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Foxp3+ Treg Cells Are Associated with Pathological Process of Autoimmune Hepatitis by Activating Methylation Modification in Autoimmune Hepatitis Patients

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BCDEF 1 **Jiang Chen**
BCDF 1 **Wen Liu**
BF 2 **Wenjing Zhu**

1 Yibin Traditional Chinese Medicine Hospital, Yibin, Sichuan, P.R. China
2 The No. 2 People's Hospital of Yibin, Yibin, Sichuan, P.R. China

Corresponding Author: Jiang Chen, e-mail: hognquanhue@yeah.net

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Background: Autoimmune hepatitis (AIH) is a chronic hepatic disorder. This study investigated role of Foxp3+ regulatory T cells (Treg) and methylation-regulated Tregs in AIH pathological processes.

Material/Methods: Forty consecutive patients diagnosed with hepatitis were enrolled and divided into a virus hepatitis (n=20) group and an AIH group (n=20). Twenty healthy individuals were assigned to the healthy control group (HC, n=20), Liver function biomarkers were detected on an automatic biochemical analyzer. Serum auto-antibodies were evaluated using immunofluorescence method. Histopathological evaluation was conducted with liver tissues. Treg cells were counted using FACS flow cytometry. Peripheral lymphocytes surface/intracellular biomarkers, $CD4^+CD25^+$, CD127, and Foxp3, were examined. Serum cytokines were evaluated using cytometric bead array. Methylation-specific PCR (MS-PCR) was conducted to identify the status of Foxp3 gene methylation.

Results: Levels of liver function biomarkers were significantly increased in the AIH group compared to the HC group ($p<0.05$). Levels of ANA and ASMA were significantly enhanced in the AIH group compared to the HC group ($p<0.05$). Other auto-antibodies, including anti-AHA, anti-ribosome P protein, and anti-RO-52, were also discovered in the AIH group. Severe lymphocytic infiltration and inflammatory cells clustering were discovered in AIH patients. There were significantly fewer $CD4^+CD25^+$ T cells in the AIH group, and interleukin 6 (IL-6) and IL-10 levels were significantly decreased compared to the HC group ($p<0.05$). $CD127^+$ Treg and $Foxp3^+$ Treg expressions were decreased in the AIH group compared to the HC group ($p<0.05$). Foxp3 in Treg cells of AIH patients exhibited higher methylation frequency compared to that of HC patients ($p<0.05$).

Conclusions: $Foxp3^+$ regulatory T cells were involved in pathological processes by activating methylation modification in autoimmune hepatitis patients.

MeSH Keywords: **Autoimmune Diseases • Hepatitis • Methylation • T-Lymphocytes, Regulatory**

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Background

Autoimmune hepatitis (AIH) is a chronic hepatic disorder caused by unknown pathogenesis or etiology, and is characterized by autoimmune response, abundant polyclonal immunoglobulin G (IgG), transaminases, hypergammaglobulinemia, and specific auto-antibodies [1–3]. AIH is morphologically characterized by chronic and inflammatory liver disease in the liver parenchyma [4,5]. A previous study [6] conducted in the Netherlands reported that dysregulation of adaptive responses, such as infiltration of T cells and plasma cells, cause clinical AIH. Therefore, immunomodulatory abnormality is also an important factor in AIH progression and development. In recent years, regulatory T cells, natural killer T cells (NKT), regulatory B cells, and follicular T cells have been proven to be involved in AIH pathogenesis [7,8]. Immunosuppressive regulatory T cells (Treg cells) have been become an especially important topic in AIH investigations [9].

Treg cells regulate the functions of antigen-presenting cells (APCs) and immune effector cells, and play critical roles in homeostasis regulation in the immune system. Treg cells express the transcription factor Foxp3, which mainly derives from the thymus and supports immunological tolerance [10]. There are a few surface markers for Treg cells, including $CD4^+$ and $CD25^+$; therefore, Treg cells are also referred to as $CD4^+CD25^+$ Foxp3 T cells. Sakaguchi et al. [11] reported that transplantation of $CD4^+CD25^+$ knocked-out T cells into nude mice can induce several autoimmune diseases, including AIH. However, treatment with Treg cells significantly inhibits the occurrence of autoimmune diseases. Treg cells inhibit the function of APCs and immune effector cells by releasing transforming growth factor beta (TGF-β) and secreting interleukin (IL), or by interacting with dendritic cells (DCs) to suppress immune effector cell activation [12]. Recently, Treg cells have been extensively applied in investigation of autoimmune diseases to explore pathogenic mechanisms. A previous study [13] conducted in the UK also illustrated that Treg cell levels in AIH patients were significantly lower than in normal health individuals. The Foxp3 gene mutation always causes severe autoimmune diseases, and plays key roles in regulation of immunological homeostasis. Foxp3 also regulates Treg cell activity by directly modulating some downstream genes.

