

# TH1/TH2 Cytokine Profile and Their Relationship with Hematological Parameters in Patients with Acute Limb Ischemia

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## Abstract

**Background:** The progression of acute limb ischemia (ALI) is being significantly influenced by changes in immune system function. The study aimed to determine the dominant immune cell responses (Th1 or Th2) in ALI patients by measuring serum levels of IL-4, IL-12, and IFN- $\gamma$ . Previous studies indicate altered cytokine levels in cerebral ischemia, but there is no prior research on these cytokines in ALI patients.

**Methods:** This study involved 34 patients with ALI and 34 healthy controls. Blood samples were analyzed for hematological factors such as erythrocyte sedimentation rate (ESR), white blood cell (WBC) count, red blood cell (RBC) count, platelet (Plt) count, hemoglobin (Hb), and hematocrit (HCT). The levels of serum cytokines IL-4, IL-12, and IFN- $\gamma$  were measured in both patients and control subjects using enzyme-linked immunosorbent assay (ELISA). The statistical analyses were conducted using SPSS and GraphPad Prism.

**Results:** The results showed that serum levels of IL-4 in ALI patients did not significantly differ from those in control groups. Acute limb ischemia exhibited significantly elevated levels of IL-12 and IFN- $\gamma$  compared to healthy individuals. In addition, no correlation between the production of cytokines and the hematological parameters was found.

**Conclusion:** Th1 responses are believed to play a role in the pathogenesis of ALI, but further research is needed to fully understand their exact role.

**Keywords:** Fibrinogen, ORF-9b protein, SARS-CoV-2, Serum Albumin.

## Introduction

The condition known as acute limb ischemia (ALI) occurs when a thrombus or an embolus prevents blood flow to a limb. Severe ischemia can lead to serious emergencies, such as limb loss and death. However, early intervention can achieve limb salvage by restoring perfusion (1). In medical terminology, ALI is defined as a condition in which individuals have had symptoms of limb ischemia for less than two weeks. Individuals may exhibit the "6 Ps": pain, paralysis, paresthesia, pulselessness, poikilothermia,

and pallor. A key part of the pathophysiology of ALI is the complex inflammatory interactions that happen in the vascular bed after an acute ischemia event (2). Most inflammatory lesions involve lymphocytes, which are drawn to the affected area for a duration of 24 to 48 hours by chemokines, cytokines, and other stimuli. Furthermore, lymphocytes control immunity by secreting immunoglobulins, inducing cytotoxicity, triggering apoptosis and balancing cytokines (3-5). Ischemia impacts both innate and

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adaptive immunity, but its impact on immune system components is unclear, possibly due to limited tissue damage (6, 7).

Based on the pattern of cytokine production, T cells can be divided into four effector types: Th1, Th2, Th17, and regulatory T cells (Treg) (8, 9). The main cytokine of Th1 cells is IFN- $\gamma$ , which also inhibits the differentiation of Th2 cells while causing Th0 cells to differentiate into Th1 cells, indicating a positive feedback loop. Moreover, macrophages, NK cells, cytotoxic T cells, and mucosal epithelial cells all release IFN- $\gamma$  (10-13). Lambertsen et al.'s study revealed that IFN- $\gamma$  increases ischemia-induced brain damage independently of TNF or through synergistic neurotoxic interactions of IFN- $\gamma$  and TNF- $\alpha$  (14).

Th2 cells further stimulate IL-4, initiating a positive feedback loop of increased IL-4 production. IL-4 decreases the production of Th1 cells, IFN- $\gamma$ , IL-12, and macrophages (15, 16). Tissue macrophages are crucial in the processes of wound healing and chronic inflammation. IL-4 levels in extravascular tissues increase the number of alternatively activated macrophages (M2 cells) (17, 18). The increase in M2 is linked to its secretion, which in turn reduces pathological inflammation (19). IL-4 regulates pro-inflammatory mediator secretion and decreases anti-inflammatory immune cell secretion (20). The study by Kim et al. found that patients with acute cerebral ischemia showed increased serum levels of IL-4 (21).

