

Interleukin-33/ST2-Mediated Inflammation Plays a Critical Role in the Pathogenesis and Severity of Type I Autoimmune Hepatitis

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Interleukin (IL)-33 was recently described as a new member of the IL-1 family; members of this family have proinflammatory activity. IL-33 and its soluble receptor ST2 (sST2) have been implicated in the pathogenesis of autoimmune diseases. This study investigated serum IL-33 and sST2 in type I autoimmune hepatitis (AIH) and the relationship of these molecules with clinical and pathologic parameters. Subjects included 65 patients with AIH who were diagnosed in our hospital. The control population included 17 healthy individuals and 36 patients with primary biliary cholangitis (PBC). Mean age at AIH diagnosis was 55.5 years, and the male-to-female ratio was 6:59. Serum IL-33 and sST2 levels were significantly higher in patients with AIH than in those with PBC or controls. Importantly, immunohistochemistry revealed high IL-33 expression in liver sections from patients with AIH. In particular, serum IL-33 and sST2 levels were significantly higher in acute-onset AIH than in chronic-onset AIH. Serum IL-33 levels were positively correlated with serum total bilirubin (TB), alanine aminotransferase (ALT), and necroinflammatory activity in AIH. We performed multivariate logistic regression analysis and found serum IL-33 levels to be independent factors for severe activity. Serum sST2 levels were positively correlated with serum TB and ALT and negatively correlated with serum albumin and prothrombin time in AIH. In particular, serum sST2 levels were significantly higher in severe symptoms of AIH. Serum IL-33 and sST2 levels in patients with AIH responsive to treatment with prednisolone were significantly decreased after treatment. Interestingly, serum IL-33 level was associated with a significantly increased risk of relapse. **Conclusion:** IL-33/ST2 may play an important role in the pathogenesis and severity of AIH and may be a promising target for AIH therapy. (*Hepatology Communications* 2019;3:670-684).

Autoimmune hepatitis (AIH) manifests as chronic liver inflammation of unknown cause. AIH is generally associated with the presence of autoantibodies and hypergammaglobulinemia.⁽¹⁾ Histologic features of interface hepatitis, i.e., infiltration of lymphocytes, plasma cells, and macrophages, suggest that an aggressive cellular immune response is involved in the pathogenesis of AIH.⁽²⁾

Abbreviations: AIH, autoimmune hepatitis; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANA, antinuclear antibody; AST, aspartate aminotransferase; CD, clusters of differentiation; CI, confidence interval; ConA, concanavalin A; ELISA, enzyme-linked immunosorbent assay; H&E or HE, hematoxylin and eosin; HBV, hepatitis B virus; HC, healthy control; IAIHG, International Autoimmune Hepatitis Group; Ig, immunoglobulin; IHC, immunohistochemical; IL, interleukin; LKM-1, liver/kidney microsomal 1; NLRP3, NLR family pyrin domain containing 3; OR, odds ratio; PBC, primary biliary cirrhosis; PLT, platelet; PSL, prednisolone; PT, prothrombin time; sST2, soluble receptor ST2; TB, total bilirubin; T_h, T helper.

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Recent studies have demonstrated that the incidence peaks at approximately 70 years of age in both men and women and that the incidence is lower in early adulthood.⁽³⁾ In Japan, the age distribution of both sexes showed a single peak at approximately 60 years of age.⁽⁴⁾ Human leukocyte antigen DR status affects the clinical features of patients with type I AIH. In Japanese patients, DR4 is dominantly associated with this disease. AIH is also associated with predominant T helper 1 (Th1) responses and decreased function and numbers of regulatory T cells (Tregs).^(5,6)

Interleukin (IL)-33 was recently described as a new member of the IL-1 family; members of this family exhibit proinflammatory activity. Following cell stress or damage, IL-33 is released in either its full length or cleaved form; however, in contrast to IL-1 β , IL-33 is not cleaved by caspase-1 and cleavage is not necessary for the secretion, biological activity, or release of IL-33, further suggesting that IL-33 plays a role as an alarmin. IL-33 is released by damaged or necrotic cells, leading to the activation of the immune system through IL-33/ST2 signaling.⁽⁷⁾

The soluble receptor ST2 (sST2) molecule lacks transmembrane and intercellular domains and acts as a binding decoy receptor for IL-33; thus, sST2 regulates IL-33 activity during inflammatory responses. *In vitro*, sST2 production has been shown to be enhanced by proinflammatory cytokines (IL-1 β and tumor necrosis factor [TNF]- α) in human lung epithelial cells and cardiac myocytes. In humans, sST2 can be produced not only spontaneously by cells in the lung, kidney, heart, and small intestine but also after activation by IL-33 in mast cells and clusters of differentiation (CD)4 and CD8 T cells.⁽⁷⁾

IL-33 and sST2 have been implicated in the pathogenesis of autoimmune diseases. Elevated levels of

IL-33 have subsequently been reported in many autoimmune diseases, including type 1 diabetes,⁽⁸⁾ rheumatoid arthritis,⁽⁹⁾ inflammatory bowel diseases,⁽¹⁰⁾ and autoimmune pancreatitis.⁽¹¹⁾

IL-33 has been reported to correlate with liver injury in patients with primary biliary cirrhosis (PBC).⁽¹²⁾ Notably, serum levels of IL-33 were closely associated with the degree of liver injury in patients with hepatitis B virus (HBV) infection.⁽¹³⁾ Moreover, the evaluation of dynamic changes in sST2 levels in HBV acute-on-chronic liver failure showed that serum sST2 levels increased over time in patients who died during follow-up but decreased in those who survived. In addition, serum sST2 levels correlated with disease severity, as assessed by total bilirubin (TB), prothrombin time (PT), and the Model for End-Stage Liver Disease score.⁽¹⁴⁾ In a mouse model in which acute hepatitis was induced by the administration of either CCl₄ or concanavalin A (ConA), IL-33 was clearly expressed in vascular and sinusoidal endothelial cells in CCl₄-induced mice but was strongly expressed in hepatocytes in ConA-induced mice.⁽¹⁵⁾ Moreover, other studies demonstrated that IL-33-deficient mice exhibited more severe ConA-mediated liver injury than wild-type controls, suggesting a protective effect of IL-33 in ConA-induced hepatitis.⁽¹⁶⁾

A few studies have linked serum IL-33 levels to clinical outcomes in patients with AIH. Liang et al.⁽¹⁷⁾ reported that the concentrations of serum IL-33 in patients with acute-onset AIH were positively correlated with hypergammaglobulinemia (immunoglobulin [Ig]G, IgM, and IgA), liver injury (gamma-glutamyltransferase [GGT]/alkaline phosphatase [ALP]), and proinflammatory cytokine levels (IL-17A and IL-4). In the liver, IL-33 is assumed to be secreted by hepatocytes and vascular and sinusoidal

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endothelial cells; this secretion is elevated following liver injury, and further inflammation is induced and becomes severe. Thus, IL-33 is considered related to the severity after onset rather than involved in AIH onset. However, the roles of IL-33/sST2 in AIH remain poorly understood. In this study, we assessed the role of serum IL-33/sST2 in the pathogenesis and severity of type I AIH in Japanese patients.

