]. The main TNF-α producing cells are activated macrophages, T-lymphocytes and dendritic cells [24]. Tumor necrosis factor alpha (TNF-α) triggers Mtb killing mechanisms in infected macrophages. Substantial redundancy amongst host receptors allows release of this pro-inflammatory cytokine promptly upon infection. Sensing of Mtb by the aryl hydrocarbon receptor (AhR) is critical for early production of TNF-α and also contributes to both autocrine macrophage activation and TB dissemination. TNF-α grants Mtb access to newly recruited macrophages thereby fostering TB progression. Mtb-beneficial effects have also been reported for exuberant TNF-α release, which induces cell lysis, tissue destruction and facilitates extracellular Mtb growth [25].

We further observed a significant rise in serum TNF-α level in MDR TB patients. Eumsy et al. [26] also reported association of TNF-α level with MDR TB. Giosue et al. [23] studied the role of aerosolized IF-α treatment in patients with MDR TB and found that TNF-α & IF-γ levels dropped in Broncheo alveolar lavage after IF-α treatment in MDR TB patients. These findings suggest the potential role of TNF-α as a marker of response to anti-tubercular therapy and a marker for successful therapy in MDR TB patients. We believe, TNF-α can be a potential marker of response to anti-tubercular treatment in MDR extra-pulmonary TB where sample collection might be a problem.

We found no significant difference in serum IL-4 levels among new and under-treatment TB cases as compared to controls. Some previous studies have shown increased production of IL-4 in human TB patients, especially those with cavitary disease [27]. According to others, it still remains to be determined whether IL-4 causes or merely reflects disease activity in human TB [28,29]. Some studies showed no detectable IL-4 in any TB patient and no significant difference in IL-4 level between TB patients and controls [30-34]. IL-4 represents as one of the cytokines produced by Th2 cells and acts as a co-factor in activation of humoral immunity by activation of B-cells and T-cells proliferation and differentiation [19].

Our study revealed significantly raised IL-4 levels in MDR TB patients. Shahemabadi et al. [9] found elevated levels of IL-4 in TB patients. The stimulated $CD4^+$ T cells by Mtb may be shifted to T helper 2 responses in MDR-TB patients [9].

In addition, MDR-TB infection up-regulated IL-4, IL-6, and TNF-α expression [7]. Our data suggest that the disturbance between protective and pathogenic effects induced by the immunosup-pression of Th1- and Th2-type responses is a substantial characteristic of MDR-TB infections.

TNF-α & IL-4 levels showed no significant variations according to the site of involvement in pulmonary or extra pulmonary TB cases.

5. CONCLUSION

We believe that the, types of cellular immune response may affect presentation, outcome and treatment response in TB. TNF-α and IL-4 can be used as markers of response to anti-tubercular treatment in pulmonary & extra-pulmonary TB. We suggest that a TNF-α marker at the end of therapy could provide an early indication for discontinuation of therapy, a significant problem for MDR-TB where treatment courses often exceed 2 years. However, the sample size in the present study is too small to

make a definitive conclusion and this hypothesis will have to be validated in larger patient cohorts.

Stability of these structures is driven by ratios of pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines along with cellular and molecular events inside the granuloma (25). A better understanding of pathways converging to cavitations could pave the way for rational design of host-directed therapies aiming at rescuing the intact organ.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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