Dendritic cells are major sources of IL-12, which induces Th1 immune deviation by stimulating TNF- $\alpha$  and IFN- $\gamma$  secretion from T and NK cells and decreasing IL-4-mediated IFN- $\gamma$  inhibition (22). IL-12 exhibits anti-angiogenic activity, inhibiting the formation of new blood vessels by increasing IFN- $\gamma$  production, which in turn increases the production of inducible protein-10 (IP-10) (23). IL-12 is crucial in causing hepatic ischemia/reperfusion injury by regulating IFN- $\gamma$  and TNF- $\alpha$  production (24). Furthermore, exogenous administration of IL-12 in healthy mice activates Kupffer cell activation, increases hepatic vascular cell adhesion

molecules, and accumulates of leukocytes in the liver (14).

Elevated ESR, HB, Plt, erythrocyte, and leukocyte counts, along with cytokine induction, significantly impact ischemic damage and clinical prognosis in acute inflammation (25). Therefore, we examined the relationship between serum cytokine levels and blood parameters secreted in response to acute inflammation in patients with ALI.

## Materials and Methods

### Patients

The study analyzed 34 ALI patients admitted to Velayat Hospital, Mashhad University of Medical Science, Iran, between February and August 2022 and compared them with 34 control age-matched subjects. The study has been approved by the Ethical Committee for Mashhad University of Medical Sciences under the code IR.MUMS.MEDICAL.REC.1400.464. All inpatients who participated in the study were given written informed consent after explaining the study's purpose.

Adult patients aged 18 years or older, demonstrating adequate cognitive ability to participate, and presenting clinically with first ALI symptoms were the main requirements for inclusion. The symptoms of acute artery occlusion, which included pain, pallor, coldness, and a lack of pulse, were classified as ALI if they lasted for two weeks. The exclusion criteria encompassed dissatisfaction with study participation, age below 18 years, kidney and heart failure, Buerger's disease, vasculitis, popliteal artery entrapment syndrome, cancer, receiving thrombolytic therapy, pregnancy, high blood pressure, and unclear symptom onset. Clinical and demographic data of patients were gathered about the patients, including age, sex, clinical symptoms, presentation of upper and lower limb acute ischemia, level of upper and lower limb ischemia (lower limb: from the top of the thigh down, below the knee down, from the ankle down, and upper limb: from the shoulder down, from the elbow down, from the wrist

down), other diseases, history of smoking and drug use, history of medication use, and other conditions.

### **Control subjects**

Control serum samples were prepared by collecting blood from 34 healthy donors at Mashhad University of Medical Sciences in Iran. To create a biobank comparable to the serum from our ALI patients, we selected healthy donors with an even distribution of male and female individuals aged between 18 and 70 years. None of the donors were taking immunosuppressive drugs.

### **Blood collection and serum preparation**

Blood samples were taken from all healthy subjects and patients. For cytokine measurements, blood was collected in 6-ml serum gel tubes with a clot activator (FL Medical, Italy) and stored for 30-120 minutes at room temperature before centrifugation at 1372 G for 5 minutes. Subsequently, the serum samples were immediately stored at -70 °C for cytokine analysis.

### **Hematological parameter measurement**

Blood samples were immediately analyzed for hematology using heparin sodium (1%) anticoagulation. Blood counts for RBC, WBC, Plt, Hb, and HCT were determined using the Sysmex XP 300 Automated Hematology Analyzer (Sysmex Corporation, China), expressed as a percentage of total blood volume, and replicated counts were made for each sample.

### **Cytokine measurement**

The concentrations of IL-4, IL-12, and IFN- $\gamma$  were measured in both patients and healthy subjects using commercial ELISA kits (Invitrogen, Frederick, Maryland, USA) following the manufacturer's instructions. Intra- and inter-assay coefficients for three cytokines were less than 3% and 8%, respectively. Sensitivities of determination for IL-4, IL-12, and IFN- $\gamma$  were 0.1, 2.1, and 4.0 pg/ml, respectively. All samples were analyzed in duplicate.