DNA methylation is one of the first discovered and most studied epigenetic regulation mechanisms [14]. The TSDR segment in the Foxp3 gene is a highly-conserved and CpG-enriched region and is also the promoter region of the Foxp3 gene. The TSDR region in natural Treg cells is completely demethylated, but is completely methylated in non-Treg cells [15]. A previous study [16] performed in Germany also showed that methylation of the TSDR gene segment is associated with stable expression of Foxp3. However, methylation of the Foxp3 gene in autoimmune diseases, chronic infection, and tumors also needs to be clarified. Moreover, the levels of Foxp3 gene expression and Foxp3 gene methylation in AIH pathogenic processes are also elusive. To investigate these Treg cell-mediated mechanisms, as well as to assess the development and prognosis of AIH, it is important to assess Foxp3 gene expression and DNA methylation levels. In this study, we investigated the levels of Treg cells and Foxp3 gene methylation in the AIH mouse model and discovered the mechanism of Foxp3 gene expression in autoimmune hepatitis.

Material and Methods

Subjects

From October 2015 to March 2017, we enrolled 40 consecutive patients (22 males and 18 females) with proven hepatitis virus infection in our department, and divided them into a hepatitis virus-infected group (VH, n=20) and an AIH group (n=20). We also enrolled 20 healthy subjects as a control group (HC, n=20). The diagnosis of AIH was made according to internationally accepted criteria [17]. The diagnosis for hepatitis virus infection was based on the national criteria of China (No. WS-2008). For comparison, a control group of healthy volunteers presenting at our medical examination center was included.

All of the procedures were in accordance with the ethics standards of the local Committee on Human Experimentation and with the Helsinki Declaration of 1975, as revised in 2008 [18]. The present study protocols were approved by the Ethics Committee of People's Hospital of Yibin, Yibin, China. All of the patients signed the written informed consents for this study.

Liver function analysis

The peripheral blood was collected into serum separation tubes and centrifuged at 3500 r/min for 15 min. Then, the supernatant was harvested and stored at –20°C for further tests. Serum samples were analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (γ-GT), alkaline phosphatase (ALP), albumin (ALB), total protein (TP), total bilirubin (TBi), and direct bilirubin (DBi) on an automatic biochemical analyzer (Mode: 7600-020, Hitachi Inc., Tokyo, Japan). All experiments were performed strictly according to the manufacturer's instructions of the liver function test kits (Nanjing Jian Cheng Ke Ji, Nanjing, China).

Auto-antibodies evaluation

The serum auto-antibodies, including anti-nuclear antibody (ANA), anti-smooth muscle antibody (ASMA), anti-mitochondrial antibody (AMA), anti-nuclear ribonucleoprotein antibody

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(anti-nRNP), anti-smith extractable nuclear (anti-SM), anti-Sjogren's A (anti-SSA), anti-Sjogren's B (anti-SSB), anti-acute hepatitis A (anti-AHA), anti-double-stranded DNA (anti-dsDNA), JO-1, Sc-70, and anti-RO-52 antibodies, were tested using indirect immunofluorescence kits (Euroimmun Company, Germany). The dilution more than 1: 100 (not including 1: 100, only including 1: 320 and 1: 1000) was considered as positive for the ANA, ASMA, AMA, and other auto-antibodies.

Histopathological analysis

Histopathological evaluation was conducted using liver tissues obtained from needle biopsy of livers of AIH patients. Briefly, liver tissues were fixed with 10% paraformaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA). Then, the fixed liver tissues were cut into 4-μm sections and stained using hematoxylin and eosin (HE) using the standard processes described in a previous published study [19]. Finally, the images were captured using an inverted microscope (Mode: CKX41, Olympus, Japan).

Peripheral lymphocytes isolation and surface/intracellular markers examination

Cell surface and intracellular markers expression was evaluated on the fresh whole EDTA-anti-coagulated peripheral blood according to the method described in a previous study [20] conducted in Sweden. The lymphocytes were isolated using density gradient centrifugation at 900×g at room temperature for 20 min over Lymphoprep™ (Nycomed, Oslo, Norway) according to the manufacturer's instructions. Briefly, for the surface staining, the lymphocytes were incubated using FITC-conjugated anti-human $CD4^+$ antibody (BD Biosciences, San Jose, CA, USA), PE-conjugated anti-human $CD25^+$ antibody (BD Biosciences), PerCP-labeled anti-human CD127 antibody (BD Biosciences), APC anti-human $CD3^+$ monoclonal antibody (BD Biosciences), and eBioscienceTM Anti-Human Foxp3 Staining St FITC (Cat. No. 71-5776, Thermo Fisher Scientific, Hudson, NH, USA) for 20 min at 4°C in the dark. Then, the lymphocytes were centrifuged (1200 r/min) for 5 min and re-suspended in 2 ml PBS. The above samples were analyzed using an FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). All flow cytometry data were analyzed using BD DIVA software 4.1 (BD Biosciences, San Jose, CA, USA). The results are presented as the percentage of $CD4^+CD25^+$ T cells, Treg $CD127^+$ T cells, or Treg $Foxp3^+$ cells of total lymphocytes.

