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Research article

Dynamics of CD40 ligand levels in newly diagnosed and treated tuberculosis patients: An evaluation of sCD40L concentration and $CD4^+$ T cell surface expression

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Abstract: Background/objective: CD40 ligand (CD40L/CD154), a member of the TNF superfamily, plays a critical role in immune regulation. Primarily expressed on $CD4^+$ T cells, the CD40-CD40L interaction is essential for B-cell activation, antibody production, and isotype class switching. CD40L expression on T cells has been linked to *Mycobacterium tuberculosis* (MTB)-stimulated IFN-γ production by peripheral blood mononuclear cells. This study aimed to determine the serum concentration of soluble CD40L (sCD40L) and its surface expression on $CD4^+$ T cells in newly diagnosed tuberculosis (TB) patients and in TB patients undergoing anti-tuberculosis treatment (ATT) for 2–3 months. Materials and methods: Ninety-two subjects infected with MTB were recruited and divided into two groups; Group I consisted of 46 newly diagnosed TB patients without prior treatment, and Group II included 46 TB patients receiving ATT for the last 2–3 months. Serum sCD40L concentration of each participant was measured using ELISA, while its surface expression on $CD4^+$ T cells was analyzed via flow cytometry. Results: Serum sCD40L levels were significantly lower in (Group I) newly diagnosed TB patients (median = 11.1 ng/mL, IQR = 1.19–20.8) compared to (Group II) patients undergoing ATT (median = 17.4 ng/mL, IQR = 8.9–27.8), with a statistically significant difference between the groups ($p = 0.000$). The surface expression of CD40L on $CD4^+$ T cells was also lower in

Group I (median = 2.9, IQR = 0.16–13.99) compared to Group II (median = 3.22, IQR = 0.01–12), though the difference was not statistically significant (p = 0.893). Conclusion: Newly diagnosed TB patients exhibited reduced levels of sCD40L, which increased following 2–3 months of anti-tuberculosis treatment. These findings suggest that CD40L may play a role in the immune response to tuberculosis and that its levels are modulated by treatment.

Keywords: tuberculosis; mycobacterium; MTB; CD40; CD40L; CD154

1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (MTB), a gram-positive acid-fast bacillus of the Actinomycetes family [1]. While approximately one-third of the global population is infected with MTB, only a small proportion (5%–10%) of infected individuals develop active TB during their lifetime [2]. In 2021, there were an estimated 10.6 million newly diagnosed TB cases, resulting in at least 1.4 million deaths among non-HIV-infected individuals and an additional 187,000 deaths in those with HIV infection. Pakistan ranks as the fifth-highest country in terms of TB burden. The WHO Eastern Mediterranean Region alone accounts for 61% of the global TB burden, with an estimated 611,000 new cases and a mortality rate of 48,000. Over 36,000 newly diagnosed TB cases were multidrug-resistant (MDR-TB) [3].

Transmission of MTB occurs when individuals with pulmonary TB release airborne droplets by coughing, sneezing, or spitting. TB symptoms include a cough lasting more than three weeks, low-grade fever (often worse at night), unintentional weight loss, and resistance to standard medication regimens [4]. Diagnosis is confirmed when MTB organisms are identified in patient samples such as sputum, pus, or tissue biopsy. A positive culture for MTB is the gold standard for confirming TB [5].

CD40 ligand (CD40L/CD154), a member of the Tumor Necrosis Factor (TNF) superfamily, plays a crucial role in immune function. The *CD40L* gene is located on the X chromosome (q26.3–q27.1). A soluble form of CD40L (sCD40L) is primarily expressed on activated $CD4^+$ T cells. Beyond T lymphocytes, CD40L is also present in non-hematopoietic cells like endothelial, smooth muscle, and epithelial cells, as well as in basophils, NK cells, platelets, B lymphocytes, mast cells, and macrophages [6]. CD40L expression on T cells is transient following activation and is scarcely detectable in resting cells [7]. The interaction between CD40 and CD40L is critical for B-cell activation, antibody production, and isotype class switching. CD40L expression on T cells correlates with MTB-induced IFN-γ production by peripheral blood mononuclear cells [8].