### **Statistical analysis**

The means were compared using the independent sample t-test. Pearson's correlation analysis was used to examine the correlations between serum cytokine levels and hematological parameters. All data were expressed as the mean  $\pm$  standard deviation (SD). The statistical software SPSS 16.0 (SPSS Inc., USA) was utilized to analyze the data. A value of  $P < 0.05$  was considered statistically significant.

### **Results**

The analysis of all clinical, experimental, and demographic data for ALI patients showed that the mean age and gender distribution did not significantly differ between the AIS and control groups (Table 1). The study involved 21 male and 13 female patients, with 23 aged over 65 years and 11 under 65 years. It also included 14 patients with lower extremity ischemia, including two above the knee and five below the knee, and 20 patients with upper extremity ischemia, including three above the arm, 10 below the elbow, and 7 below the wrist. The mean WBC, RBC, and Plt counts in ALI patients were  $9.81 \pm 4.19$  ( $10^3/\text{mcL}$ ),  $4.02 \pm 0.93$  ( $10^6/\text{mcL}$ ), and  $258.5 \pm 130$  ( $10^3/\text{mcL}$ ), respectively. The mean Hb, HCT, and ESR levels were recorded as  $11.33 \pm 3.02$  (g/dL),  $33.51 \pm 7.57$  (%), and  $43.8 \pm 34.8$  (mm), respectively. Additionally, our findings showed that the mean levels of IL-4 in serum of ALI patients did not significantly change ( $1.7 \pm 0.2$  pg/ml,  $P = 0.6$ ) compared to the healthy control group ( $1.68 \pm 0.19$  pg/ml). Patients showed significantly higher mean concentrations of IL-12 ( $37.09 \pm 2.4$  pg/ml,  $P < 0.001$ ) compared to healthy subjects ( $29.41 \pm 2.08$  pg/ml). The patients also exhibited significantly higher mean IFN- $\gamma$  levels ( $14.51 \pm 2.23$  pg/ml,  $P < 0.001$ ) compared to the control group ( $9.36 \pm 1.14$  pg/ml) (Figure 1). Additionally, significant positive correlations ( $P < 0.001$ ) were found between Hb and RBC count, Hb and HCT, and ESR and Plt in ALI patients. Significant negative correlations were observed between ESR and HCT ( $P = 0.033$ ), ESR and Hb ( $P = 0.034$ ), and ESR and RBC count ( $P = 0.013$ ).

**Table 1.** Clinical and hematological variables of the acute limb ischemia (ALI) patients and cytokine serum levels of these and control subjects.

<i>Characteristic</i>	<i>ALI patients (n=34)</i>	<i>Control subject/Reference interval (n=34)</i>
<b>Gender, n (%)</b>		
Man	21 (61.76%)	21 (61.76%)
Woman	13 (38.23%)	13 (38.23%)
<b>Age</b>		
Man	70.4±3.5 <sup>a</sup>	71.04±3.4
Woman	54.8±9.2	53.46±9.3
<65	11	11
>65	23	23
<b>Upper extremity, n (%)</b>		
Above the arm	3 (8.8%)	-
Below the elbow	10 (29.4%)	
Below the ankle	7 (20.5%)	
<b>Lower extremity, n (%)</b>		
Above the knee	2 (5.8%)	-
Below the knee	7 (20.5%)	
Below the ankle	5 (14.7%)	
<b>WBC</b> (10 <sup>3</sup> /mcL)	9.81±4.19	4.5-11
<b>RBC</b> (10 <sup>6</sup> /mcL)	4.02±0.93	4.2-6.1
<b>Plt</b> (10 <sup>3</sup> /mcL)	258.5±130	150-400
<b>ESR</b> (mm/h)	43.88±34.8	<30
<b>Hb</b> (g/dL)	11.33±3.02	12-18
<b>HCT</b> (%)	33.51±7.57	36-50