Treg cells counting

Lymphocytes in the liver were used to count Treg cells according to the method described in a previous study [21]. Treg cells were stained and counted using the eBioscience™ Human Regulatory T Cell Staining Kit (Cat. No. 88-8999, Thermo Fisher Scientific, Hudson, NH, USA). Briefly, a new Treg cells counting process was used as follows. The concentration of lymphocytes was adjusted to 1×10^7/ml. A total of 100 μl hepatic lymphocytes suspension was treated with 20 μl FITC anti-human $CD4^+$ monoclonal antibody, 20 μl PE anti-human $CD25^+$ monoclonal antibody, 20 μl PerCP-labeled anti-human $CD45^+$ monoclonal antibody, and 20 μl APC anti-human $CD3^+$ monoclonal antibody (BD Biosciences, San Jose, CA, USA) and incubated for 20 min in the dark. Then, the lymphocyte suspension was incubated with 450 μl FACS lysing solution (Catalogue No. 349202, BD Biosciences, San Jose, CA, USA) at room temperature for 15 min in the dark. The lymphocyte suspension was also centrifuged at 1500 r/min and re-suspended in 2 ml phosphate buffer solution (PBS, Sigma-Aldrich), centrifuged again, and then the supernatant was discarded. Finally, the pillets were dissolved in PBS and prepared for FACS flow cytometry (BD Biosciences, San Jose, CA, USA).

Cytometric bead array analysis

To evaluate the levels of serum cytokines, including interleukin 2 (IL-2), IL-4, IL-6, IL-10, and tumor necrosis factor α (TNF-α), the cytometric bead array was conducted according to the instructions of the manufacturer (Cell Gene Bio., Hangzhou, China) with a few minor modifications. In brief, 25 μl of serum sample was analyzed using cytometric bead array kits on an FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). Serum cytokine concentrations were quantified using Cytometric Bead Array software version 1.4 (BD Biosciences, San Jose, CA, USA).

DNA isolation

Peripheral mononuclear cells (Treg cells) were isolated from peripheral blood using a GE Healthcare Ficolli-Hypaque density gradient kit (Cat. No. 17144002, GE Healthcare Life Science, Little Chalfont, UK), and we magnetically separated them using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Cat. No. 130-091-301, Milteny Biotec. Bergish Gladbach, Germany) according to the manufacturer's instruction. Total RNA was extracted from Treg cells using RNA extracting mini kits (Qiagen, Hilden, Germany). Then, DNA was synthesized with RNA as the template using a cDNA synthesis kit (Qiagen, Hilden, Germany). Simultaneous purification for the genomic DNAs and total RNAs was conducted from the single-biological sample. The quantity and the quality of the last DNAs were evaluated and determined using a NanoDrop spectrophotometer 3300 (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Methylation-specific PCR (MS-PCR)

The methylation-specific PCR (MS-PCR) was conducted to identify the methylation status of targeting genes. Treg cells pretreated with Bisulfite Genomic Sequence (BSP) method were

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Table 1. Primers for the Foxp3 gene.

Primers	Sequences	Length (bp)
B183_Foxp3_P1F	TTTYGGTATTAGTGGGTGTGG	329
B183_Foxp3_P1R	AACTTCCTTTTACRACCACC	
B183_Foxp3_P2F	GGTGGTYGTAAAAGGAAGTTTAG	302
B183_Foxp3_P2R	AAAAAAAACTTACCCCRA	
B183_Foxp3_P3F	TGAGGGTTYGGGGTAAGT	307
B183_Foxp3_P3R	TATAACRCRTACRACCCCTATA	
B183_Foxp3_P4F	TATAGGGGTYGTAYGYGTTATAT	346
B183_Foxp3_P4R	AACTATCTACTTCTATTTTCTTCATCA	

Table 2. Liver function examination for the patients in health control, virus hepatitis and autoimmune hepatitis groups (mean ±SD).

