Accepted Article

Possible involvement of $CCR7^{-}PD\text{-}1^{+}$ follicular helper T cell subset in the pathogenesis of autoimmune hepatitis

Naruhiro Kimura, Satoshi Yamagiwa, Tomoyuki Sugano, Toru Setsu, Kentaro Tominaga, Hiroteru Kamimura, Masaaki Takamura and Shuji Terai

Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan

Tel: +81-25-227-2207, Fax: +81-25-227-0776

Correspondence to: Satoshi Yamagiwa, M.D., Ph.D.
Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences,
1-757 Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan
E-mail address: syamagi@med.niigata-u.ac.jp
Tel: +81-25-227-2207
Fax: +81-25-227-0776

Disclosure statement: The authors have no conflict of interests.
This study was supported in part by Grants-in-Aid for Scientific Research (C) (15K08991 to S.Y.) and (B) (26293175D to S.T.) from Japan Society for the Promotion of Science (JSPS).

Acknowledgement: We thank T. Tsuchida for his excellent technical assistance. We also thank all the patients who participated in the study.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jgh.13844

This article is protected by copyright. All rights reserved.

Accepted Article

Abstract

Background & Aim: Recent studies have demonstrated that B cells and follicular helper T (Tfh) cells, which are central regulators of humoral immune response, contribute to the development and progression of autoimmune diseases. Because Tfh cells can be divided into several subsets with distinct functional properties, this study aimed to examine the roles of different subsets of circulating Tfh cells in the immune pathogenesis of autoimmune hepatitis (AIH).

Methods: Thirty-five patients with AIH, 28 patients with primary biliary cholangitis, 24 patients with chronic hepatitis B (CHB), and 44 health controls (HC) were enrolled. The frequencies of different Tfh subsets in the blood and liver were examined by flow cytometry and immunohistochemical staining. The function of circulating Tfh subsets was examined after *in vitro* stimulation.

Results: In newly diagnosed AIH patients, the frequency of circulating chemokine C-C receptor 7 (CCR7)$^-$ programmed cell death-1 (PD-1)$^+$ Tfh subset was significantly increased compared with that in CHB patients and HC, significantly correlated with clinical parameters, including serum IgG,

This article is protected by copyright. All rights reserved.

prothrombin time and albumin levels, and significantly decreased after corticosteroid treatment. In the liver of AIH patients, the frequencies of activated Tfh subsets were significantly increased and positively correlated with those in the blood. Moreover, the ability to produce interleukin (IL)-21 and IL-17 from circulating Tfh cells was significantly increased in AIH patients compared with HC.

Conclusions: These results significantly extend our understanding of Tfh subsets in AIH, and suggest a potential role of dysregulated CCR7$^-$PD-1$^+$ Tfh subset in the pathogenesis and disease progression of AIH.

Electric word counts: 250 words

Key words: follicular helper T cells; autoimmune hepatitis; primary biliary cholangitis; programmed cell death-1; interleukin-21.

Accepted Article

This article is protected by copyright. All rights reserved.

Introduction

Autoimmune hepatitis (AIH) shows varied clinical manifestations raging from asymptomatic, mild chronic hepatitis to acute-onset fulminant liver failure.[1,2] AIH typically presents as asymptomatic or mild chronic hepatitis, and the majority of patients initially respond well to corticosteroid. After initial remission, long-term maintenance therapy is required, but even after liver inflammation disappears completely, 13% of the patients eventually experience a relapse.[3,4] Besides the difficulty in sustaining remission, AIH progressing to acute liver failure often responds poorly to corticosteroid treatment and needs liver transplantation. Therefore, elucidating the pathogenesis of AIH is still essential for the management of patients with AIH.

Although liver-infiltrating T cells are considered as the primary mediators of inflammatory liver damage, it is unclear how the dysregulated T cells trigger the development of AIH.[1,2,4] Recent studies have implicated that B cells also contribute to the development and progression of AIH.[5] Activated B cells differentiate into plasma cells that secrete antibodies, including autoantibodies such as anti-nuclear antibodies (ANA) or anti-smooth muscle

This article is protected by copyright. All rights reserved.

Accepted Article

antibodies (SMA). Activated B cells also can function as antigen presenting cells to present antigen determinants for inducing T cell activation.[6] However, the mechanism of dysregulation of B cells and the interaction between T cells and B cells in AIH remains to be elucidated.

The activation and functional differentiation of B cells are regulated by $CD4^+$ T cells, particularly by follicular helper T (Tfh) cells, which are central regulators of humoral immune response.[7] Tfh cells are characterized by increased expression of chemokine C-X-C receptor (CXCR) 5, inducible co-stimulator (ICOS), programmed cell death-1 (PD-1), CD40-ligand and transcription factor B-cell lymphoma 6 (Bcl-6), and secrete interleukin (IL)-21, which are important for their function.[8] Dysregulated Tfh cells have been show to play an important role in the induction and progression of murine model of AIH,[4,9] and the frequency of Tfh cells was reported to be significantly increased in human AIH and primary biliary cholangitis (PBC).[10,11]

Recent studies have shown that Tfh cells were composed of heterogeneous cell populations and can be divided into several subsets with distinct functional properties, such as Tfh1, Tfh2, and Tfh17,[12-16] but the precise evaluation of those Tfh subsets in autoimmune liver diseases has not

Accepted Article

This article is protected by copyright. All rights reserved.

been performed yet. Therefore, in the present study, we examined the frequencies of Tfh cells and different Tfh subsets in the blood and liver of patients with AIH and PBC in order to evaluate their potential role in the immune pathogenesis of AIH and PBC.

