ORIGINAL ARTICLE

Serum interleukin-17 levels predict inflammatory activity in patients with autoimmune hepatitis

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KEY WORDS

autoimmune hepatitis, hepatic inflammation, histologic activity index, interleukin 17

ABSTRACT

INTRODUCTION The etiology of autoimmune hepatitis (AIH) is unclear, with molecular mimicry between host and viral/drug antigens being the most plausible mechanism initiating the immune cascade that induces hepatocyte injury. Finding a serologic parameter that closely relates to the liver histology would be beneficial for monitoring AIH activity and optimizing treatment.

OBJECTIVES We studied serum interleukin (IL)-17 levels and IL-17 activators (IL-6 and transforming growth factor β1 [TGF-β1]) in treatment-naive and immunosuppressed patients with AIH. We also analyzed the relationships between these cytokines and histological inflammation scores.

PATIENTS AND METHODS A total of 44 patients with confirmed AIH were enrolled to the study (22 treatment-naive patients and 22 patients in clinical remission after at least 3 years of immunosuppression). Liver biopsies were performed, and the histological grading of inflammatory activity was performed by a single pathologist. The control group comprised 30 healthy age- and sex-matched subjects. Serum IL-17, IL-6, and TGF-β1 levels were measured by a quantitative sandwich enzyme immunoassay.

RESULTS Serum IL-17, IL-6, and TGF-β1 levels were higher in treatment-naive patients compared with controls (23.2 pg/ml vs 15.3 pg/ml, $P = 0.0001$; 5.20 pg/ml vs 1.42 pg/ml, $P = 0.0001$; and 40.5 ng/ml vs 30.1 ng/ml, $P = 0.04$; respectively). In treatment-naive patients, serum IL-17 negatively correlated with hepatic inflammation ($r = –0.63$, $P = 0.01$). A reduced serum IL-17 concentration correlated with an increased TGF-β1 concentration in patients in clinical remission ($r = –0.51$, $P = 0.03$).

CONCLUSIONS Serum IL-17 levels may be a useful parameter for assessing disease activity in patients with AIH.

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INTRODUCTION The etiology of autoimmune hepatitis (AIH) is unclear. Molecular mimicry between host and viral/drug antigens is suggested to be the most probable mechanism that leads to the immune cascade resulting in hepatocyte injury. The activation of T-helper (Th) cells is a critical step in this process. Activated Th cells differentiate into the Th_1 and Th_2 subsets as well as the Th_{17} subset, which produces interleukin 17 (IL-17). IL-17 induces matrix metalloproteases that regulate collagen degradation and promotes chemokine and proinflammatory cytokine production with activation of macrophages and neutrophils in the liver tissue.[1-4] IL-6 and transforming growth factor β1 (TGF-β1) activate naive Th cells to differentiate into pathogenic Th_{17} lymphocytes. Several investigators have shown that naive CD^+ T cells generate anti-inflammatory regulatory T cells when exposed to TGF-β1 alone, whereas in the presence of both TGF-β1 and IL-6 they generate Th17 cells.[5-7] The role of IL-17 in patients with AIH, however, is not well established, especially in relation to the histological activity of this disease.[8-11]

TABLE 1 Clinical, laboratory, and histological characteristics of patients with active autoimmune hepatitis

