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ARTICLE ORIGINAL

High levels of proinflammatory cytokines IL-6, IL-8, TNF-A, IL-23, and IFN-γ in Tunisian patients with type 1 autoimmune hepatitis

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ABSTRACT. Autoimmune hepatitis (AIH) is a chronic hepatitis of unknown etiology and several cytokines have been implicated in its pathogenesis and onset. Our objective was to determine the profile of pro and anti-inflammatory cytokines, including IL-1β, IL-6, IL-8, IL-23, IFN-γ, TNF-α, IL-10 in autoimmune hepatitis and their association with HLA gene polymorphisms. Serum cytokine levels were determined in 50 autoimmune hepatitis patients and one hundred fifty controls using chemiluminescence and ELISA techniques and HLA genotyping performed by PCR SSP. The levels of IL-6 (12 pg/mL *vs.* 5.5 pg/mL, p = 0.017), IL-8 (24.1 pg/mL *vs.* 7.8 pg/mL, p = 0.006), and TNF-α (61.1 pg/mL *vs.* <4.00 pg/mL, p = 0.002) were significantly higher in AIH patients in pretreatment phase compared to levels after remission and in controls. HLA*DRB15 was significantly associated with higher levels of IL-8. IL-6, IL-8, and TNF-α may be biomarkers of AIH activity. HLA gene expression may play a role in higher cytokine production and could allow an earlier diagnosis and better management of the disease.

Key words: autoimmune hepatitis, proinflammatory cytokines, pathogenesis

INTRODUCTION

Autoimmune hepatitis (AIH) is a progressive inflammatory disease of the liver of unknown etiology, characterized by increase in aminotranferases levels, hypergammaglobulinemia, and presence of autoantibodies in serum. AIH results from a loss of tolerance mechanisms against the hepatocyte antigens that leads to hepatic parenchyma destruction [1]. Hepatocyte damage is mainly due to interface hepatitis characterized by lymphocyte infiltration into the liver eventually followed by fibrosis and cirrhosis [2]. The etiology and pathologic mechanism of AIH has not yet been fully known, although its immunological features, environmental triggers, failure of immune tolerance mechanisms, and genetic predisposition interact to induce a T-cell-mediated immune response against liver antigens, leading to a progressive necroinflammatory and fibrotic process damaging the liver [3].
Almost 1/3 of patients with AIH are asymptomatic, which may contribute to a late diagnosis of the disease usually in the phase of cirrhosis (25% of cases) [4]. The onset is often insidious with nonspecific symptoms such as asthenia (85%), jaundice (80%), nausea (47%), abdominal pain (38%), and arthralgias (30%) at presentation, but the clinical spectrum of AIH is wide, ranging from an asymptomatic presentation to an acute fulminant hepatitis [5]. The diagnosis is based on histologic abnormalities, characteristic clinical and biological parameters, abnormal levels of serum globulins, and the exclusion of other conditions that cause chronic hepatitis and cirrhosis. Cytokines are bioactive substances that play an important role in the pathologic mechanism of many metabolic and autoimmune diseases, and may be indicators of AIH activity or occurrence. Cytokines affect mechanisms of humoral and cellular immunity, and disturbances in cytokine production have been described in autoimmune hepatitis patients (Trautwein and Manns, 1995). The immunoregulatory cytokines such as interleukin 2 (IL-2), interferon γ (IFN-γ), and tumor necrosis factor (TNF-α) promote the cellular immune response, and the proinflammatory cytokines such as IL-4, IL-5, IL-6, IL-8, IL-10, and IL-13 promote the humoral immune response [6]. Early studies have indicated that autoimmune hepatitis exhibits a humoral cytokine response [7]. Several cytokines have been linked to the pathogenesis and severity of AIH [8, 9]. Czaja *et al.* (2000) measured the serum levels of IL-2, IL-4, IL-10, and IFN-γ in AIH patients and showed that IL-2 and

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IL-4 were significantly lower than healthy subjects [10]. The expression of IL-12 in the liver and peripheral blood was found to be significantly increased in AIH children compared to healthy controls [11]. Other studies demonstrated that the serum levels of IL-1β, IL-6, TNF-α, and IFN-ɣ were increased in chronic hepatitis and not really affected by the type of liver disease. Cytokine production by mitogen stimulated T lymphocytes from the peripheral blood and cytokine production by liver infiltrating T lymphocytes was detected in patients with autoimmune hepatitis and demonstrated increased levels of secretion of IL-4 and IL-10 [12] Mononuclear cells from patients with autoimmune hepatitis showed also higher production of IL-4 and IL-6 than similar cell populations from patients with chronic hepatitis B or healthy subjects [13]. The cytokines are involved in the induction of inflammatory and immune responses, and would likely play a critical role in the development of autoimmune diseases. IL-1β , IL-6, TNF-α, and IFN-γ enhance the cellular immune mechanisms by activating lymphocytes and macrophages, IL-6 stimulate the cellular cytotoxicity in part by increasing expression of class II antigens of the major histocompatibility complex (MHC) on monocytes and IL-1α and β and their receptor antagonist IL-1RA play major roles in initiating and modulating immune responses [14]. Other cytokines such as IL-4, IL-10, IL-13 influence the humoral immunity by activating B cells and stimulating antibody production [15]. They can also stimulate NK cell activity and downregulate class II MHC expression on monocytes. The immune stimulatory and inhibitory effects of these two types of cytokines responses cross-regulate each other and modulate the immune response [16]. Cytokine expression has been reported to be involved in the development of various inflammatory and immune diseases in Tunisian patients including Inflammatory bowel disease [17], Graves' disease (GD) [18], type 1 Diabetes [19], Behçet's disease [20], and rheumatoid arthritis [21]. So far, few studies have investigated the involvement of cytokine expression in autoimmune hepatitis in Tunisian or north African patients, Accordingly, in this study, we aimed to investigate cytokine profile of IL-1β, IL-6, IL-8, IL-10, IL-23, IFN-ɣ, and TNF-α in patients with type 1 autoimmune hepatitis in both pretreatment and remission following treatment phases compared to healthy control subjects, then we investigated the association between increase in cytokines levels and HLA polymorphic genotypes in AIH patients to evaluate the role of cytokine expression and production in the physiopathologic mechanism of autoimmune hepatitis.