Methods

Subjects

A total of 35 patients with AIH, 28 patients with PBC, and 22 patients with chronic hepatitis B (CHB) were sequentially enrolled in this study at the Niigata University Medical and Dental Hospital. The patients with AIH were categorized into two groups as follows: a group of patients who were newly diagnosed and corticosteroid treatment-naive (AIH at onset, n = 12) and a group of patients who were previously diagnosed and under the treatment (AIH with treatment, n = 23). All the patients with AIH were diagnosed as definite type I AIH.[17,18] All the PBC patients were previously diagnosed and treated by ursodeoxycholic acid (UDCA) (according to the weight-based dose, 13-15mg/kg/day). Forty-four healthy individuals who had no history of any chronic inflammatory disease were used as healthy controls (HC). Liver

Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article

biopsy samples were also obtained from 19 patients with AIH, 26 patients with PBC, and 12 patients with CHB. This work was conducted in accordance with the Declaration of Helsinki. Written informed consent under institutional review board-approved protocols (approval no. 1906) at Niigata University Medical and Dental Hospital was appropriately obtained from all the individuals enrolled in the study.

Clinical examination, follow-up and outcome measures

The demographic and clinical characteristics of these subjects are indicated in Table 1. All the treatment-naïve AIH patients received standard prednisolone (PSL) therapy at a daily dose of 0.5 to 0.8 mg/kg of body weight. At the onset of PSL treatment, and at 6 weeks after treatment when their laboratory data were improved within normal ranges, peripheral blood was collected at each visit and frequency of Tfh cells and their surface expression were analyzed.

Flow cytometry analysis

Venous blood samples were collected from individual subjects. Liver specimens were pressed through 200-gauge stainless steel mesh and suspended in Eagle's MEM medium (Life Technologies, Grand Island, NY, USA),

This article is protected by copyright. All rights reserved.

supplemented with 5 mM HEPES and 10% heat-inactivated fetal calf serum (FCS). Mononuclear cells were then separated by density-gradient centrifugation using Ficoll-Paque Plus (GE Healthcare UK Ltd, Little Chalfont, UK). Cells were stained by following monoclonal antibodies: FITC-conjugated anti-CD4 (SK3); PE-conjugated anti-CD56 (B159), anti-CCR7 (3D12), anti-CXCR3 (1C6), anti-PD-1 (EH12.1), anti-CD45RA (HI100) (BD Pharmingen, San Diego, CA, USA), and anti-ICOS (669222, R&D Systems Inc., Minneapolis, MN, USA); PerCP-Cy5.5-conjugated anti-PD-1 (SH12.1) and anti-CCR6 (11A9) (BD Pharmingen); PerCP-eFluor710-conjugated anti-CD3 (SK7); APC-conjugated anti-CXCR5 (MU5UBEE) (eBiosicence, San Diego, CA, USA) for 30 min. After being washed with phosphate-buffered saline (PBS), the cells were characterized on a FACSCalibur (Beckton-Dickinson, San Diego, CA, USA) and at least 50,000 events were analyzed by Flow-Jo software (v5.7.2, TreeStar Inc., Ashland, OR, USA).

Accepted Article

This article is protected by copyright. All rights reserved.

Cell culture and in vitro stimulation

Peripheral blood mononuclear cells were initially rested in DMEM medium supplemented with 10% FCS for 30 min at 37°C and then activated with (1) 50 ng/ml phorbol myristate acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA) and 500 ng/ml ionomycin (Sigma-Aldrich) for 5 h at 37°C for IL-21 production assays; or (2) with 5 ng/ml PMA and 100 ng/ml ionomycin for 5 h at 37°C for IL-17 and interferon (IFN)-γ production assays. In both stimulation conditions, 0.67 μl/ml BD GolgiPlug (BD Biosciences, San Jose, CA, USA) was added when 3 h after starting cell culture. After activation, PBMCs were harvested, and stained with Fixable Viability Dye eFluor780 (eBiosicence) for 20 min at 4°C. Cells were stained with fluorochrome-conjugated antibodies against surface receptors, including FITC-conjugated anti-CD4 (SK3), PE-conjugated anti-IL-21R (17A12) (BD Biosciences), and APC-conjugated anti-CXCR5 antibodies (MU5UBEE, eBiosicence), for 30 min at 4°C. Fixation and permeabilisation was performed using Fix/Perm Buffer set (BioLegend, San Diego, CA, USA) and cells were then stained with the respective intracellular antibodies, including PE-conjugated anti-IL21 (3A3-N2.1), anti-IFN-γ (B27), and anti-IL-17 (SCPL1362) antibodies (BD Biosciences), for 30 min at 4°C.

This article is protected by copyright. All rights reserved.

Accepted Article

Immunohistochemical analysis

Tissue sections were first deparaffinized with xylene and the rehydrated through graded alcohol. To retrieve antigenicity, sections were immersed in pH 6.0 citrate buffer. After autoclaving and blocking endogenous peroxidase activity, sections were then incubated with a series of antibodies at a 1:500 dilution in PBS supplemented with 3% BSA at 4ºC overnight. Immunohistochemical detection was performed according to the avidin-biotin-peroxidase complex method using the Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA). Sections were finally developed with diaminobenzidine substrate (Muto Pure Chemicals, Tokyo, Japan). Specimens then were counterstained with Hematoxylin-eosin and mounted.

Statistical analysis

All statistics were performed using SPSS software (Ver.18, SPSS Inc., Chicago, IL, USA). The significance of differences was analyzed statistically by the compared *t* test with Welch's correction, or Mann-Whitney *U* test, whereas comparisons between the same individual were performed with Wilcoxon's matched-pairs test. Kruskal-Wallis test followed by the

Accepted Article

This article is protected by copyright. All rights reserved.

Steel-Dwass post-hoc test was used to compare more than two groups. The relationship between two variables was evaluated using the Spearman rank correlation test. In all cases, the level of significance was set at P value <0.05.