Groups	Items							
	ALT (U/L)	AST (U/L)	ALP (U/L)	ALB (g/L)	TP (g/L)	γ-GT (U/L)	TBi (μmol/L)	DBi (μmol/L)
HC group	17.5±9.8	16.7±6.9	14.9±4.4	43.8±3.7	68.9±17	14.3±39	31.1±4.6	6.7±1.9
VH group	147.6±24.5**	127.4±101.9**	50.9±30.6*	35.7±5.6	68.2±3.9	56.1±43.8*	40.54±23.7*	21.5±13.4*
AIH group	179.2±59.6**,#	168.4±55.8**,#	170.3±44.1**,##	40.4±4.01	66.9±5.6	173.9±55**,##	77.5±31.5*,#	46.3±16.7**,##

HC group – health control group; VH group – virus hepatitis group; AIH group – autoimmune hepatitis group; ALT – alanine aminotransferase; AST – glutamic acid transferase; ALP – alkaline phosphatase; ALB – albumin; TP – total protein; γ-GT – glutamyltransferase; TBi – total bilirubin; DBi – direct bilirubin. * $p<0.05$, ** $p<0.01$ *vs.* HC group, # $p<0.05$, ## $p<0.01$ *vs.* VH group.

used to prepare samples for MS-PCR. The 50 μl MS-PCR multiplex PCR system (Cat. No. GK8015, Shanghai Generay Biotech. Co., Shanghai, China) included Taq DNA polymerase (1 μl), Mg^{2+} (2 μl), dNTPs (25 nM, 2.5 μl), multiplex PCR buffer (10×, 5 μl), Q solution, primers (300 ng/μl sense primer 1 μl, 300 ng/μl antisense primer 1 μl), RNAse-free water (35.5 μl), and the bisulfite-converted template DNA (2 μl). All PCR processes were conducted using 96-well PCR plates (Thermo Scientific, Waltham, MA, USA). The thermal cycling parameters for amplification were set as follows: denaturation step at 95°C for 4 min, followed by 40 cycles of amplification of 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s, with a final extension step at 72°C for 10 min. The primer sequences of MS-PCR are listed in Table 1. The MS-PCR products were analyzed by agarose gel electrophoresis. The PCR product was calculated using the Real-time PCR Detection System (Shanghai Generay Biotech. Co., Shanghai, China). The samples were defined as methylated Foxp3 depending on the amplified visual bands with the methylated primers. Relative quantification of the Foxp3 gene expression was conducted using the $2^{-\Delta\Delta ct}$ method.

Statistical analysis

All data were analyzed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) and are presented as percentages for categorical variables and as mean ± standard deviation (SD) for continuous variables. Continuous variables among multiple groups were compared using ANOVA, and categorical variables among groups were compared with the chi-square test. $p<0.05$ was considered as statistically significant.

Results

Liver functions were damaged in AIH patients

The liver function parameters, including ALT, AST, ALP, ALB, TP, γ-GT, TBi and DBi, were examined. The results showed that levels of ALT, AST, ALP, γ-GT, TBi, and DBi of AIH patients were significantly increased compared to patients in the HC group (Table 2, $p<0.05$). However, there were no significant differences in the above liver function parameters between the VH group and AIH group (Table 2, $p>0.05$). These results suggest

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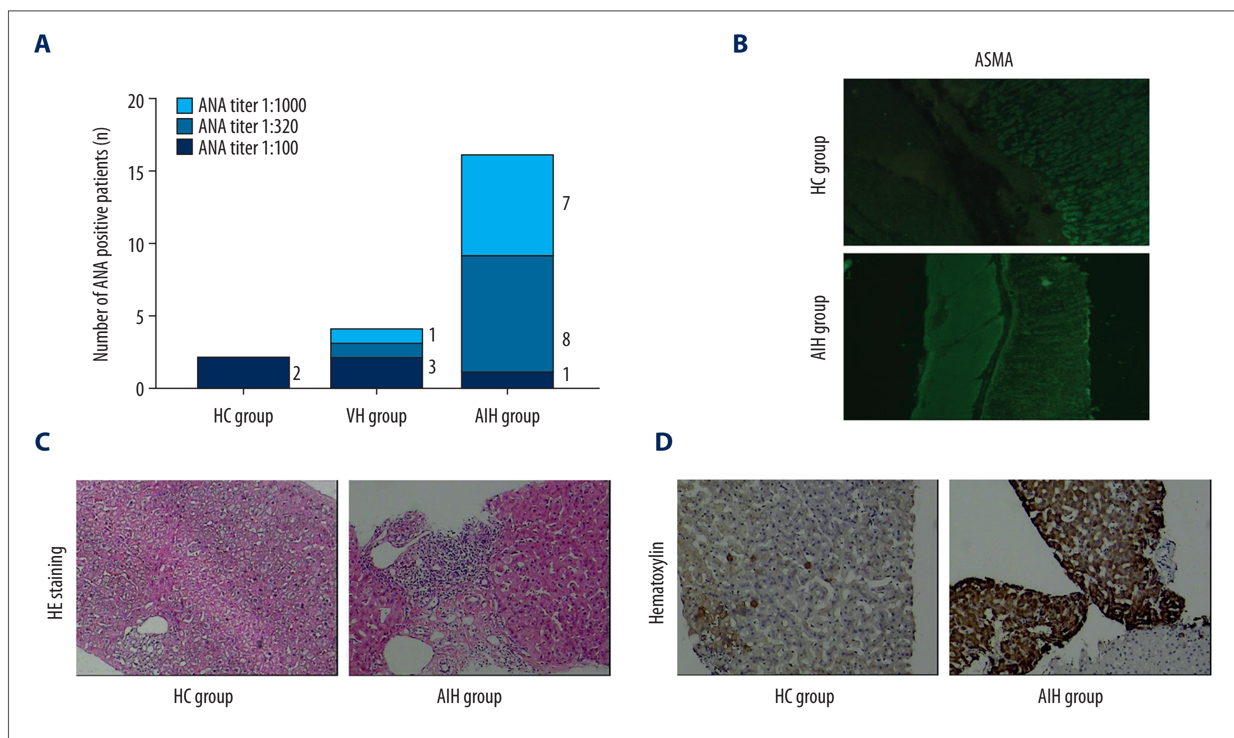