MATERIALS AND METHODS

Patients and controls

A total of 50 unrelated patients with definite AIH were recruited from the Gastroenterology department of Military hospital, Charles Nicolle and La Rabta in Tunis between September 2013 and April 2015. These cases were diagnosed on the basis of International AIH Group criteria using scoring calculator (10-15). A score of > 15 was taken as definite AIH, and ≥ 10 as probable AIH. Patients with a score less than 10 were excluded from the analysis. Thirty six (86%) were female and their ages ranged from 18 to 78 years (mean age 44.2 years). AIH patients had concurrent immune disorder type 1 diabetes (16%), primary biliary cirrhosis (16%), and Sjogren's syndrome (3%). Sera were stored at -80 °C and were collected from AIH patients at presentation before treatment (pretreatment phase) and after remission phase following corticosteroid therapy. Remission was defined as the sustained return of transaminase and bilirubin values to normal for at least 6 months. We therefore compared cytokine levels in 22 patients with active AIH (44%) and 28 patients (58%) after total remission from the total 50 patients included in our study. Clinical and biological features were obtained from the medical records of patients. One hundred and fifty unrelated healthy donors were included in our study (129 women and 21 men, mean age 46.74 yrs. ± 14.17) and matched for gender and age with AIH cases. Study participants have signed an informed consent before the study, and the Ethics Committee of the Pasteur Research Institute in Tunis approved the study protocol. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in *a priori* approval by the institution's human research committee.

Cytokine assessments

Interleukine 1β, IL-6, IL-8, and TNF-α were simultaneously tested by an automated solid-phase chemiluminescent immunometric assay (Immulite 1000® DPC Los Angeles, CA). A polystyrene bead coated with murine monoclonal antibodies (anti-IL-1β, anti-IL6, anti-IL8, and anti-TNF-α) specific for the cytokines to be measured (IL-1β, IL-6, IL-8, and TNF-α) serves as the solid phase. The beads are incubated with at least 100 µl of serum from patients for thirty minutes when the antigen/antibody reaction occurs. Polyclonal sheep IgG antibodies labeled with alkaline phosphatase enzyme are used as detection antibodies. Unbound components were removed after 30 min using a patented centrifugal washing technique. Automatically added chemiluminescence substrate (AMPPD,3-(2'-spirodamantane)-4-methoxy-4-(3'-phophoryloxy) phenyl-l,2-dioxetane) is converted by the bound enzyme during the following 10-min incubation period to an unstable intermediate. The resulting light emission is directly proportional to the concentration of the analyte in the samples. The control reference ranges of the selected cytokines, according to the given reference on the manual, were as follows: IL-1β: 0-5 pg/mL, TNF-α: 0-8.1 pg/mL, IL-6: 0-5.9 pg/mL, IL-8: 0-5.2 pg/mL. The value of index was defined as higher one if it was beyond the reference range.
The calibration slope is adjusted by the user using two sera (Adjustors; DPC) at least every two weeks. The adjustment was validated in each run using a high- and low-quality control (DPC). Calibration ranges were: for IL-6, 2–2,000 pg/mL; for IL-8, 10–7,500 pg/mL; for

IL-1β, 5–1,000 pg/mL; and for TNF, 4–1,000 pg/mL. The basic control values of the selected cytokines, according to the given reference on the manual, were as follows: IL-1β: 0-5 pg/mL, TNF-α: 0-8.1 pg/mL, IL-6: 0-5.9 pg/mL, IL-8: 0-5.2 pg/mL. The value of index was defined as higher one if it was beyond the reference range. The detection limits were: IL-1β: 1.5 pg/mL, TNF-α: 1.7 pg/mL, IL-6: 5 pg/mL, IL-8: 2 pg/mL and the sensitivity levels for each cytokine were: IL-1β: 1.5 pg/mL, TNF-α: 1.7 pg/mL, IL-6: 1 pg/mL, IL-8: 2 pg/mL. The sample and the test units (one for each cytokine to be tested) were simply loaded on the platform. The test procedure was fully automated. The data acquisitions were completed within 40 (TNF) to 70 (IL-6, IL-8, IL-1β) min.

IL-23, IFN-ɣ, and IL-10 levels were measured in serum by ELISA, a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiterplate in accordance with the manufacturer's instructions (DIA source IL-10-EASIA, human l'IL-23 ELISA (Invitrogen Thermo Fisher scientific) and human IFN-ɣ High Sensitivity ELISA (Invitrogen Thermo Fisher scientific)). The assay uses microtiter plates with immobilized monoclonal antibodies directed against distinct epitopes of the human cytokine of interest (IL-10, IL-23, and IFN-ɣ).