Results

Increased frequency of Tfh cells in AIH at onset and PBC

As shown in Fig. 1A, Tfh cells were identified as cells expressing $CXCR5^{+}CD4^{+}$ cells among $CD3^{+}$ cells (Tfh/$CD3^{+}$) and PD-$1^{+}CXCR5^{+}$ cells among $CD4^{+}$ cells (PD-$1^{+}CXCR5^{+}/CD4^{+}$). The proportion of Tfh/$CD3^{+}$ was significantly increased in patients with AIH at onset and PBC compared with those in other groups (all P values <0.05) (Fig. 1B). The proportion of PD-$1^{+}CXCR5^{+}/CD4^{+}$ was also increased in patients with AIH at onset and PBC compared with those in other groups all P values <0.05) (Fig. 1C). These results indicate an increase in the circulating Tfh cells in patients with AIH at onset and PBC as well as a difference between patients with AIH at onset and AIH with treatment.

This article is protected by copyright. All rights reserved.

Accepted Article

Changes in frequencies of Tfh subsets

Tfh cells can be subdivided by the expression of CXCR3 and PD-1.[19] PD-1^{+} Tfh subsets can be further subdivided into two subgroups according to the expression of ICOS, and ICOS^{+}PD-1^{+} subpopulation represents activated Tfh cells, which were barely present in healthy individuals.[20] We found that the expression of CXCR3 on CD4^{+}CXCR5^{+}PD-1^{+} cells was significantly decreased in the AIH patients with treatment compared to other groups (all P values <0.05) (Fig. 2A). However, the expressions of other surface markers, including ICOS, chemokine C-C receptor (CCR) 6 and CD45RA, on CD4^{+}CXCR5^{+}PD-1^{+} and CD4^{+}CXCR5^{+}PD-1^{-} Tfh cells were not significantly different among the patient groups (Fig. 2).

Human peripheral Tfh cells can be also subdivided into three major subsets with distinguished biological functions according to expression of CXCR3 and CCR6; CXCR3^{+}CCR6^{-} cells that share properties with Th1 cells (Tfh1), CXCR3^{-}CCR6^{-} cells resembling Th2 cells (Tfh2), and CXCR3^{-}CCR6^{+} cells resembling Th17 cells (Tfh17).[12] Moreover, previous studies have shown that CCR7^{-}PD-1^{+} Tfh cells are defined as an activated subset and can produce IL-21 and promote antibody responses.[21] Although no significant changes were found between the patients with AIH and other groups (Fig. 3A), the

This article is protected by copyright. All rights reserved.

Accepted Article

frequencies of Tfh2 was significantly decreased in patients with PBC compared to those of AIH with treatment and HCs (P = 0.005 and 0.048, respectively) (Fig. 3A). The frequencies of activated $CCR7^{-}PD\text{-}1^{+}$ Tfh subsets were significantly increased in patients with AIH at onset and PBC compared to those in patients with AIH with treatment and HCs (all P values <0.05). These results indicate that an activated Tfh subset was increased in patients with AIH at onset and PBC, ant that there was an imbalance of Tfh subsets in patients with PBC.

Clinical relevance of $CCR7^{-}PD\text{-}1^{+}$ Tfh subset in AIH

As shown in Fig. 3B, the frequencies of $CCR7^{-}PD\text{-}1^{+}$ Tfh subset was positively correlated with the levels of serum IgG (r = 0.672, P <0.01) in patients with AIH. We also found that the frequency of $CCR7^{-}PD\text{-}1^{+}$ Tfh subset was negatively correlated with the serum prothrombin time (PT) (r = -0.517, P = 0.028) and albumin levels (r = -0.390, P = 0.025) in patients with AIH. Moreover, although the number of examined patients was limited (n = 6), we investigated the changes in frequencies of $CCR7^{-}PD\text{-}1^{+}$ Tfh subsets in the blood of patients with AIH before and after PSL treatment when the levels of serum asparate aminotransferase (AST), alanine transaminase (ALT) and IgG

This article is protected by copyright. All rights reserved.

Accepted Article

decreased to within normal ranges. The frequency of activated $CCR7^{-}PD-1^{+}$ Tfh subset significantly decreased after PSL treatment *(P* = 0.040) (Fig. 3E). These results suggest that activated $CCR7^{-}PD-1^{+}$ Tfh subset plays an important role in the pathogenesis and disease progression of AIH, and that the frequency of $CCR7^{-}PD-1^{+}$ Tfh subset may be a useful predictor of effectiveness of PSL treatment and relapse of inflammation in patients with AIH.

Increased cytokine production from Tfh cells in AIH patients

$CD4^{+}CXCR5^{+}$ Tfh cells in the blood of AIH patients with treatment produced significantly higher levels of IL-21, IFN-γ, and IL-17 compared to those in HC after PMA/ionomycin stimulation (*P* = 0.044, <0.01, and 0.019, respectively) (Fig. 4A-C). In addition, the expression of IL-21R on Tfh cells was significantly increased after PMA/ionomycin stimulation compared to that in HCs (*P* = 0.044) (Fig. 4D). These results indicate that Tfh cells in patients with AIH were more sensitive to stimulation and could produce higher amount of cytokines upon stimulation than Tfh cells in HC.

This article is protected by copyright. All rights reserved.

Activated Tfh cells were increased in the liver of patients with AIH

Immunohistochemical staining showed that $CXCR5^+$ cells, which include Tfh cells, were significantly increased in the liver of patients with AIH and PBC compared to patients with CHB (both *P* values <0.05) (Fig. 5). We then investigated detailed characteristics of Tfh cells in the liver of AIH patients at onset by flow cytometry. The frequencies of activated $PD\text{-}1^+$, $ICOS^+$ and $ICOS^+PD\text{-}1^+$ Tfh subsets were significantly increased in the liver compared with the blood of patients with AIH (P = 0.041, P <0.01 and P <0.01, respectively) (Fig. 6A). The frequency of $PD\text{-}1^+CXCR5^+$ cells among $CD4^+$ cells in the liver was positively correlated with that in the blood of patients with AIH (r = 0.785, P = 0.003) (Fig. 6B). Interestingly, the frequency of $PD\text{-}1^+CXCR5^+$ cells among $CD4^+$ cells in the liver was positively correlated with serum ANA titer levels (r = 0.693, P = 0.016) (Fig. 6C). These findings suggest that activated Tfh cells were increased in the liver of patients with AIH, and the increase of activated Tfh cells in the blood might result from the increase of these cells in the liver of patients with AIH.

