

Assessment of Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) Levels as an Immune Checkpoint in Toxoplasmosis: A Clinical Study

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ABSTRACT

Background: The acute phase of *Toxoplasma gondii* infection is critical for parasite proliferation and dissemination within the host. During this phase, the immune system plays a vital role in controlling the infection. However, *T. gondii* employs various immune evasion strategies, including manipulation of host cell signaling, inhibition of apoptosis, and cytokine regulation, to establish infection. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), an immune checkpoint molecule, regulates T-cell activation and immune homeostasis. Increased CTLA-4 expression during *T. gondii* infection creates an immunosuppressive environment that helps the parasite evade immune clearance. **Objective:** This study aimed to evaluate the serum levels of CTLA-4 in patients during the acute phase of toxoplasmosis and to investigate its correlation with serological markers of *T. gondii* infection. **Methods:** A total of 350 individuals suspected of toxoplasmosis, aged 18–48 years, were included in the study. Serum samples were collected and analyzed using quantitative ELISA to measure CTLA-4 levels and *T. gondii* IgM. Statistical analyses were performed to assess differences in CTLA-4 levels and their correlation with infection markers. **Results:** CTLA-4 levels were significantly higher in patients with toxoplasmosis compared to the control group ($P = 0.0001$). A significant difference in CTLA-4 concentrations was observed across the three categories of toxoplasmosis symptoms ($P = 0.0001$). However, the correlation analysis revealed a non-significant negative relationship between CTLA-4 levels and *T. gondii* IgM ($P = 0.8$). **Conclusion:** The study highlights a significant increase in serum CTLA-4 levels during acute toxoplasmosis, suggesting its role in the immunosuppressive environment that facilitates *T. gondii* survival. These findings provide insights into immune checkpoint mechanisms in toxoplasmosis and underscore the potential of CTLA-4 as a biomarker for immune regulation in this infection.

Keywords: Immune Checkpoint, CTLA-4, Toxoplasmosis, T. Gondii

INTRODUCTION

Toxoplasma gondii is known to manipulate host immune responses to establish a persistent infection [1]. This manipulation involves modulation of immune regulatory pathways, including those mediated by checkpoint molecules like CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) [2]. The parasite employs various strategies to evade host immune surveillance and promote its survival within the host organism [3]. CTLA-4 is a critical immune checkpoint protein that negatively regulates T cell responses. Research suggests that *Toxoplasma gondii* infection can influence CTLA-4 expression and function [4]. This influence may contribute to immune suppression and parasite persistence by dampening T cell-mediated immune responses against the parasite [5].

MATERIAL AND METHODS

Study Design

Samples were collected from suspected 350 individuals infected and non-infected with the *Toxoplasma gondii* were selected from the Hospitals and external laboratories, with an age range from (18-48) years suffering from cervical lymphadenopathy and flu-like symptoms (fever, headache, joint pain, cough, malaise, anorexia, nausea and pain in the body), women with aborted and delayed pregnancy. The diagnosis was under the supervision of a consultant. This study was conducted over a duration of Eight months, specifically from September 2023 to June 2024. The approval I.R.B for this study was (20230904) granted by the Ethics Committee of the Department of Microbiology at the College of Medicine, University of AL-Nahrain,

Iraq. A total of over from suspected 350 individuals infected and non-infected with the *Toxoplasma gondii* were selected from the Hospitals and external laboratories.

Including criteria: Patient with cervical lymphadenopathy, abortion, delayed pregnancy and flu-like symptoms (fever, headache, joint pain, cough, malaise, anorexia, nausea and pain in the body) will subjection for blood collection. Exclusion criteria: Patients with other diseases such as (DM. Under chemotherapy. Other immune diseases).

Samples

Five milliliters (5 ml) of blood were obtained through venous puncture by the technicians. The serum was separated from the blood, and divided into three portions. The first portion was prepared for first analysis (rapid test or several laboratory devices for detection) was performed, and the second portion was for second analysis by ELISA assay to detect *Toxoplasma gondii* IgM positive and the third portion for ELISA assay to detect level of concentration to CTLA-4 proteins. Serum was transferred to a blank tube for serology tests. Finally, in order to prevent cross-contamination and unnecessary thawing, the samples were promptly frozen and stored at -20 C.

