AMERICAN JOURNAL OF BIOLOGY AND NATURAL SCIENCES
https://biojournals.us/index.php/AJBNS
ISSN: 2997-7185

Article

Comprehensive Evaluation of TLR4 and Cytokine Profiles in Patients Infected with *Entamoeba histolytica*

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Citation: Hasan T. A. H. Comprehensive Evaluation of TLR4 and Cytokine Profiles in Patients Infected with *Entamoeba histolytica*. American Journal of Biology and Natural Sciences 2025, 2(10), 57-67.

Received: 30th Aug 2025
Revised: 11th Sept 2025
Accepted: 26th Sept 2025
Published: 07th Oct 2025

Abstract: The current research aimed at systematically identifying the values of Toll-Like Receptor four (TLR4) and the trend of expression of some immune cytokines (IL-1b, IL-6, TNF-a, IL-10, IFN-g) in an entamoeba histolytica-infected patient with amebic liver abscess (ALA) and with amebic colitis (AC) versus healthy controls. The blood sample analyses were conducted on the blood samples using the sophisticated techniques such as enzyme immunoassay (ELS), the western blot and the flow cytometry processes to correlate these disease markers depending on the intensity of the disease. Twenty healthy people were used as a control group, 30 patients with ALA and 30 patients with AC were incorporated. The findings indicated that the ALA patients had a greater up-regulation of TLR4 along with the expression of pro-inflammatory cytokines (IL-1b, IL-6, TNF-a, IFN-g) in comparison with the AC patients and controls which meant that there was a high inflammatory immune response which was associated with severe infection. However, the anti-inflammatory cytokine IL-10 was relatively increased in AC patients, which indicated that it may play important roles in inflammation control for the defense of tissue. Moreover, clinical symptoms correlated positively with higher inflammatory markers, respectively proving a stronger correlation in severe ALA cases.These results indicate that TLR4 and the studied cytokines are potential biomarkers for severity of onset of Entamoeba histolytica infection and could be useful in the implementation of future diagnostic and therapeutic approaches based on modulation of immune responses to limit tissue damage and preserve an effective host defence. These results enrich the importance of pro-and anti-inflammatory cytokine balance for determining the end of the disease and this study suggests internalization of these biomarkers for early diagnosis and immune-target therapy.

Keywords: Entamoeba Histolytica, Cytokine Profiles, Toll-Like Receptor 4, Amebic Liver Abscess

Introduction

Infection by the intestinal protozoan *E. histolytica* is a principal cause of mortality globally. The condition results from the parasite's capacity to infiltrate the colon, leading to amebic colitis. *Entamoeba histolytica* can spread to the liver through the portal venous system, leading to amebic liver abscess (ALA). Approximately 90% of infected individuals are asymptomatic cyst carriers [1,2,3]. The

molecular mechanisms by which this parasite induces invasive amebiasis remain incompletely elucidated. *E. histolytica* possesses adhesion and cytotoxicity elements crucial for its survival; however, these factors do not directly contribute to ALA production. The limiting and prevention of recurrent invasive amebiasis necessitate the establishment of a robust immune response. Consequently, the initial inflammatory response linked to *E. histolytica* infection is probably a crucial role in the onset of ALA" [4].

"Immune responses specific to parasites are modulated by cytokines and chemokines, which facilitate the establishment of immunity; nevertheless, same responses can exacerbate infection, promoting pathogenesis and the persistence of parasites. Limited information exists concerning the amoebic cues that trigger an acute inflammatory response [5]. Reports indicate that in mice infected with *E. histolytica*, tissue damage in the host is predominantly due to the lectin activity of galactose/N-acetyl-D-galactosamine (Gal/GalNAc) from *E. histolytica*, which facilitates the aggregation of mononuclear cells, such as neutrophils, inflammatory monocytes, and macrophages, at the infection site [6]. The application of the lectin Gal/GalNAc from both pathogenic and nonpathogenic entamoebas (*E. histolytica* and *E. dispar*) to cultured human intestinal cells induces the secretion of chemoattractant and proinflammatory cytokines, indicating that these cells and cytokines may also play a role in tissue damage, influencing the mechanisms of initiation, amplification, or attenuation of inflammatory processes during invasive amebiasis" [7].