Accepted Article

This article is protected by copyright. All rights reserved.

Discussion

Accepted Article

In the present study, we found that the frequencies of circulating activated PD-1^{+} Tfh cells, especially circulating CCR7^{-}PD-1^{+} Tfh subset that is a main producer of IL-21, were significantly increased in patients with AIH at onset than in HC. In addition, we found significant associations between the frequency of circulating CCR7^{-}PD-1^{+} Tfh subset and clinical parameters, including the levels of serum IgG, PT, and albumin, in patients with AIH at onset. Because Tfh cells, especially CCR7^{-}PD-1^{+} Tfh subset, could promote naïve and memory B cells to differentiate into plasma cells and lead to the IgG production,[22,23] it may be compatible that the frequency of CCR7^{-}PD-1^{+} Tfh subset was correlated with serum IgG levels. Our results also suggest that the increase of circulating CCR7^{-}PD-1^{+} Tfh subset was associated with the severity and progression of inflammation in the liver of patients with AIH. Moreover, the frequencies of CCR7^{-}PD-1^{+} Tfh cells were significantly decreased after the level of serum ALT was normalized with PSL treatment. Therefore, these results suggest a potential role of dysregulated Tfh subset in the pathogenesis of AIH.

This article is protected by copyright. All rights reserved.

In humans, blood Tfh cells are currently considered to represent a circulating memory compartment of Tfh lineage cells,[16,24] and blood memory Tfh cells can be subdivided into three major subsets: Tfh1, Tfh2, and Tfh17 subsets.[12] Previous studies have suggested that an inactivated Tfh2 and/or Tfh17 subsets and a decrease of Tfh1 cell subsets within Tfh cells might be shared by multiple autoimmune diseases.[13-16] In the present study, we found an imbalance of Tfh1/Tfh2 subsets in patients with PBC although we did not find any significant change in the balance of Tfh1/Tfh2 subsets in AIH patients. Wang *et al.* reported that the frequency of circulating Tfh cells was significantly increased in patients with PBC, and that the frequency of these cells was significantly decreased in UDCA responders compared to UDCA-treated nonresponders.[11] Because we first focused on the alterations of Tfh subsets in patients with AIH and could not include enough number of patients with newly diagnosed PBC, we described the frequencies of Tfh subsets only in UDCA-treated patients with PBC. Therefore, we should consider the effect of UDCA treatment on our results. However, we found an increased frequency of Tfh2 subset and a decrease of Tfh1 subset in patients with PBC, and we believe that our results provide further information on the immunopathogenesis of PBC.

This article is protected by copyright. All rights reserved.

Among the Tfh subsets, the expression of CCR7 is the lowest in ICOS^{+}PD-1^{+} Tfh cells,[19,21] and the differential expression levels of CCR7 in circulating memory Tfh subsets might reflect their distinct propensity to enter B cell follicles *in vivo*.[12] Upon subsequent antigenic stimulation, these memory cells may quickly form Tfh cells and promote germinal center (GC) responses.[25] Uncontrolled generation of circulating Tfh cells may reflect GC dysregulation and play an important role in amplifying autoreactive B cells, promoting pathogenic autoantibody production, the onset of clinical symptoms, continued immune responsiveness, and eventually irreversible tissue damage.[26] We found that the frequencies of activated Tfh cells in the liver of patients with AIH were significantly increased compared to those in the blood, and they were positively correlated with each other. Although the precise mechanisms by which Tfh cells accumulate in inflammatory sites in humans remain largely unknown,[24] we speculate that both the circulating Tfh cells and accumulating Tfh cells in the liver could reflect GC dysregulation and play an important role in the induction and progression of AIH.

Accepted Article

This article is protected by copyright. All rights reserved.

Besides the increased frequency of $CCR7^{-}PD\text{-}1^{+}$ Tfh subset, we found that stimulated circulating Tfh cells in patients with AIH produced higher levels of IL-21, IFN-γ and IL-17 compared to those in HC. These results suggest that Tfh cells in patients with AIH were more sensitive to stimulations than those in HC. IL-21 is predominantly produced by Tfh cells and regulates humoral responses, and has also been shown to be an important factor for the differentiation of the Th17 lineage.[27-29] Therefore, the increase in IL-17 producing Tfh cells in patients with AIH might be a consequence of increased IL-21 production. Because patients with autoimmune diseases have increased activated memory Tfh17 cells in the blood, the increase of IL-17 production from Tfh cells might also be associated with the autoimmune mechanisms in AIH.

There are several limitations associated with the present study. First, we noticed that the number of enrolled subjects was relatively small. Second, we could not analyze functions of Tfh cells in the liver because of a small number of cells obtained from the liver. Third, we did not examine the numbers of different subsets of B cells and the functional interaction between Tfh cells and B cells. As the associations between the frequencies of Tfh cells and B cells and the ability of Tfh cells to promote B cell maturation were reported in

Accepted Article

This article is protected by copyright. All rights reserved.

patients with AIH and PBC,[10,11,30] further investigations of associations of different Tfh subsets with the number of B cell subsets and the function of B cells in a larger cohort of patients with AIH are necessary. However, to the best of our knowledge, this is the first study that revealed an increase of circulating CCR7^{-}PD-1^{+} Tfh subset in the blood and an increase of activated Tfh subsets in the liver of patients with AIH at onset, and an imbalance of Tfh1/Tfh2 subsets in patients with PBC. Therefore, we believe that these results significantly extend our understanding of Tfh subsets especially in AIH, and suggest a potential role of dysregulated Tfh subsets in the pathogenesis of AIH.

Acknowledgement: We thank T. Tsuchida for his excellent technical assistance. We also thank all the patients who participated in the study.