A measured volume of sample or standard was added to each test well and incubated to allow the cytokine to be bound by the antibodies on the microtiter plate. Once the calibrators and serum samples are added to their appropriate wells in each separate kit, IL-10, IL-23, and IFN-ɣ are captured by these monoclonal antibodies. After the incubation period, the wells were washed with a buffer solution and a biotinylated antibody solution was added to each well. In this instance, an antibody conjugated with horseradish peroxidase (HRP) was applied allowing the formation of a sandwish: coated antibody- human cytokine- HRP-coated antibody. After another incubation period and washing, a peroxidase-labeled streptavidin reagent was added to each well of the IL-23, IFN-ɣ, and IL-10 microtiter plates. The plates were allowed to incubate for a time defined by the individual cytokine kit. Thereafter, the wells were washed and a substrate solution was added. The appearance of a blue color indicated the presence of a cytokine. Acid was then added into the well until the mixture turned yellow. The amount of substrate was determined colorimetically by measuring the absorbance which is proportional to the cytokine concentration in wells. The absorbance of each well was read at 450 nm and a standard curve was constructed to quantify the amount of cytokine present in the serum samples. Each serologic analysis utilized a newly constructed standard curve. Standard curves for IL-23, IFN-ɣ, and IL-10 were constructed and the equation of this curve was used to determine the concentration of each cytokine in each well.

The basic control values of the selected cytokines, according to the given reference on the manual, were as follows: IL-10: 0-3.3 pg/mL, for IFN-ɣ: 0-3 pg/mL and for IL-23: 0 pg/mL.

The detection limits were: for IL-10: 1.6 pg/mL, for IFN-ɣ: 0.03 pg/mL and for IL-23: 5 pg/mL.

HLA genotyping

Only patients with significantly higher levels of cytokine were included in HLA genotyping. Genomic DNA was extracted from lymphocytes separated from whole blood using a Ficoll–Hypaque solution (density 1.077 ± 0.001 g/mL). DNA extraction was performed using QIAamp® DNA Blood Mini Kit (Qiagen®), following the manufacturer's instructions. HLA-DRB1 and -DQB1 antigens were detected by Single-specific-primer polymerase chain reaction (SSP-PCR) technique, according to Micro SSP Generic HLA class II DNA Typing Trays DRB1/DQB1 (One lambda®). Amplified DNA fragments were analyzed on 2.5% agarose gel stained with ethidium bromide. HLA Fusion Software (2005, One lambda®) was used to detect specific DRB1 and DQB1 alleles.

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 software (IBM, Armonk, NY). Comparisons of cytokine levels in pretreatment phase with those in remission phase were calculated using the nonparametric Wilcoxon rank sum test. Comparisons of cytokines in AIH patients and controls were calculated using the Mann–Whitney U-test. Correlations between two quantitative variables were studied using the Pearson coefficient test. A *p-value* of less than 0.05 was considered statistically significant.

RESULTS

Clinical and immunological features in AIH patients

Demographic and clinical characteristics of patients are shown in *table 1*.

Proinflammatory cytokines levels in AIH patients and controls

Four cytokines (IL-6, IL-8, IL-23, and IFN-ɣ) were significantly higher in AIH patients than in healthy controls. The levels of IL-6, IL-8, and TNF-α were also significantly higher in patients in the pretreatment phase than those detected in AIH patients after remission (*figure 1*).

IL-6 was detected in a total of 32 AIH patients (64%) with an average rate of 11.42 pg/mL. The higher rates of cytokines were revealed in the sera samples from patients in the pretreatment phase during the disease activity (22 cases, 44% of total 50 patients) (mean level of 12,00 pg/mL) compared to the values found in AIH patients after remission (28 cases, 58% of total 50 patients) (mean level of 5.59 pg/mL) with a p-value of 0.017 and this difference is considered statistically significant. The level of IL-6 was also significantly increased in AIH patients compared to the healthy controls (11,42 pg/mL *vs.* 1,29 pg/mL, $p < 0.001$) (*figure 1*).

IL-8 was detected in 36 AIH patients (72%) with an average rate of 19.16 pg/mL. Mean serum levels of IL-8 found in (22 cases, 44% of total 50 patients) AIH patients in the pretreatment phase (24.10 pg/mL) was significantly higher than the mean serum level found in

Table 1
Characteristics of Tunisian autoimmune hepatitis cases and controls.

Parameters	Cases[1] (n = 50)	Controls[a] (n = 150)	*P*-value
Gender, n (%)			
Women	36 (84.0)	129 (86.0)	0.112
Age[b]	50.48 ± 16.05	46.47 ± 14.17	0.188
Smoking, n (%)			
Yes	6 (12)	32 (21.30)	0.145
Alcohol use, n (%)			
Yes	2 (4)	19 (12.30)	0.083
Age at onset[b]	44.22 ± 14.33	NA	-
Disease duration[c]	5.0 (2.0-8.0)	NA	-
Clinical presentations, n (%)	NA	-	
Asthenia	33 (66)		
Arthralgia	10 (20)		
Nausea	8 (16)		
Anorexia	10 (20)		
Weight loss	13 (26)		
Abdominal pain	13 (26)		
Jaundice	40 (80.0)		
Pruritus	23 (46)		
Hepatomegaly	17 (34)		
Splenomegaly	26 (52)		
Antibodies presence, n (%)			
ANA	35 (70.0)		
SMA	29 (58)		
LKM1	2 (4)		
SLA	2 (4)		
AMA-M2	11 (22)		
Infections, n (%)			
EBV infection	1 (3.3)		
CMV infection	3 (6)		

AMA-M2: anti-mitochondrial antibody-M2; ANA: antinuclear antibodies; CMV: cytomegalovirus; EBV: Epstein - Barr virus; LKM1: anti-Liver/kidney microsomal antibodies type 1; NA: not applicable; SLA: antibodies against soluble liver antigen; SMA: smooth-muscle antibodies.
[a] A total of 50 AIH cases and 150 healthy controls were included.
[b] t-Student's test for the variable "Age" with normal distribution (mean ± standard deviation).
[c] Variable without normal distribution (median [interquartile range]).