Previous studies have reported altered CD40L expression in TB patients. A study in 2000 showed reduced CD40L expression on peripheral blood mononuclear cells (PBMCs) when B cells were exposed to soluble factors from MTB-infected monocytes, but CD40L expression increased two- to five-fold after six months of anti-TB therapy [9]. Another study in Korea in 2014 reported increased sCD40L levels in TB patients after at least two months of anti-tuberculosis treatment (ATT), coinciding with MTB clearance [10]. Furthermore, CD40L expression was found to be elevated in MTB patients, including in effector memory T cells, compared to uninfected individuals [11]. NK cells also demonstrated increased CD40L expression, contributing to enhanced innate defense against MTB [12]. Additionally, $CD4^+CD40L^+$ T lymphocyte levels were elevated following stimulation with MTB antigens such as early secreted antigen target-6 (ESAT-6) or BCG, compared to negative controls [13].

Active TB patients have shown elevated levels of sCD40L, as well as other cytokines like EGF, IL-10, IL-12p40, IL-13, and monocyte chemoattractant proteins (MCP-1, MCP-1α, and MCP-1β), after initiating ATT, with these levels normalizing by the end of treatment [14].

Given the pivotal role of CD40L in the immune response to TB, this study aims to investigate the serum concentration of sCD40L and its surface expression on $CD4^+$ T cells in newly diagnosed TB patients (without treatment) and those undergoing ATT for 2–3 months.

2. Materials and methods

2.1. Study setting and ethical approval

This study was conducted at the Department of Immunology, University of Health Sciences (UHS), Lahore, Pakistan. The approval (UHS/Education/126-21/3385) to conduct this research study was granted by the Advanced Studies and Research Board, UHS, Lahore, Pakistan.

2.2. Study design and population

A cross-sectional comparative study design was employed, using a convenience sampling technique. This research study was conducted from October 29, 2021, to June 30, 2022. Written informed consent was obtained from each participant. The study included 92 participants, divided into two groups: 46 newly diagnosed TB patients and 46 TB patients who had been receiving ATT for ≥2 and ≤3 months. Both male and female participants aged between 18 and 50 years were included. Exclusion criteria comprised patients with immunodeficiencies, malignancies, or autoimmune diseases.

2.3. TB diagnosis

TB was diagnosed using three criteria:

1. Sputum smear microscopy for AFB.
2. Chest radiograph consistent with active pulmonary TB.
3. Clinical symptoms (cough >3 weeks, fever, night sweats, weight loss).

Each study participant had documented sputum smear testing and chest X-rays. Corresponding positivity rates are included here (see Table 1).

Group II: At the time of sample collection (after 2–3 months of ATT), sputum smear and X-ray results were recorded to determine whether disease activity persisted. These results are included in Table 1.

2.4. Measurement of serum sCD40L levels

Serum levels of soluble CD40 ligand (sCD40L) were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Optical density (OD) was measured at 450 nm using Microplate Manager® Software Version 5.2.1 from Bio-Rad Laboratories, California, USA.

Table 1. Age, number, percentage, and comparison of gender and clinical findings between the two groups. Values are mean ± SD.

Variables	Group I (newly diagnosed TB patients) N = 46	Group II (patients on ATT) N = 46	p-value
Age (mean ± SD)	34.3 ± 11	31 ± 7.8	0.092
Gender			
Male n (%)	29 (50.9)	28 (49.1)	1.0
Female n (%)	21 (48.8)	22 (51.2)	1.0
Cough n (%)	42 (54.5)	35 (45.5)	0.153
Weight loss n (%)	19 (44.2)	24 (55.8)	0.419
Smoking status n (%)	18 (47.4)	20 (52.6)	0.083
Fever n (%)	32 (66.7)	16 (33.3)	0.003*
Sputum smear positive (%)	46 (100)	46 (100)	
Chest X-ray consistent with TB (%)	46 (100)	46 (100)	

*p-value ≤ 0.05 as statistically significant.

2.5. Immunophenotyping and flow cytometry

Surface expression of CD40L on $CD4^+$ T cells was assessed through flow cytometry using the lyse-wash method. Anti-CD4-FITC and anti-CD40L-PE antibodies (BD Pharmingen, USA) were used for immunophenotyping. Data acquisition and analysis were performed on a FACSCalibur flow cytometer (BD Biosciences, USA) (Figures 1–3). CD40L expression is transient and is typically low in resting, unstimulated peripheral blood T cells. As no in vitro stimulation was performed**,** only a small proportion of $CD4^+$ T cells were expected to express CD40L, explaining the low percentage in dot plots.