This study was supported in part by Grants-in-Aid for Scientific Research (C) (15K08991 to S.Y.) and (B) (26293175D to S.T.) from Japan Society for the Promotion of Science (JSPS).

Accepted Article

This article is protected by copyright. All rights reserved.

References

1 Krawitt EL. Autoimmune hepatitis. *N. Engl. J. Med.* 2006; **354**: 54-66.

2 Manns MP, Czaja AJ, Gorham JD, Krawitt EL, Mieli-Vergani G, Vergani D *et al.* Diagnosis and management of autoimmune hepatitis. *Hepatology*. 2010; **51**: 2193-213.

3 Montano-Loza AJ, Carpenter HA, Czaja AJ. Improving the end point of corticosteroid therapy in type 1 autoimmune hepatitis to reduce the frequency of relapse. *Am. J. Gastroenterol.* 2007; **102**: 1005-12.

4 Maruoka R, Aoki N, Kido M, Iwamoto S, Nishiura H, Ikeda A *et al.* Splenectomy prolongs the effects of corticosteroids in mouse models of autoimmune hepatitis. *Gastroenterology*. 2013; **145**: 209-20.

5 Lapierre P, Johanet C, Alvarez F. Characterization of the B cell response of patients with anti-liver cytosol autoantibodies in type 2 autoimmune hepatitis. *Eur. J. Immunol.* 2003; **33**: 1869-78.

6 McHeyzer-Williams LJ, McHeyzer-Williams MG. Antigen-specific memory B cell development. *Annu. Rev. Immunol.* 2005; **23**: 487-513.

7 Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. *Immunity*. 2009; **30**:

Accepted Article

This article is protected by copyright. All rights reserved.

324-35.

8 King C, Tangye SG, Mackay CR. T follicular helper (T_{FH}) cells in normal and dysregulated immune responses. *Annu. Rev. Immunol.* 2008; **26**: 741-66.

9 Aoki N, Kido M, Iwamoto S, Nishiura H, Maruoka R, Tanaka J *et al.* Dysregulated generation of follicular helper T cells in the spleen triggers fatal autoimmune hepatitis in mice. *Gastroenterology*. 2011; **140**: 1322-33.

10 Ma L, Qin J, Ji H, Zhao P, Jiang Y. Tfh and plasma cells are correlated with hypergammaglobulinaemia in patients with autoimmune hepatitis. *Liver Int.* 2014; **34**: 405-15.

11 Wang L, Sun Y, Zhang Z, Jia Y, Zou Z, Ding J *et al.* $CXCR5^+$ $CD4^+$ T follicular helper cells participate in the pathogenesis of primary biliary cirrhosis. *Hepatology*. 2015; **61**: 627-38.

12 Schmitt N, Bentebibel SE, Ueno H. Phenotype and functions of memory Tfh cells in human blood. *Trends Immunol.* 2014; **35**: 436-42.

13 Che Y, Qiu J, Jin T, Yin F, Li M, Jiang Y. Circulating memory T follicular helper subsets, Tfh2 and Tfh17, participate in the pathogenesis of Guillain-Barre syndrome. *Sci. Rep.* 2016; **6**: 20963.

Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article

14 Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G *et al.* Human blood CXCR5^{+}CD4^{+} T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity*. 2011; **34**: 108-21.

15 Li XY, Wu ZB, Ding J, Zheng ZH, Li XY, Chen LN *et al.* Role of the frequency of blood CD4^{+} CXCR5^{+} CCR6^{+} T cells in autoimmunity in patients with Sjogren's syndrome. *Biochem. Biophys. Res. Commun.* 2012; **422**: 238-44.

16 Ueno H. Human circulating T follicular helper cell subsets in health and disease. *J. Clin. Immunol.* 2016; **36 Suppl 1**: 34-9.

17 Lindor KD, Gershwin ME, Poupon R, Kaplan M, Bergasa NV, Heathcote EJ. Primary biliary cirrhosis. *Hepatology*. 2009; **50**: 291-308.

18 Alvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL *et al.* International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J. Hepatol.* 1999; **31**: 929-38.

19 Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL *et al.* Human circulating PD-1^{+}CXCR3^{-}CXCR5^{+} memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity*. 2013; **39**: 758-69.

This article is protected by copyright. All rights reserved.

20 Bentebibel SE, Lopez S, Obermoser G, Schmitt N, Mueller C, Harrod C *et al.* Induction of ICOS^{+}CXCR3^{+}CXCR5^{+} T_H cells correlates with antibody responses to influenza vaccination. *Sci. Transl. Med.* 2013; **5**: 176ra32.

21 He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N *et al.* Circulating precursor CCR7^{lo}PD-1^{hi} CXCR5^{+} CD4^{+} T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity.* 2013; **39**: 770-81.

22 Odendahl M, Jacobi A, Hansen A, Feist E, Hiepe F, Burmester GR *et al.* Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. *J. Immunol.* 2000; **165**: 5970-9.

23 Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B *et al.* Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science*. 2009; **325**: 1006-10.

24 Ueno H, Banchereau J, Vinuesa CG. Pathophysiology of T follicular helper cells in humans and mice. *Nat. Immunol.* 2015; **16**: 142-52.

25 Papp G, Szabo K, Szekanecz Z, Zeher M. Follicular helper T cells in autoimmune diseases. *Rheumatology*. 2014; **53**: 1159-60.

26 Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG *et al.* Expansion of circulating T cells resembling follicular helper T cells is a

Accepted Article

This article is protected by copyright. All rights reserved.

fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum.* 2010; **62**: 234-44.

27 Marwaha AK, Crome SQ, Panagiotopoulos C, Berg KB, Qin H, Ouyang Q *et al.* Cutting edge: Increased IL-17-secreting T cells in children with new-onset type 1 diabetes. *J. Immunol.* 2010; **185**: 3814-8.

28 Bettelli E, Korn T, Kuchroo VK. Th17: the third member of the effector T cell trilogy. *Curr. Opin. Immunol.* 2007; **19**: 652-7.

