

Th2 and Regulatory Immune Reactions Are Increased in Immunoglobulin G4-Related Sclerosing Pancreatitis and Cholangitis

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Immunoglobulin G (IgG) 4-related sclerosing pancreatitis and cholangitis (autoimmune pancreato-cholangitis [AIPC]) are recently recognized disease entities characterized by high serum IgG4 concentrations and sclerosing inflammation with numerous IgG4-positive plasma cells, although the underlining immune mechanism remains only speculative. In this study, the immunopathogenesis of AIPC was examined with respect to the production of cytokines *in situ* and the possible involvement of regulatory T cells (Tregs) using fresh (5 cases) and formalin-fixed (28 cases) specimens of AIPC and related extra-pancreatobiliary lesions. Quantitative real-time polymerase chain reaction revealed that AIPC and extra-pancreatobiliary lesions had significantly higher ratios of interleukin (IL)-4/interferon- γ (IFN- γ) (45.8-fold), IL-5/IFN- γ (18.7-fold), IL-13/interferon (IFN)- γ (20.7-fold), IL-10/CD4 (45.3-fold), and tumor growth factor (TGF)- β /CD4 (39.4-fold) than did primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC). Lymphocytes with signals for IL-4 and IL-10 were frequently found in AIPC by *in situ* hybridization. The expression of Foxp3 messenger RNA, a transcription factor specific for naturally arising CD4⁺CD25⁺ Tregs, was significantly increased in AIPC and extra-pancreatobiliary lesions in comparison to PSC and PBC (36.4-fold). Immunohistochemically, CD4⁺CD25⁺Foxp3⁺ cells were frequently found in AIPC, while few were found in PSC and other disease controls. Taken together, AIPC could be characterized by the over-production of T helper (Th) 2 and regulatory cytokines. Tregs might be involved in the *in situ* production of IL-10 and TGF- β , which could be followed by IgG4 class switching and fibroplasia. **Conclusion: AIPC is a unique inflammatory disorder characterized by an immune reaction predominantly mediated by Th2 cells and Tregs. (HEPATOLOGY 2007;45:1538-1546.)**

Immunoglobulin (Ig) G4-related sclerosing diseases are rapidly emerging disease entities, the clinicopathological features of which have been studied extensively.^{1,2} In 2001, Hamano et al. reported that patients with

sclerosing pancreatitis, also called autoimmune pancreatitis (AIP), have high serum IgG4 concentrations.¹ AIP is pathologically characterized by a diffuse lymphoplasmacytic infiltration with marked interstitial fibrosis, acinar atrophy, eosinophilic infiltration, and obliterative phlebitis.² In addition, abundant IgG4-positive plasma cells can be identified by immunostaining.^{2,3} AIP is frequently associated with sclerosing cholangitis, which occurs mostly in the intra-pancreatic bile duct but can occur in other portions of the biliary tract.⁴ AIP and sclerosing cholangitis occur asynchronously in some cases. In addition, cases of IgG4-related sclerosing cholangitis without evident AIP have also been reported.⁵ Taken together, IgG4-related sclerosing lesions occurring in the pancreas and bile duct may belong to a single disease entity involving the different parts of the pancreatobiliary system (autoimmune pancreato-cholangitis [AIPC]).⁴

Recent studies have shown AIPC to be different from classical primary sclerosing cholangitis (PSC).^{6,7} Namely, AIPC is characterized by a high prevalence in middle-to-

Abbreviations: AIH, autoimmune hepatitis; AIP, autoimmune pancreatitis; AIPC, autoimmune pancreato-cholangitis; CD, cluster of differentiation; ISH, *in situ* hybridization; Foxp3, forkhead box P3; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; Tregs, regulatory T cells.

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Table 1. Summary of the Cases Used in This Study

	Number	Age (range)	Gender (male:female)	Material (n)
IgG4-related diseases				
Autoimmune pancreato-cholangitis	14*	53–78	12:2	Surgical, ¹² needle biopsy ^{2†‡}
Chronic sclerosing sialadenitis	9*	52–72	6:3	Surgical, ⁸ lip biopsy ^{1‡}
Chronic sclerosing dacryoadenitis	2	62–65	1:1	Surgical ^{2‡}
Pulmonary inflammatory pseudotumor	8	53–72	5:3	Surgical ⁸
Disease controls				
Primary sclerosing cholangitis	12	23–65	8:4	Transplantation, ^{11‡} wedge biopsy ^{1‡}
Primary biliary cirrhosis	2	58–60	0:2	Transplantation ^{2‡}
Sjögren's syndrome	5	34–46	4:1	Lip biopsy ⁵
Hepatolithiasis	5	45–79	3:2	Surgical ⁵
Sialolithiasis	5	32–55	2:3	Surgical ⁵

* Two patients had both autoimmune pancreato-cholangitis and chronic sclerosing sialadenitis.

† Biopsied from pancreas.

‡ Containing frozen sections.

old aged patients and effective steroid therapy.^{4,6} In contrast, PSC is a refractory disease which more frequently occurs in young patients or children with inflammatory bowel diseases.⁸ In addition, IgG4-related sclerosing lesions similar to AIPC have been identified in other organs such as the salivary gland (chronic sclerosing sialadenitis or Küttner's tumor),⁹ lacrimal gland (chronic sclerosing dacryoadenitis or Mikulicz's syndrome),¹⁰ liver (inflammatory pseudotumor),⁴ retroperitoneum (retroperitoneal fibrosis)^{3,11} and lung (interstitial pneumonia or inflammatory pseudotumor).¹² IgG4-related diseases of these extra-pancreatobiliary organs share the clinicopathological features of AIPC, thus suggesting that similar immune mechanisms participate in their pathogenesis.