Statistical Analysis

T-test were used to compare between test groups and control.X2 was used to compare between frequency The obtained data were subjected to one way analysis of variance (ANOVA) test to compare various groups with each other. Results were expressed as mean $\pm$

standard deviation (SD) and other descriptive statistics. LSD test was used to calculate the significant differences between tested mean, the letters (A, B, and C) represented the levels of significant, highly significant start from the letter (A) and decreasing with the last one. Similar letters mean there are no significant differences between tested mean. $P < 0.05$ considered statically significant. The statistical analysis was carried out by SPSS (v 20).

Results

The study consisted of 100 individuals, divided into two groups. Group 1 was comprised of 50 acute toxoplasmosis patients revealed

positive ELISA result for *Toxoplasma gondii* IgM antibody, Group 2 Control included 50 individuals who appeared to be healthy and revealed negative ELISA result for *Toxoplasma gondii* IgM antibody.

Serum Concentration of *Toxoplasma gondii* IgM in the Study Group

As showing in table (1) Compare between Group 1 and Group 2 according to *Toxoplasma gondii* IgM serum concentration group1 was (3.43) while in group 2 was (0.63), then in median, group1 was 3.32 and in group2 was 0.55, revealed highly significant statically deferent importance (P value 0.0001).

Table 1: Compare between Group 1 and Group 2 According to Serum Concentration of *Toxoplasma Gondii* IgM

Statistics	TOXO /IU/ml	
	Group 1	Group 2
N	50	50
Mean	3.43	0.63
Std. Error of Mean	0.11	0.04
Median	3.32	0.55
Std. Deviation	0.75	0.30
Minimum	1.90	0.09
Maximum	4.79	1.68
P Value	0.0001	

Serum Concentration of CTLA-4 in the Study Groups

Table (2) shows the concentration of CTLA-4 in both studies group. The mean of CTLA-4 in group 1 was (116) while in group 2 was (81), and a median was (114) in group 1 and (80) in group 2, the result was highly significant statically deferent importance (P value 0.0001).

CTLA-4 Concentration According to Toxoplasmosis Symptoms

Table (3) shows CTLA-4 concentration with Toxoplasmosis symptoms, the highest concentration was in patients with cervical lymphadenopathy (59 ng/ml), then patients with abortion (43 ng/ml), and the low concentration in patients with delayed pregnancy (38 ng/ml), the result revealed highly significant difference in serum concentration between the three types of Toxoplasmosis symptoms (P value 0.0001)

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Correlations Between Serological Biomarker and Serum Concentration of *Toxoplasma gondii* IgM Antibody

Table (4) Showing Correlations between serological biomarker and serum concentration of *Toxoplasma gondii* IgM, and revealed negative correlation with non-significant between CTLA-4 and Toxoplasmosis (P -value=0.8)

Table 2: Serum Concentration of CTLA-4 in Study Group

Statistics	CTLA-4/ng/ml	
	Group1	Group 2
N	50	50
Mean	116.10	81.60
Std. Error of Mean	1.48	1.85
Median	114.17	80.47
Std. Deviation	10.49	13.05
Minimum	100.09	63.17
Maximum	143.55	111.50
P value	0.0001	

Table 3: Associated CTLA-4 Concentration with Major Symptoms

CTLA-4 concentration	CTLA-4 Associated with Major Symptoms		
	Symptoms		
	Abortion	Cervical Lymphadenopathy	Delayed Pregnancy
N	23	11	16
Mean	B	A	C
	43.00	59.00	38.00
Std. Error of Mean	10.89	14.23	19.88
Median	2.40	2.48	2.61
Std. Deviation	14.46	15.24	19.69
Minimum	11.26	8.24	10.44
Maximum	100.09	104.83	102.45
P value	0.0001		

*LSD test was used to calculate the significant differences between tested mean, the letters (A, B, and C) represented the levels of significant, highly significant start from the letter (A) and decreasing with the last one. $P < 0.05$ considered statically significant.