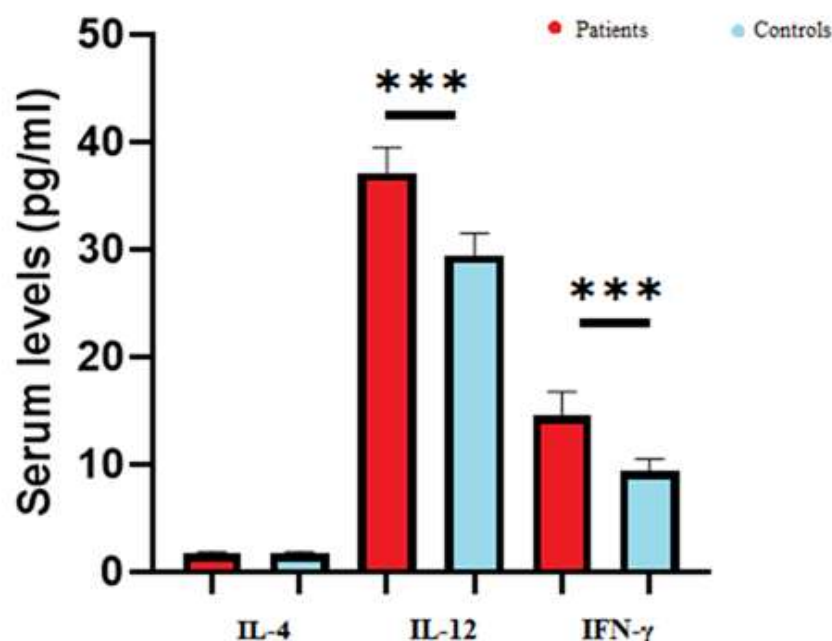
\*The 21 patients were male, and 13 patients were female. The 20 patients (3 above the arm ischemia, 10 below the elbow ischemia and 7 from below the wrist ischemia) suffered from upper extremity ischemia and 14 patients (2 above the knee ischemia, 7 below the knee ischemia and 5 the ankle ischemia) suffered from lower extremity ischemia.

<sup>a</sup>Data are shown as mean ± standard deviation.