The human immune system is controlled by subsets of T cells: T helper (Th) 1 and Th2 cells, and regulatory T cells (Tregs). Th1 cells produce interferon- γ (IFN- γ), whereas a Th2-type response is mediated by interleukin (IL) -4, IL-5, or IL-13. Tregs are capable of immune suppression, which is mediated by cell-cell contact or production of regulatory cytokines (IL-10 and tumor growth factor [TGF]- β).^{13,14} Among the several types of Tregs, those that are cluster of differentiation (CD) CD25⁺ have been the most often investigated. CD4⁺CD25⁺ Tregs are characterized by the expression of a specific transcription factor, forkhead box P3 (Foxp3), in addition to the co-expression of CD4 and CD25. CD4⁺CD25⁺ Tregs have been reported to play a key role in the prevention of autoimmune diseases, and the impairment of immune response by Tregs has been proposed in several autoimmune diseases. CD4⁺CD25⁺ Tregs are known to affected numerically and functionally in cases of autoimmune hepatitis and primary biliary cirrhosis (PBC).¹⁵

The immune mechanisms involved in IgG4-related diseases including AIPC have not yet been well clarified.

In particular, the mechanism responsible for the elevation in the serum IgG4 level or sclerosing inflammation with IgG4-positive plasma cells is still a mystery. In this study, we examined the immune mechanisms involved in AIPC with a focus on the production of cytokines *in situ* and the possible involvement of Tregs.

Patients and Methods

Patients

The cases used in this study are shown in Table 1. A total of 31 cases (33 lesions) of IgG4-related diseases were obtained from the pathology files of the Department of Human Pathology, Kanazawa University Graduate School of Medicine in Japan. They consisted of AIPC (14 cases), chronic sclerosing sialadenitis (9 cases), pulmonary inflammatory pseudotumor (8 cases), and chronic sclerosing dacryoadenitis (2 cases). Two of the patients had suffered from both AIPC and chronic sclerosing sialadenitis. Formalin-fixed and paraffin-embedded specimens obtained from the 28 cases were used for immunohistochemical studies. Frozen sections (5 cases), which were obtained during surgery for a frozen-section diagnosis from 2 cases of AIPC (needle biopsy from pancreatic head), 2 cases of chronic sclerosing dacryoadenitis (surgical excision), and one case of chronic sclerosing sialadenitis (excisional biopsy), were used for RNA extraction and *in situ* hybridization.

As disease controls, 12 cases of PSC, 2 cases of PBC, 5 cases of hepatolithiasis, 5 cases of Sjögren's syndrome (minor salivary glands), and 5 cases of sialolithiasis were used. Frozen sections of PSC (3 cases) and PBC (2 cases) were used as controls for PCR studies, and paraffin-embedded specimens from the remaining 24 cases were used for immunohistochemical studies.

All subjects provided written informed consent for surgery or biopsy and for the use of resected specimens in research.

Diagnosis of IgG4-Related Diseases

All cases of AIPC histologically showed severe lymphoplasmacytic infiltration with fibrosis, obliterative phlebitis, thickening of bile ducts, and atrophy of pancreatic acini. Immunostaining using a monoclonal antibody for human IgG4 (ZYMED Laboratories, San Francisco, CA) revealed abundant IgG4-positive plasma cells in areas of sclerosing inflammation in all AIPC specimens. In addition, two biopsied specimens (frozen sections) were found to have high serum IgG4 concentrations after biopsy (141 and 354 mg/dl; normal range <135). Out of 14 cases of AIPC, 10 showed both pancreatitis and cholangitis, while 3 cases and the remaining one case showed only cholangitis and pancreatitis, respectively. In the cases showing cholangitis, the hilar bile ducts were affected in 4 cases, the proximal common bile ducts in 2 cases, and the intra-pancreatic bile ducts in 7 cases.

Extra-pancreatobiliary lesions were also pathologically diagnosed as IgG4-related diseases according to histological features in previous studies.^{10,13} Three patients with chronic sclerosing sialadenitis had histories of AIPC. One patient was radiologically diagnosed as having AIPC, while the remaining two were surgically treated for suspected pancreatic or biliary cancer. Those specimens were included in the AIPC group in this study. The serum IgG4 concentration was measured in three cases (all frozen-section cases) of extra-pancreatobiliary lesions, and was high in all three (1420, 486, and 164 mg/dl).

RT-PCR

Total RNA was extracted from frozen sections in cases of IgG4-related diseases (5 cases) and disease controls (5 cases) using an RNeasy Mini Kit (QIAGEN, Valencia, CA). We performed RT-PCR for IFN- γ , IL-4, IL-5, IL-13, IL-10, TGF- β , Foxp3, CD4, and β -actin. The oligonucleotide sequences, product sizes, numbers of cycles and annealing temperatures of these primers were as follows: IFN- γ , forward 5'-TGACCAGAGCATCCAAAAGA-3', reverse 5'-CTCTTCGACCTCGAAACAGC-3', 236 bp, 35 cycles, 52°C; IL-4, forward 5'-TGCCTCCAAGAA-CACAACTG-3', reverse 5'-CTCTGGTTGGCTTC-CTTCAC-3', 219 bp, 35 cycles, 52°C; IL-5, forward 5'-TATGCCATCCCCACAGAAAT-3', reverse 5'-CAG-TACCCCCTTGCACAGTT-3', 199 bp, 37 cycles, 50°C; IL-13, forward 5'-GGTCAACATCACCCAGAACC-3', reverse 5'-TTTACAACTGGGCCACCTC-3', 240 bp, 37 cycles, 50°C; IL-10, forward 5'-AAGCCTGAC-CACGCTTTCTA-3', reverse 5'-ATGAAGTGGTTG-