Parameter	Mean (SD)	Median (min–max)
Demographic data		
Age, y	49.1 (13.5)	51.0 (19–70)
Weight, kg	68.2 (12.7)	69.5 (52–95)
Height, cm	162.4 (8.5)	160.5 (147–176)
BMI, kg/m^2	25.8 (3.8)	24.6 (21.4–34.2)
Laboratory data		
Total protein, g/dl	8.05 (0.78)	8.15 (6.40–9.8)
Albumin, g/dl	5.37 (7.3)	3.95 (2.9–38.0)
IgG, mg/dl	2739 (799)	2744 (1738–5327)
Prothrombin, %	72.5 (13.2)	73.5 (49.4–93.5)
Bilirubin, mg/dl	5.66 (5.31)	2.13 (0.80–15.4)
AST, U/l	477 (335)	422 (62–1236)
ALT, U/l	629 (397)	589 70–1636)
ALP, U/l	146 (57)	139 (58–322)
γ-GTP, U/l	167 (109)	168 (34–448)
CRP, mg/l	7.20 (4.45)	7.20 (0.85–16.8)
Hemoglobin, g/dl	13.1 (1.3)	13.2 (9.6–15.9)
Erythrocytes, $\times 10^6$/μl	4.35 (0.46)	4.26 (3.59–5.69)
Leukocytes, $\times 10^3$/μl	5.84 (1.44)	6.07 (3.58–8.3)
Platelets, $\times 10^3$/μl	213 (88)	210 (67–514)
Histological data		
Histological activity index, grade	6.3 (2.4)	7.0 (2–10)
Length of biopsy specimen, mm	21.1 (5.8)	20.0 (12.0–35.0)
No. of portal tracts	11.1 (4.4)	10.0 (6.0–23.0)

Conversion factors for SI units are as follows: for total protein, 10.0; for albumin, 10.0; for IgG, 0.055; for prothrombin, 1.0; for bilirubin, 17.1; for AST, ALT, ALP, and γ-GTP, 0.0167; for CRP, 0.555; for hemoglobin, 10.0; for erythrocytes, 1.0; for leukocytes, 0.001; and for platelets, 1.0.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; CRP, C-reactive protein; IgG, immunoglobulin G; IL-17, interleukin 17; IL-6, interleukin 6; TGF-β1, transforming growth factor β1; γ-GTP, γ-glutamyltranspeptidase

The inflammatory process within the liver tissue is considered the most important precursor of fibrosis in patients with AIH, and therefore it is critically important to assess inflammatory activity in these patients. The ability to assess inflammatory activity in the liver is vital for evaluating drug dose and therapeutic efficacy. The gold standard for assessing inflammatory activity is liver biopsy, but because it is an invasive procedure associated with the risk of serious bleeding complications and possible sampling error, it is not suitable for frequent monitoring of patients with AIH.[12,13] Therefore, it would be extremely valuable to determine a simple and sensitive parameter that would allow a precise assessment of inflammation in the liver. Thus, we analyzed the relationship between serum IL-17, IL-6, and TGF-β1 levels and a histological score of inflammatory activity in treatment-naive and immunosuppressed patients with AIH.

PATIENTS AND METHODS

Study population

The study population comprised 44 patients with a confirmed diagnosis of AIH (36 women, 8 men; age range, 19–67 years) divided into 2 groups. Group 1 included 22 de novo diagnosed individuals and group 2 comprised 22 patients with AIH in clinical remission who had received immunosuppressive treatment for at least 3 years. All patients underwent a thorough physical examination and abdominal ultrasound. Patients with a complete set of laboratory data and who had undergone a liver biopsy were enrolled. The diagnosis of AIH was based on the diagnostic criteria of the International Autoimmune Hepatitis Group.[14]

The characteristics of the study groups are shown in TABLES 1 and 2. The exclusion criteria for both groups were as follows: 1) concomitant autoimmune liver disease, overlapping syndromes, viral hepatitis, nonalcoholic fatty liver disease, Wilson disease, genetic hemochromatosis, α_1--antitrypsin deficiency, or focal liver lesions; 2) drinking more than 150 g (men) or 80 g (women) of alcohol weekly; 3) use of hepatotoxic drugs; or 4) history of drug abuse.

The control group included 30 healthy age- and sex-matched volunteers (21 women, 9 men; age range, 20–62 years). In all controls, medical history, complete blood count, and laboratory data were obtained to screen for acute and chronic diseases (TABLE 3).

Written informed consent was obtained from each patient enrolled in the study. The Medical University of Silesia Ethics Committee approved the study protocol, which conformed to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008).