"Identifying the mediators involved in leukocyte activation during *E. histolytica* infection is crucial for comprehending host responses in amebiasis. Cellular interactions with cytokines have been shown in amebic infections, with cytokines demonstrated to modulate monocyte function and enhance the amebicidal activity of monocytes [8]. They have advanced as successful pathogens in part because of their remarkable and sophisticated ways to evade innate host defenses. This clutches true for both intracellular and extracellular parasites that deploy multiple strategies to circumvent innate host defenses for their survival [9]. The host's body activates the standard immune response during infection with *E. histolytica*, utilising many defence mechanisms, including lymphocytes, mucosal secretions from skeletal membranes, intestinal motility, and the stomach's acidic environment [10,11]".

The cell-mediated immune response is considered the major host defense against *E. histolytica*. During the first step of infection, intestinal epithelial cells bind the carbohydrate domain of "Gal/GalNAc lectin through the Toll -like receptor (TLR)-2/4 NFKB, which functions by producing inflammatory cytokines, including IL-1b, IL-6, IL-10, IFN-g, and TNF-ą" [12]. Humoral immune responses to *E. histolytica* are completely developed and generated when amebiasis infects the patient, producing circular antibodies within seven days post-infection [13]. Examining the immunological response to *E. histolytica* is crucial for exploring the early detection of this illness.

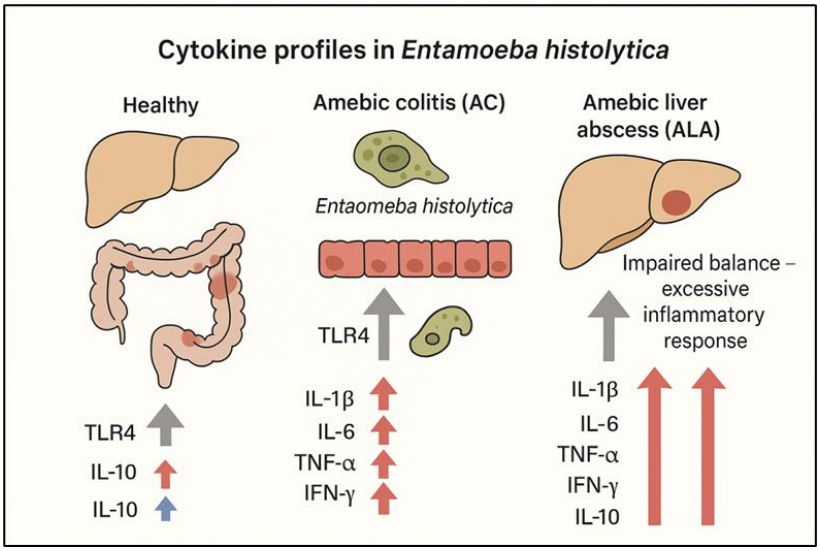

Figure 1. TLR4 and cytokine levels in Entamoeba histolytica infections indicate a normal immunological equilibrium in healthy persons, a moderate reaction in colonic amoebiasis (AC), and an exaggerated inflammatory response in amoebic liver abscess (ALA).

TLR4 is established as a sensor for recognition of microbial cell wall structures, which include the bacteria cell wall component lipopolysaccharide (LPS) in Gram-negative bacteria [14]. It has however also been implicated as being a key contributor to the response to secreted factors of parasites like Entamoeba histolytica. Basically, when TLR4 is bound on its activation, multiple pro-inflammatory cytokines arise through an array of complex signal transduction pathways through NF-kB and MAPKs [15]. It triggers a cascade response of immunity that may either install some protection against the parasite, or, to the contrary, increase inflammation and tissue injury [16].