GGAATGA-3', 193 bp, 35 cycles, 52°C; TGF- β , forward 5'-ACCAACTATTGCTTCAGCTC-3', reverse 5'-TTATGCTGGTTGTACAGG-3', 180 bp, 35 cycles, 52°C; Foxp3, forward 5'-CAAATGGTGTCTG-CAAGTGG-3', reverse 5'-CACAGATGAAGCCTTG-GTCA-3', 233 bp, 37 cycles, 51°C; CD4, forward 5'-AGGAAGTGAACCTGGTGGTG-3', reverse 5'-CTCAGCAGACACTGCCACAT-3', 190 bp, 35 cycles, 52°C; and β -actin, forward 5'-CAAGAGATGGC-CACGGCTGCT-3', reverse 5'-TCCTTCTGCATC-CTGTCCGCA-3', 334 bp, 30 cycles, 55°C. After PCR, 5- μ l aliquots of the products were subjected to either 1.5% or 2.0% agarose gel electrophoresis and then stained with ethidium bromide.

Real-time Quantitative PCR

Multiplex real-time polymerase chain reaction (PCR) was performed for quantitative analyses, according to the standard protocol using the TaqMan Universal PCR Master Mix (Applied Biosystems) and ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Specific primers and probes for IFN- γ , IL-4, IL-5, IL-13, IL-10, TGF- β , Foxp3, and CD4 were obtained from Applied Biosystems. The cycling conditions were as follows: incubation for 2 min at 50°C, for 10 minutes at 95°C, and 50 cycles of 15 seconds at 95°C and 1 min at 60°C. The Th2 cytokines (IL-4, IL-5 and IL-13) were normalized to a Th1 cytokine (IFN- γ). The regulatory cytokines (IL-10 and TGF- β) were similarly normalized to CD4. Each experiment was performed in triplicate, and the mean was adopted as the value in each experiment.

In situ Hybridization (ISH)

Single strand RNA probes for the IFN- γ , IL-4, and IL-10 genes were obtained by RT-PCR and *in vitro* transcription. The T7- or SP6-RNA polymerase promoter (T7, 5'-GTAATACGACTCACTATAGGGCGAWAS-3'; SP6, 5'-GATTTAGGTGACACTATAGA-3') was attached to each primer as follows: IFN- γ , sense 5'-T7-TTGGCTTTTCAGCTCTGCATCG-3', antisense 5'-SP6-TGCTCTTCGACCTTGAAACAGC-3', 471 bp; IL-4, sense 5'-T7-CCTCTGTTCTTCCTGCTAGCAT-3', antisense 5'-SP6-AACGTACTCTGGTTGGCTTCCT-3', 371 bp; IL-10, sense 5'-T7-AAGCCTGACCACGCTTTCTA-3', antisense 5'-SP6-TTCCATCTCCTGGGTTC-AAG-3', 463 bp. Each single-strand RNA probe complementary (antisense probe) and anti-complementary (sense probe; negative control) to the corresponding gene transcripts was obtained by *in vitro* transcription, according to the standard protocol of the Digoxigenin RNA Labeling Kit (Roche Diagnostics K.K., Indianapolis,

IN). Frozen sections obtained from those with IgG4-related diseases (5 cases) were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS, pH 7.4) for 15 min at 4°C. After rapid dehydration, the specimens were incubated with a hybridization solution mixed with the sense or antisense digoxigenin-labeled probe in a moist chamber at 50°C for 16 hours. Slides were washed in $0.2 \times$ sodium chloride-sodium citrate. After blocking reagent (Roche Diagnostics K.K.) was applied, the sections were incubated with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Roche Diagnostics K.K.) for 1 hour. Color development was achieved by adding a prepared substrate solution of chloro-3-indolyl phosphate toluidinium salt (175 μ g/ml) to the slides for approximately 7 hours in a darkroom.

Dual Fluorescent Immunostaining of CD4 and CD25

All formalin-fixed and paraffin-embedded specimens of AIPC (12 cases) and PSC (9 cases) were used for dual fluorescent immunostaining of CD4 and CD25. The deparaffinized sections were microwaved in citrate buffer (pH 6.0) for 20 min, and incubated in protein block solution (Dako Cytomation, Glostrup, Denmark) for 20 min. Specimens were incubated with a mouse monoclonal antibody to CD4 (clone 1F6, fully diluted, Nichirei, Tokyo, Japan) overnight at 4°C. The specimens were incubated for one hour at room temperature with goat anti-mouse immunoglobulins, which were conjugated to alkaline phosphatase-labeled polymers (Envision+; Dako Cytomation). The reaction product was visualized with Vector SG (Vector Laboratories, Burlingame, CA). The specimens were microwaved in citrate buffer (pH 6.0) for 5 min at 100°C to unfasten the first antibody.¹⁶ Then, the specimens were incubated with protein block solution (Dako Cytomation) for 20 min, and with a mouse monoclonal antibody to CD25 (clone 4C9, 1:100, Novocastra Laboratories, Newcastle, UK) overnight at 4°C. The reaction product was visualized with fluorescent goat anti-mouse and anti-rabbit IgG antibodies (1:500, Molecular Probes Inc., Eugene, OR), and observed under a confocal laser microscope (LSM5 PASCAL; Carl Zeiss, Oberkochen, Germany). No positive staining was obtained when the primary antibodies were omitted or replaced with normal mouse serum in the negative controls of the staining procedures.

