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ORIGINAL ARTICLE

Long-term outcomes of autoimmune pancreatitis: a multicentre, international analysis

Phil A Hart,[1] Terumi Kamisawa,[2] William R Brugge,[3] Jae Bock Chung,[4] Emma L Culver,[5] László Czakó,[6] Luca Frulloni,[7] Vay Liang W Go,[8] Thomas M Gress,[9] Myung-Hwan Kim,[10] Shigeyuki Kawa,[11] Kyu Taek Lee,[12] Markus M Lerch,[13] Wei-Chih Liao,[14] Matthias Löhr,[15] Kazuichi Okazaki,[16] Ji Kon Ryu,[17] Nicolas Schleinitz,[18] Kyoko Shimizu,[19] Tooru Shimosegawa,[20] Roy Soetikno,[21] George Webster,[22] Dhiraj Yadav,[23] Yoh Zen,[24] Suresh T Chari[1]

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For numbered affiliations see end of article

Correspondence to
Dr Terumi Kamisawa, Department of Internal Medicine, Tokyo Metropolitan Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan; kamisawa@cick.jp

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ABSTRACT

Objective Autoimmune pancreatitis (AIP) is a treatable form of chronic pancreatitis that has been increasingly recognised over the last decade. We set out to better understand the current burden of AIP at several academic institutions diagnosed using the International Consensus Diagnostic Criteria, and to describe long-term outcomes, including organs involved, treatments, relapse frequency and long-term sequelae.

Design 23 institutions from 10 different countries participated in this multinational analysis. A total of 1064 patients meeting the International Consensus Diagnostic Criteria for type 1 (n=978) or type 2 (n=86) AIP were included. Data regarding treatments, relapses and sequelae were obtained.

Results The majority of patients with type 1 (99%) and type 2 (92%) AIP who were treated with steroids went into clinical remission. Most patients with jaundice required biliary stent placement (71% of type 1 and 77% of type 2 AIP). Relapses were more common in patients with type 1 (31%) versus type 2 AIP (9%, p<0.001), especially those with IgG4-related sclerosing cholangitis (56% vs 26%, p<0.001). Relapses typically occurred in the pancreas or biliary tree. Retreatment with steroids remained effective at inducing remission with or without alternative treatment, such as azathioprine. Pancreatic duct stones and cancer were uncommon sequelae in type 1 AIP and did not occur in type 2 AIP during the study period.

Conclusions AIP is a global disease which uniformly displays a high response to steroid treatment and tendency to relapse in the pancreas and biliary tree. Potential long-term sequelae include pancreatic duct stones and malignancy, however they were uncommon during the study period and require additional follow-up. Additional studies investigating prevention and treatment of disease relapses are needed.

Significance of this study

What is already known on this subject?
- Autoimmune pancreatitis (AIP) is a treatable form of chronic pancreatitis that is felt to be responsive to steroid treatment.
- There are few long-term data regarding response to treatment and subsequent disease sequelae.

What are the new findings?
- Disease relapses are common after steroid discontinuation, and typically occur in the pancreas and/or biliary tract.
- Pancreatic duct stones are relatively uncommon, but are seen more frequently in patients with at least one disease relapse.
- The occurrence of incident cancers following AIP diagnosis appears to be uncommon.

How might it impact on clinical practice in the foreseeable future?
- Since disease relapses are common, additional studies are needed to compare different treatment strategies for maintaining disease remission.
- Further investigations are needed to understand if the risk of cancer is increased compared with the general population.

INTRODUCTION

Autoimmune pancreatitis (AIP) is a unique form of chronic pancreatitis with characteristic histological features, frequent elevations of serum IgG4 levels and a predictable response to steroid therapy. Although the identification of a steroid-responsive form of chronic pancreatitis was initially reported in 1995 by Yoshida *et al*, there was minimal progress in understanding this disease until a serum biomarker (IgG4 antibody) was identified by Hamano *et al*.[1 2] Over the last decade significant progress has been made in understanding this disease, including identification of two distinct histological subtypes, with different clinical phenotypes (termed type 1 and type 2 AIP), incorporation of seemingly unrelated diseases within the spectrum of IgG4-related disease (of which AIP is the pancreas manifestation) and treatment of refractory patients with rituximab.[3–6]

Despite these advances, many questions remain unanswered. Although patients respond initially to steroid therapy, many patients will develop disease relapse either during steroid taper or following

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steroid discontinuation. Reported rates of disease relapse have ranged from 15–60% in various series.[4 7–10] Although there is general agreement that steroids are the ideal initial treatment, there is no clear consensus regarding treatment for disease relapses.