AIH patients after remission (7.85 pg/mL) (28 cases, 58% of total 50 patients), p= 0.006. The mean serum level of IL-8 found in AIH patients decreased signif icantly from 19,16 pg/mL in AIH patients to 3,70 pg/mL in healthy controls, p <0.001 (*figure 1*).

The tumor necrosis factor-α was detected in 19 AIH patients (38%) with a mean serum level of 61.15 pg/mL. The levels of TNF-α were significantly higher in AIH patients during the pretreatment phase (mean of 55.5 pg/mL, 28 cases, 58% of patients) and were not detected in patients after remission with values <4.00 pg/mL (p = 0.002) in 28 cases, 58% of initial patients. Serum TNF-α showed no significant differences between AIH patients and controls, the mean values differed from 61,15 pg/mL to 2,01 pg/mL, p = 0.17 (*figure 1*).

IL-23 was detected in 86% of AIH patients. The IL-23 levels ranged from 15.14 pg/mL to 300 pg/mL with a mean of 108.59 pg/mL, which was considered high compared to levels detected in healthy controls. A mean serum level of 96.22 pg/mL was found in patients in pretreatment phase (22 cases, 44% of patients) compared to 81.44 pg/mL found in AIH patients after remission (28 AIH patients, 58%). A slight decrease was observed in patients after remission but this difference was not statistically significant, p = 0.355. The levels of IL-23 were significantly higher in autoimmune hepatitis patients compared to the levels found in control subjects (88,75 *vs.* 17,05 pg/mL, p< 0,001**)** **(***figure 2*).

IFN-ɣ was detected in all patients with AIH. The levels of IFN-ɣ ranged from 2.45 pg/mL to 35.1 pg/mL with a mean serum level of 13.41 pg/mL. The mean level of IFN-ɣ detected in patients in pretreatment phase was 13.83 pg/mL (22 AIH patients, 44%) compared to a mean level of 12.52 pg/mL found in patients after total remission (28 AIH patients, 58% of total patients). This difference was not statistically significant using the nonparametric Wilcoxon test since p value = 0.615. But, the level of IFN-ɣ was significantly higher in AIH patients compared with the levels detected in healthy control subjects (13,34 pg/mL *vs.* 3,08 pg/mL, p< 0,001) (*figure 2*).

IL-1 β was not detected in any of the total 50 AIH patients included in our study, in the pretreatment phase or after remission. A positive correlation between IL-6 and IL-8 was found p= 0,004, correlation of Pearson (r) = 1.