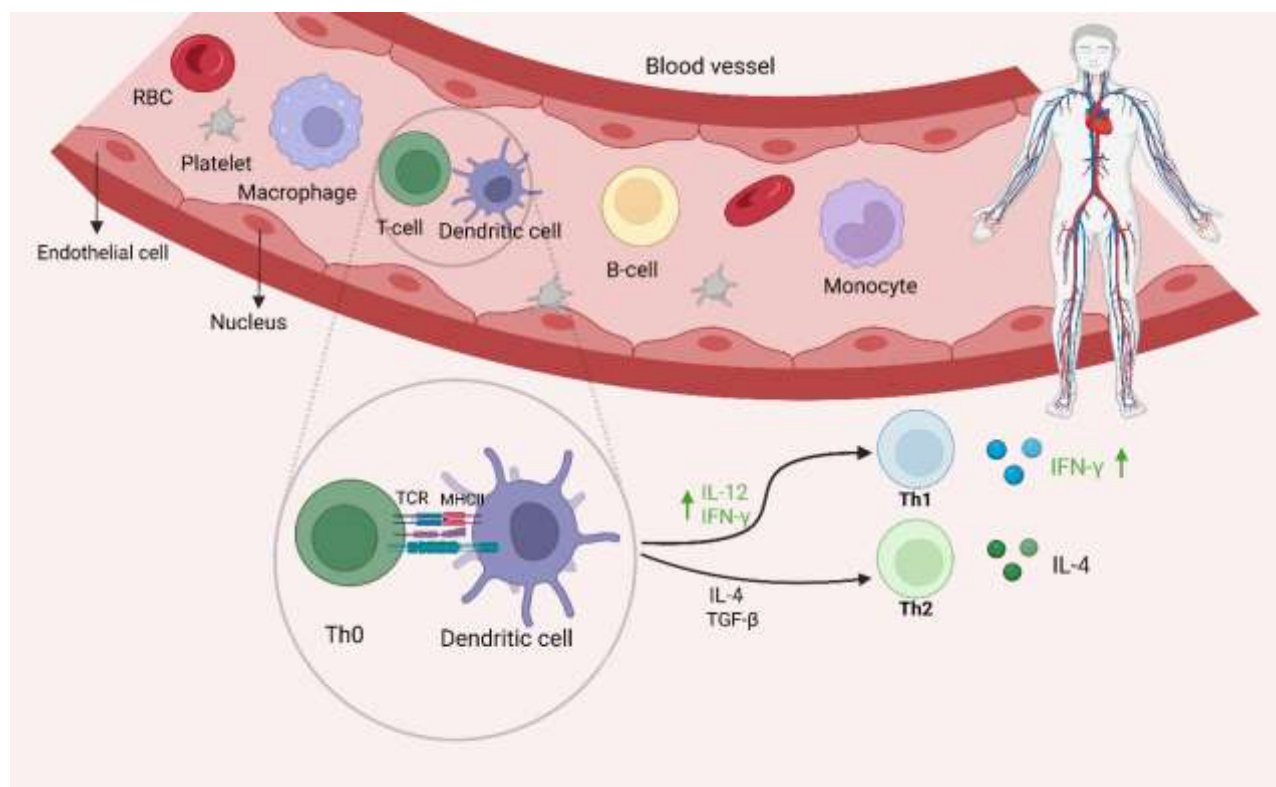
**Table 2.** Pearson correlation coefficient (upper value) and p-value (lower value) of the hematological parameters and cytokine levels in ALI patients.

<b>IL4</b>	1								
<b>IL-12</b>	0.03	1							
	0.842								
<b>IFN-<math>\gamma</math></b>	0.09	0.14	1						
	0.596	0.439							
<b>ESR</b>	0.04	-0.02	-0.20	1					
	0.841	0.906	0.321						
<b>Hb</b>	-0.27	-0.05	0.24	<b>-0.42</b>	1				
	0.162	0.794	0.209	<b>0.034</b>					
<b>WBC</b>	0.19	-0.10	-0.27	-0.05	-0.21	1			
	0.347	0.604	0.171	0.797	0.285				
<b>RBC</b>	-0.21	-0.12	0.25	<b>-0.50</b>	<b>0.91</b>	-0.11	1		
	0.303	0.544	0.227	<b>0.013</b>	<b>&lt;0.001</b>	0.595			
<b>HCT</b>	-0.27	-0.05	0.25	<b>-0.42</b>	<b>0.98</b>	-0.11	0.93	1	
	0.172	0.785	0.208	<b>0.033</b>	<b>&lt;0.001</b>	0.572	<b>&lt;0.001</b>		
<b>Plt</b>	0.09	-0.07	-0.19	<b>0.70</b>	-0.14	0.22	-0.04	-0.11	1
	0.650	0.741	0.355	<b>&lt;0.001</b>	0.501	0.295	0.833	0.590	
	<b>IL-4</b>	<b>IL-12</b>	<b>IFN-<math>\gamma</math></b>	<b>ESR</b>	<b>Hb</b>	<b>WBC</b>	<b>RBC</b>	<b>HCT</b>	<b>Plt</b>

Significant positive correlation was found between ESR and Plt, Hb and RBC count, Hb and HCT ( $P < 0.001$ ) in ALI patients. Significant negative correlation was found between ESR and HCT ( $P = 0.033$ ), ESR and Hb ( $P = 0.034$ ), ESR and RBC count ( $P = 0.013$ ).