Figure 1. ANA and ASMA levels examination and lymphocytic infiltration observation. (**A**) ANA examination using Euroimmune immunofluorescence assay. (**B**) ASMA examination using Euroimmune immunofluorescence assay. (**C**) Hepatic tissue inflammation examination by using hematoxylin staining method and eosin staining method (HE staining). (**D**) Hepatic tissue inflammation examination using hematoxylin staining method. ANA – anti-nuclear antibody; ASMA – anti-smooth muscle antibody; HE – hematoxylin and eosin.

that the damaged liver functions of autoimmune hepatitis are as serious as in viral hepatitis.

ANA and ASMA levels were enhanced in AIH patients

According to the Euroimmune immunofluorescence results, the blood samples were diluted to 1: 100, 1: 320, and 1: 1000. The results indicated that there were 15 ANA-positive samples in the AIH group (including 8 cases of 1: 320 and 7 cases of 1: 1000 dilution) (Figure 1A). However, there was only 1 ANA-positive sample of 1: 320 dilution in the VH group and no ANA-positive samples in the HC group (Figure 1A). Moreover, immunofluorescence images showed significantly more ASMA-positive stained cells in liver tissues in the AIH group than in the HC group (Figure 1B).

Severe lymphocytic infiltration appeared in liver tissues of AIH patients

The liver needle biopsy and HE staining results showed that 3 out of 8 AIH patients had severe lymphocytic infiltration and inflammatory cells clustering (Figure 1C, 1D). Hepatocytes showed obvious cell swelling, denaturation, and necrosis. Liver tissues in AIH patients showed the characteristics of chronic active hepatitis.

Multiple auto-antibodies were discovered in blood of AIH patients

We isolated the blood of the patients and examined the auto-antibodies in patients in the HC, VH, and AIH groups. The results indicated that no high-titer auto-antibodies were discovered in the HC and VH groups (Table 3). However, auto-antibodies of AIH patients were diverse, and 8 patients had specific auto-antibodies. Among all of the specific auto-antibodies, the positive rates of anti-nRNP, anti-AHA, and anti-ribosome P protein were higher significantly (Table 3).

Changes in $CD4^+CD25^+$ Treg cells and lymphocytes in AIH patients

The results showed that the total lymphocytes and T cells in the AIH group were significantly increased compared to the HC group (Figure 2A, $p<0.05$). However, the amounts or percentages of $CD4^+CD25^+$ T cells in the AIH group were significantly lower compared to the HC group (Figure 2A, $p<0.05$).

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Table 3. The autoantibodies of the patients in health control, virus hepatitis and autoimmune hepatitis groups.

Items	Groups		
	HC group (n=20)	VH group (n=20)	AIH group (n=20)
Anti-SM	0	0	0
Anti-SS-A	0	0	0
Anti-SS-B	0	0	0
Anti-dsDNA	0	0	0
Anti-AHA	0	0	**5**
JO-1	0	0	0
Sc-70	0	0	0
Anti-AMA	0	0	0
Anti-ribosome P protein	1	0	2
Anti-RO-52	1	1	**3**

HC group – health control group; VH group – virus hepatitis group; AIH group – autoimmune hepatitis group.

IL-6 and IL-10 levels were increased in AIH patients

We assessed levels of cytokines IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ. The results showed that the levels of both IL-6 and IL-10 were significantly lower in the AIH group than in the HC group (Figure 2B, $p<0.05$).

CD127+ Treg and Foxp3+ Treg cells expressions were lower in AIH patients

Because dysfunction of Foxp3 causes severe autoimmune diseases, we examined Foxp 3 and CD127 expression in Treg cells. The results indicated that the expression of CD127 in Treg cells in the AIH group was significantly lower than in the HC group (Figure 2C, $p<0.05$), while the expression of Foxp3 was significantly decreased in Treg cells in the AIH group compared to the HC group (Figure 2D, $p<0.05$).