Laboratory analysis

All subjects underwent a routine laboratory workup including complete blood count and liver function tests. An automated hematology analyzer (Sysmex XT-2000i; Sysmex Corporation, Kobe, Japan) was used to measure the blood count. An autoanalyzer (Olympus AU 650; Olympus Corporation, Tokyo, Japan) was used to assess the serum levels of albumin, total bilirubin, total protein, aspartate transaminase, alanine trasaminase, alkaline phosphatase, γ-glutamyltranspeptidase, immunoglobulin G, and C-reactive protein. A coagulation analyzer (ACL TOP 500; Werfen Company, Barcelona, Spain) was used to calculate the prothrombin index.

Liver histology

All patients underwent a blind percutaneous liver biopsy under local anesthesia (lidocaine, 2%). Liver samples were obtained with a 1.6-mm diameter Menghini needle (HEPAFIX® G16, B. Braun Melsungen AG, Germany), using standard procedures.[15] The biopsy materials were fixed in 4% buffered formalin, embedded in paraffin blocks, and stained with hematoxylin and eosin. An experienced pathologist blinded to the clinical data of the patients

TABLE 2 Clinical, laboratory, and histological characteristics of patients with autoimmune hepatitis in remission

Parameter	Mean (SD)	Median (min–max)
Demographic data		
Age, y	42.0 (15.1)	41.0 (21–67)
Weight, kg	70.9 (14.3)	69.8 (48.5–102)
Height, cm	166.3 (7.8)	164.5 (158–185)
BMI, kg/m^2	25.5 (4.3)	25.4 (19.0–34.9)
Duration of treatment, mo	51.6 (10.7)	52 (38–70)
Laboratory data		
Total protein, g/dl	7.69 (0.48)	7.70 (6.80–8.8)
Albumin, g/dl	4.49 (0.32)	4.55 (4.0–5.3)
IgG, mg/dl	1351(264)	1366 (868–1941)
Prothrombin, %	92.4 (9.0)	92.9 (75.5–113.0)
Bilirubin, mg/dl	1.03 (0.4)	1.00 (0.40–1.96)
AST, U/l	26 (9)	24 (14–49)
ALT, U/l	25 (17)	20 (9–76)
ALP, U/l	63 (18)	64 (34–99)
γ-GTP, U/l	31 (25)	22 (10–115)
CRP, mg/l	1.00 (0.6)	0.80 (0.18–2.33)
Hemoglobin, g/dl	14.1 (1.3)	14.0 (12.2–17.6)
Erythrocytes, ×10^6/μl	4.55 (0.49)	4.53 (3.73–5.68)
Leukocytes, ×10^3/μl	5.53 (1.47)	5.15 (3.42–9.3)
Platelets, ×10^3/μl	196 (70)	185 (91–358)
Histological data		
Histological activity index, grade	1.3 (1.0)	1.0 (0–3)
Length of biopsy specimen, mm	19.4 (7.1)	18.0 (10.0–35.0)
No. of portal tracts	8.7 (3.4)	8.0 (5.0–21.0)

Abbreviations and conversion factors: see TABLE 1

TABLE 3 Clinical and laboratory characteristics of the control group

Parameter	Mean (SD)	Median (min–max)
Demographic data		
Age, y	41.9 (12.1)	40.5 (20–62)
Weight, kg	70.3 (11.0)	70.5 (52–91)
Height, cm	169.1 (6.9)	168.0 (157–182)
BMI, kg/m^2	24.5 (3.0)	25.5 (18.4–29.0)
Laboratory data		
AST, U/l	20 (4)	20 (13–28)
ALT, U/l	18 (8)	17 (7–40)
CRP, mg/l	0.15 (0.13)	0.09 (0.01–0.56)
Hemoglobin, g/dl	13.6 (1.3)	13.3 (11.6–16.3)
Erythrocytes, ×10^6/μl	4.69 (0.38)	4.59 (4.11–5.41)
Leukocytes, ×10^3/μl	6.11 (1.42)	5.67 (4.04–9.6)
Platelets, ×10^3/μl	242 (51)	242 (154–341)

Abbreviations and converstion factors: see TABLE 1

graded the liver specimens for inflammatory activity using the 18-point Knodell score as modified by Ishak (histological activity index grade).[16]

Sample collection and storage For the measurement of IL-6 and IL-17 levels, a sample of peripheral blood (5 ml) was obtained from each subject and placed into a serum separator tube, allowed to clot for 30 minutes at room temperature, and then centrifuged for 15 minutes at 1000 × *g*. The serum was removed immediately, decanted into Eppendorf tubes, and stored at a temperature of –70°C.