Table 4: Showing Correlations between serological biomarker and serum concentration of Toxoplasmosis IgM

Correlations		TOXO	CTLA-4
TOXO	Pearson Correlation	1	-.028-
	Sig. (2-tailed)		.848
	N	50	50
CTLA-4	Pearson Correlation	-.028-	1
	Sig. (2-tailed)	.848	
	N	50	50

Correlations between Serological Biomarker and Toxoplasmosis Positive Women with Abortion

Table (5) Shows Correlations between serological biomarker and serum concentration of *Toxoplasma gondii* IgM in women with abortion. There is negative correlation with non-significant between CTLA-4 and Toxoplasmosis (P-value=0.3)

Table 5: Showing Correlations between Serological Biomarker and Serum Concentration of *Toxoplasma Gondii* IgM in Aborted Women

		Correlations	
Abortion		TOXO	CTLA-4
TOXO	Pearson Correlation	1	-.260-
	Sig. (2-tailed)		.369
CTLA-4	Pearson Correlation	-.260-	1
	Sig. (2-tailed)	.369	

*Correlation is significant at the 0.05 level (2-tailed).

Table 6: Showing Correlations between Serological Biomarker and Serum Concentration of *Toxoplasma Gondii* IgM in Cervical Lymphadenopathy

		Correlations	
Cervical Lymphadenopathy		TOXO	CTLA-4
TOXO	Pearson Correlation	1	-.283-
	Sig. (2-tailed)		.348
CTLA-4	Pearson Correlation	-.283-	1
	Sig. (2-tailed)	.348	

*Correlation is significant at the 0.05 level (2-tailed).

Table 7: Showing Correlations between Serological Biomarker and Serum Concentration of *Toxoplasma Gondii* IgM in Delayed Pregnancy

		Correlations	
Delayed Pregnancy		TOXO	CTLA-4
TOXO	Pearson Correlation	1	.064
	Sig. (2-tailed)		.778
CTLA-4	Pearson Correlation	.064	1
	Sig. (2-tailed)	.778	

*Correlation is significant at the 0.05 level (2-tailed).

Correlations between Serological Biomarker and Toxoplasmosis Positive Women with Delayed Pregnancy

In table (7) shows Correlations between serological biomarker and serum concentration of Toxoplasmosis IgM in women Delayed pregnancy. There are positive correlation with non-significant correlation between CTLA-4 and Toxoplasmosis (P-value=0.7)

Discussion

1- In table 1, group 1 had a high concentration of IgM than group 2, revealed highly significant statically deferent importance (P value 0.0001). In group 1 the presence of *Toxoplasma gondii* IgM antibodies are a key marker in diagnosing acute toxoplasmosis. During an acute infection, the immune system produces IgM antibodies as an early response to the parasite [6]. In Iraq, Mikaeel (2020) his conclusion the presence of IgM antibodies was used to identify women with recent *Toxoplasma gondii* infections [7]. Also Ameer (2022) said the presence of *Toxoplasma gondii* IgM antibodies indicates acute or recent infection [8]. Both studies utilized the presence of *Toxoplasma gondii* IgM antibodies to identify recent infections and explore potential health impacts, these studies confirm the importance of monitoring *Toxoplasma gondii* infections and their potential health effects [9]. In group 2 the presence of Toxoplasmosis IgM in uninfected people can be attributed to several factors, including cross-reactivity, persistence of IgM antibodies, non-specific binding, and Laboratory Error [10].

2- The distribution of Checkpoint Biomarkers CTLA-4 in the studied groups in table 2, demonstrated a highly statically deferent importance in the levels or presence of CTLA-4 between the patients with toxoplasmosis IgM and the control group (P value 0.0001).

Correlations between Serological Biomarker and Toxoplasmosis Positive Women with Cervical Lymphadenopathy

Table (6) Shows Correlations between serological biomarker and serum concentration of *Toxoplasma gondii* IgM in Cervical lymphadenopathy. There is a negative correlation with non-significant in the relation Toxoplasmosis and CTLA-4 (P-value=0.3).

Studies into the specific concentration of CTLA-4 expression in individuals with acute toxoplasmosis is limited, but there are some studies that provide insights into the role and expression levels of CTLA-4 in the subject of toxoplasmosis. A study published in "The Journal of Immunology" investigated the role of CTLA-4 in the immune response to *Toxoplasma gondii* infection. The studies found that CTLA-4 expression was significantly upregulated in CD4+ T cells during acute toxoplasmosis. While the studies did not specify the exact percentage of cells expressing CTLA-4, it demonstrated that CTLA-4 plays a crucial role in modulating the immune response to the parasite [11, 12]. To provide a more precise percentage, further detailed studies focusing specifically on CTLA-4 expression levels in acute toxoplasmosis are required. As of now, the available literature indicates that CTLA-4 expression is indeed upregulated during acute infection, playing a role in immune regulation and potentially contributing to the chronicity and immune evasion mechanisms of *Toxoplasma gondii*.