Patients and Methods

STUDY POPULATION

The subjects included 65 patients with AIH and 36 patients with PBC diagnosed at Fukushima Medical University Hospital between 1986 and 2017; 17 healthy controls (HCs) were also included. Normal serum was drawn from staff members at our hospital as HCs.

The diagnosis of AIH was based on the revised and simplified International Autoimmune Hepatitis Group (IAIHG) scoring system.⁽¹⁸⁻²⁰⁾ The presence of serum anti-liver/kidney microsomal 1 (anti-LKM-1) antibody was detected by enzyme-linked immunosorbent assay (ELISA) using commercially available kits. Patients with chronic liver disease from other causes, particularly alcohol abuse and chronic HBV or hepatitis C virus infection, were excluded from the AIH group. Patients were diagnosed as having PBC features if they met at least two of the following three criteria: 1) chronic elevation of the cholestatic liver enzymes ALP and GGT for at least 6 months; 2) presence of serum anti-mitochondrial antibody, detected by either indirect immunofluorescence or ELISA, using commercially available kits; and 3) typical histologic findings in biopsied liver specimens.⁽²¹⁾ Assessed data that were extracted retrospectively from the patients' medical charts included patient background characteristics (age, sex, onset type of disease, IAIHG score), clinical parameters at presentation (aspartate aminotransferase [AST], alanine aminotransferase [ALT], ALP, TB, albumin [ALB], platelet [PLT] count, PT, IgG, antinuclear antibodies [ANAs]) and at remission (ALT, ALP, TB, IgG), presence of relapse, presence of cirrhosis, disease severity, and therapeutic methods. Acute AIH was defined by the presence of acute-onset symptoms (e.g., jaundice and/or fatigue and/or anorexia) in conjunction with bilirubin >5 mg/dL

and/or serum ALT levels more than 10-fold higher than the upper limit of normal. The relapse of AIH was defined as an increase in serum transaminases to greater than twice the upper limit of normal (ALT level >90 U/L). Biochemical remission was defined as the complete normalization of aminotransferases and IgG.⁽²²⁾

Liver cirrhosis was diagnosed based on histologic findings, radiologic findings of cirrhosis by computed tomography or magnetic resonance imaging, and the presence of complications, such as portal hypertension. The demonstration of histologic cirrhosis at the subsequent liver tissue examination, radiologic findings of cirrhosis, and the development of portal hypertension or its complications in patients without these findings at presentation indicated progression to cirrhosis. Disease severity was assessed in accordance with the diagnosis and treatment guide for AIH in Japan.⁽²³⁾ Severe cases were defined as those that fulfilled at least one of the following criteria: (1) clinical signs (hepatic encephalopathy or a reduction in or disappearance of hepatic dullness); (2) clinical laboratory test results (AST/ALT >200 U/L, bilirubin >5 mg/dL, or PT <60%); and (3) imaging test findings (hepatic atrophy or a heterogeneous liver parenchyma pattern). Twenty patients had severe symptoms (TB >5.0 mg/dL and/or PT <40%).

Serum samples were obtained from 65 patients with AIH at the time of diagnosis before immunosuppressive treatment. Serum samples from 14 patients with AIH who had achieved biochemical remission (defined as normal serum aminotransferases and IgG levels) and 7 patients who did not achieve biochemical remission (defined as the lack of normalization of serum aminotransferases and IgG levels) were obtained during the immunosuppressive treatment and after at least 6 months of treatment. All serum samples were frozen and stored in multiple tubes at -20°C until analysis. The period from the collection of serum samples to liver biopsy was on average 9.8 ± 12.5 days.

ETHICS STATEMENT

All patients agreed to serum and histologic testing, and written informed consent was obtained. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved for the use of opt-out consent by the ethics committee

of Fukushima Medical University School of Medicine. This study was performed in accordance with relevant guidelines and regulations, and all patients and control subjects agreed to undergo serum and histologic testing and have their blood stored for future research.

IMMUNOASSAYS

Concentrations of IL-33 in serum were measured using a Luminex Bio-Plex 200 system (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Concentrations of sST2 in serum were measured using an ELISA kit (R&D Systems, Minneapolis, MN). Each sample was measured in duplicate.

HISTOLOGIC EVALUATION

Fifty-nine patients with AIH and 36 patients with PBC underwent ultrasound-guided liver biopsy. Six patients were excluded from the pathologic analysis; 2 patients were excluded because of cirrhosis and the other patients were excluded because of poor health conditions, such as hepatic failure. Liver sections were stained with hematoxylin and eosin (H&E). Slides were coded and read by two pathologists blinded to patient identity and history. Histologic evaluation was performed according to the classification of Scheuer⁽²⁴⁾ and Desmet et al.⁽²⁵⁾ Grades for necroinflammatory activity (G) and stages of fibrosis (S) ranged from G0 to G4 and from S0 to S4, respectively.

Immunohistochemical (IHC) staining was performed on 4- μ m sections obtained from formalin-fixed paraffin-embedded tissue samples from 26 patients with AIH and 36 patients with PBC. In most cases, immunostained samples were available from diagnostic biopsies from the original examination. A polyclonal rabbit antibody (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan) against human IL-33 was used. Microscopic analysis of IL-33 was performed independently by two observers in a blinded manner; there was no discrepancy between the assessments of the two investigators. Three visual fields were chosen randomly with a high-power lens (40 \times magnification). All cells that were morphologically consistent with positive IL-33 staining (vascular endothelial cells or sinusoidal endothelial cells) in high-power fields of portal tracts or hepatic lobules were counted. The final evaluation was derived from the average of the nuclear staining results.

STATISTICAL ANALYSES

Results were expressed as means \pm SDs. Differences were compared using the Mann-Whitney U test and Wilcoxon matched-pairs signed-rank test. Correlations between variables were assessed using Spearman's rank correlation coefficient. To find the optimal IL-33 and ST2 cut-off level that could distinguish between severe and nonsevere necroinflammatory activity, receiver operating characteristic curves were used. Cut-off levels for the parameters were set at the points closest to 100% sensitivity and specificity. Univariate and multivariate logistic regression analyses were performed to analyze the factors related to grading of the necroinflammatory activity and relapse. All statistical analyses were performed using Prism 6.0 software (GraphPad Software, Inc.) and JMP Pro 13.1 (SAS Institute Inc., Cary, NC). $P < 0.05$ was considered significant.