For the measurement of TGF-β1 levels, a sample of peripheral blood (6 ml) was obtained from each subject and placed into a serum separator tube and allowed to clot for 30 minutes at room temperature. To ensure the complete release of TGF-β1, we incubated the samples overnight at a temperature of 2°C to 8°C before centrifuging them for 15 minutes at 1000 × *g*. The serum was then decanted and the samples stored at a temperature of –70°C.

All serum cytokines were quantified using sandwich enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, Minnesota, United States) specific for each cytokine. The microplates were precoated with a monoclonal antibody specific for IL-6, IL-17, or TGF-β1. Standards, controls, and samples were pipetted into the microplate wells. Unbound substances were washed off and enzyme-linked polyclonal antibody specific for IL-6, IL-17, or TGF-β1 was then added to the wells. Following a wash to remove the unbound antibody-enzyme reagent, the substrate solution was added to the wells. Color development was stopped, and the color intensity was measured spectrophotometrically (wavelength, 450 nm) in a microplate reader (μQiand, BioTek Instruments, Winooski, Vermont, United States).

Statistical analysis The data were presented as medians, means, and standard deviations. All statistical tests were carried out using STATISTICA (StatSoft, Kraków, Poland). Differences in serum cytokine concentrations between the analyzed groups were assessed using the Mann–Whitney test. Correlations between the histological activity index grade and serum cytokine concentrations were assessed using the Spearman rank correlation test. A *P* value of less than 0.05 was considered significant.

RESULTS

Serum cytokine concentrations Serum IL-6 and IL-17 concentrations were significantly higher in patients in the active stage of AIH (group 1) than in group 2 or controls. Serum IL-6 and IL-7 concentrations did not differ significantly between group 2 and controls. Serum TGF-β1 concentrations were higher in group 1 compared with controls. The TGF-β1 concentration did not differ between group 2 and group 1 or controls (TABLE 4).

The only significant correlation was detected between reduced serum IL-17 concentrations and an increased TGF-β1 concentration in patients in clinical remission (r = –0.51, P = 0.03; TABLE 5, FIGURE 1).

TABLE 4 Serum cytokine levels in patients with active autoimmune hepatitis (group 1), patients in remission (group 2), and controls

Cytokine	Control			Group 1			Group 2			*P* value
	Mean	Median	SD	Mean	Median	SD	Mean	Median	SD	
IL-17, pg/ml	16.4	15.3	6.7	30.8	23.2	18.5	16.0	13.9	9.2	0.0001
IL-6, pg/ml	1.69	1.42	1.28	6.82	5.20	5.71	2.22	1.27	2.28	0.0001
TGF-β1, ng/ml	30.4	30.1	7.5	40.0	40.5	15.0	37.9	38.7	15.4	0.04

Abbreviations: IL-6, interleukin 6; IL-17, interleukin 17; others, see **TABLE 1**

TABLE 5 Correlations between cytokine concentrations among patients with active autoimmune hepatitis (group 1), patients in remission (group 2), and controls

Cytokine	Control			Group 1			Group 2		
	IL-17	IL-6	TGF-β1	IL-17	IL-6	TGF-β1	IL-17	IL-6	TGF-β1
IL-17, pg/ml	1	0.27	–0.03	1	–0.10	0.29	1	–0.18	–0.51[a]
IL-6, pg/ml	0.27	1	–0.02	–0.10	1	0.37	–0.18	1	–0.19
TGF-β1, ng/ml	–0.03	–0.02	1	0.29	0.37	1	–0.51[a]	–0.19	1