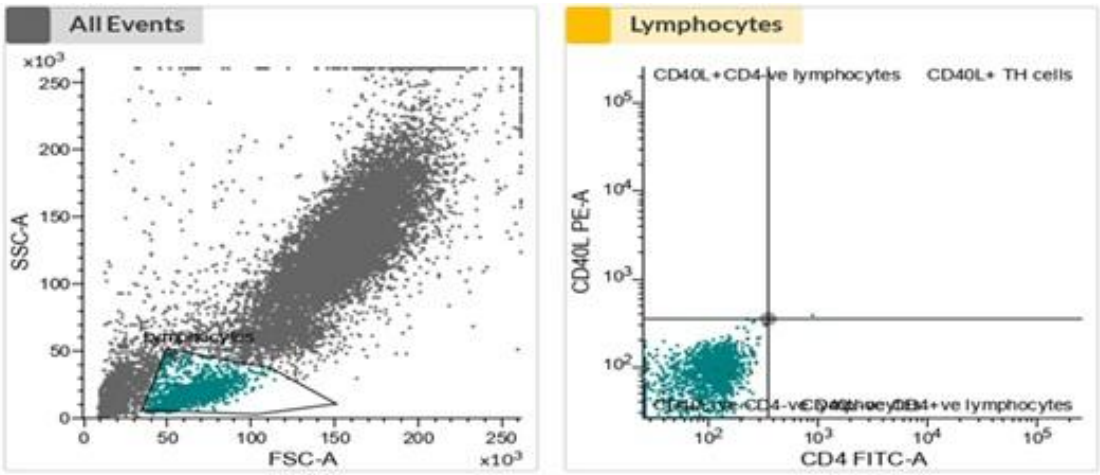

Figure 1. Unstained flow cytometry plots (quality control/background control). (1a) Forward scatter (FSC-A) versus side scatter (SSC-A) dot plot of an unstained peripheral blood sample from a newly diagnosed TB patient before treatment, showing three major cell populations based on size and granularity, with the lymphocyte gate indicated. (1b) CD40L (PE-A) versus CD4 (FITC-A) dot plot of the same unstained sample, presented as a representative negative control to demonstrate background autofluorescence and to define gating thresholds for subsequent stained analyses.

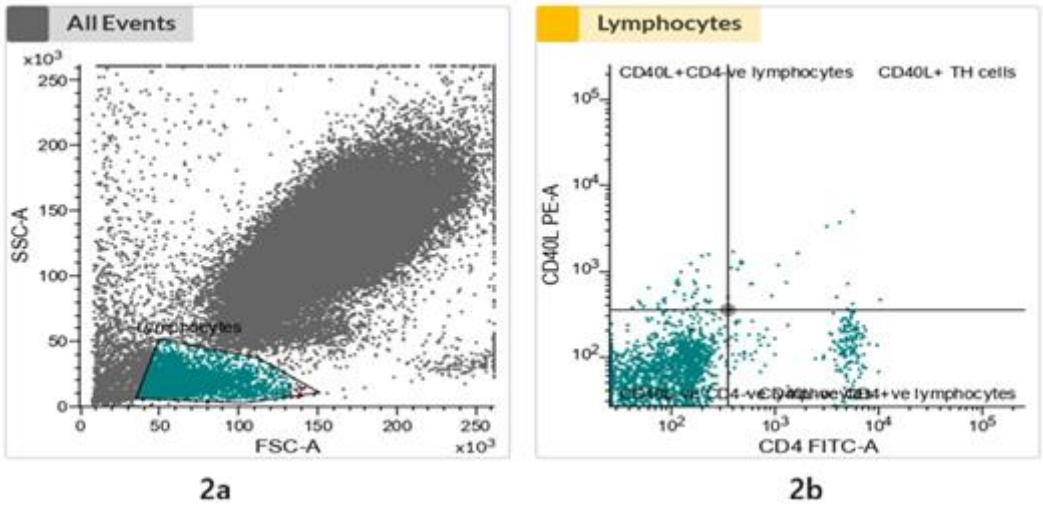

Figure 2. Stained flow cytometry plots of a TB patient after treatment (representative plot). (2a) FSC-A versus SSC-A dot plot of a stained peripheral blood sample from a diagnosed TB patient after treatment, showing gated lymphocytes selected for further immunophenotypic analysis. (2b) CD40L (PE-A) versus CD4 (FITC-A) dot plot of gated lymphocytes from the stained sample, illustrating $CD4^+$ T cells and the distribution of CD40L expression following antibody staining.