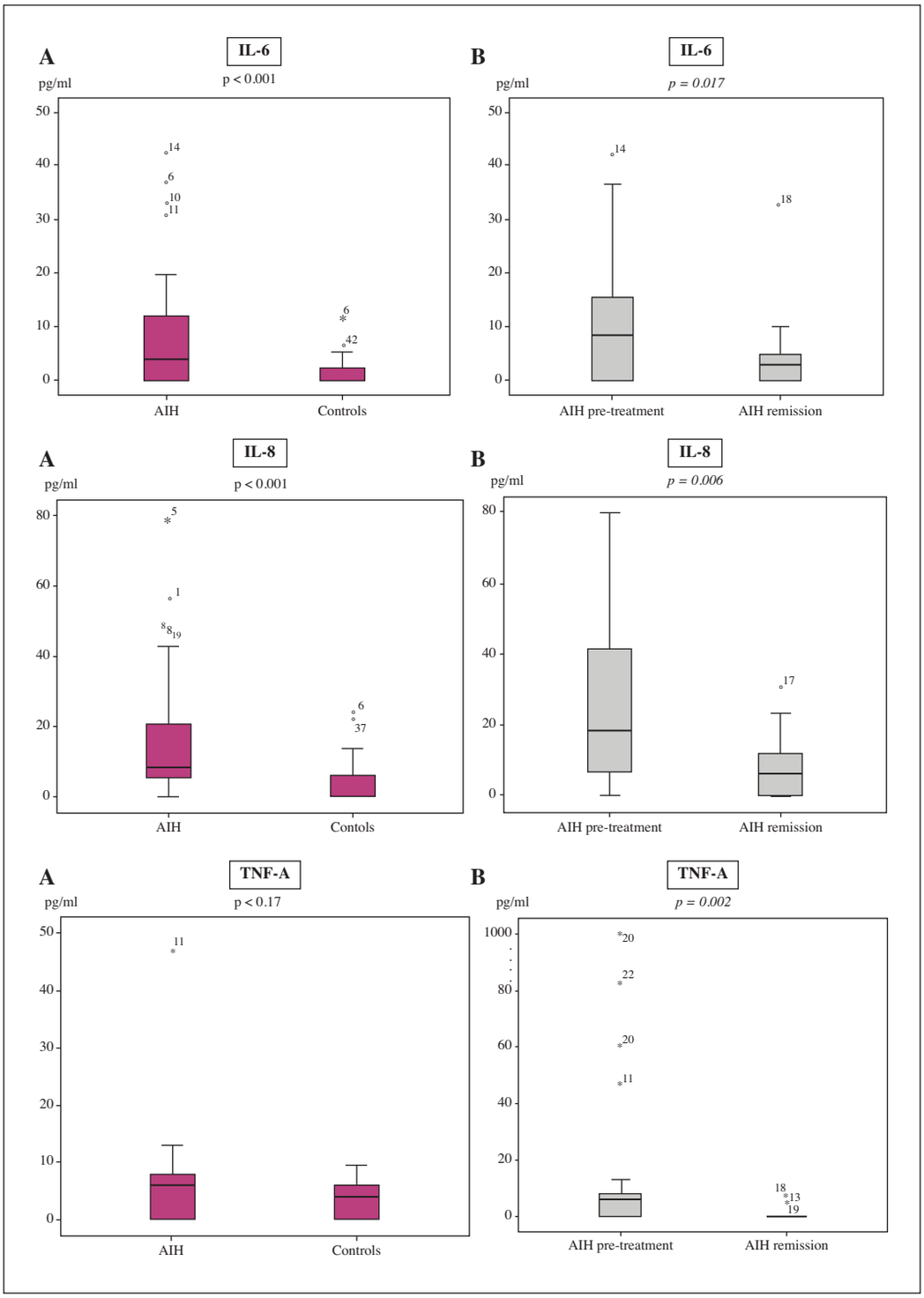

Figure 1

Association of proinflammatory cytokines IL-6, IL-8 and TNF-α with autoimmune hepatitis active phase. (A) shows increase in IL-6, IL-8 and TNF-α levels in AIH patients compared to healthy control subjects and (B) shows increase in IL-6, IL-8 and TNF-α levels in AIH patients during pretreatment phase compared to remission following corticosteroid therapy: AIH: 50 patients, Controls: 150 healthy donors, AIH pre-treatment: 22 patients (44%), AIH remission: 28 patients (58%).

Association of cytokine high levels with HLA genes

The study of HLA genes associations with increased levels of proinflammatory cytokines IL-6, IL-8, TNF-α, and IFN-γ (levels > 10 pg/mL) was investigated in AIH patients compared to healthy controls and results showed no significant association of the different HLA genes with increased levels found of the selected cytokines. Only HLA DRB1*15 showed a significantly higher frequency in AIH patients showing increased levels of IL-8 ($p = 0.04$) while frequency of HLA DRB1*13 was higher in controls compared to AIH patients (*table 2*).

DISCUSSION

Several cytokines are important mediators of the inflammatory response in infectious, autoimmune, and

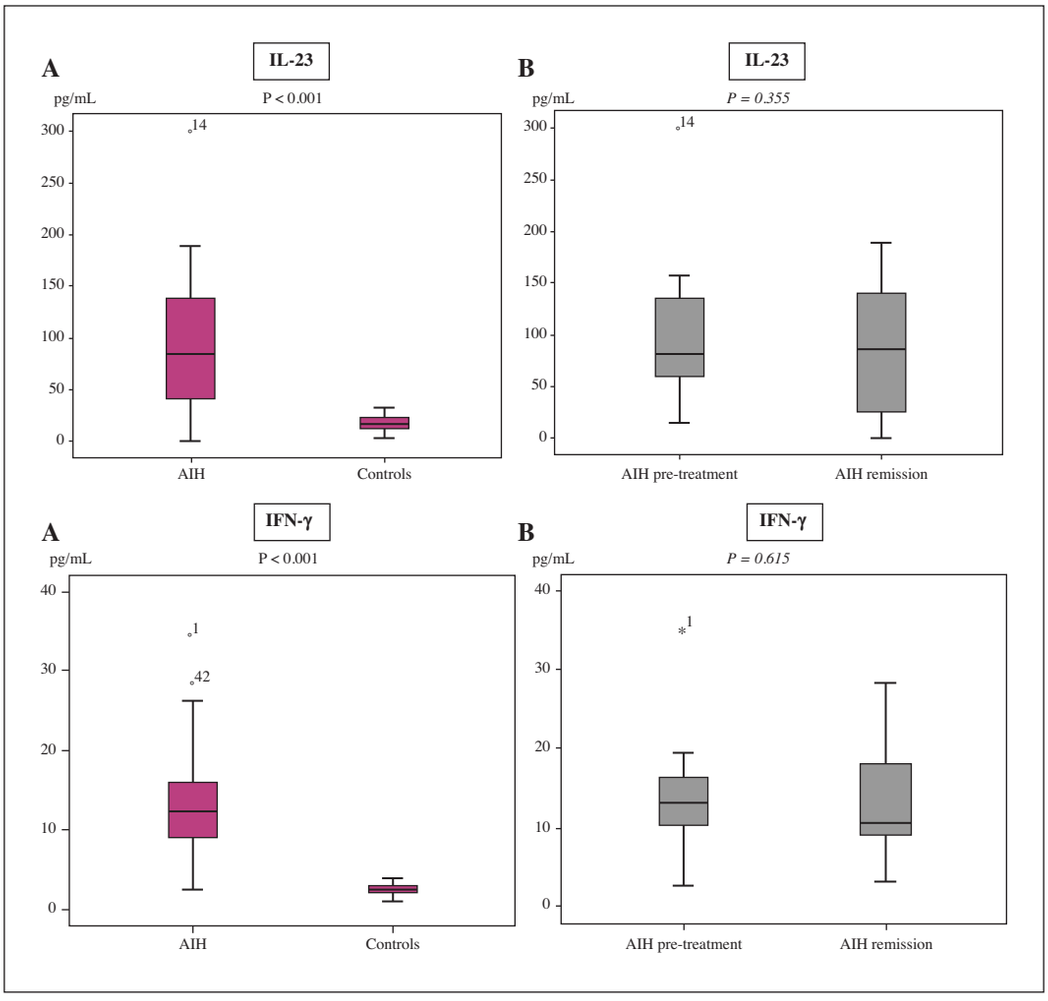

Figure 2
Association of IL-23 and IFN-ɣ levels with autoimmune hepatitis occurrence. (A) shows increase in IL-23 and IFN-ɣ levels in AIH patients compared to healthy control subjects and (B) shows modifications in IL-23 and IFN-ɣ levels in AIH patients during pretreatment phase and remission following corticosteroid therapy. AIH: 50 patients, Controls: 150 healthy donors, AIH pre-treatment: 22 patients (44%), AIH remission: 28 patients (58%).

malignant disorders. In AIH, these cytokines are produced by the immune cells and circulate in abnormal concentrations, inflicting tissue injury, inflammation, and pathophysiologic changes in the liver. Their secretion by the immune system is directly related to the factors that cause abnormalities in cell-to-cell interactions, proliferation, and function, which may be characteristic features of AIH [22]. The

Table 2
Association of increased cytokine levels with HLA gene expression in AIH patients.