29 Yang L, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M *et al.* IL-21 and TGF-beta are required for differentiation of human T_H17 cells. *Nature*. 2008; **454**: 350-2.

30 Zhu C, Ma J, Liu Y, Tong J, Tian J, Chen J *et al.* Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease. *J. Clin. Endocrinol. Metab.* 2012; **97**: 943-50.

Accepted Article

This article is protected by copyright. All rights reserved.

Table 1. Patient characteristics

Parameters (Median, range)	AIH		PBC	CHB	Healthy Controls
	At onset	Treated			
n	12	23	28	22	44
Age (years)	66 (52-76)	64 (31-87)	69 (45-90)	58 (32-81)	31 (23-83)
Gender (M / F)	1 / 11	1 / 22	5 / 23	13 / 9	19 / 25
Treatments					
PSL	0 (0%)	13 (56.5%)	0 (0%)	0 (0%)	
UDCA	5 (41%)	22 (95.7%)	28 (100%)	2 (10.0%)	
Laboratory data					
AST (IU/l)	106 (23-699)	21 (8-195)	30.0 (17-49)	24.0 (14-59)	-
ALT (IU/l)	116 (12-1001)	14 (7-403)	24.5 (6-59)	24.5 (12-103)	-
T.Bil (IU/l)	1.1 (0.5-3.6)	0.7 (0.2-1.8)	0.6 (0.4-2.0)	0.75 (0.3-2.0)	-
Alb (g/dl)	3.9 (2.7-4.6)	4.0 (3.2-4.6)	4.1 (3.4-4.7)	4.3 (3.7-5.2)	-
PT (%)	84.5 (46-101)	107 (89-120)	104.5 (83-116)	99 (75-107)	-
IgG (mg/dl)	2005 (1265-3968)	1499 (835-2674)	1430 (868-2764)	1356 (695-1976)	-
ANA (titer)	16.0 (5.1-65.6)	16.8 (5-133.4)	39.95 (5-134.3)	-	-

M, male; F, female; PSL, prednisolone; UDCA, ursodeoxycholic acid; AST, asparate aminotransferase; ALT, alanine transaminase; T.Bil, total bilirubin; Alb, albumin, PT, prothrombin time; ANA, antinuclear antibody.

This article is protected by copyright. All rights reserved.

Fig. 1A

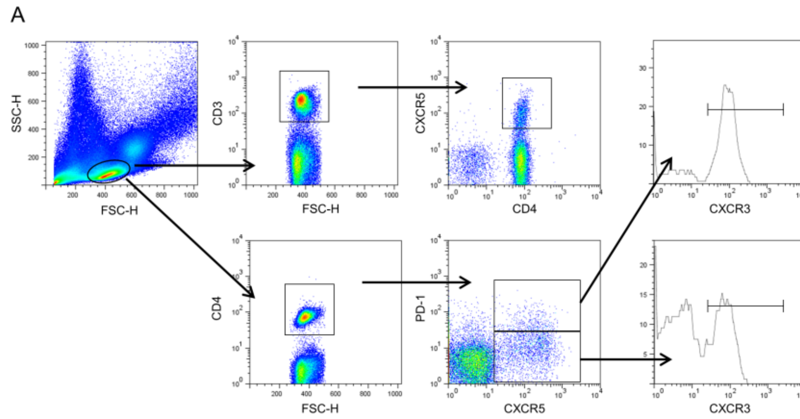

Fig. 1BC

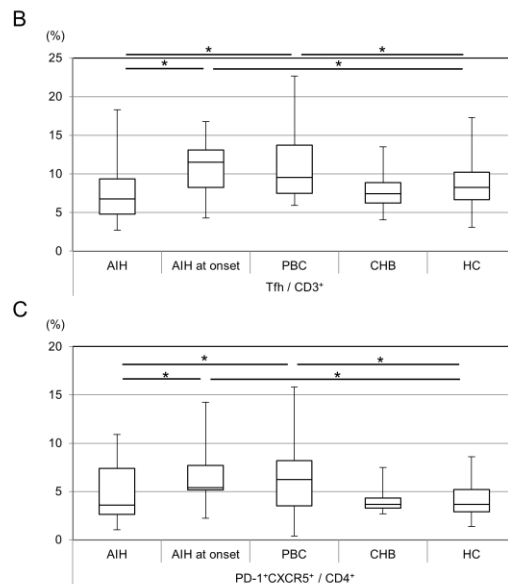

Figure 1. Increased frequencies of follicular helper T (Tfh) cells and PD-1$^+$ Tfh cells in AIH at onset and PBC. (**A**) Representative results of CXCR5$^+$CD4$^+$ among CD3$^+$ cells (Tfh/CD3$^+$) and CXCR5$^+$PD-1$^+$ cells among CD4$^+$ cells (CXCR5$^+$PD-1$^+$/CD4$^+$), which were detected by flow cytometry, are shown. (**B, C**) The frequencies of Tfh cells (**B**) and PD-1$^+$Tfh cells (**C**) were significantly increased in the blood of AIH at onset and PBC compared to those in patients with CHB and HC. $^*P$ <0.05.

This article is protected by copyright. All rights reserved.