Single Immunostaining

All formalin-fixed and paraffin-embedded specimens of IgG4-related diseases (28 cases) and disease controls (24 cases) were used for the immunostaining of Foxp3 and CD4. The immunostaining of Foxp3 or CD4 was

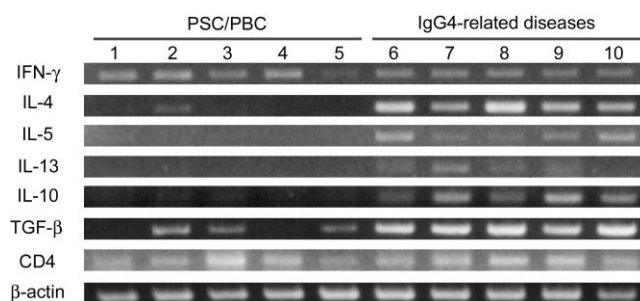


Fig. 1. Cytokine expression in IgG4-related diseases, PSC, and PBC (RT-PCR). The Th1 cytokine (IFN- γ) was similarly expressed in all cases examined. The Th2 cytokines (IL-4, IL-5, and IL-13) and regulatory cytokines (IL-10 and TGF- β) were more intensely expressed in IgG4-related diseases than in PSC and PBC. Lane 1-3, PSC; lane 4-5, PBC; lane 6-7, AIPC; lane 8-9, chronic sclerosing dacryoadenitis; lane 10, chronic sclerosing sialadenitis.

performed using a mouse monoclonal antibody against human Foxp3 (clone 136A/E7, 1:50, Abcam, Cambridge, UK) or CD4 (clone 1F6, fully diluted, Nichirei, Tokyo, Japan), respectively. The deparaffinized sections were pretreated in ethylenediaminetetraacetic acid buffer (pH 8.0) in a pressure-cooker at 100°C for 5 min (sections for Foxp3) or microwaved in citrate buffer (pH 6.0) for 20 minutes (sections for CD4). 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen. Cells positive for Foxp3 or CD4 were counted under 10 different high-power fields (hpf: 10 $\times$ eyepiece and 40 $\times$ lens) with intense inflammation, and the ratio (percentage) of the Foxp3-positive plasma cells/CD4-positive plasma cells was calculated in each case.

Statistics

A statistical analysis was performed using the Mann-Whitney U test to analyze continuous variables. The Pearson product-moment correlations were used to compare the expression ratios of Foxp3/CD4 obtained from real-time quantitative PCR and the serum IgG4 concentrations. A probability of $P < 0.05$ was considered to be statistically significant.

Results

Cytokine Expression in AIPC and Extra-Pancreatobiliary Lesions

The expression of Th1 (IFN- γ), Th2 (IL-4, IL-5 and IL-10), and regulatory cytokines (IL-10 and TGF- β) was examined in IgG4-related diseases (AIPC, 2 cases; chronic sclerosing dacryoadenitis, 2 cases; chronic sclerosing sialadenitis, one case) and 5 cases of autoimmune biliary diseases (PSC, 3 cases; PBC, 2 cases) by RT-PCR (Fig. 1). IFN- γ was similarly expressed in IgG4-related diseases, PSC, and PBC. IL-4, IL-5, and IL-13 were frequently expressed in IgG4-related diseases, whereas their expres-

sion was not identifiable in PSC and PBC except for one PSC case with weak expression of IL-4. The expression of IL-10 and TGF- β mRNA was also more frequent and intense among the IgG4-related diseases in comparison to PSC and PBC.

The ratio of Th1/Th2 cytokines and the expression of regulatory cytokines were quantitatively analyzed by real-time PCR. IL-4, IL-5, and IL-13 mRNA expression was not observed in most PSC and PBC cases by RT-PCR, while weak expression of IL-4, IL-5, and IL-13 was identifiable in two cases of PSC and one case of PBC by real-time PCR. The expression of IL-10 and TGF- β mRNA was also observed in all cases of PSC and PBC by real-time PCR. As shown in Fig. 2, IgG4-related diseases showed significantly higher expression ratios of IL-4/IFN- γ (45.8 fold), IL-5/IFN- γ (18.7 fold) and IL-13/IFN- γ (20.7 fold) than did PBC and PSC ($P < 0.001$). Similarly, the expression ratios of IL-10/CD4 and TGF- β /CD4 were significantly higher in IgG4-related diseases (45.3 and 39.4 fold, respectively; $P < 0.001$). These expression profiles of cytokines were similar in AIPC and related extra-pancreatobiliary lesions (Fig. 2).

ISH for IFN- γ , IL-4, and IL-10 in AIPC and Extra-Pancreatobiliary Lesions

ISH for IFN- γ , IL-4, and IL-10 was performed using frozen sections of AIPC (2 cases) and extra-pancreatobiliary lesions (3 cases). In addition to IFN- γ -positive lymphocytes, many lymphocytes positive for IL-4 and IL-10 were randomly identifiable in all cases of IgG4-related diseases (Fig. 3). Interestingly, lymphocytes positive for IL-10 surrounded a pancreatic duct branch in one AIPC case (Fig. 3). Except for lymphocytes, positive signals for these cytokine mRNAs were not evident in either epithelial or mesenchymal cells.