The supposed cytokines that are involved in this process are:

IL-1b: It is an early pro-inflammatory cytokine and mediator to drive the recruitment of immune cells.

IL-6: A measurement of the extent of inflammation and acute phase reaction.

TNF-a: This is one of the strongest-inflammatory cytokines that are linked to tissue destruction.

IFN-g: This is released by T cells and activates cellular (Th1) immunity.

IL-10: An anti-inflammatory cytokine that possesses an inhibitory regulation capacity in preventing over production of the immune system [17]. The analysis of concentrations of these cytokines and the level of TLR4 can help create a significantly more comprehensive image of what happens to the immune system at each phase of amoebic infection [18]. This will give possibilities to develop improved diagnostic and prognostic methods and potential therapeutic interventions based on the immune response change to restrict tissue pathology and improve clinical outcomes [19].

Materials and Methods

Collection and Diagnosis of the samples.

Blood and stool samples were taken on all of the patients. Direct microscopic analysis was carried out on stool samples to indicate the presence of the parasite. The study enrolled 60 patients in outpatient clinics in selected districts and sub-districts of Kirkuk city, each of whom had one-third of the entire population (30 patients' amoebic colitis and 30 patients amoebic liver abscess (ALA). Also, the sample size used as a control group was 20 healthy persons.

Isolation of Immune Cells:

The blood components were separated by centrifugation and the white blood cells (plasma and cellular components) could be isolated [20]. They were used to determine the TLR4 protein levels and the concentration of cytokines.

TLR4 Protein Measurement

In order to prepare immune cells, centrifugation and cell separation were used to isolate them using blood samples. The surface expression of TLR4 protein on the immune cells was then measured in two different ways: Flow Cytometry: TLR4 surface expression of the immune cells could be quantified and analysed by using fluorescently labelled antibodies specific to TLR4 [21].

Western Blot: The protein separation was performed based on the molecular weight by gel electrophoresis and then the protein content was determined and identified using TLR4-specific antibodies [22].

Cytokine Measurement

The levels of pro-inflammatory cytokines (IL-1b, IL-6, TNF- a, IFN- g) and the anti-inflammatory cytokine (IL- 10) in the serum samples were established by applying ELISA analysis. This method relies on the affinity of some antibodies to all cytokines, which gives a colorimetric response, which is detected using a microplate reader [23].

Statistical Analysis

The SPSS software was used to discuss data. The ANOVA tests were applied to compare the means of study groups, t-tests were applied to compare the groups in pairs. Appropriate correlation coefficients were used to determine correlations between TLR4 levels and levels of cytokines. A p-value below 0.05 was regarded as significant.

Results and Discussion

The data in Table 1 demonstrate the concentration of TLR4 protein and some major cytokines in three groups of the study participants: patients with amoebic liver abscess (ALA), patients with amoebic colitis (AC), and healthy people (Control) as a control group.

Table 1. Levels of TLR4 and Cytokines in the Studied Groups.

Group	TLR4 (ng/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)	IL-10 (pg/ml)	IFN-γ (pg/ml)
Amoebic Liver Abscess (ALA)	7.85 ± 0.4	190 ± 12	310 ± 15	220 ± 10	45 ± 6	180 ± 8
Amoebic Colitis (AC)	5.10 ± 0.3	110 ± 10	180 ± 12	140 ± 9	70 ± 5	120 ± 7
Healthy Controls	2.30 ± 0.2	35 ± 6	55 ± 5	40 ± 5	85 ± 6	45 ± 4

As can be seen in the table, the TLR4 level is highest in the ALA group (7.85 +- 0.4 ng/ml), which suggests that the innate immune system is highly activated in this situation. TLR4 is also important in the detection of the existence of the parasite and the initiation of inflammatory signaling pathways. Comparatively, the TLR4 concentration is lower in the AC (5.10 +- 0.3) and lowest in the healthy controls (2.30 +- 0.2), which are the baseline levels in healthy people.