Results

PATIENT CHARACTERISTICS

Baseline characteristics of the patients with AIH or PBC and HCs are summarized in Table 1. No significant differences were found among the AIH, PBC, and HC groups in age and sex. Liver enzyme (ALT and ALP) levels in patients with AIH were significantly higher than levels in the HCs. Compared with patients with PBC, levels of AST, ALT, TB, and IgG were significantly higher while levels of ALB and PT and the PLT count were significantly lower in patients with AIH. Among the patients with AIH, 60 of the 65 patients with AIH tested positive for ANAs, while 58 of the 63 patients tested negative for anti-LKM-1 antibodies. We were unable to examine the anti-LKM-1 antibody in two patients because the serum samples were in short supply. These patients were positive for ANA and diagnosed as type I AIH. Acute-onset AIH accounted for 44.6% of patients. Twenty patients (30.8%) had severe disease symptoms (TB >5.0 mg/dL and/or PT $<40\%$); 11 patients (16.9%) had cirrhosis at diagnosis, and 3 patients (4.6%) developed cirrhosis. Four patients (6.2%) developed decompensation. Ten patients (15.4%) had relapse during the immunosuppressive treatments; 3 of the 10 patients with AIH relapsed after immunosuppressive

TABLE 1. CLINICAL CHARACTERISTICS OF PATIENTS WITH AIH

Characteristics	AIH	PBC	HC
n	65	36	17
Age at diagnosis, years	55.5 ± 14.1	57.6 ± 10.8	54.2 ± 6.8
Sex, male/female	6/59	7/29	2/15
Laboratory data			
Platelet count, ×10 ⁴ /μL	17.7 ± 7.6 [†]	19.9 ± 6.0	-
Albumin level, g/dL	3.4 ± 0.6 [†]	4.1 ± 0.4	-
Bilirubin level, mg/dL	6.2 ± 8.6 [†]	1.0 ± 0.7	-
Prothrombin time, %	73 ± 24 [†]	97 ± 20	-
AST level, U/L	478 ± 681 [†]	59 ± 36	-
ALT level, U/L	452 ± 558 ^{*,†}	57 ± 39	5 ± 2
ALP level, U/L	450 ± 252 [*]	554 ± 354	158 ± 54
IgG level, mg/dL	2,674 ± 987 [†]	1,952 ± 592	-
ANA			
< ×40	4 (6.2)	17 (47.2)	-
×40	1 (1.5)	1 (2.8)	-
×80	7 (10.8)	0 (0)	-
> ×80	53 (81.5)	18 (50.0)	-
Anti-LKM-1 (n = 63)			
Index <17, (-)	58 (92.1)	-	-
Index 17-49, (±)	4 (6.3)	-	-
Index ≥50, (+)	1 (1.6)	-	-
Mean observation period, months	92.2 ± 84.4	-	-
Scoring			
IAIHG score	16.8 ± 3.4	-	-
Simplified score	6.8 ± 1.6	-	-
Definite AIH	40 (61.5)	-	-
Acute onset			
Acute hepatitis phase	6 (20.7)	-	-
Acute exacerbation phase	23 (79.3)	-	-
Chronic onset			
Severity (mild/moderate/severe)	24/23/18	-	-
Severe symptoms at diagnosis [†]	20 (30.8)	-	-
Cirrhosis at diagnosis	11 (16.9)	-	-
Grading of activity (0/1/2/3/4) (n = 59)	1/6/28/21/3	10/9/15/2/0	-
Staging of fibrosis (0/1/2/3/4) (n = 59)	8/7/16/19/9	9/9/15/3/0	-
Stage F3 or F4 at diagnosis (n = 61)	30 (46.2)	-	-
Complication of other autoimmune diseases	9 (13.8)	-	-
Clinical outcomes			
Relapse	10 (15.4)	-	-
Relapse during continuous PSL therapy	7 (10.8)	-	-
Development of cirrhosis	3 (4.6)	-	-
Development of decompensation	4 (6.2)	-	-
Liver transplantation	2 (3.1)	-	-
Liver-related death	2 (3.1)	-	-
Therapy			
PSL therapy (%)	57 (87.7)	-	-
Initial dose of 15-30 mg/day	51 (78.5)	-	-
Initial dose of 40-60 mg/day	6 (9.2)	-	-
PSL/AZA combination therapy (%)	15 (23.1)	-	-

TABLE 1. Continued

Characteristics	AIH	PBC	HC
Initial dose 50 mg/day	14 (21.5)		
Initial dose 100 mg/day	1 (1.5)		
PSL pulse (%)	8 (12.3)	-	-

* $P < 0.05$ was considered significant (AIH versus HCs); † $P < 0.05$ was considered significant (AIH versus PBC); ‡Severe symptoms: total bilirubin >5.0 mg/dL and/or prothrombin time $<40\%$. Abbreviation: AZA, azathioprine.

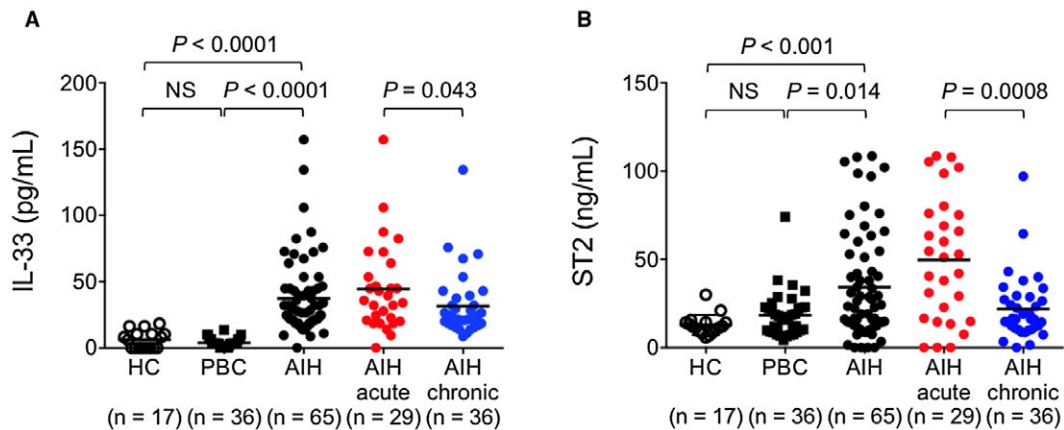


FIG. 1. Patients with AIH ($n = 65$) have higher serum IL-33 and sST2 levels than patients with PBC ($n = 36$) or healthy controls ($n = 17$). Comparison of patients with AIH with acute- ($n = 29$) or chronic- ($n = 36$) onset disease at presentation. (A) Serum IL-33. (B) Serum sST2. Horizontal line indicates the mean. P values were calculated with the Mann-Whitney U test; $P < 0.05$ was considered significant. Abbreviation: NS, not significant.

treatment withdrawal, and 7 patients relapsed during continuous immunosuppressive treatment. The necro-inflammatory activity grades were as follows: G0 (1/59, 1.6%), G1 (6/59, 10.2%), G2 (28/59, 47.5%), G3 (21/59, 35.6%), and G4 (3/59, 5.1%). The stages of fibrosis were as follows: F0 (8/59, 13.5%), F1 (7/59, 11.9%), F2 (16/59, 27.1%), F3 (19/59, 32.2%), and F4 (9/59, 15.3%). In therapy, most patients (87.7%) were treated with prednisolone (PSL) and 15 patients (23.1%) were treated with azathioprine in combination with PSL.