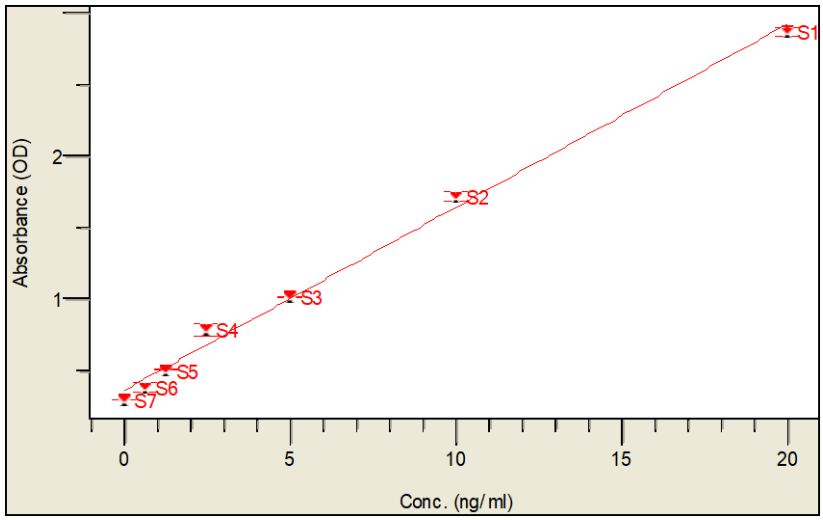

Figure 3. Standard curve of CD40L.

2.6. Statistical analysis

Data was entered and analyzed using SPSS version 24.0. Normality of the data was assessed using Kolmogorov–Smirnov and Shapiro–Wilk tests. For normally distributed quantitative variables, means and standard deviations (mean ± SD) were reported, while medians and interquartile ranges (IQR) were provided for non-normally distributed data. To compare group differences in sCD40L levels and CD40L surface expression on T cells, an independent-samples t-test was employed for normally

distributed data, while the Mann–Whitney U test was used for non-normal distributions. Spearman's rho test was used to evaluate correlations between sCD40L levels and CD40L surface expression. A p-value of ≤ 0.05 was considered statistically significant.

3. Results

The mean age of the subjects in Group I (newly diagnosed TB patients) was slightly higher: 34.3 ± 11 years, compared to Group II (patients on ATT), where it was 31 ± 7.8 years. However, this difference was not statistically significant (p = 0.092). Regarding gender distribution, Group I had 50.9% males, while Group II had 49.1%, with no statistically significant difference between the two groups (p = 1.0). A history of smoking was more prevalent in Group II (56.6%) compared to Group I (47.4%), but this difference was not statistically significant (p = 0.83).

In terms of clinical symptoms, a significantly higher proportion of Group I subjects (66.7%) reported a history of fever compared to Group II (33.3%), and this difference was statistically significant (p = 0.003). However, other symptoms, such as weight loss, were more common in Group II (55.8%) than in Group I (44.2%), but the difference was not statistically significant (p = 0.419). Similarly, the prevalence of cough was higher in Group I (54.5%) than in Group II (45.5%), though this difference was also not statistically significant (p = 0.153) (Table 1).

3.1. CD40L expression and serum levels

The surface expression of CD40L on $CD4^+$ T cells was slightly lower in Group I (median = 2.9, IQR = 0.16–13.99) compared to Group II (median = 3.22, IQR = 0.01–12). However, the difference was not statistically significant (p = 0.893). In contrast, the serum levels of sCD40L were significantly lower in newly diagnosed TB patients (Group I) with a median of 11.1 ng/mL (IQR = 1.19–20.8) compared to patients receiving ATT in Group II, who had a median of 17.4 ng/mL (IQR = 8.9–27.8). This difference was statistically significant (p = 0.000).

3.2. Correlation analysis

The surface expression of CD40L on T cells showed a positive correlation with serum sCD40L levels, but this correlation was not statistically significant (p = 0.2). Additionally, CD40L surface expression had a negative correlation with age, though this too was not statistically significant (p = 0.817). Serum sCD40L levels also demonstrated a positive correlation with $CD4^+$ T cells expressing CD40L and a negative correlation with age, but neither of these correlations was statistically significant (p = 0.23 and p = 0.552, respectively) (Table 2).

Table 2. Serum levels of CD40L and its surface expression between the two study groups.