**Fig. 1.** Comparing serum levels of IL-4, IL-12 and IFN- $\gamma$  in ALI patients compared to healthy patients. Student's t-test was used for comparison between groups. Comparison of IL-4 levels in ALI patients were not changed significantly compared to healthy patients' group ( $P = 0.6$ ). The serum levels of IL-12 were increased significantly in ALI patients compared to healthy patients. The levels of IFN- $\gamma$  were also enhanced in ALI patients compared to healthy patients. \*\*\* $P < 0.001$ . ALI patients are shown with red color and healthy patients are shown with blue color.



**Fig. 2. Cytokines production in the serum of ALI patients.** The interaction between dendritic cell (DC) and T cell leads to T cell activation and immune response including ALI diseases. The MHC-antigen complex in the surface of DC activates the TCR and the T cell cytokines secretion. The serum levels of IL-4 in ALI patients were not changed compared to control group. The serum levels of IFN- $\gamma$  and IL-12 were increased in patients compared to healthy control group, that induce Th1 immune response. \*The figure was created with BioRender.com.

## Discussion

Our findings revealed that patients had higher serum levels of IL-12 and IFN- $\gamma$  when compared to a healthy control group, both of which induce a Th1 immune response. Although a few studies have looked at cytokine production in ALI patients, none have investigated into Th1 cytokine production (26). A growing body of evidence suggests that inflammation plays a critical role in the pathogenesis of acute ischemic stroke (27-29). Yassin et al. assayed plasma concentrations of TNF- $\alpha$  in male Wistar rats and found a significant elevation of TNF- $\alpha$  levels in animals who were affected by 3 hours of bilateral hind limb ischemia and 1 hour of reperfusion compared to the control group (30). Yu et al. discovered increased Th17 and Th1 cells in acute ischemic stroke patients compared to controls, but not Th2 cells.

Furthermore, they found a link between Th1 and Th17 cells and the severity of acute ischemic

stroke (31). Bellavia et al. investigated the Th1/Th2 balance to assess the peripheral immune response to cerebral ischemia. The results revealed that there was no change towards Th1 (32). Ormstad et al. observed higher blood levels of IL-12 in acute ischemic stroke patients, which is consistent with our findings (27). According to Zaremba et al., serum IL-12 levels were higher in stroke patients than in controls. The authors also reported that IL-12 levels exhibited a strong connection with ESR values in stroke patients (33). Some animal studies have found IL-12 involvement in cardiac ischemia-reperfusion injury (34-36). Yan et al. discovered that dectin-2 deficient mice can reduce IL-12 expression and alleviate Th1 immune responses, potentially protecting against cardiac ischemia-reperfusion injury (37, 38). Ischemic brain injury may be exacerbated by IL-12. IL-12 can boost the production and activity of several pro-inflammatory chemokines and

cytokines (39). After retinal ischemia, animals were given a neutralizing IFN- $\gamma$  antibody, which enhanced retinal ganglion cell survival (40). IL-4 can increase Th2 differentiation by activating the transcription factors GATA3 and STAT6 (41). To date, there have been no investigations into ALI patients' secretion of IL-4. In our investigation, there was no significant difference in IL-4 levels between ALI patients and controls.

The present study found a positive correlation between ESR and Plt count in ALI patients, while a negative correlation was found with Hb, ESR, and RBC, indicating that Hb, Plt, RBC, and WBC count are key predictors of ESR. ESR levels are affected by a variety of confounding circumstances. Several investigations have found a substantial relationship between ESR levels and RBC, Hb, and HCT (28), as well as platelet count (29). Elevated ESR levels should not be used as a direct indicator of inflammation in ALI but should be considered alongside other strong inflammation markers (42,43). The study suggests that elevated serum levels of IL-12 and IFN- $\gamma$ , but not IL-4, may indicate a Th1

dominant response in ALI patients.

The study suggests that the Th1 response may be involved in the pathogenesis of ALI, as serum levels of IFN- $\gamma$  and IL-12 increased in ALI patients compared to healthy controls. To better understand immune system alterations in ALI patients, a larger study population and assaying other cytokines and immunological markers are recommended.

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## Conflict of interest

No potential conflict of interest was reported by the authors.

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