PATIENTS WITH AIH HAVE HIGHER SERUM IL-33 AND sST2 LEVELS THAN PATIENTS WITH PBC OR HCs

The mean titer of IL-33 as measured by the BioPlex system was significantly higher in serum samples from patients with AIH (37.3 pg/mL) than in those from patients with PBC (3.7 pg/mL; $P <$

0.0001) or HCs (6.2 pg/mL; $P < 0.0001$) (Fig. 1A). Similarly, sST2 levels were significantly increased in the serum of patients with AIH compared with the serum in patients with PBC or HCs (Fig. 1B). In the comparison between patients with AIH with acute- ($n = 29$) or chronic- ($n = 36$) onset disease at presentation, serum IL-33 and sST2 levels were significantly higher in patients with acute AIH than in those with chronic AIH ($P = 0.043$, $P = 0.0008$, respectively).

RELATIONSHIP BETWEEN SERUM IL-33/sST2 AND CLINICAL PRESENTATION

The Spearman's rank coefficient analysis is shown in Table 2. Serum IL-33 levels showed a significantly weak positive correlation with levels of TB and ALT. However, serum sST2 levels showed a weak positive correlation with levels of AST and ALT, a moderate positive correlation with levels of TB, and a weak negative correlation with levels of ALB and the PT.

TABLE 2. RELATIONSHIP BETWEEN SERUM IL-33 AND sST2 LEVELS AND CLINICAL PRESENTATION IN PATIENTS WITH AIH

Variable	IL-33		sST2	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
AST (U/L)	0.1377	0.2899	0.3168	0.0129*
ALT (U/L)	0.2514	0.0433*	0.4008	0.0009*
TB (mg/dL)	0.3596	0.0035*	0.5175	<0.0001*
ALP (U/L)	0.2166	0.0830	0.1089	0.3880
ALB (g/dL)	-0.2395	0.0677	-0.4103	0.0012*
PT (%)	-0.1371	0.2839	-0.2544	0.0442*
PLT (×10 ⁴ /μL)	-0.1208	0.3455	-0.1119	0.3827
IgG (mg/dL)	-0.1935	0.5967	-0.1935	0.1232
Staging of fibrosis	0.001608	0.9903	0.005869	0.9645
Grading of activity	0.2758	0.0345*	0.1518	0.2511

**P* < 0.05 was considered significant.

A comparison of the serum IL-33 and sST2 levels between patients with AIH presenting severe symptoms and those presenting nonsevere symptoms is shown in Fig. 2. Serum sST2 levels were significantly higher in patients with severe symptoms of AIH than in those with nonsevere symptoms (Fig. 2A,B).

RELATIONSHIP BETWEEN SERUM IL-33/sST2 LEVELS AND HISTOLOGY

Although serum IL-33 levels did not correlate with the stage of fibrosis (*r* = 0.0016; *P* = 0.99), they showed a significantly weak positive correlation with

necroinflammatory activity grade (*r* = 0.28; *P* < 0.05) (Table 2). Patients with AIH were also stratified by the progression of necroinflammatory activity (Fig. 3). Comparing serum IL-33 and sST2 levels between patients exhibiting severe necroinflammatory activity (G3-G4) and those exhibiting nonsevere necroinflammatory activity (G0-G2) using liver histology, serum IL-33 levels in the group with severe necroinflammatory activity were significantly higher (28.0 pg/mL versus 50.8 pg/mL; *P* < 0.01) (Fig. 3A,B). Results of the univariate and multivariate logistic regression analyses for factors significantly associated with severe necroinflammatory activity are shown in Table 3. Multivariate analysis was performed with the significant factors identified by univariate analysis; IL-33 and IgG were found to be independent factors. However, no significant differences in serum sST2 levels were found between the groups with severe and nonsevere necroinflammatory activity and fibrosis (Fig. 3C,D).

NUMBER OF IL-33-EXPRESSING CELLS IN LOBULES AND PORTAL AREAS OF LIVER IN PATIENTS WITH AIH AND PATIENTS WITH PBC

IHC staining for IL-33 was performed on liver tissue from 26 patients with AIH and 36 patients with PBC. H&E and nuclear staining of IL-33 in the hepatic lobules, portal areas, and hepatocytes of liver in patients with AIH and those with PBC is

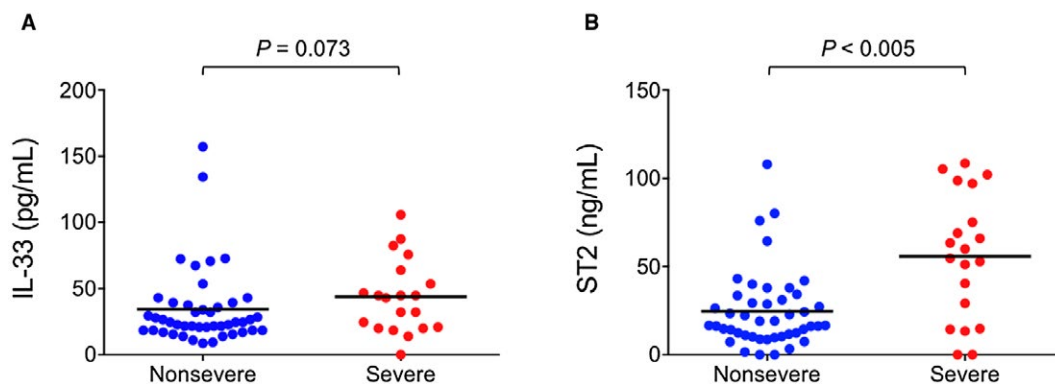


FIG. 2. Relationship between serum IL-33/sST2 levels and severe symptoms in patients with AIH. (A) Comparison of serum IL-33 levels of patients with AIH exhibiting severe and nonsevere symptoms. (B) Comparison of serum sST2 levels of patients with AIH exhibiting severe and nonsevere symptoms. Horizontal line indicates the mean. *P* values were calculated with the Mann-Whitney U test; *P* < 0.05 was considered significant.