The ALA group has a high concentration of pro-inflammatory cytokines (IL-1b, IL-6 and TNF-a), indicating the presence of acute and severe inflammatory state, probably caused by the tissue damage caused by the parasite in the liver [24]. The concentrations of these cytokines are lower with the AC group, however, higher than the control group, which suggests a moderate inflammatory response in the colon [25]. Nevertheless, in AC patients, (70 +- 5 pg/ml) anti-inflammatory cytokine IL-10 is higher than in ALA patients (45 +- 6 pg/ml), which may be the result of an attempt to restrain excessive inflammation of the colon [26]. The ALA population demonstrates a higher level of IFN-g (180 +- 8 pg/ml), a biomarker of Th1-cellular immunity activation, meaning that this pattern of immunity is associated with the intense parasitic response in the liver, but is smaller in AC and lowest in healthy controls [27]. The general pattern of the table is that the immune response to amoebic liver abscess is graver and more acute and is characterised by substantial stimulation of pro-inflammatory cytokines and TLR4, and reduction in IL-10. Conversely, amoebic colitis has a more balanced cytokine profile (both pro- and anti-inflammatory), which could justify the relatively mild clinical manifestations of this type of infection.

High TLR4 levels in the ALA group are an indicator of great activation of innate immune system. TLR4 is among important receptors that identify pathogenic substances and promote the release of a series of inflammatory response via the NF-kB pathway, resulting in the synthesis of cytokines [28].This is due to the pro-inflammatory cytokines, including IL-1b, IL-6, and TNF-a that are high in patients with liver abscess, which signifies a severe inflammatory reaction that correlates with the worsening of symptoms and the destruction of tissues [24]. Although it is necessary to combat the infection, it can be devastating when not controlled effectively, particularly in the presence of the high levels of IFN-g (T cell (Th1)-driven activation of cellular immunity and augmented tissue damage [29]. On the contrary, higher IL-10 levels of patients with amoebic colitis without abscess indicate that the body tries to control the level of inflammation and avoid worsening. The anti-inflammatory cytokine IL-10 is also important in terms of balancing the immune system and preventing over-inflammation of the tissue [30].

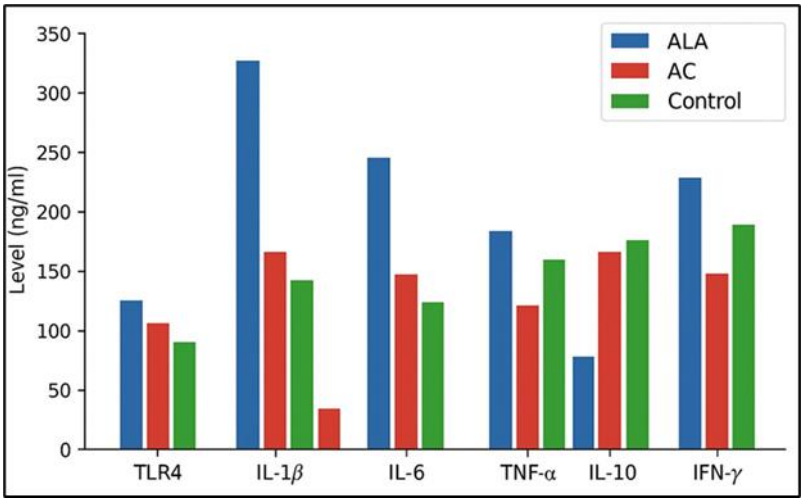

Figure 2. "TLR4 and Cytokines Levels in Amebic Liver Abscess, Amebic Colitis and healthy controls.

The statistical analysis of the levels of immune markers between the three investigated groups patients with amebic liver abscess (ALA) and patients with amebic colitis (AC) as well as healthy individuals (Control) can be observed in Table 2.

Table 2. Statistical Differences Among Studied Groups in TLR4 and Cytokine Levels.