HLA Alleles	**DRB1*03**		**DRB1*13**		**DRB1*15**		**DRB1*01**		**DRB1*08**		**DRB1*07**		**DRB1*09**	
Cytokine Levels (pg/mL)	**AIH (%)**	**C (%)**	**AIH (%)**	**C (%)**	**AIH (%)**	**C (%)**	**AIH (%)**	**C (%)**	**AIH (%)**	**C (%)**	**AIH (%)**	**C (%)**	**AIH (%)**	**C (%)**
IL-6 > 6	43	39	16	26	13	24	6	14	6	5	13	24	3	2
P value	0.67		0.29		0.21		0.28		0.71		0.21		0.76	
IL-8 > 6	24	35	16	23	16	14	10	18	3	13	10	19	3	8
P value	0.29		0.22		**0.04**		0.14		0.65		0.15		0.79	
TNF-α > 8	10	30	10	39	6	23	15	11	13	6	2	25	6	5
P value	0.12		**0.03**		0.13		0.80		0.06		0.23		0.89	
IFN-γ > 3	11	36	14	25	14	23	7	18	9	5	8	23	5	1
P value	0.11		0.21		0.34		0.22		0.76		0.16		0.85	

AIH: 50 AIH patients; Basic control cytokine levels (IL-6: 0-5.9 pg/mL; IL-8: 0-5.2 pg/mL; IFN-ɣ: 0-3 pg/ml; TNF-α: 0-8.1 pg/mL); C: 150 healthy controls.

pathological immune response in AIH is generally characterized by increased expression of HLA class II antigens in hepatocytes, a predominance of CD4+ T cells in the portal infiltrate and might indicate the involvement of Th cells and their related cytokine secretions in the pathogenesis of AIH.

Our study indicated that patients with type 1 autoimmune hepatitis had significantly higher serum levels of IL-6 (11,42 pg/mL *vs.* 1,29 pg/mL, $p < 0.001$) and IL-8 (19,16 pg/mL *vs.* 3,70 pg/mL, $p < 0.001$) than healthy controls subjects. The levels of IL-6 (12.00 pg/mL *vs.* 5.59 pg/mL, $p = 0.017$) and IL-8 (24.10 pg/mL to 7.85 pg/mL, $p = 0.006$) were also significantly higher in AIH patients during the pretreatment phase detected in 22 AIH patients, 44% of total patients, compared to levels detected in 22 AIH patients, 44% of total patients after total remission. These results could indicate that IL-6 and IL-8 may be biomarkers of the onset of autoimmune hepatitis especially of the activity phase of the disease. The levels of TNF-α showed no significant differences between AIH patients and controls but were significantly higher in AIH patients during the pretreatment phase (mean of 55.5 pg/mL) and were not detected in patients after remission, $p = 0.002$. The proinflammatory cytokine TNF-α may consecutively play a role as a biomarker of the disease activity state. The levels of IL-23 and IFN-ɣ were also significantly higher in AIH patients compared to levels found in healthy control subjects (88,75 *vs.* 17,05 pg/mL, $p < 0,001$ and 13,34 pg/mL *vs.* 3,08 pg/mL, $p < 0,001$ respectively) but did not show any significant difference in AIH patients during the pretreatment phase and after clinical remission. This could show that IL-23 and IFN-ɣ may only be biomarkers of AIH occurrence and onset.

The IL-1β was not detected in any of the total 50 AIH patients included in our study, in the pretreatment phase or after remission. No difference was also detected in the levels of IL-10 between AIH patients and healthy controls ($p = 0.73$) or in the activity phase of the disease and after remission 22 cases, 44% *vs.* 28 cases, 58%, $p = 0.4$).