Foxp3 Expression in AIPC and Extra-Pancreatobiliary Lesions

The above-mentioned expression profile of cytokines suggested that IgG4-related diseases were characterized by an intense expression of Th2 and regulatory cytokines. Th2 cytokines are usually produced from CD4⁺ helper T cells, whereas the expression of regulatory cytokines is mainly mediated by Tregs.^{13,14} Thereafter, the expression of Foxp3, a transcription factor specific for CD4⁺CD25⁺ Tregs, was examined. RT-PCR for Foxp3 revealed a more frequent and intense expression of Foxp3 in IgG4-related diseases than in PSC or PBC (Fig. 4A). Next, the expression ratios of Foxp3/CD4 were quantitatively analyzed by real-time PCR. IgG4-related diseases showed significantly higher expression ratios of Foxp3/CD4 than PBC and PSC (36.4 fold, $P < 0.001$) (Fig. 4B). Regarding the

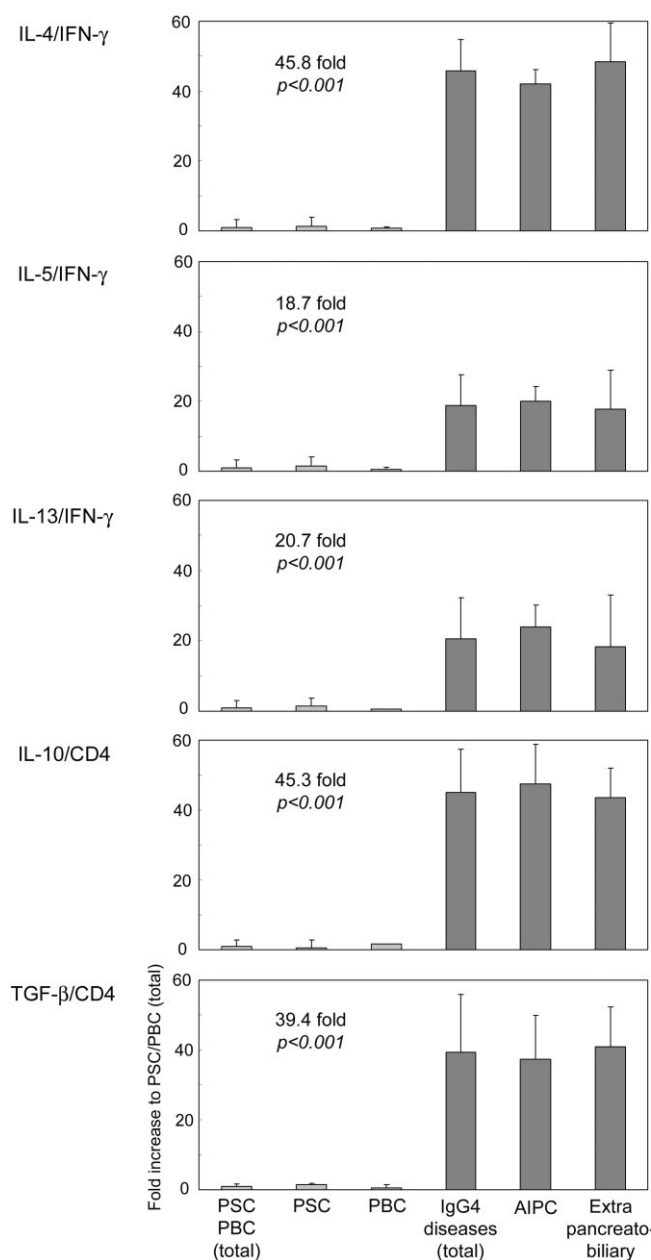


Fig. 2. Results of real-time quantitative PCR. The expression ratios of IL-4/IFN- γ , IL-5/IFN- γ , IL-13/IFN- γ , IL-10/CD4, and TGF- β /CD4 were significantly higher in IgG4-related diseases than in PSC and PBC. IgG4-related diseases include AIPC, chronic sclerosing dacryoadenitis, and chronic sclerosing sialadenitis.

expression ratios of Foxp3/CD4 and the serum IgG4 concentrations in IgG4-related diseases, no significant correlation was observed in the 5 cases examined ($r = 0.736$, $P = 0.156$).

Immunostaining of paraffin-embedded specimens revealed the nuclear expression of Foxp3 in relatively many lymphocytes in all cases of IgG4-related diseases, irrespective of the organs affected (Fig. 5). Foxp3-positive cells were randomly distributed within each lesion. In contrast,