Biomarker	ALA vs. AC	ALA vs. Control	AC vs. Control	(p-value)
TLR4	Significant ↑	Highly significant ↑	Significant ↑	$p < 0.001$
IL-1β	Significant ↑	Highly significant ↑	Significant ↑	$p < 0.001$
IL-6	Significant ↑	Highly significant ↑	Significant ↑	$p < 0.001$
TNF-α	Significant ↑	Highly significant ↑	Significant ↑	$p < 0.001$
IL-10	Decreased ↓	Not significant	Significant ↑	$p = 0.02$
IFN-γ	Significant ↑	Highly significant ↑	Significant ↑	$p < 0.001$

As can be seen, the differences ($p < 0.001$) between the ALA group and either of the other two groups are highly significant in most markers which means that the inflammatory response in the case of liver abscess is strong. TLR4 was highly elevated in ALA group, compared to the AC and healthy subjects who exhibited a robust activation of innate immunity in such severe infection.

Pro-inflammatory cytokines (IL-1b, IL-6 and TNF-a) also remained significantly elevated in the ALA group, which further supported the severity of symptoms and the degree of inflammation. Another markedly increased in ALA was IFN-g associated with cellular immunologic stimulation and as such it plays a considerable role in cellular immunity in the attack against the parasite--but the overactivation of IFN-g may be implicated in tissue destruction [31]. On the other hand, the IL-10 (an anti-inflammatory cytokine) was observed to be lower in ALA compared to AC and there was no statistically significant difference between ALA and healthy persons. The decrease of IL-10 may signify the failure of the immune system to prevent excess inflammation when it comes to abscesses of the liver [32]. These findings suggest that immune balance in ALA is severely disrupted and pro- inflammatory cytokines are dominant in the response. Such imbalance is able to improve the progression of the disease and worsen clinical symptoms [33].

The study results have revealed that TLR4 and several other pro-inflammatory cytokines were significantly greater in patients with amebic liver abscess (ALA) compared to patients with amebic colitis (AC) and controls. The implications of this increase on the direction of the intensity of the immune response and the course of infection are severe [34]. TLR4 is one of the primary receptors of the innate immune system and its stimulation triggers the cascade of signals, the ultimate outcome of which is the production of strong inflammatory cytokines [35]. Therefore, the elevated levels of TLR4 within the ALA group point to the existence of a robust immune activation, most likely due to the fact

that the parasite has expanded to the liver which necessitates more intense immune activation [16]. Similarly, the concentrations of inflammatory cytokines such as IL-1b, IL-6, and TNF-a were substantially increased in the group of ALA, which evidences an acute inflammatory response and can be the source of tissue damage and the acuteness of the symptoms of liver abscess [24]. Previous research has linked these cytokines to the intensity of symptoms and the development of abscesses in invasive amebiasis because they are considered to be one of the signs of the excessive activation of the immune system [36].Conversely, anti-inflammatory cytokine IL-10 was highly depleted in ALA as opposed to AC, which demonstrates a deficit in immune regulatory processes that would otherwise suppress injury that occurs as a result of excessive inflammation. This is in favor of the IL-10 to pro-inflammatory cytokine imbalance which leads to the hypothesis of immune dysregulation as an important determinant of disease severity [37]. Further, IFN-g, as well, was highly increased in ALA, indicating improved cellular (Th1) immunity, critical to parasite clearance but also capable of increasing inflammations, especially when the IL-10 does not regulate it [38].

As shown in Table 3, TLR4 was significantly elevated in the ALA group than it was in the AC group and highly elevated than it was in the control group, showing strong activation of innate immune receptors in liver abscess cases. The statistically significant difference between AC and Control was also significant to a lesser degree. The IL-1b levels were significantly high in the ALA patients compared to the AC and Control group indicating an acute inflammatory response. There was also a significant difference between the AC and Control, which means that IL-1b plays a role in both types of diseases, albeit with different degrees of intensity.