a $P < 0.05$

Abbreviations: see **TABLES 1** and **4**

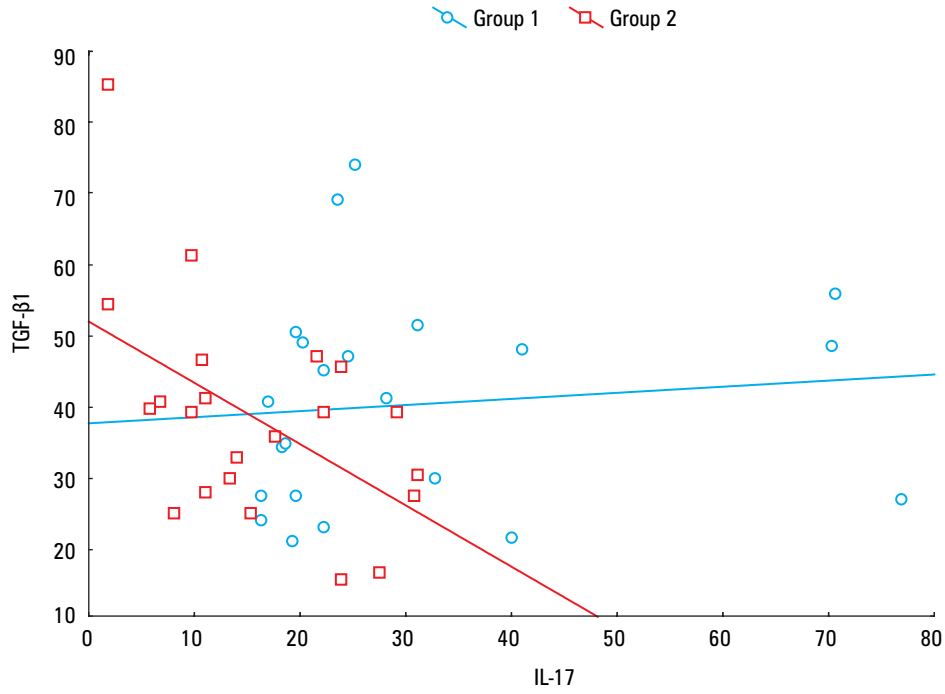

FIGURE 1 Correlations between cytokine concentrations of interleukin 17 (IL-17) and tumor growth factor β1 (TGF-β1) in patients with active autoimmune hepatitis (group 1) and in those in remission (group 2)

Correlations between cytokine concentrations and inflammatory activity index A negative correlation was detected between the index of inflammatory lesions and serum IL-17 concentrations. Higher inflammatory activity in liver biopsy samples was closely correlated with a significant reduction in serum IL-17 concentrations ($r = –0.63$, $P = 0.01$; **FIGURE 2**).

DISCUSSION

Based on an extensive review of the literature published in the last 20 years (PubMed, www.ncbi.nlm.nih.gov, 1997–2017), this study is the first to evaluate serum IL-6, IL-17, and TGF-β1 concentrations in patients with AIH at different stages of disease in relation to the inflammatory activity in the liver tissue. Although cytokine concentrations in the peripheral blood do not fully reflect the processes taking place in the liver, together with histopathological analysis they are a valuable source of information, allowing for evaluation of immunological phenomena in the liver. In clinical practice, serum transaminase and immunoglobulin G levels are routinely used to assess hepatitis activity, but normal transaminasemia does not exclude the presence of mild to moderate liver inflammation.[17-19] Therefore, it would be very helpful to discover a simple serologic parameter that closely relates to liver histology in patients with AIH. Such a parameter would be beneficial for monitoring AIH activity and for optimizing treatment.