3- The distribution in table (3) revealed highly significant deferent importance (P value 0.0001). The most statistically significant is cervical lymphadenopathy, and then abortion, and finally delayed pregnancy. CTLA-4 is crucial in regulating T cell responses during infections, including *Toxoplasma gondii*. It generally acts to inhibit T cell activation, which can help control immune-mediated tissue damage in infections that cause lymphadenopathy [13]. Studies have shown that blockade of CTLA-4 leads to enhanced resistance against *T. gondii* but also increases immune-mediated pathology, suggesting a balancing role of CTLA-4 in immune regulation [14, 15]. The general understanding is that CTLA-4 expression increases significantly in response to infection,

potentially affecting more than half of the T cell population involved in the response. CTLA-4 is known to be upregulated during *Toxoplasma gondii* infection to modulate the immune response. This upregulation can help control the inflammatory response, which is crucial in preventing tissue damage but might also be implicated in the pathology of abortion when the immune response to the infection is dysregulated [11]. CTLA-4 plays a significant role in maintaining immune tolerance during pregnancy. Its expression can affect pregnancy outcomes by regulating maternal immune responses to fetal antigens. In the context of infections, CTLA-4 expression can be altered, potentially impacting fertility and pregnancy maintenance [16]. Henriquez said the preventing excessive inflammation and tissue damage during infection, which can be critical of pregnancy and fertility [17]. There are no studies on delayed pregnancy and CTLA-4, and this study is the first study in this regard.

4- Negative correlation between CTLA-4 and Toxoplasmosis with non-significant statically distribution (P-value=0.8), were demonstrated in Table (4). The concept of a negative correlation between CTLA-4 expression and the severity of *Toxoplasma gondii* infection suggests that higher levels of CTLA-4 might be associated with reduced severity or better control of the infection due to its immune-inhibitory functions. The study results negative correlation with non-significant between CTLA-4 and Toxoplasmosis also in agreement with Souza (2021), who said the non-significant statistical distributions indicate that observed correlations between CTLA-4 expression and *Toxoplasma gondii* infection outcomes do not meet predefined thresholds for statistical significance. This may reflect the need for larger sample sizes, host genetics, parasite strain variability, and immune history, more precise measurements, or additional controls to detect meaningful associations [18].

5- According to the results of Table 5, there was a negative correlation between CTLA-4 and Toxoplasmosis abortion women with non-significant statically distribution (P-value=0.3)

6- In patients cervical lymphadenopathy symptoms and Toxoplasmosis positive Table 6 indicate negative correlation non-

significant between CTLA-4 and Toxoplasmosis (P-value=0.3). Direct studies specifically linking CTLA-4 expression to cervical lymphadenopathy due to *Toxoplasma gondii* infection may be sparse [19]. However, their roles in immune regulation and lymphoid tissue responses suggest potential implications for inflammatory conditions affecting cervical lymph nodes. Buchbinder demonstrated that CTLA-4 can function synergistically or independently to modulate immune responses in lymphoid tissues such as cervical lymph nodes [19]. Their expression levels and activities influence the balance between immune activation and tolerance, which is crucial for regulating inflammatory responses and maintaining tissue integrity.

7- Table 7 shows positive with non-significant correlation between CTLA-4 and Toxoplasmosis (P-value=0.7). While direct studies on CTLA-4 in delayed pregnancy specifically due to *Toxoplasma gondii* infection may be limited, their dysregulation in immune responses can indirectly impact reproductive outcomes.

Understanding how these immune checkpoints influence immune tolerance and inflammation regulation in infections like *Toxoplasma gondii* is crucial for elucidating their roles in delayed pregnancy or infertility. CTLA-4 are critical checkpoints that act to suppress immune responses and prevent autoimmunity. CTLA-4 competes with CD28 for binding to B7 molecules on antigen-presenting cells, resulting in the inhibition of T-cell activation and proliferation [20].

The study result in agreement with Piccinni, et al shown that CTLA-4 can interact synergistically or independently to modulate immune responses in various subject, including infections and autoimmune diseases. Their expression levels and activities can influence the balance between immune activation and tolerance, which are critical for normal reproductive function [21].

Conclusions

There is a significant serum level (CTLA-4) in acute Toxoplasmosis.

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