Fig. 2

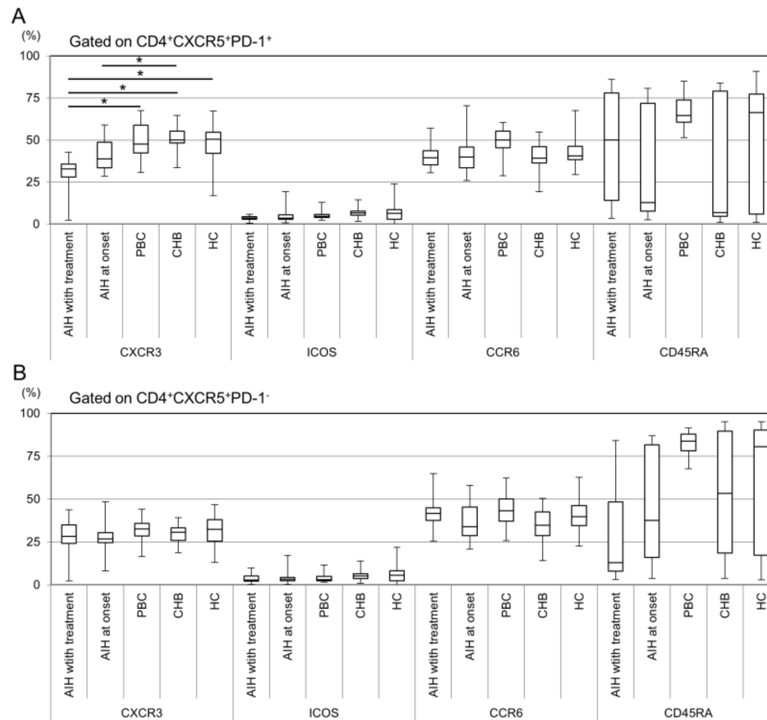

Figure 2. Expression of surface markers in PD-1$^+$ and PD-1$^-$ Tfh cells. (**A**) Representative results of flow cytometric analysis of CD4$^+$CXCR5$^+$PD-1$^+$ and PD-1$^-$ cells are shown. (**B, C**) Proportions of positivity of surface markers, including CXCR3, ICOS, CCR6, and CD45RA, in PD-1$^+$ (**B**) and PD-1$^-$ Tfh cells (**C**) in the blood are shown. Although the expression of CXCR3 was higher in PD-1$^+$ Tfh cells than PD-1- Tfh cells, no significant differences were found in the expression of those markers on Tfh cells among the groups.

Accepted

This article is protected by copyright. All rights reserved.

Fig. 3A

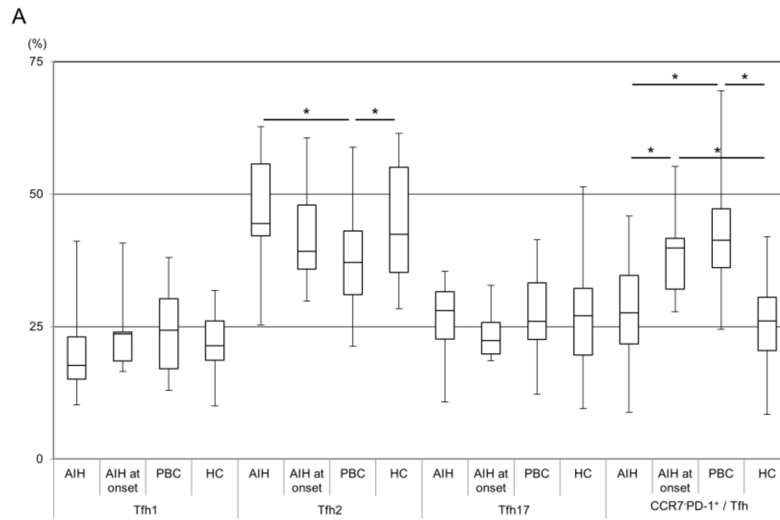

Fig. 3B-E

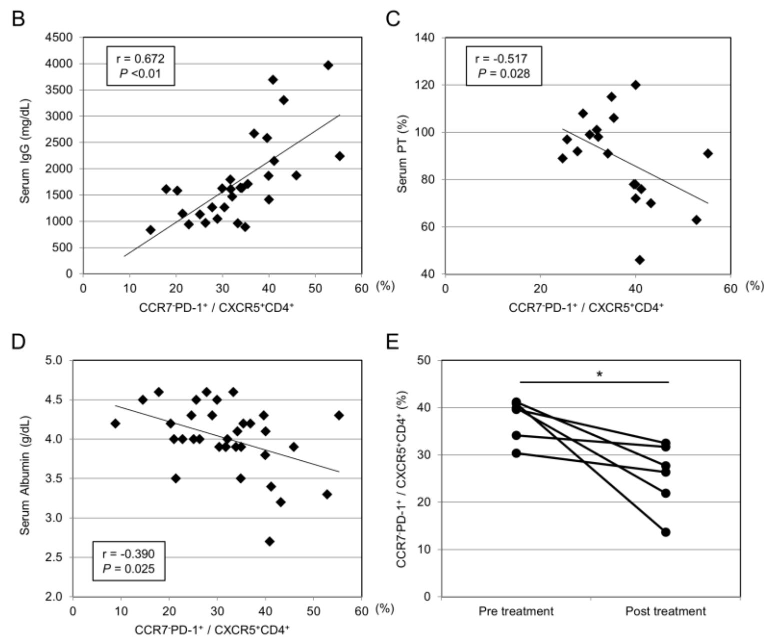

Figure 3. Changes in Tfh1 and Tfh2 subsets and increase of CCR7^{-}PD-1^{+} Tfh subset in AIH at onset and PBC. (**A**) Frequencies of Tfh1 (CXCR3^{+}CCR6^{-} cells), Tfh2 (CXCR3^{-}CCR6^{-} cells), Tfh 17 (CXCR3^{-}CCR6^{+} cells), and activated CCR7^{-}PD-1^{+} Tfh subsets among total Tfh cells are shown. In

This article is protected by copyright. All rights reserved.

patients with PBC, the frequency of Tfh2 subset was significantly decreased. The frequencies of activated $CCR7^{-}PD\text{-}1^{+}$ Tfh subset were significantly increased in patient with AIH at onset and PBC. (**B-D**) The frequency of $CCR7^{-}PD\text{-}1^{+}$ Tfh subset was positively correlated with serum IgG (**B**) and negatively correlated with PT-INR levels (**C**) and serum albumin levels in patients with AIH (**D**). (**E**) The frequency of $CCR7^{-}PD\text{-}1^{+}$ Tfh subset was significantly decreased in patients with AIH (n = 6) after treatment with PSL. $^{*}P = 0.040$.