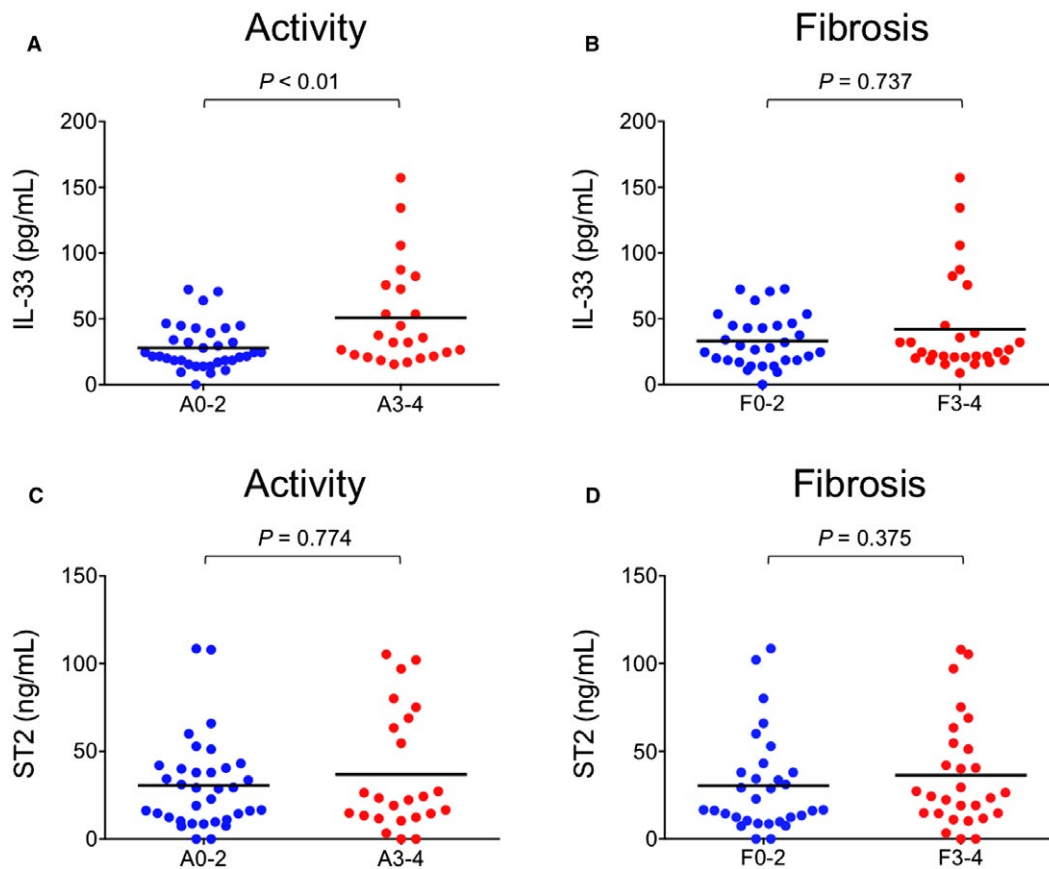


FIG. 3. Relationship between serum IL-33/sST2 levels and histology in patients with AIH. Serum IL-33/sST2 levels according to the degree of necroinflammatory activity and liver fibrosis. (A) Comparison of serum IL-33 levels between severe and nonsevere necroinflammatory activity grades in liver histology. (B) Comparison of serum IL-33 levels between severe and nonsevere stages of fibrosis in liver histology. (C) Comparison of serum sST2 levels between severe and nonsevere necroinflammatory activity grades in liver histology. (D) Comparison of serum sST2 levels between severe and nonsevere stages of fibrosis in liver histology. Horizontal line indicates the mean. *P* values were calculated with the Mann-Whitney U test; *P* < 0.05 was considered significant.

TABLE 3. UNIVARIATE AND MULTIVARIATE ANALYSES OF FACTORS ASSOCIATED WITH AN ACTIVITY GRADE OF A3-A4

A3-A4 vs. A0-A2	Univariate		Multivariate	
	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
Age (years)	1.00 (0.96-1.03)	0.885		
AST (U/L)	1.00 (1.00-1.00)	0.372		
ALT (U/L)	1.00 (1.00-1.00)	0.568		
TB (mg/dL)	1.06 (1.00-1.14)	0.066		
ALP (U/L)	1.00 (1.00-1.00)	0.198		
ALB (g/dL)	0.31 (0.12-0.81)	0.016*	0.80 (0.23-2.81)	0.732
PT (%)	0.97 (0.95-1.00)	0.02*	0.98 (0.94-1.02)	0.238
PLT ($\times 10^4/\mu\text{L}$)	0.93 (0.85-1.01)	0.103		
IgG (mg/dL)	1.00 (1.00-1.00)	0.003*	1.00 (1.00-1.00)	0.009*
IL-33 (pg/mL)	1.03 (1.01-1.06)	0.012*	1.04 (1.01-1.08)	0.025*
sST2 (ng/mL)	1.01 (1.99-1.03)	0.411		

**P* < 0.05 was considered significant.

shown in Fig. 4A-L. IL-33-positive cells (vascular endothelial cells or sinusoidal endothelial cells) in high-power fields of portal tracts or hepatic lobules were counted. A comparison of the number of IL-33-positive cells in the hepatic lobules and portal areas of liver in patients with AIH and those with PBC

is shown in Fig. 4M,N. IL-33 was visually localized in the nucleus of hepatocytes. Rates of positive IL-33 staining in liver sections from patients with AIH and patients with PBC were 61.5% and 13.9%, respectively (Table 4). Immunohistochemistry revealed high IL-33 expression in liver sections from patients with