AIH patients had more methylated Foxp3 in Treg cells

MS-PCR results showed there were obviously more Foxp3-methylated Treg cells in the AIH group than in the HC group (Figure 3A). The ratio of methylation in the AIH group was also significantly higher compared to the HC group (Table 4, $p<0.05$) for the P1, P2, and P3 methylation sites.

Foxp3 in Treg cell of AIH patients exhibited higher methylation frequency

At methylation sites P1, P2, and P3, there were significantly more methylation sites in the AIH group more compared to the HC group (Figure 3B, $p<0.05$). The results also indicated that the methylation frequency of Foxp3 in the AIH group was significantly higher compared to that in the HC group (Figure 3C, $p<0.05$).

Discussion

AIH is an autoimmune liver disease characterized by infiltration of plasma cells, positive serum antibodies, and serum hypergammaglobulinemia [22,23]. The pathophysiology for the AIH is a complex process affected by environmental factors, auto-antigens, genetic factors, and immune dysfunction [24]. Previously, research on AIH mainly focused on immune system regulation, gene polymorphism, inflammation, and hepatocyte apoptosis [25]. However, in recent years, studies reported that autoimmune T cell activation and immunological regulation dysfunction play critical roles in the pathophysiology of AIH [26].

In this study, we first assessed liver function changes in AIH patients. The results showed that AIH patients had significantly higher levels of ALT, AST, ALP, γ-GT, TBi, and DBi compared with the healthy patients and viral hepatitis patients, which suggests that the liver function damage caused by AIH is more serious than that caused by viral hepatitis. This result is consistent with a previous study [27] conducted by New York University, which reported that viral hepatitis slowly progresses to autoimmune hepatitis. Auto-antibodies have been considered to be relatively specific biomarkers for autoimmune diseases in recent years [28,29]. The detection of auto-antibodies, such as ANS, SMA, anti-RO-52, and anti-F-action, has been become the hallmark for the AIH diagnosis in clinical practice [30]. In the present study, we also detected auto-antibodies in the blood of AIH patients, showing that the positive rates of anti-nRNP, anti-AHA, and anti-ribosome P protein were significantly higher in the AIH patients compared to the HC group. ANA and ASMA levels in AIH patients were also significantly higher compared to healthy individuals. However, there no auto-antibodies were discovered in the healthy individuals and viral hepatitis patients.

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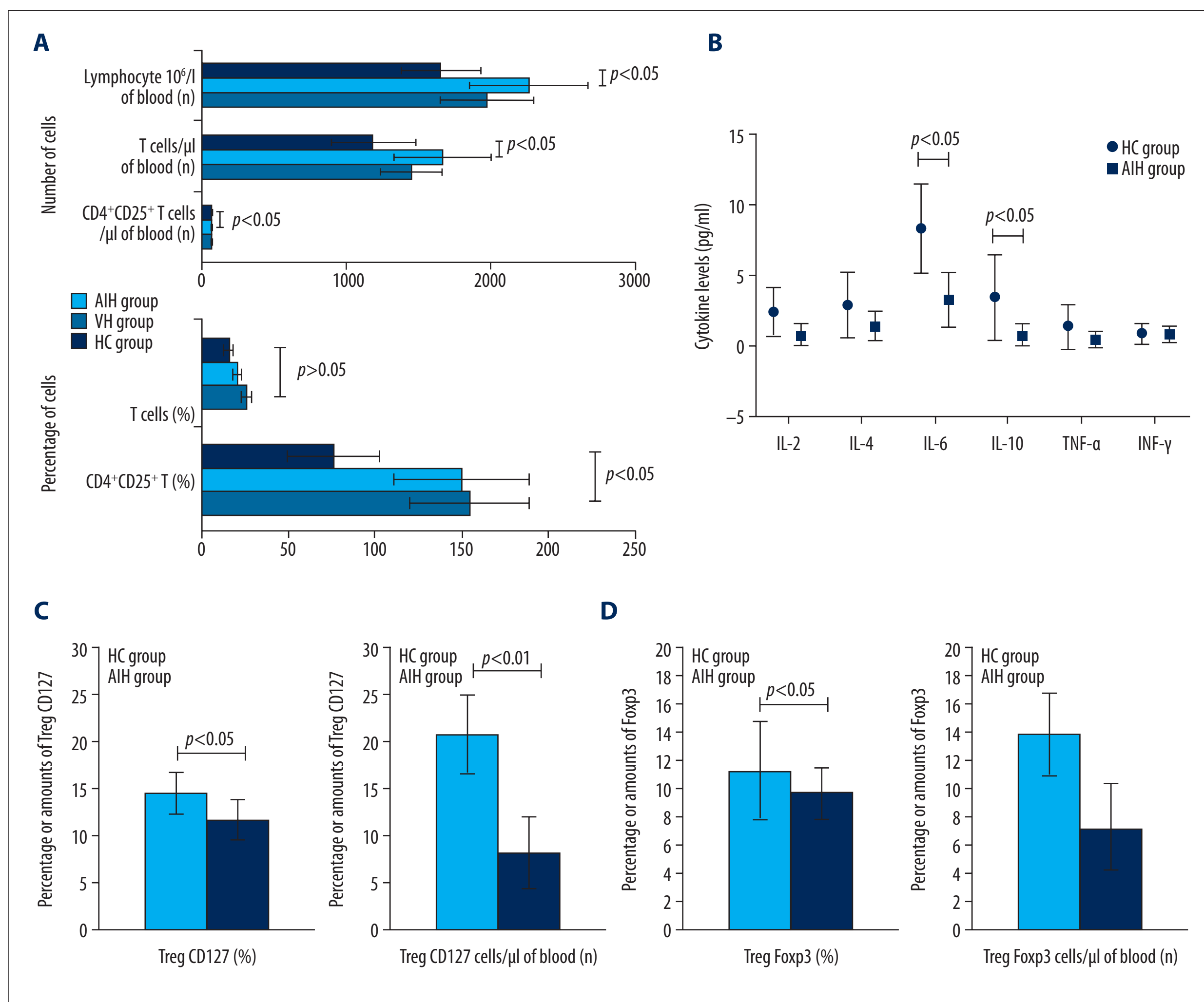