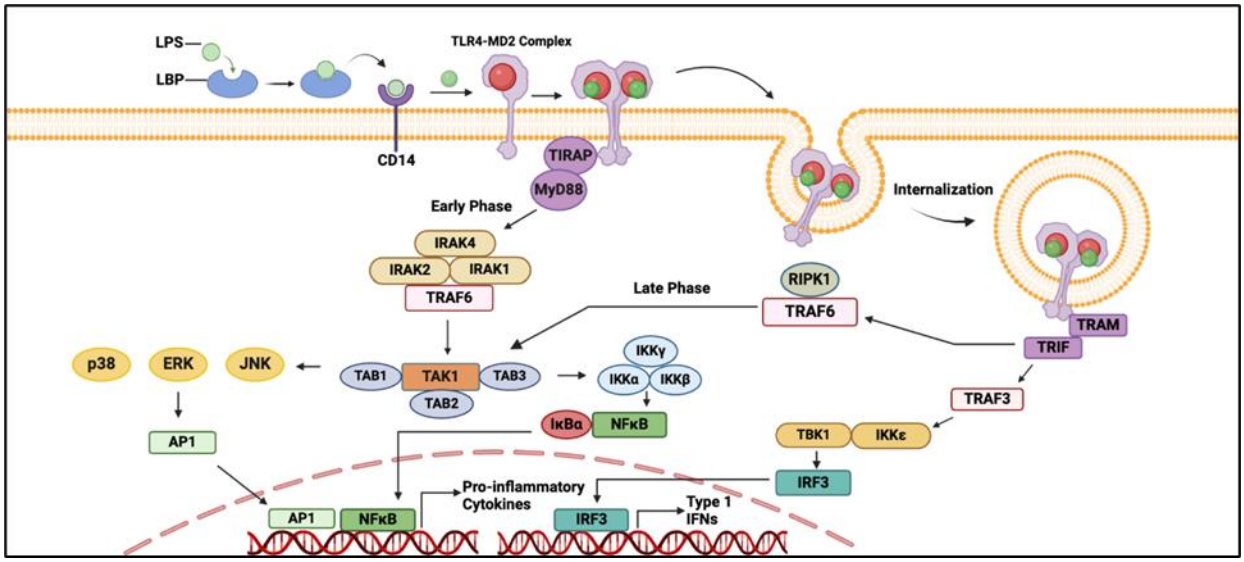

Figure 3. illustrates the pathways through which TLR-4 expresses pro-inflammatory cytokines. The image illustrates a MyD88-dependent pathway that activates NF-κB and MAP kinases, resulting in the expression of pro-inflammatory cytokines. On the other hand, it illustrates a TRIF-dependent pathway, which is crucial for the production of interferon gamma in response to viral attacks. Created with Biorender.

Table 3. Association between Clinical Symptom Severity and Means of Immune Markers in patients with Amoebic Liver Abscess (ALA).

Symptom Severity	TLR4 (ng/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)	IL-10 (pg/ml)	IFN-γ (pg/ml)
Mild	6.1 ± 0.3	130 ± 10	220 ± 12	160 ± 9	60 ± 6	140 ± 7
Moderate	7.5 ± 0.4	180 ± 11	300 ± 14	210 ± 10	48 ± 5	175 ± 8
Severe	8.8 ± 0.5	240 ± 13	390 ± 18	270 ± 12	35 ± 4	210 ± 9

The same was similarly observed in the case of IL-6, where the levels were significantly high in ALA patients, lower in AC, and lowest in healthy individuals. This indicates that IL-6 is an indicator of

the level of inflammation [39]. TNF-a levels had the same pattern as well- high at ALA, the lowest at AC, and the lowest at Control- indicating the central role of TNF-a in the inflammatory process and aggravation of symptoms in severe cases [40].

Unlike the other markers, IL-10 was reduced in ALA than AC, implying the poor regulation of anti-inflammatory actions in the severe cases. The difference between ALA and Control was not significant whereas the difference between AC and Control was significant, which evidenced a more pronounced regulatory role in case of colitis [41]. The levels of IFN-g were significantly high in ALA, which showed the presence of the cellular immune response. All the groups showed statistically significant differences, which highlights the role of this cytokine in the control of the infection. The statistical differences are a confirmation that the majority of the researched biomarkers relate to the severity of the disease and show specific patterns in liver abscess and colitis. These data indicate that the cytokine profiling could be used to differentiate the stages of diseases and the effectiveness of the immune reaction [42].