Accepted Article

This article is protected by copyright. All rights reserved.

Fig. 4

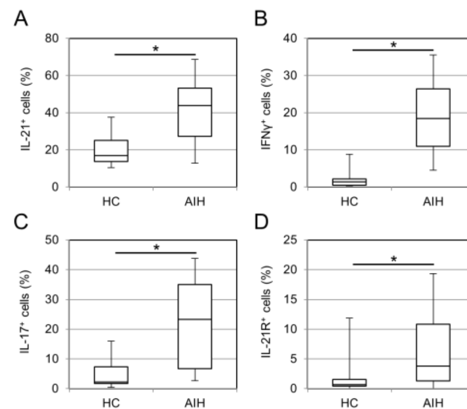

Figure 4. Cytokine production by Tfh cells. The frequencies of IL-21$^+$ cells (**A**), IFN-γ^+ cells (**B**), IL-17$^+$ cells (**C**), and IL-21R$^+$ cells (**D**) among CD4$^+$CXCR5$^+$ Tfh cells are shown. CD4$^+$CXCR5$^+$ Tfh cells in AIH patients with treatment (n = 11) produced higher levels of IL-21, IFN-γ, and IL-17 than those in HC (n = 14) when stimulated with PMA/ionomycine. The expression of IL-21R was significantly increased in patients with AIH compared to HC. $^*P$ <0.05.

Accepted

This article is protected by copyright. All rights reserved.

Fig. 5

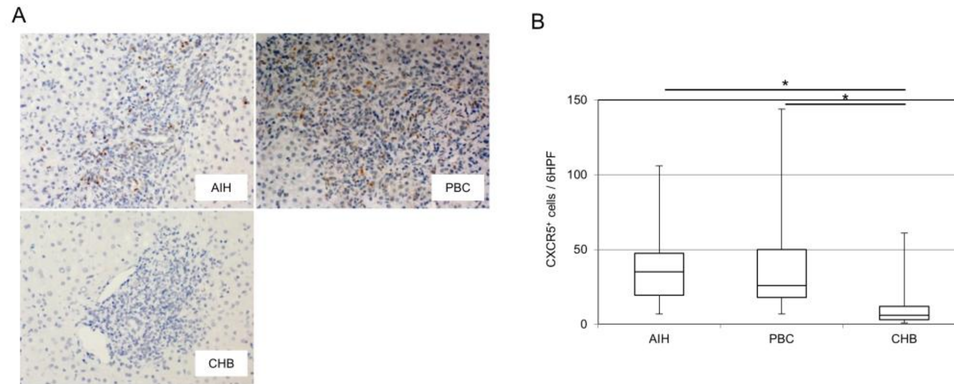

Figure 5. Increase in total Tfh cells in the liver of patients with AIH. (**A**) Representative results of immunohistochemical staining of CXCR5 in the liver of patients with AIH (n = 19), PBC (n = 26) and CHB (n = 12) are shown. (**B**) The number of $CXCR5^{+}$ cells, which indicate Tfh cells, per 6 high power fields (HPF) were significantly increased in the liver of patients with AIH and PBC compared to patients with CHB. $^{*}P < 0.05$.

Accepted

This article is protected by copyright. All rights reserved.

Fig. 6

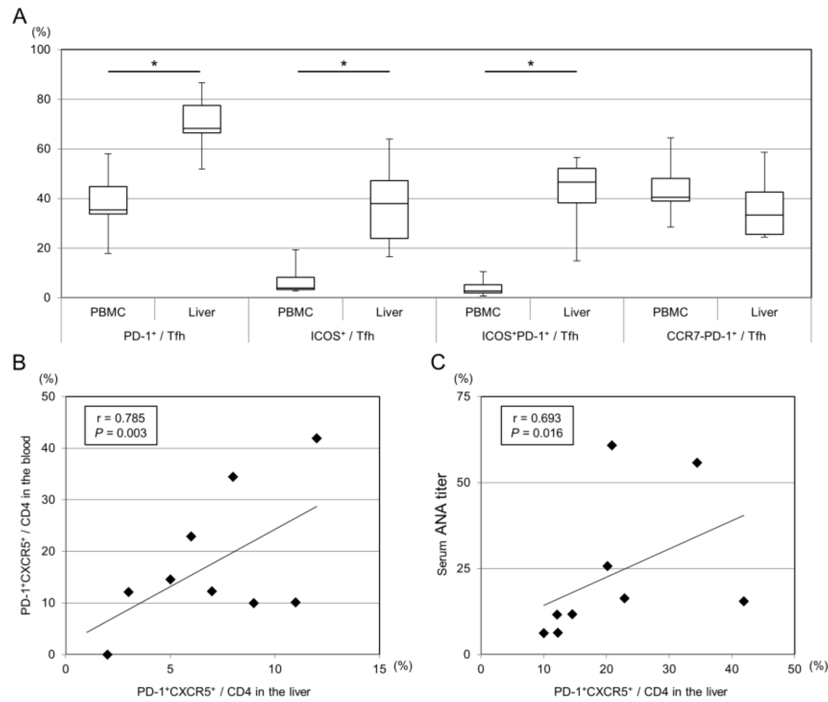

Figure 6. Increase in total Tfh cells and activated Tfh subset in the liver of patients with AIH. (**A**) Flow cytometry analysis showed the frequency of Tfh cells in the liver were significantly increased compared to that in the blood of patients with AIH (n = 10). Especially, the frequencies of activated PD-1^+, $ICOS^+$, and PD-1^+ICOS^+ Tfh subsets were significantly increased in the liver compared to those in the blood of patients with AIH. (**B**) The frequency of PD-1^+CXCR5^+ cells among $CD4^+$ cells in the liver was positively correlated with that in the blood of patients with AIH. (**C**) The frequency of PD-1^+CXCR5^+ cells among $CD4^+$ cells in the liver was positively correlated with serum ANA titer levels in patients with AIH. $^*P$ <0.05.

This article is protected by copyright. All rights reserved.