Variables	Group I n = 46	Group II n = 46	p-value
sCD40L (ng/mL)	11.1 1.19–20.8	17.4 8.9–27.8	0.000*
Surface expression of CD40L (%)	2.9 0.16–13.99	3.22 0.01–12	0.893

Data is expressed as median with interquartile range (IQR), *p-value ≤ 0.05 is considered statistically significant.

4. Discussion

The current study demonstrated a statistically significant increase in serum concentrations of sCD40L in patients of Group II (on ATT) compared to Group I (newly diagnosed TB patients) who had not received ATT yet. This finding aligns with the conclusions of Hur et al. and Saiwaya et al. [15]. CD40L is known to stimulate TNF-α-dependent antimicrobial activity in macrophages, playing a critical role in host defense mechanisms, especially when IFN-γ signaling is compromised [16].

However, this study's results differ from those reported by Samten et al. where no significant effect of sCD40L on the intracellular growth of MTB was found. These studies also noted that blocking CD40L on $CD4^+$ T cells with monoclonal antibodies did not influence MTB clearance, implying that other lymphocyte surface ligands may be involved in the immune response to MTB. Mizusa et al. also documented significantly higher sCD40L levels in TB patients with cavitary lesions compared to those without such lesions. The difference in findings could be attributed to the distinct age demographics between studies; the mean age of participants in their study was 59 ± 22 years, whereas in our study, it was significantly younger (34.3 ± 11 years in Group I and 31 ± 7.8 years in Group II) [17].

In the current study, CD40L surface expression on T cells was lower in newly diagnosed TB patients compared to those receiving ATT, though the difference was not statistically significant. CD40L expression on T cells has been directly correlated with MTB-stimulated IFN-γ production in TB patients and healthy tuberculin reactors [18]. Samten et al. similarly reported reduced CD40L expression on peripheral blood mononuclear cells (PBMCs) after exposure to MTB, with a two- to five-fold increase observed after six months of ATT, a finding that aligns with our results [17]. However, this study contrasts with a 2020 report that suggested elevated CD40L expression on T cells and effector memory cells in MTB patients compared to uninfected contacts, highlighting the need for further investigation [10].

Further, this study did not find a statistically significant correlation between CD40L surface expression on T cells and serum sCD40L levels in TB patients. While a weak positive correlation was observed, it was not statistically significant. None of the available literature documents a correlation between these two markers in TB patients. However, studies in other inflammatory conditions have demonstrated increased CD40L expression. Berner et al. observed enhanced CD40L surface expression on T cells in rheumatoid arthritis patients [18], while Liu et al. noted elevated CD40L levels in patients with inflammatory bowel disease [19]. Similarly, Clodi et al. reported increased CD40L expression on B cells in Hodgkin's disease [20], and elevated CD40L levels have been found in multiple sclerosis patients compared to healthy controls [21].

One of the limitations of the current study was its small sample size and cross-sectional design. Future studies with larger sample sizes involving both newly diagnosed and treated TB patients are needed to further confirm these findings. Additionally, longitudinal studies may provide a clearer understanding of the role of CD40L and sCD40L in TB. If confirmed in larger-scale studies, sCD40L could serve as a potential biomarker for monitoring treatment response in TB patients.

5. Conclusions

This study demonstrated decreased serum levels of sCD40L in newly diagnosed TB patients and significantly increased levels in patients on ATT for a period of 2–3 months. Although an increase in CD40L surface expression on T cells after ATT treatment was observed, it was not statistically

significant. The observed rise in sCD40L levels post-treatment suggests that sCD40L could potentially serve as a valuable biomarker for monitoring treatment response in TB patients undergoing ATT. Further studies with larger sample sizes and longitudinal designs are needed to confirm its utility as a reliable indicator of therapeutic efficacy.

6. Limitations

Baseline levels of sCD40L and CD40L for Group II before beginning ATT were not available. There was an absence of healthy control participants and a modest sample size. Further, flow cytometric measurements of CD40L in unstimulated ex vivo T cells were naturally low.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Author contributions

(I) Conception and design: SS, RT, NA, MK, FS; (II) Administrative & supervision support: NA, RT, FS, VS, AR; (III) Provision of study materials or patients: MK, AS, NA, SS, VS, AR; (IV) Collection and assembly of data: SS, AR, MK, AR; (V) Data analysis and interpretation: SS, VS, MK, FS; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Data availability

Data supporting the findings of this research article can be provided on demand by contacting the corresponding author.

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