Severity limits of clinical symptoms in amoebic liver abscess (ALA) patients is clearly related to the mean levels of immune markers detected in the blood [43]. The levels of TLR4, the receptor that triggers a cascade of immune signals that leads to the activation of the innate immune system, increase gradually with the severity of the disease, which is mild, moderate, and severe. Such an increase means that the immune system is strengthening and the infection becomes more severe [44].

The same applies to pro-inflammatory cytokines, like IL-1b, IL-6 and TNF-a, the levels of these cytokines increase with a progressive aggravation of symptoms and are evidence of an increasing immune activation that serves to contribute to tissue damage [45]. This rise can be explained by the effort of the body to eliminate the parasite and can also be one of the reasons of the drastic symptoms [46].

Conversely, the anti-inflammatory cytokine IL-10 causes decline in relation to the severity of the disease [47]. This degradation indicates that the system is unable to provide the same defense against inflammation or inhibit it as the infection deepens, thus permitting harm done by the overreacting immune system to increase [48]. Lastly, in severe cases, there is a significant increase in the cellular immune response (Th1), i.e. IFN- γ, which reflects extreme activation of cellular immunity. Though it will help in combating the parasite, it can also make the disease complex [49].

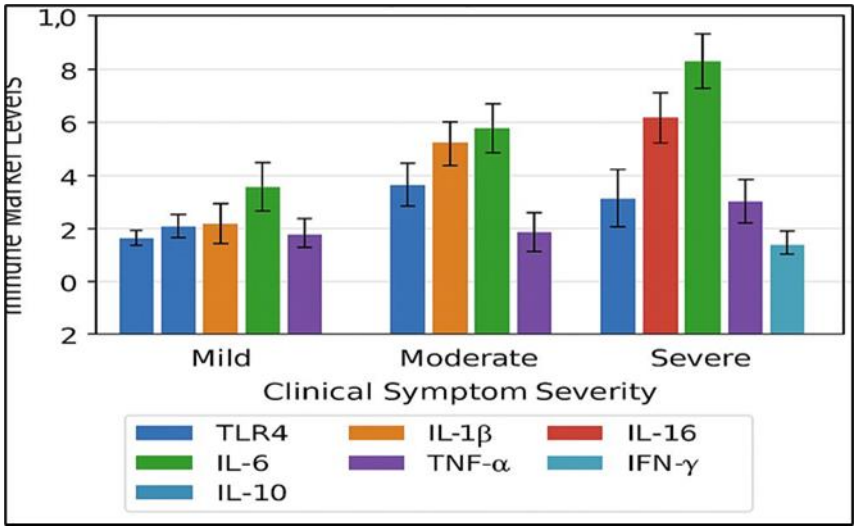

Figure 4. Immune marker levels by clinical symptom severity in patients with Amoebic liver abscess (ALA).

Conclusion

Ambient liver abscess patients have strong activation of innate immune activity, which is correlated with elevated TLR4 levels, which contributes to the development of serious inflammatory processes. The levels of pro-inflammatory cytokines (IL-1b, IL-6, TNF-a, IFN-g) are significantly higher

in cases of liver abscess and determine the severity of the inflammation and the severity of the symptoms. The elevated levels of IL-10 in amebic colitis patients show that it has anti-inflammatory functions to mediate the immune responses and inhibit tissue damage. The proportion between the pro- and anti-inflammatory cytokines are significant in deciding the severity and outcome of a disease. The levels of TLR4 and cytokines may be useful biomarkers to analyse the severity of infections and immunomodulatory therapy in Entamoeba histolytica infection. More studies are suggested to create treatment protocols that would adjust the immune responses that would enhance patient outcomes.

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