Figure 2. Evaluation for the CD4⁺CD25⁺ T cells, CD127⁺ Treg, Foxp3⁺ Treg cells, and cytokines. (**A**) CD4⁺CD25⁺ T cells evaluation. (**B**) Cytokines evaluation. (**C**) Percentage or amounts of CD127⁺ Treg. (**D**) Percentage or amounts of Foxp3⁺ Treg. Treg – regulatory T cells. * $p<0.05$: AIH group *vs.* HC group.

A previous study [31] reviewing data of AIH patients in European countries reported that there were significantly fewer $CD4^+CD25^+$ Treg cells and cytokines (IFN-γ and IL-4) in type I AIH patients compared to healthy subjects. In the present study, we assessed changes of $CD4^+CD25^+$ Treg cells and lymphocytes in AIH patients, showing that the AIH group had significantly fewer total lymphocytes and T cells, and the AIH group had significantly fewer $CD4^+CD25^+$ T cells compared to the HC group. These results suggest that the pathological process causes the decrease in $CD4^+CD25^+$ T cells. We also found that the levels of both IL-6 and IL-10 were significantly lower in the AIH group compared to the HC group. These results suggest that IL-6 and IL-10 participate in AIH, which is consistent with a previous study [32] in the United States.

The forkhead/winged helix transcription factor, Foxp3, acts as a specific transcription molecule for Treg cells and is a critical factor for development and function of T cells [33]. We found that the Treg cell count and the levels of Foxp3 protein in AIH patients were significantly lower than in the healthy control group, which is also consistent with the results of Wang et al. [4]. Our results also showed that the CD127 levels were significantly lower in the AIH group compared to the HC group. Therefore, we speculate that there may be a correlation between Foxp3 and CD127 expression in AIH patients, which should be further investigated in subsequent studies.

The methylation process always participates in the pathogenesis of diseases; therefore, we speculate that Foxp3 methylation may be involved in the progression of AIH. Our results indicate that more Foxp3-methylation occurs in the Treg cells of AIH patients, and there were significantly more Foxp3-ethylated Treg cells in AIH patients than in the HC group. The ratio of methylation in the AIH group was significantly higher