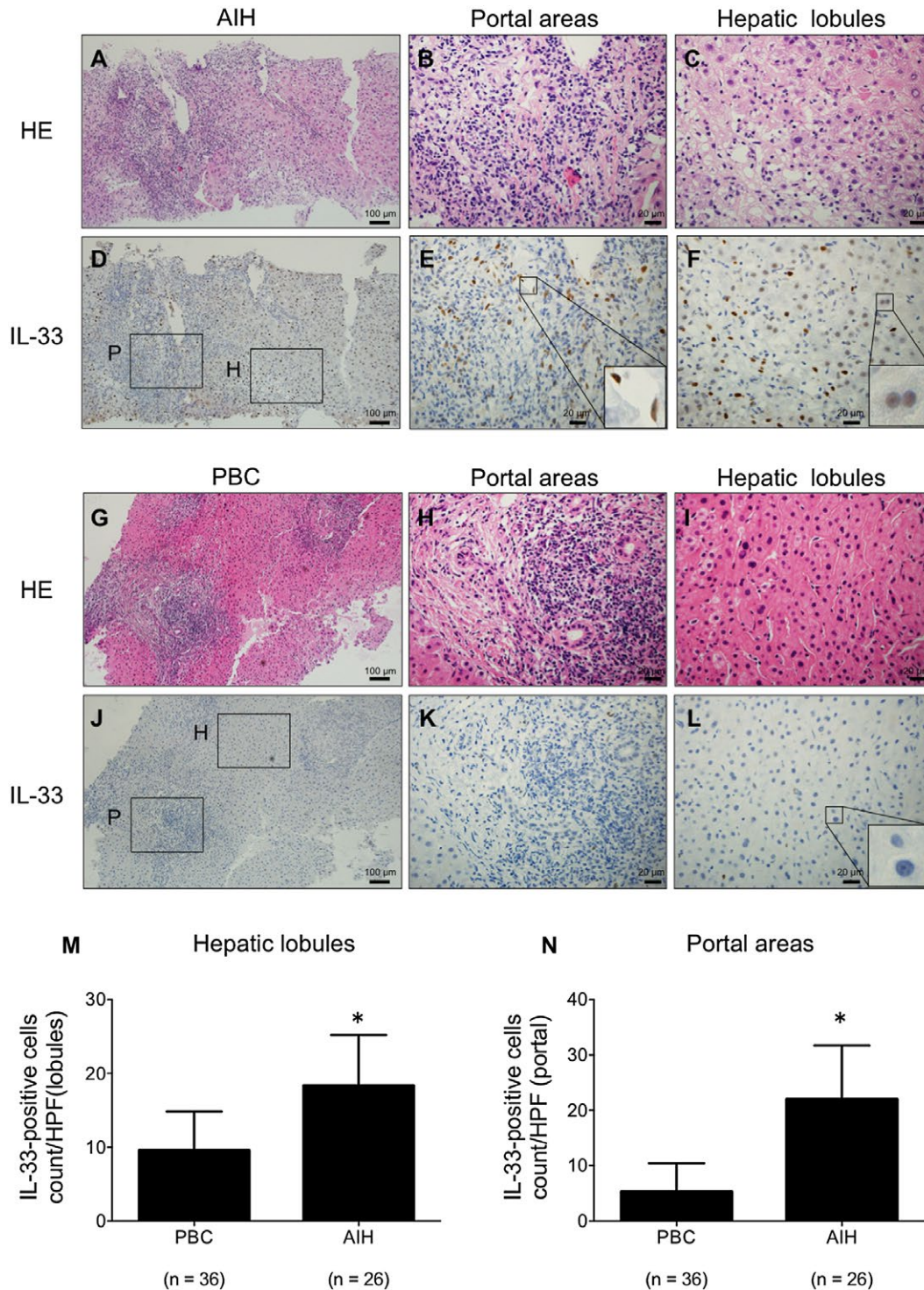


FIG. 4. Immunohistochemical analyses of IL-33 expression in sections of different liver tissues from AIH and patients with PBC. (A) H&E staining of liver from a patient with AIH. (B) H&E staining of portal areas from a patient with AIH. (C) H&E staining of lobules from a patient with AIH. (D) Nuclear staining of IL-33 in the liver of a patient with AIH. (E) Nuclear staining of IL-33 in portal areas of a patient with AIH (endothelial cells). Typical vascular endothelial-positive cells observed in portal areas are further enlarged in the squared area. (F) Nuclear staining of IL-33 in lobules from a patient with AIH (sinusoidal cells and hepatocytes). Typical hepatocyte-positive cells observed in lobules are further enlarged in the squared area. (G) H&E staining of liver from a patient with PBC. (H) H&E staining in portal areas from a patient with PBC. (I) H&E staining in lobules from a patient with PBC. (J) Nuclear staining of IL-33 in portal areas from a patient with PBC (endothelial cells). (K) Nuclear staining of IL-33 in portal areas of liver from a patient with PBC (endothelial cells). (L) Nuclear staining of IL-33 in lobules of liver from a patient with PBC (sinusoidal cells and hepatocytes). Typical hepatocyte-negative cells observed in lobules are further enlarged in the squared area. (A,D,G,J) Scale bars, 100 μ m. (B,C,E,F,H,I,K,L) Scale bars, 20 μ m. (M) Comparison of the number of IL-33-expressing cells in lobules of liver in patients with AIH and PBC. (N) Comparison of the number of IL-33-expressing cells in portal areas of liver in patients with AIH and PBC. *P* values were calculated with the Mann-Whitney U test; *P* < 0.05 was considered significant. Data in (M,N) show mean \pm SD. Abbreviations: H, hepatic lobule; HPF, high-power field; P, portal.

TABLE 4. NUCLEAR EXPRESSION OF IL-33 IN HEPATOCYTES

	n	IL-33		Significance (χ^2 Test)
		-	+	
AIH	26	10 (38.5%)	16 (61.5%)	<0.0001*
PBC	36	31 (86.1%)	5 (13.9%)	

**P* < 0.05 was considered significant.

AIH compared with IL-33 expression in liver sections from patients with PBC.

SERUM LEVELS OF IL-33 AND sST2 IN PATIENTS WITH AIH FOLLOWING TREATMENT

We analyzed levels of serum IL-33/sST2 in 14 patients with AIH who had achieved biochemical remission and 7 patients who had not. Comparison of serum IL-33/sST2 levels at onset and posttreatment among patients with AIH (Fig. 5A,B) showed that serum IL-33 and sST2 levels in patients with AIH who were responsive to PSL treatment were significantly decreased after treatment. Serum IL-33/sST2 levels in patients with AIH who were resistant to treatment with PSL were not significantly decreased after treatment (Fig. 5C,D).

COMPARISON OF SERUM IL-33 LEVELS BETWEEN RELAPSE AND NONRELAPSE CASES OF PATIENTS WITH AIH

Comparisons of clinical markers and serum IL-33/sST2 levels between relapse and nonrelapse cases

of patients with AIH are shown in Table 5. Serum IL-33 levels and levels of TB and ALT were significantly higher in patients with AIH who relapsed compared with those who did not. The cut-off value of serum IL-33 used to predict relapse in patients with AIH during continuous immunosuppressive therapy was 30.03 pg/mL; this value showed a sensitivity of 85.7% and a specificity of 67.4%. The area under the curve for relapse in patients with AIH was 0.76 (Fig. 6A). Additionally, the cumulative incidence of relapse in patients with AIH with serum IL-33 levels of >30 pg/mL or \leq 30 pg/mL is shown (Fig. 6B). Serum IL-33 levels of >30 pg/mL were associated with a significantly higher risk of developing relapse than serum IL-33 levels of \leq 30 pg/mL. Results of the multivariate logistic regression analysis with the factors significantly associated with relapse are shown in Table 6. Adjusting for confounding variables, such as age and sex, the odds ratio (OR) of developing relapse with serum IL-33 levels >30 pg/mL versus serum IL-33 levels \leq 30 pg/mL was 16.8 (95% confidence interval [CI], 1.90-147.85). After adjusting for confounding variables, such as age, sex, TB, and PSL treatment withdrawal, the OR was 11.8 (95% CI, 1.01-136.46). The serum IL-33 level was an independent factor for relapse in patients with AIH.