Our study showed similar results to a study conducted in Germany by Landi *et al.* (2014) in which significantly high serum levels of proinflammatory cytokines such as IL-6, IL-1β, IL-8, and TNF-α were detected in a total of 40 patients with autoimmune hepatitis type 1 compared to healthy controls. In fact, the median value of IL-6 was 10 pg/mL in AIH patients compared to a median of 0.5 pg/mL found in the control subjects with $p < 0.0001$ and the median serum level of IL-8 found in AIH patients was almost twice (38 pg/mL) the level found in healthy controls (15 pg/mL ($p < 0.0001$) [23]. These high levels could be consistent with a possible infectious etiology for AIH and a TH1 state of autoimmune activation in autoimmune hepatitis. A previous association of polymorphic genes coding cytokines was revealed previously in autoimmune patients specifically with TNF-α [24] and with molecules implicated in signal transduction inside hepatocytes or immune cells such as CTLA [25] SH2B3 and PTPN22 [26]. Another study realized by Tilg *et al.* (1992) showed a statistically significant elevation in serum levels of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and IFN-ɣ in patients with chronic liver diseases compared to healthy control subjects [27]. According to Tilg *et al.* (1992), the elevation of serum cytokine levels in these patients is a consequence of hepatic dysfunction and promotes the proinflammatory markers that would stimulate the activation of the monocytes, lymphocytes, and the autoimmune state [27]. A more recent study realized by Maggiore *et al.* (1995) in Italy revealed similar results to our study [28]. This study aimed to determine proinflammatory cytokines levels of IL-6, IL-8, and TNF-α in children with AIH before immunosuppressive treatment. The results showed a significant elevation of TNF-α levels in patients with type 1 AIH (mean of 45 pg/mL) compared to control subjects ($p < 0.005$). The IL-6 levels were also significantly increased in 5 among 7 patients with AIH compared to controls ($p < 0.02$). The levels of IL-8 were also significantly increased in patients with type 1 AIH (mean of 83.5 pg/mL) compared with controls ($P = 0.06$). IL-6 and TNF-α levels have decreased and have even been undetected in all patients with AIH after remission following immunosuppressive therapy. This study also showed significantly higher concentrations of IL-8 in patients presenting with cirrhosis compared to noncirrhotic patients. Since the liver is the main organ of clearance of circulating cytokines, high serum levels have been observed in AIH patients with cirrhosis due to the disruption of this function. This increase in cytokine production was also revealed by a study conducted in Saudi Arabia by Elwabel *et al.* (1993) which included 10 patients with AIH. Cytokine production in vitro by peripheral blood T cells from patients with AIH was detected before and after immunosuppressive treatment. This study indicated a predominance of CD 4^+ T cells with a significant increase in production of IL-6 in AIH patients compared to controls (1100 pg/mL *vs.* 390 pg/mL, $p < 0.001$) or compared to patients with viral hepatitis ($p < 0.01$). The production of TNF-α by peripheral blood lymphocytes was higher in AIH patients compared to healthy controls but was not statistically significant. An increase in IFN-ɣ production was also statistically significant in patients with AIH compared to healthy control subjects (350 pg/mL *vs.* to 210 pg/mL, $p < 0.05$). In fact, the levels of many cytokines are not stable in serum because of the action of proteases and inhibitors, which can degrade cytokines and might produce aberrant values. Also increase of various cytokines produced by immune cells is a true indicator of cytokine involvement in the pathophysiology of the human disease. The interleukin 6 is a monomeric and pleiotropic cytokine production of which is stimulated by TNF-α and IL-1β. Also the role of IL-6 in the stimulation of IL-17 production was well demonstrated by previous studies. The gene implicated in IL-6 expression is located on the short arm of chromosome 7. Previous studies have shown the presence of 4 polymorphisms at the promoter site of IL-6 gene but only the (G/C) polymorphism at -174 position of the IL-6 gene was associated with chronic liver disease onset in particular AIH. This polymorphism was also associated with increased liver transaminase levels and an intensive disease flare. A correlation between the activity of IL-6 and TNF-α strengthened the hypothesis of the close connection and interaction between the various

pro-inflammatory cytokines implicated in AIH pathogenesis [29]. We also conducted a study aiming to investigate HLA genes associations with higher levels of proinflammatory cytokines IL-6, IL-8, TNF-α, and IFN-γ in AIH patients compared to healthy controls and results showed no significant association of the different HLA genes with higher levels of cytokine profile. Only HLA DRB1*15 showed a significantly higher frequency in AIH patients having higher levels of IL-8 ($p = 0.04$) while frequency of HLA DRB1*13 was higher in controls. In fact, Hla genes could therefore not have a significant role in proinflammatory processes but rather be a major susceptibility factor for AIH onset [30].

Higher serum levels of TNF-α and IFN-ɣ in type 1 AIH patients have also been revealed by many previous studies compared to levels detected in healthy controls or to type 2 AIH patients. In our study, TNF-α levels were higher in the pretreatment phase patients than the levels detected in AIH patients after treatment and following remission with corticosteroid therapy and the levels of IFN-ɣ were significantly higher in AIH patients compared to levels detected in healthy control subjects ($p < 0.001$). These results were similar to those found by a previous study that showed that the lymphocytes predominating in the livers of the patients with AIH were characterized by higher production of IL-4 and IFN-ɣ [31]. In fact, the significant difference in TNF-α levels between patients and controls was due to a polymorphism affecting the TNF-α gene, which occurs more frequently in patients with type 1 AIH than in healthy controls. The gene coding for TNF-α is located within the HLA class III region in the short arm of chromosome 6. Four substitutions of guanine (G) to adenine (A) affect the gene promoter in positions -163, -238, -308, and -376, but only those detected in the -238 and -308 positions directly affect the expression of TNF-α. Czaja *et al.* (1999) showed that only the substitution of G to A at position -308 was significantly associated with type 1 AIH [32]. This polymorphism can be related to the high production of TNF-α, the lower frequency of remission after corticosteroid treatment and the higher occurrence of treatment failure and cirrhosis. IL-23 is a central cytokine in autoimmunity and it is involved in the differentiation of Th17 cells in a pro-inflammatory context especially in the presence of TGF-β and IL-6. Activated Th17 cells produce IL-17A, IL-17F, IL-6, IL-22, TNF-α, and GM-CSF which cause tissue inflammation and recruit other pro-inflammatory mediators and leukocytes to the hepatic lesion milieu [33]. IL-23 plays an essential role in the expansion and maintenance of Th17 cells *in vitro* and *in vivo*. A study realized in 2011 focused on the determination of cytokines levels in 39 patients with type 1 AIH and showed that the serum IL-17 was significantly higher in patients with AIH compared to controls ($P < 0.05$); also the level of serum IL-23 was significantly higher in AIH patients (1050 pg /mL) than the level detected in healthy controls (850 pg /mL), ($P < 0.05$) [34]. A significant increase in the mean serum levels of IL-23 was also revealed by the study Kamijo et al (2011) in Japanese AIH patients [35]. In fact, levels of IL-23 were significantly higher in 40 AIH patients in the pretreatment phase (26.3 pg/mL) compared to those detected in patients following remission and treatment with corticosteroids (18,5 pg/mL), ($p = 0.04$). IL-17F, and not IL-17A, was also increased in the pretreatment phase of AIH in this study. Thus, Th17 cells may factor in AIH pathogenesis in concert with IL-23 [35].