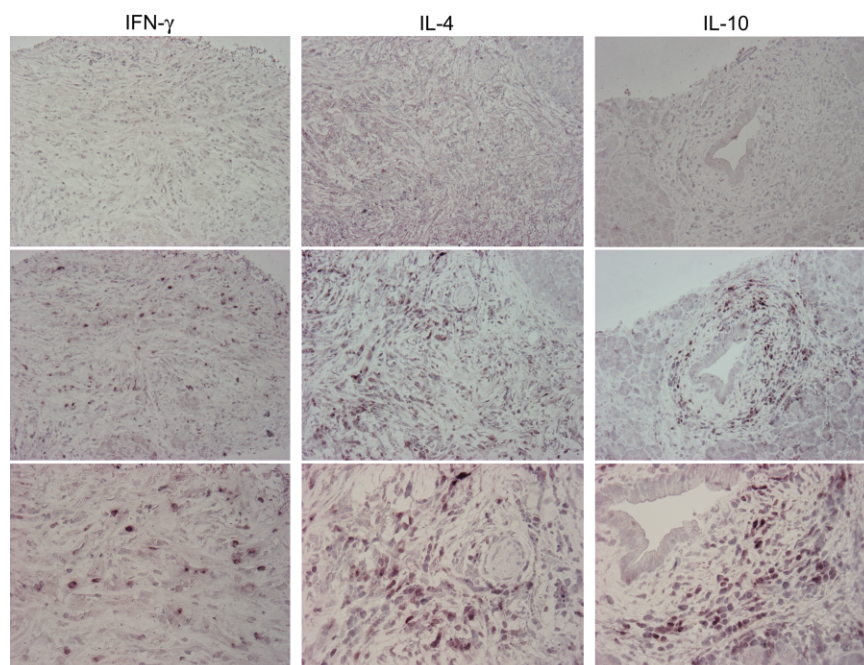


Fig. 3. *In situ* hybridization for IFN- γ , IL-4, and IL-10 in AIPC. In addition to IFN- γ -expressing cells, many mononuclear cells expressing IL-4 and IL-10 were observed in areas of sclerosing inflammation in cases of AIPC. Upper photos, negative control, $\times 200$ (sense probe); middle photos, $\times 200$ (antisense probe); lower photos, $\times 400$ (antisense probe). The upper and middle photos were obtained from almost the exact same fields.

Foxp3-positive cell infiltration was not prominent in disease controls including autoimmune diseases (PSC and Sjögren's syndrome) and non-autoimmune diseases (hepatolithiasis and sialolithiasis). In particular, Foxp3-positive cells were rare in PSC cases.

The ratios of Foxp3/CD4-positive cells were higher in IgG4-related diseases than in disease controls (Fig. 6). Among the disease controls, autoimmune diseases (PSC and Sjögren's syndrome) showed lower ratios of Foxp3/CD4-positive cells than the non-autoimmune diseases

(hepatolithiasis and sialolithiasis). In addition, the ratio of Foxp3/CD4 was significantly lower in PSC than in Sjögren's syndrome (Fig. 6).

Dual Immunostaining for CD4/CD25

CD4⁺CD25⁺ cells in AIPC and PSC were examined by dual immunostaining for CD4 and CD25 using paraffin-embedded specimens from AIPC and PSC patients. In AIPC, CD4-positive or CD25-positive cells were distributed diffusely within the lesions. Among them, a relatively large number of CD4⁺CD25⁺ cells were identifiable in AIPC (Fig. 7). CD4⁺CD25⁺ cells were randomly distributed in areas of pancreatitis and cholangitis. In PSC, many CD4-positive cells were observed around the injured bile ducts, whereas only a few CD4⁺CD25⁺ cells could be identified (Fig. 8).

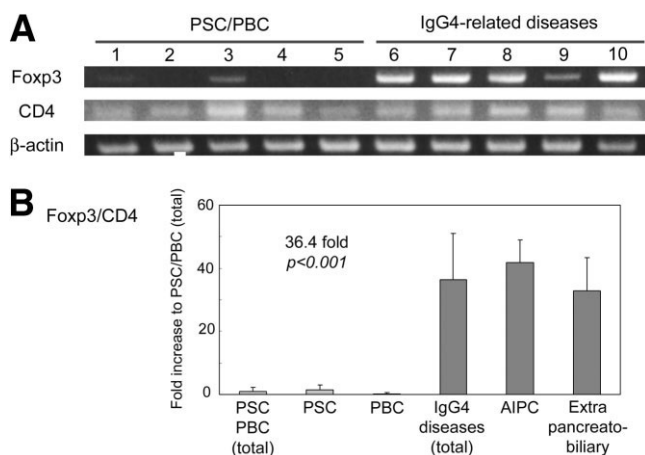


Fig. 4. Foxp3 expression in IgG4-related diseases, PSC, and PBC. (A) RT-PCR revealed a more intense expression of Foxp3 in IgG4-related diseases than in PSC and PBC. Lane 1-3, PSC; lane 4-5, PBC; lane 6-7, AIPC; lane 8-9, chronic sclerosing dacryoadenitis; lane 10, chronic sclerosing sialadenitis. (B) In the real-time PCR, the expression ratio of Foxp3/CD4 was significantly higher in IgG4-related diseases than in PSC and PBC.

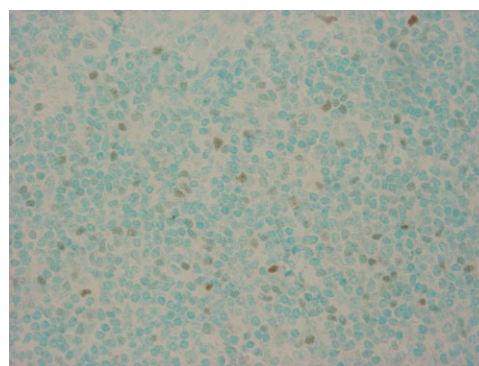


Fig. 5. Immunostaining of Foxp3 in AIPC. The nuclear expression of Foxp3 was observed in several mononuclear cells infiltrating the sclerosing lesion. ($\times 400$)

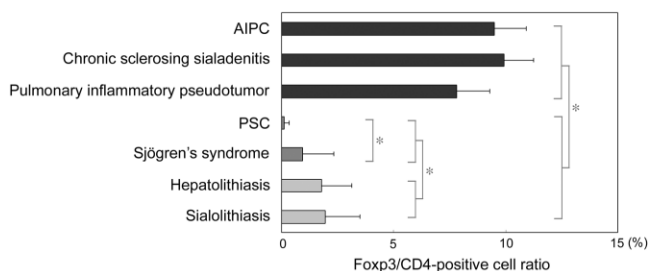


Fig. 6. Ratios of Foxp3/CD4-positive cells in IgG4-related diseases and disease controls (immunohistochemistry). Each IgG4-related disease showed a significantly higher ratio of Foxp3/CD4 in comparison to the corresponding disease control ($P = 0.0002$ - 0.012). Among the disease controls, PSC and Sjögren's syndromes had significantly lower ratios of Foxp3/CD4 than hepatolithiasis and sialolithiasis ($P = 0.002$ - 0.007). In addition, the Foxp3/CD4 ratio in PSC was significantly lower than that in Sjögren's syndrome ($P = 0.007$). *, $P < 0.05$.