Discussion

Previous studies have shown that IL-33 functions as an alarmin that is released following cell necrosis to alert the immune system to tissue damage.⁽²⁶⁾ Additionally, IL-33 exerts its biological effects by activating the mitogen-activated protein kinase and nuclear factor kappa B signaling pathways.^(7,27) Other

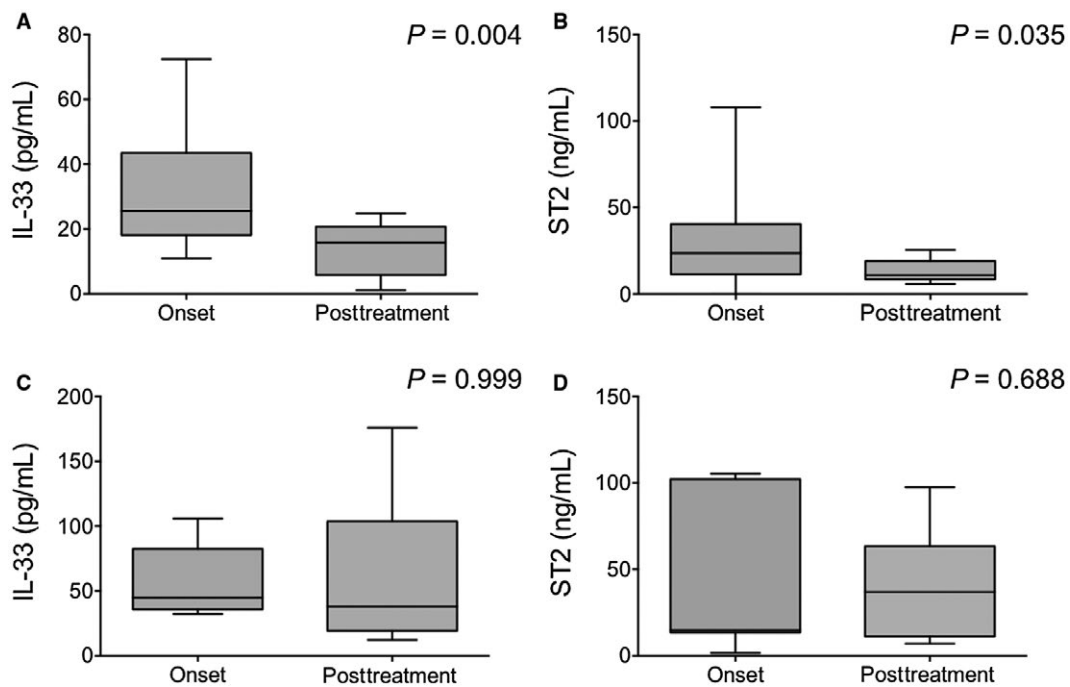


FIG. 5. Serum levels of IL-33 and sST2 in patients with AIH following PSL treatment. (A) Comparison of serum IL-33 levels at onset and posttreatment among patients with AIH who achieved biochemical remission. (B) Comparison of serum sST2 levels at onset and posttreatment among patients with AIH who achieved biochemical remission. (C) Comparison of serum IL-33 levels at onset and posttreatment among patients with AIH who did not achieve biochemical remission. (D) Comparison of serum sST2 levels at onset and posttreatment among patients with AIH who did not achieve biochemical remission. *P* values were calculated with the Wilcoxon matched-pairs signed-rank test; *P* < 0.05 was considered significant. Data represent mean ± SD.

TABLE 5. COMPARISONS OF CLINICAL MARKERS AND SERUM IL-33/sST2 LEVELS ACCORDING TO DISEASE RELAPSE IN PATIENTS WITH AIH

	Relapse (n = 10)	Nonrelapse (n = 43)	<i>P</i>
Age (years)	56.3 ± 15.8	55.6 ± 13.2	0.9153
Sex (female)	10 (100%)	37 (86.0%)	0.2097
IAIHG score	15.9 ± 3.8	16.9 ± 3.6	0.3474
ALT (U/L)	947 ± 936	411 ± 439	0.0451*
TB (mg/dL)	11.6 ± 9.6	5.3 ± 6.8	0.0098*
ALP (U/L)	383 ± 221	492 ± 268	0.4905
ALB (g/dL)	3.1 ± 0.5	3.4 ± 0.7	0.2321
PT (%)	56 ± 19	72 ± 24	0.0513
PLT (×10 ⁴ /μL)	16.3 ± 7.6	18.5 ± 7.7	0.2824
IgG (mg/dL)	2,760 ± 901	2,685 ± 974	0.9014
Grading of activity (0/1/2/3/4) (n = 50)	0/1/3/4/1	0/4/20/15/2	0.4910
IL-33 (pg/mL)	52.5 ± 26.5	32.1 ± 25.1	0.007*
sST2 (ng/mL)	37.0 ± 33.0	35.0 ± 28.7	0.8992
PSL withdrawal	3 (30.0%)	3 (7.0%)	0.0385*

**P* < 0.05 was considered significant.

studies have suggested that increased IL-33 levels correlate with the acute-phase inflammatory response in autoimmune diseases, such as systemic lupus erythematosus.⁽²⁸⁾ In our study, we found that serum IL-33 and sST2 levels were higher in patients with AIH than in HCs. Additionally, serum IL-33 and sST2 levels were significantly higher in patients with AIH with acute presentation than in those with chronic presentation. In the most recent nationwide survey of patients with AIH in Japan, patients with acute hepatitis had clinical features different from those of patients with chronic hepatitis.⁽⁴⁾ Patients with the recently established entity of AIH with acute presentation often display atypical clinical features that mimic those of forms of acute hepatitis even though AIH is categorized as a chronic liver disease.⁽²⁹⁾ Several studies have revealed immunoserologic and histologic differences between AIH with acute presentation and chronic AIH.^(30,31)

In addition, our study revealed that the elevated levels of serum IL-33 were weakly positively correlated

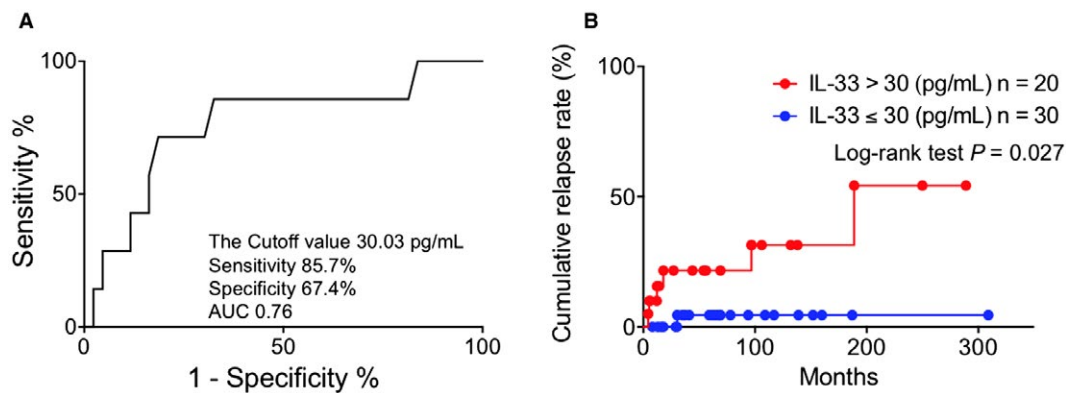


FIG. 6. Relationship between serum IL-33 levels and relapse in patients with AIH. (A) ROC curves of the serum IL-33 level for predicting relapse in patients with AIH. (B) Cumulative incidence of relapse in patients with AIH with a serum IL-33 level >30 or ≤30 is shown. Serum IL-33 levels >30 pg/mL were associated with a significantly higher risk of relapse compared to serum IL-33 levels ≤30 pg/mL. *P* values were calculated with the log-rank test; *P* < 0.05 was considered significant. Abbreviations: AUC, area under the curve; ROC, receiver operating characteristic.