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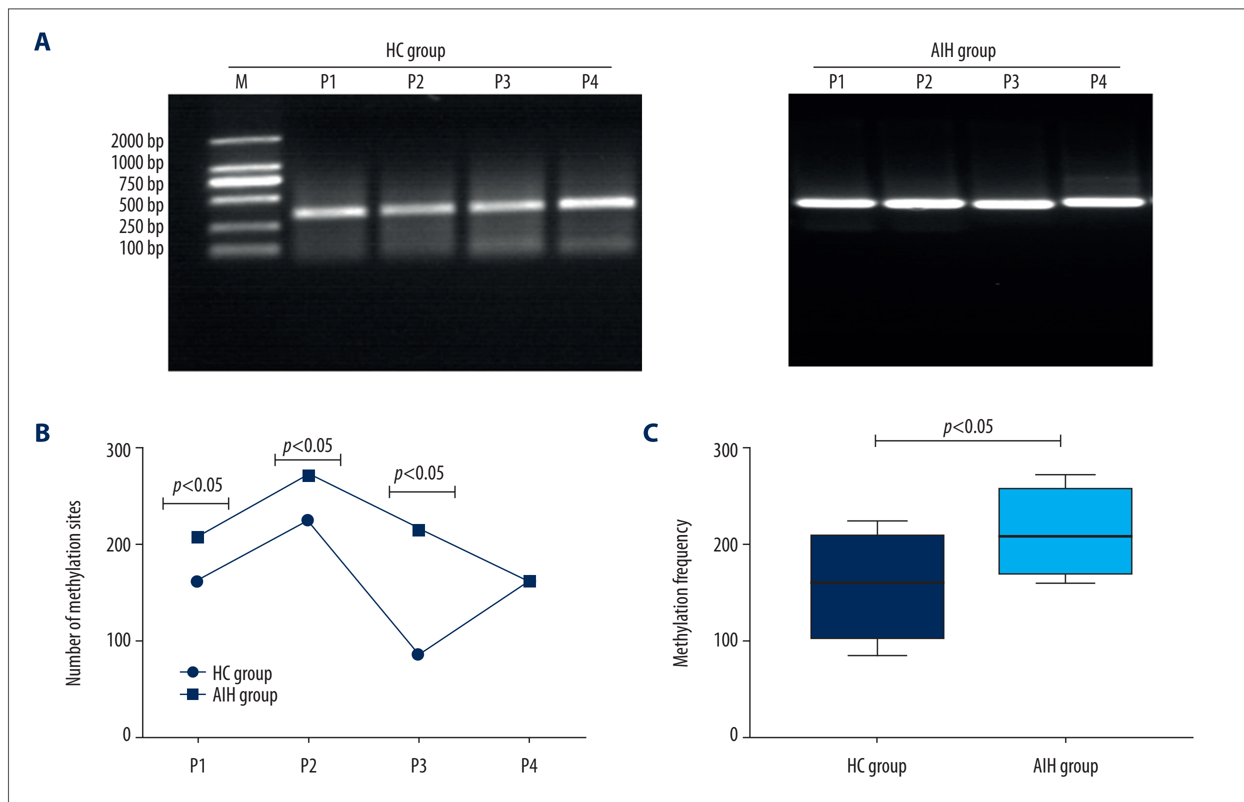

Figure 3. Foxp3 gene methylation in Treg cells of AIH patients. (**A**) Fox3 gene methylation observation using methylation-specific PCR (MS-PCR) assay. (**B**) Statistical analysis for the number of methylation sites. (**C**) Statistical analysis for the methylation frequency. * $p<0.05$: AIH group *vs.* HC group.

Table 4. Methylation detection data for Foxp3 gene of Treg cells.

Methylation sites	HC group (number of methylation site)			AIH group (number of methylation site)		
	Methylation	Non-methylation	Ratio	Methylation	Non-methylation	Ratio
P1	161	159	50.30%	207	113	64.80%*
P2	224	126	64.00%	273	77	78.00%*
P3	85	365	18.80%	214	236	47.50%**
P4	161	159	50.30%	160	160	50.00%

HC group – health control group; AIH group – autoimmune hepatitis group. * $p<0.05$, ** $p<0.01$ *vs.* HC group.

compared to the HC group. There were significantly more methylation sites in the AIH group compared to HC group; therefore, the methylation frequency of Foxp3 in AIH group was higher significantly compared to that in the HC group. These results suggest that Foxp3 methylation participates in the pathogenic processes of AIH.

There have been few investigations to clarify the roles of Tregs in the pathological mechanisms of autoimmune hepatitis. Liang et al. [34] reported that an imbalance between Tregs and Th1-Th17-Th22 cells might contribute to the pathogenic process of AIH. Hao et al. [35] found that impaired Tregs differentiation participates in the pathogenesis of AIH. John et al. [36] discovered that the apoptosis of Tregs was increased in active autoimmune hepatitis patients. Although our study and all 3 of the above studies demonstrated the roles of Tregs in the pathological progression of AIH, they proved their conclusions with different approaches and methods. All of these conclusions may represent novel strategies for protecting against damage caused by AIH.

Although our study produced some interesting results, it also has a few limitations. First, the laboratory models were not established and were not compared with the results of AIH

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patients. In our next study, we plan to establish animal models for the further investigation of AIH. Second, the mechanism for the methylation of Foxp3^{+} regulatory T cells has not been fully clarified, and this needs to be investigated. Third, the methylation of VH patients was not tested because it is indirectly associated with the focus of this study. Fourth, the counting of cells with Treg markers was not sufficient to illustrate the biological activity. In the future, we plan to conduct functional tests to accurately evaluate the cell counts. Fifth, the clinical relevance of Tregs in pathological processes in AIH has not been fully clarified, and we intend to explore the clinical association between Treg cells and pathological processes of AIH in future research.

Conclusions

Our data shows that autoimmune hepatitis patients had damaged liver function, presented a series of auto-antibodies, and exhibited the downregulation of Foxp3, CD127, and 2 inflammatory cytokines (IL-6 and IL-10). Foxp3 methylation frequency was significantly lower in AIH patients. Therefore, Foxp3^{+} regulatory T cells were associated with the pathological processes by activating the methylation modification in autoimmune hepatitis patients.

Conflict of interest

None.

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