Discussion

The main findings of this study are: (1) The expression of Th2 cytokines (IL-4, IL-5, and IL-13) and regulatory cytokines (IL-10 and TGF- β) was up-regulated in the affected tissues of AIPC. (2) The lymphocytes infiltrating AIPC produced IL-4 and IL-10 in addition to IFN- γ . (3) Significant numbers of CD4⁺CD25⁺Foxp3⁺ Tregs infiltrated the affected tissues in cases of AIPC. (4) These cytokine profiles and the infiltration of Tregs were similarly found in AIPC and related extra-pancreatobiliary lesions.

Th1/Th2 Balance in AIPC. Inflammatory conditions show a characteristic Th1/Th2 balance, and most autoimmune diseases are reported as Th1-predominant disorders.¹⁷ Interestingly, the cytokine profile of AIPC shown in this study suggested that the immunopathogenesis of AIPC is mediated by a Th2-predominant reaction. There have been two reports regarding cytokine production in IgG4-related diseases. In 2000, Okazaki et al. examined the Th1/Th2 balance in patients with AIP using peripheral blood lymphocytes.¹⁸ The number of CD4⁺ cells producing IFN- γ in peripheral blood and the se-

creted levels were significantly increased in AIP patients in comparison with the controls, whereas the number of IL-4-producing CD4⁺ cells was not increased in AIP patients. They concluded that AIP could be mediated by a Th1-predominant immune reaction. Recently, Yamamoto et al. serologically examined the IFN- γ /IL-4 ratio in patients with Mikulicz's disease (an IgG4-related lesion) and classical Sjögren's syndrome.¹⁰ The IFN- γ /IL-4 ratio was significantly higher in Mikulicz's disease than in Sjögren's syndrome. These results regarding peripheral blood lymphocytes and serum cytokine levels are not in line with our data. The discrepancy may be due to the specimens examined. The serum concentration of cytokines and peripheral blood lymphocytes might not be sufficient to precisely reflect cytokine production in the affected organs.

Tregs in AIPC. Large numbers of Tregs were found in the affected tissues in cases of AIPC in comparison with the autoimmune or non-autoimmune disease controls. While the mechanism of their induction or proliferation in the pancreatobiliary systems in cases of AIPC remains speculative, the following two possibilities should be considered. First, Tregs might be secondarily induced to inhibit Th2-predominant immune reactions in AIPC, because Tregs are usually activated by excessive immune reactions in certain types of infectious or allergic disorders and are known to prevent a Th2-type immune response.^{19,20} Second, the Tregs infiltrating the affected organs might be functionally abnormal. Namely, Tregs might proliferate abnormally such as via autocrine or paracrine pathways in the affected organs in cases of IgG4-related diseases. Viral infection is also known to induce a marked proliferation of lymphocytes. In fact, Chen et al. reported that HTLV-I-infected leukemia cells from a subset of patients with adult T cell leukemia expressed Foxp3.²¹ In this sense, AIPC could be a proliferative or potentially neoplastic disease. Excessive production of regulatory cytokines by proliferating Tregs

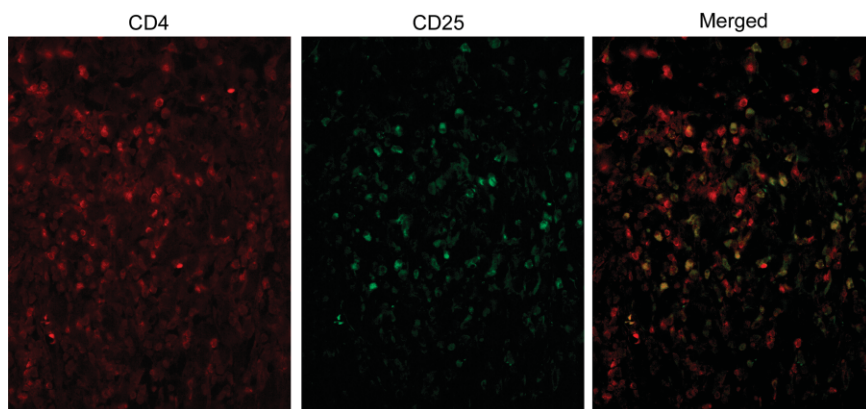


Fig. 7. Double immunostaining of CD4 and CD25 in AIPC. Mononuclear cells positive for CD4 or CD25 were detected. The merged image demonstrated many mononuclear cells double positive for CD4 and CD25 in areas of sclerosing inflammation. ($\times 200$)