Due to the recent recognition of patients with this condition, the long-term sequelae of the disease are largely unknown. Follow-up data are recently becoming available, permitting the present analysis. In an effort to better understand these knowledge gaps we set out to perform an international analysis of patients with type 1 and type 2 AIP. One previous study evaluated the distribution of AIP subtypes worldwide, however multiple diagnostic criteria were used based on the country of origin.[11] Recently a multinational group met and agreed upon diagnostic criteria, termed International Consensus Diagnostic Criteria (ICDC).[12] This classification scheme categorises diagnostic evidence into one of two levels of confidence in the following categories: pancreatic parenchymal imaging, imaging of the pancreatic duct (ie, endoscopic retrograde pancreatogram), serum IgG4 level, other organ involvement of IgG4-related disease, histology of the pancreas (from core biopsy or resection) and response to steroid treatment. We specifically set out to gain additional understanding of the current burden of AIP at several large, academic institutions using the ICDC, and to describe the long-term outcomes of this disease including organs involved, treatments, relapse frequency and long-term sequelae.

METHODS

A total of 31 institutions were invited to participate in this study on the basis of their scientific merit in this field, or established experience in management of AIP; ultimately 23 institutions from 10 different countries participated. The Tokyo Metropolitan Komagome Hospital in Japan and Mayo Clinic in the USA served as the coordinating centres. The study was approved by the institutional review board of Tokyo Metropolitan Komagome Hospital and was in compliance with the Declaration of Helsinki.

In centres with existing patient databases, patient follow-up data were updated and retrieved. If data were not available, investigators retrospectively reviewed paper and/or electronic medical records or contacted patients by telephone for data collection. Each centre independently reviewed histological, radiographic, and clinical records of subjects with suspected AIP. Subjects classified as either definite or probable type 1 or type 2 AIP according to the ICDC were selected for this study (see online supplementary tables S1–S4).[12] The two subtypes are definitively distinguished based on their histology in which type 1 AIP (also known as lymphoplasmacytic sclerosing pancreatitis) demonstrates lymphoplasmacytic infiltration, obliterative phlebitis, storiform fibrosis and abundant IgG4-postive cells, while type 2 AIP (also known as GEL+ pancreatitis or idiopathic duct-centric chronic pancreatitis) shows granulocytic infiltration of the duct wall (termed GEL) and absent or minimal IgG4-postive cells. Additionally, type 2 AIP patients are unlikely to have serum IgG4 elevation or other organ involvement. Site data through 1 January 2012 were compiled using a standardised data collection form, then submitted to the lead investigator (TK) for analysis.

Definitions

For the purposes of this study, proximal biliary was defined as involvement of either intrahepatic bile ducts or the extrahepatic common bile duct proximal to the head of the pancreas. When it occurred in the context of type 1 AIP it was referred to as IgG4-related sclerosing cholangitis (IgG4-related SC)). On the other hand, distal biliary disease referred to disease isolated to the intrapancreatic portion of the common bile duct. Serum IgG4 values vary depending on the assay used, so normal levels were recognised as those less than the upper limit of normal for the lab where the test was performed. Pancreatic duct stones were identified with the use of either cross-sectional imaging or endoscopic retrograde pancreatography.

Treatment regimens

A wide variety of steroid regimens were employed for induction and maintenance of remission. For the initial dose of steroids, all centres used either a weight-based strategy (0.6 mg/kg/day of prednisolone) or fixed-dose regimen (30–40 mg/day) that were roughly equivalent for treatment of a 70 kg individual. Tapering strategies ranged from 5–10 mg decrease every 1–2 weeks. All Asian centres (n=10) used a maintenance strategy of low-dose (2.5–5 mg/day) prednisolone, which was continued for anywhere from 6 months to 3 years. In general, the European and North American groups tapered the steroids off within 3 months and did not provide maintenance doses of steroids. Multiple centres elected to use immunomodulator drugs instead of low-dose steroids for maintenance therapy (n=5). In the four centres treating more than five subjects with this strategy, azathioprine (2 mg/kg/day) was the preferred agent and was used for a variable duration of time (1–3 years).

Many patients initially underwent surgery either due to the absence of typical features of AIP or clinical presentation prior to the recognition of AIP as a disease entity. Surgeries were performed for resection of mass-forming lesions (ie, pancreaticoduodenectomy or distal splenectomy) or palliative bypass (ie, gastrojejunostomy) for those with an apparently unresectable cancer. Surgery was not intentionally performed as primary treatment for AIP. A number of patients were treated conservatively, without the use of steroids or surgery. Supportive care was provided for a variety of reasons including asymptomatic disease, severe comorbid disease (eg, metastatic cancer) or patient preference.

Statistical analyses

Continuous variables were compared using Student t test, χ^2 test and Fisher's exact t test (when one or more expected cell frequencies were <5) were used for comparison of proportions. A p value <0.05 was considered statistically significant.