TABLE 6. MULTIVARIATE ANALYSES OF FACTORS ASSOCIATED WITH RELAPSE IN PATIENTS WITH AIH

Relapse Versus Nonrelapse		Model 1 [†]		Model 2 [‡]	
		OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
IL-33 (pg/mL)	>30	16.8 (1.90-147.85)	0.0112*	11.8 (1.01-136.46)	0.0487*
	≤30	1 (Ref.)		1 (Ref.)	
TB (mg/dL)	>5			6.0 (0.89-40.07)	0.0660
	≤5			1 (Ref.)	
PSL withdrawal	Yes			14.9 (0.87-256.79)	0.0628
	No			1 (Ref.)	

**P* < 0.05 was considered significant; [†]after adjusting for age, sex; [‡]after adjusting for age, sex, TB, PSL treatment withdrawal. Abbreviation: Ref., reference group.

with liver injury, as indicated by levels of ALT and TB and necroinflammatory activity grade. Multivariate analysis, performed with the significant factors identified by univariate analysis, showed that serum IL-33 level was an independent factor for severe necroinflammatory activity. Our study is the first to show a correlation between serum IL-33 levels and liver histologic findings in patients with AIH; however, we did not find a positive correlation between serum IL-33 and IgG levels as was observed in the previous study. Serum IgG levels were generally low in patients with AIH with acute presentation; these patients accounted for 44.6% of our patient cohort.

Results of IHC staining for IL-33 were similar to the findings of previous reports.^(13,14) IL-33

staining was concentrated in the nuclei of vascular endothelial cells and sinusoidal endothelial cells in areas of inflammation. By counting IL-33-positive cells (vascular endothelial cells or sinusoidal endothelial cells) in high-power fields of portal tracts or hepatic lobules, we found higher IL-33 expression in liver sections from patients with AIH than in those from patients with PBC. Interestingly, we found that hepatocytes expressed IL-33 with nuclear localization in more than half of patients with AIH. In contrast, IL-33 was not detected in intrahepatic bile ducts. Consistent with the IHC staining results, serum IL-33 levels were higher in patients with AIH than in patients with PBC. PBC and AIH are classically viewed as distinct autoimmune liver diseases. PBC is

a progressive autoimmune liver disease characterized by immune-mediated destruction of the intrahepatic bile ducts. The histologic findings in AIH are known to be plasma cell/lymphocyte infiltration and interface hepatitis in the portal tract. Thus, the induction of IL-33 in hepatocytes is concomitant with cellular death or hepatic injury.

In addition, our study indicated that serum IL-33 levels were significantly higher in patients with AIH who relapsed than in those with sustained remission. Additionally, serum IL-33 levels of >30 pg/mL were associated with a significantly higher risk of relapse. Montano-Loza et al.⁽³²⁾ reported that serum levels of aminotransferase, IgG, and hepatitis activity were higher in patients with relapse than in those with sustained remission prior to corticosteroid therapy, and our prior studies^(33,34) showed that an IAIHG score of 17 or greater and rapid corticosteroid tapering were associated with relapse in patients with AIH. Patients with AIH who relapse more frequently progress to cirrhosis, the development of esophageal varices, and death from hepatic failure compared to those with sustained remission.⁽³⁵⁻³⁸⁾ Serum IL-33 levels may help predict relapse during AIH onset.

sST2 acts as a decoy receptor for IL-33, attenuating Th2 inflammatory responses by preventing the binding of IL-33 to transmembrane ST2 receptors, which are involved in several T-cell-mediated diseases.⁽³⁹⁾ Previous studies have concluded that sST2 may be a useful biomarker for cardiovascular disease and inflammation, for example, heart failure and sepsis.⁽⁴⁰⁻⁴²⁾ In addition, serum sST2 levels predict mortality in HBV-related acute-on-chronic liver failure.⁽¹⁴⁾ In our study, we found the sST2 level to be significantly increased in serum of patients with AIH compared with that of patients with PBC and HCs. Compared with patients with nonsevere symptoms, serum sST2 levels were significantly higher in cases with severe symptoms of AIH, although there was no significant difference in necroinflammatory activity or fibrosis between the severe and nonsevere cases. Lipopolysaccharide activates macrophages through toll-like receptor 4, inducing proinflammatory cytokines, such as IL-1 β and TNF- α , which activate fibroblasts and other cell types to produce sST2, which then binds macrophages and represses the expression of proinflammatory cytokines.⁽⁴³⁾ Soluble CD163, a specific macrophage activation marker, is reported to be markedly elevated in acute-phase

AIH.⁽⁴⁴⁾ Therefore, sST2 may function as a negative regulator of severe symptoms in patients with AIH. sST2 is considered a decoy receptor neutralizing IL-33; thus, sST2 could perform functions opposing IL-33. The enhanced sST2 presence has been shown to inhibit the production of the type 2 cytokines IL-4 and IL-5 but not the type 1 cytokine interferon- γ .⁽⁴⁵⁾ sST2 could act as a moderator of inflammation, but more evidence is needed to confirm the extent of the ability of sST2 to moderate inflammation in AIH.

Previous reports have demonstrated the implication of several cytokines in the pathogenesis and severity of AIH.^(46,47) The complex interplay of several cytokines, especially proinflammatory and Th17 cytokines, and Treg suppression by IL-12p40 play a pivotal role in the pathogenesis of AIH. We previously found that serum IL-21 levels were significantly higher in severe AIH cases compared to nonsevere cases and that serum IL-21 level correlated positively with TB level and necroinflammatory activity grade.⁽⁴⁸⁾ Moreover, a recent study revealed that IL-21 and IL-33 are potent regulators of HBV clearance.⁽⁴⁹⁾ This relationship between IL-21 and IL-33 is also expected in the pathogenesis of AIH. Furthermore, the NLR family pyrin domain containing 3 (NLRP3) inflammasome pathway has been proposed to be involved in the maturation of IL-33.⁽⁵⁰⁾ Thus far, no evidence supports the activation of the NLRP3 inflammasome in AIH. It appears that both the NLRP3 inflammasome and the IL-33 pathway can become activated in sterile inflammation-associated liver diseases; therefore, components of this inflammatory pathway represent promising new therapeutic targets.⁽⁵⁰⁾

Our study has some limitations. First, the sample population was relatively small. Second, we used a retrospective design, and thus our results will need to be confirmed in a prospective study.

To our knowledge, this study is the first to determine the relationship between serum IL-33 levels and histologic necroinflammatory activity in patients with AIH. Moreover, higher serum IL-33 levels were associated with a significantly higher risk of relapse. These findings suggest that IL-33/ST2 may play an important role in mediating the pathogenesis and severity of AIH. Further research on the systemic and localized effects of IL-33/ST2 in AIH will provide a basis for targeted therapy that could benefit this patient population.

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