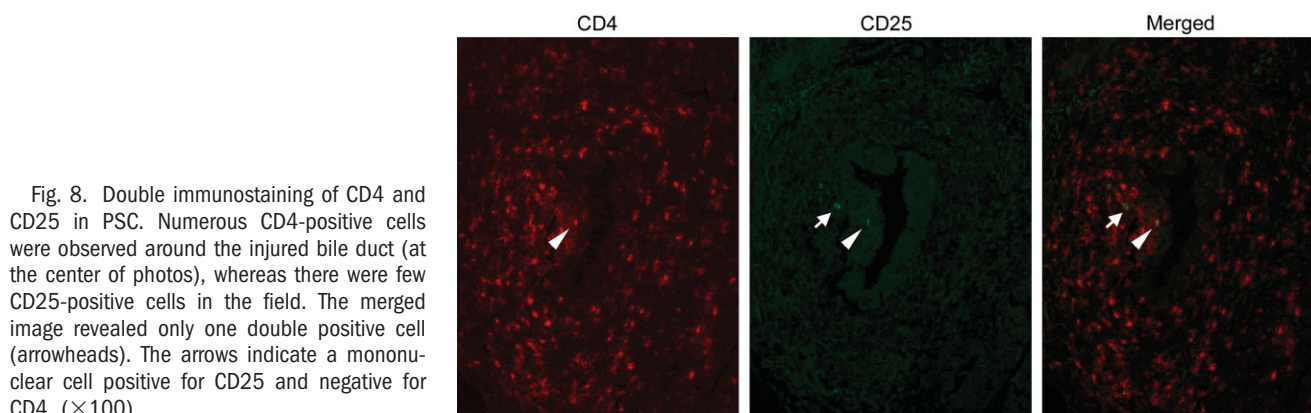


Fig. 8. Double immunostaining of CD4 and CD25 in PSC. Numerous CD4-positive cells were observed around the injured bile duct (at the center of photos), whereas there were few CD25-positive cells in the field. The merged image revealed only one double positive cell (arrowheads). The arrows indicate a mononuclear cell positive for CD25 and negative for CD4. ($\times 100$)

may be involved in the unique pathological features of IgG4-related diseases.

Relationship Between Cytokine Production and Histopathology. AIPC is histologically characterized by diffuse lymphoplasmacytic infiltration, irregular fibrosis, eosinophilic infiltration, obliterative phlebitis, and IgG4-positive plasma cell infiltration.^{2,4} Patients with IgG4-related diseases frequently showed peripheral blood eosinophilia or high serum concentrations of IgE.²² In addition, numerous mast cells have been reported to be found in pulmonary inflammatory pseudotumors (one type of pulmonary IgG4-related disease), although there have been no reports on mast cells in AIPC.²³ These pathological and immunological features of AIPC and related lesions might be mediated by IL-4, IL-5, and IL-13, because IL-4 is known to direct naive human B cells to switch to IgE production, and IL-5 is important for eosinophilic infiltration and activation.²⁴ IL-13 is another T-cell-derived cytokine involved in the activation of IgE production and eosinophilic infiltration.²⁴

Regulatory cytokines such as IL-10 and TGF- β might also be closely involved in the pathogenesis of AIPC. IL-10 secreted by Tregs may participate in the elevation of serum IgG4 concentrations and IgG4-positive plasma cell infiltration, because IL-10 has a major role in directing B cells to produce IgG4.^{25,26} In addition, TGF- β , which is a powerful fibrogenic cytokine, might participate in fibroplasia in the pancreas and bile ducts in AIPC. Therefore, it seems likely that IL-10 and TGF- β are involved in two major pathological findings of AIPC: IgG4-positive plasma cell infiltration and diffuse fibrosis.

Is AIPC an Autoimmune Disease or Not? IgG4 is the rarest IgG subclass and accounts for only 3%-6% of all the IgG in normal serum. High serum IgG4 concentrations have been reported in a limited number of diseases including bronchial asthma, pemphigus vulgaris, and pemphigus foliaceus.^{27,28} Pemphigus vulgaris and pemphigus foliaceus are autoimmune skin diseases caused by

autoantibodies against desmoglein 3 and desmoglein 1, respectively. IgG4 is the predominant type of autoantibody detected in pemphigus.²⁷ In contrast, antigen-injection immunotherapy for allergic diseases has also been reported to relate to IgG4 production. Exposure to a high dose of allergen during immunotherapy results in the generation of Tregs.^{20,28} Tregs can prevent Th2-type immune responses through cell-cell contact or the production of regulatory cytokines including IL-10 and TGF- β . IL-10 can direct B cells to switch from IgE to IgG4 antibody production.^{20,28} Therefore, IgG4 behaves as a pathogenetic antibody in pemphigus and as a suppressive antibody in allergic diseases.

AIP was first reported to be an autoimmune disease based on the presence of antinuclear antibodies, hyper γ -globulinemia, steroid sensitivity, and an association with other autoimmune diseases such as Sjögren syndrome and primary sclerosing cholangitis.²⁹ However, the evidence supporting the autoimmune hypothesis is now being reconsidered. Anti-nuclear antibodies or hyper γ -globulinemia are not detectable in some cases of AIPC.²² In addition, the sialadenitis and sclerosing cholangitis associated with AIPC had been described as Sjögren syndrome and PSC earlier on; however, the extra-pancreatobiliary lesions were elucidated to be IgG4-related diseases, not classical autoimmune diseases.^{4,9,10} Furthermore, the autoantigens of AIPC have not been demonstrated until now. The immune reactions predominantly mediated by Th2 cells and Tregs are not common in classical autoimmune diseases, but rather, closely involved in the pathogenesis of allergic disorders such as bronchial asthma and atopic dermatitis. Taken together, it seems premature to conclude that AIPC and related diseases are autoimmune disorders. The predominant Th2 and regulatory immune reactions in AIPC might reflect an allergic nature in their pathogenesis.

In conclusion, this study revealed that Th2 and regulatory immune reactions are up-regulated in the affected

tissues in cases of AIPC and related disorders. Th2 and regulatory cytokines may therefore be involved in the pathogenesis of these diseases.

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