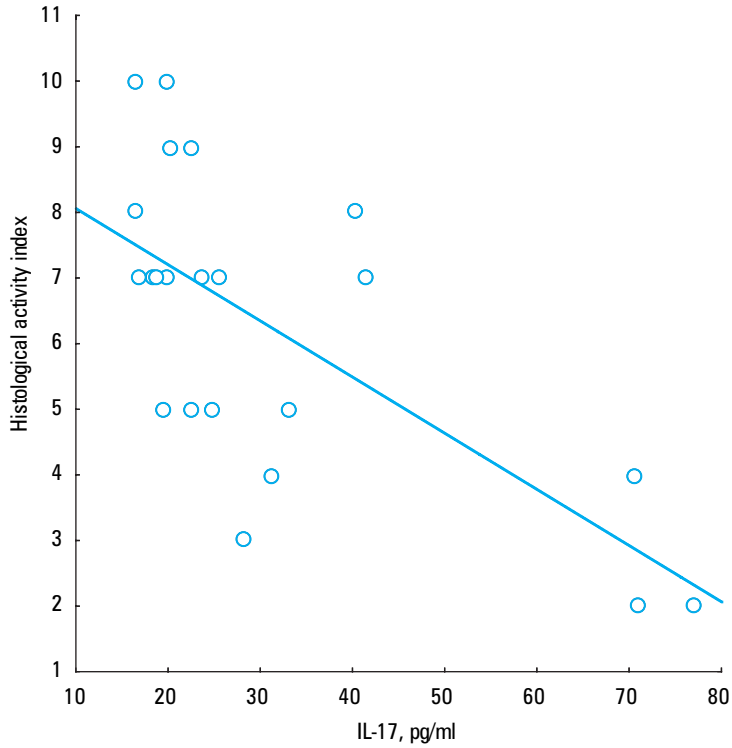

FIGURE 2 Correlations between interleukin 17 (IL-17) levels and histological activity index in patients with active autoimmune hepatitis

Only a few studies have examined the involvement of IL-17 in the pathogenesis of AIH. The most important observation in the present study is the significantly higher serum IL-17 concentration in patients in the active phase of AIH compared with the control group and patients in remission. Higher IL-17 concentrations in an active stage of the disease result from the activation of lymphocytes producing IL-17, as confirmed by Yu et al,[20] who evaluated IL-17 mRNA expression in mononuclear cells in the peripheral blood of 12 patients with AIH (10 women, 2 men; mean age, 45.3 years) and a group of 10 healthy volunteers (8 women, 2 men; mean age, 29.7 years). The results confirmed that IL-17 mRNA expression is increased in patients with AIH compared with controls.

Another important observation of the present study is the negative correlation between the serum IL-17 concentration and the severity of inflammatory lesions in the liver tissue in patients with active AIH. The analysis of immunological phenomena occurring during exacerbation of AIH revealed that exacerbation of inflammatory lesions may result from an increased concentration of Th_{17} cells in the liver. This hypothesis was confirmed in animal models by Yu et al,[20] and in clinical trials by Lan et al[21] and Lemmers et al.[22] The results reported by the Yu's group were based on the use of a mouse model of AIH that allowed them to gain insight into the condition of the liver tissue after parenteral administration of monoclonal antibodies that block IL-17. In the experiment in which 2 groups of 25 animals were evaluated, AIH was experimentally induced. After 10 days, mice from only one group were given anti–IL-17 antibodies. Fourteen days after starting the experiment, autopsies of all the animals were conducted, with liver tissue taken for histopathology. In the blood of the animals that received the anti–IL-17 antibody, alanine transaminase activity was significantly reduced, and the number of mononuclear cells was reduced in the biopsy sample, indicating the IL-17 expression and significantly fewer inflammatory necrotic lesions compared with the control group.

Using immunohistochemical staining, Lan et al[21] evaluated the number of IL-17$^+$ mononuclear cells in the portal spaces of biopsy samples of 17 patients with primary biliary cholangitis, 15 patients with nonalcoholic steatohepatitis, 26 patients with a different chronic disease of the liver, and 4 patients with AIH. Despite the small number of patients with AIH, the high value of this research must be emphasized (one of two studies in which IL-17$^+$ cell expression was evaluated in human liver biopsy samples of patients with AIH). This study demonstrated that the number of IL-17$^+$ cells in portal spaces is the lowest in patients with primary biliary cholangitis, then higher in patients with a different chronic disease of the liver followed by patients with nonalcoholic steatohepatitis, and finally the highest in patients with AIH.