IL-1β was not detected in AIH patients included in our study, in the pretreatment or after remission. A same result was revealed by a previous Japanese study which showed undetectable IL-1β levels in the pretreatment AIH phase or in remission. This low level of IL-1β could be explained by the presence of specific inhibitors such as IL-RA, a natural inhibitor that competes with IL-1β for its specific receptor and counterbalances its biological effects. The semiquantitative evaluation of IL-1β serum levels and of IL-1RA mRNA, suggests that the progression of a more aggressive form of AIH may be associated with an excess of IL-1β production compared to IL-1RA in tissues and, conversely, the overexpression of IL-1RA may be associated with less severe liver damage which could be due to the

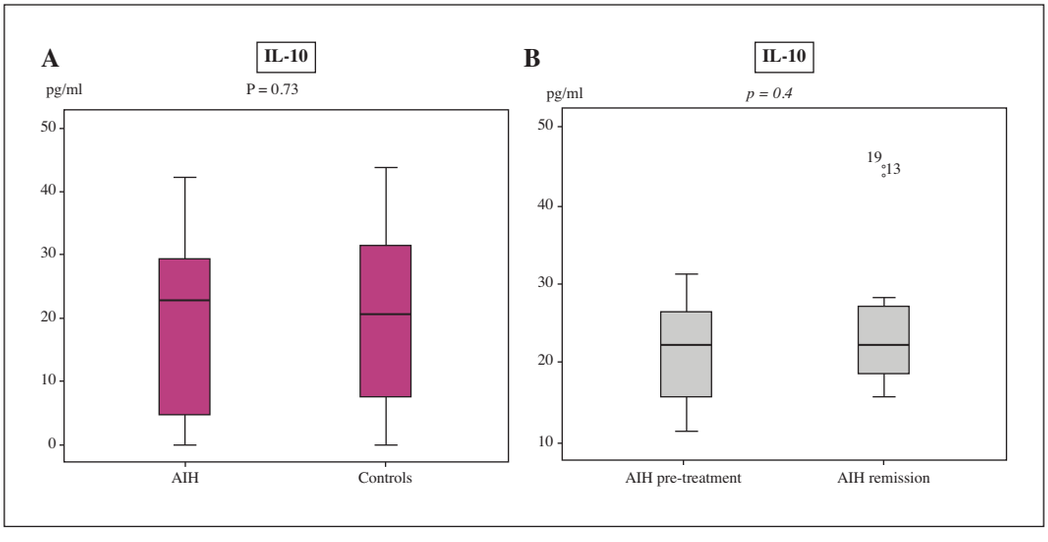

Figure 3

Association of anti-inflammatory cytokine IL-10 with autoimmune hepatitis state. (A) shows IL-10 levels in AIH patients and healthy control subjects and (B) shows IL-10 levels in AIH patients during pretreatment phase and remission following corticosteroid therapy. AIH: 50 patients, Controls: 150 healthy donors, AIH pre-treatment: 22 patients (44%), AIH remission: 28 patients (58%).

protective role of IL-1 RA within the liver [10]. IL-10 was detected in all patients with AIH and had an average level of 46.35 pg/mL which was high compared to controls, but the p value was not statistically significant. IL-10 was also higher in patients in pretreatment phase compared to those after remission (*figure 3*). A similar result was found by a previous study which included 43 patients with type 1 AIH and revealed that IL-10 was detected in all patients at AIH onset and before initiation of immunosuppressive therapy. By comparing the IL-10 levels found in patients with controls, the difference was not statistically significant. These results could confirm the hypothesis that the humoral immune response predominates during the active course of autoimmune hepatitis type 1 [10]. Similarly, a Japanese study by including 40 patients with AIH showed that IL-10 was only detected in two patients (5%) before receiving treatment with levels ranging from 0 to 22.94 pg/mL IL-10 then became undetectable in the same patients following therapy [35]. In contrast, other studies showed an elevation in IL-10 levels in patients with AIH before treatment. High concentrations of IL-10 could be explained by an activation of T regulatory cells secreting IL-10 especially during disease activity and due to excessive exposure to hepatic antigens which amplify the immune response or as a result of a suppressive action on pro-inflammatory cytokines, which could explain the low serum concentrations of IL-1β, IFN-ɣ, and TNF-α revealed in our study. Further studies are needed to design novel therapeutic approaches for autoimmune hepatitis based on cytokine manipulations.

CONCLUSION

Our study showed significantly higher levels of proinflammatory cytokines IL-6 and IL-8 in AIH patients than in healthy controls. The levels of IL-6, IL-8, and TNF-α were higher in AIH patients during the pretreatment phase compared to levels detected after clinical remission showing that they may be diagnostic biomarkers of AIH activity phase. IL-23 and IFN-ɣ were higher in AIH patients than in controls and may be biomarkers of disease occurrence and onset. IL-1β was not detected in AIH patients at presentation or after disease treatment and levels of IL-10 were not statistically significant between AIH patients and controls. Proinflammatory cytokines may permit an earlier diagnosis of AIH allowing the better management of the disease and therapeutic follow-up avoiding liver transplantation.

CONFLICT OF INTEREST

All authors have no conflict of interest.

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