The second study which evaluated the number of IL-17$^+$ lymphocytes in liver biopsy samples of 7 patients with active AIH (5 women, 2 men), was conducted by Lemmers et al.[22] Using immunohistochemical staining, the authors stained and counted the IL-17$^+$ lymphocytes (CD3$^+$, CD3$^+$CD4$^+$) present in the inflammatory infiltrate. The results were compared with analogical results obtained from the evaluation of biopsies from 40 patients with alcoholic liver disease (ALD; 31 in the inflammatory

stage, 9 in the cirrhotic stage). The mean number of IL-17$^+$ lymphocytes found in the field of view was the highest in patients with AIH (38.2), lower in patients with ALD in the inflammatory stage (20.4), and the lowest in patients with ALD at the cirrhotic stage (7.9). Moreover, the authors demonstrated that IL-17$^+$ lymphocytes are concentrated mainly on the developing fibrous portal walls.

Another important observation in our study was the higher TGF-β1 concentration in the serum of patients with active AIH compared with the control group. Similar results were obtained by Sakaguchi et al,[23] who evaluated TGF-β1 concentrations in the serum of 22 patients with AIH and 20 healthy volunteers. The results showed a significantly higher TGF-β1 concentration in patients with AIH.

Of note, TGF-B1 exerts double effect on the inflammatory fibrotic process in the liver tissue. Depending on the conditions of the microenvironment, cytokines exert both proinflammatory and anti-inflammatory actions. TGF--β1 enhances the expression of the FOXP3 nuclear transcription agent, an important inductor of regulatory lymphocytes, which blocks the inflammatory response. The number of positive FOXP3 cells is inversely proportional to the IL-6 concentration in the microenvironment. Its presence drastically changes the action of TGF-β1, which promotes the expression of RORγt—a receptor belonging to the family of so called orphan receptors. RORγt is a key element responsible for the differentiation of auxiliary lymphocytes into the Th_{17} line.[24]

Paladino et al[25] reported that genetic TGF--β1 polymorphisms may determine the clinical course of AIH. The authors demonstrated that patients with type 1 AIH, which progresses more slowly than type 2, are characterized by the presence of 2 characteristic codons (10CC and 25CC), resulting in lower TGF-β1 profibrogenic and proinflammatory activity.

Further, the effect of glucocorticoid therapy on TGF-β1 activity was evaluated. Glucocorticoid steroids may block the promoter region of the TGF-β1 coding gene, thereby reducing its synthesis. Moreover, such medications inhibit TGF--β1 activation by weakening the protein binding activating the cytokine with its membrane receptor.[26] These observations indicate that by blocking TGF-β1 function while inhibiting the inflammatory cascade this drug class exerts the antifibrotic effect in patients with AIH.[27,28]

The higher serum IL-6 concentration observed in our study in patients with active disease in comparison with the control group and patients in remission supports the proinflammatory activity of IL-6. The only study evaluating serum IL-6 concentrations in patients with AIH was reported by Maggiore et al.[29] The authors measured serum IL-6 concentrations in 13 children with AIH in active stage and during remission (mean age, 7.5 years; range, 1.6–14.3 years). The population comprised 7 children with type 1 AIH and 6 children with type 2 AIH. Twenty healthy children (mean age, 7.5 years; range, 3–15 years) were included as the control group. Among children in active stage, the serum IL-6 concentration in was significantly higher than the mean IL-6 concentration in the control group and normalized after achieving disease remission. In 5 children with type 2 AIH, the mean serum IL-6 concentration did not differ from the mean concentration in the control group. It is interesting that among children with type 1 AIH, but not type 2, the concentration of IL-6, which is an important inflammatory mediator, was significantly higher. Type 2 AIH, however, is characterized by a more aggressive course, higher inflammatory activity, and faster progression to severe fibrosis and cirrhosis, which results in a higher percentage of end-stage liver failure and indications for liver transplantation.[30]

In conclusion, serum IL-17 levels may be a simple and useful marker of disease activity in patients with AIH.

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CONTRIBUTION STATEMENT KG and MH conceived the concept for the study. All authors were involved in data collection. MK reviewed liver samples. TK-H quantified the cytokines. KG, DG, MP, and JK analyzed the data and wrote the paper. KG and MH revised the manuscript for final submission. All authors edited and approved the final version of the manuscript.

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