RESULTS

AIP subject characteristics

A total of 1064 subjects were identified, 978 with type 1 AIP and 86 with type 2 AIP. The average age of subjects at diagnosis was 61.4 and 39.9 years for type 1 and type 2 subjects, respectively. The proportion of males was 77% in type 1 subjects and 55% in type 2 subjects (p<0.001). The proportion of patients diagnosed with type 2 AIP was lower in Asian countries (3.7%) compared with European (12.9%, p<0.001) and North American (13.7%, p<0.001) countries (figure 1).

Treatment response

The majority (74%) of subjects with type 1 AIP were initially treated with steroids, rather than surgical or conservative treatments, in comparison with type 2 subjects in which only 62% were treated with steroids (p=0.01). Remission was successfully induced in almost all subjects with type 1 and type 2 AIP (table 1). The per cent of subjects achieving remission was higher in type 1 subjects who received intervention (either

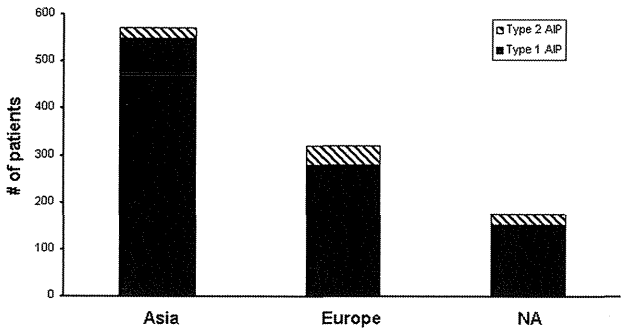

Figure 1 Regional distribution of type 1 and type 2 autoimmune pancreatitis based on the country of diagnosis. NA, North America.

steroids or surgery) (99.2%) compared with those who were managed conservatively (55.2%, p<0.001). However, initial remission rates were similar in patients with type 2 AIP who received intervention compared with conservative management (83.5% vs 66.7%, respectively, p=0.29). Interestingly many of the patients who underwent palliative surgical bypass achieved successful clinical remission; however the total number of cases was small. Initial treatment strategies and indications for treatment and concurrent therapies used in those receiving steroid treatment are also shown in table 1. Treatment for diabetes mellitus was given to a minority of patients prior to steroid treatment. However, biliary stenting was performed for most subjects presenting with jaundice. In subjects with type 1 AIP, jaundice (63%) was the most common indication, followed by abdominal pain with or without biochemical pancreatitis. In those with type 2 AIP, abdominal pain and inflammatory bowel disease were major indications.

In subjects with type 1 AIP and abnormal serum IgG4 levels (n=446) prior to steroids, the serum level decreased in 427 (95.7%) subjects and returned to within normal limits for 204 (45.7%). Of 609 type 1 AIP subjects with pancreatic enlargement at the time of diagnosis, the parenchyma appeared normal in 400 (65.7%), atrophic in 173 (28.4%) and persistently enlarged in 35 (5.9%) subjects following steroid treatment. In contrast, 50 type 2 AIP subjects had pancreatic enlargement at diagnosis. Following treatment the appearance returned to normal in the majority (43/50, 86%) with progression to atrophy in the remaining seven subjects.

Relapse data

Of the 978 subjects with type 1 disease, a total of 302 (31%) subjects experienced at least one disease relapse during the study period, compared with 8 (9%, p<0.001) subjects with type 2 AIP (table 2). The vast majority of relapse episodes occurred in steroid treated subjects following steroid discontinuation (67%), as compared with during the steroid taper (15%) or while on maintenance steroids (18%). Most relapses occurred in the biliary system or pancreas for type 1 AIP, while relapses in type 2 AIP were limited to the pancreas.

Table 1 Initial treatment strategies and treatment details for those treated with steroids

	Type 1 AIP (n=901†)		Type 2 AIP (n=85†)		
	Successful remission, n	%	Successful remission, n	%	
Initial treatment					
Steroids	681/684	99.6	48/52	92.3	
Surgical resection	125/127	98.4	17/25	68.0	
Palliative surgical bypass	22/23	95.7	1/2	50.0	
Conservative	37/67	55.2	4/6	66.7	
	Type 1 AIP (n=724)		Type 2 AIP (n=53)		
	n	%	n	%	p Value*
Indications for steroid treatment					
Jaundice	458	63	13	25	<0.001
Pancreatitis/abdominal pain	198	27	34	64	<0.001
Abnormal imaging (diffuse pancreatic enlargement, pancreas mass)	71	10	0	–	0.01
Salivary gland enlargement	49	7	0	–	0.04
Diagnostic steroid trial	46	6	4	8	0.77
Retroperitoneal fibrosis	17	2	0	–	0.62
IgG4-related renal disease	9	1.2	0	–	0.99
Lymphadenopathy	6	0.8	0	–	0.99
IgG4-related lung disease	4	0.6	0	–	0.99
Inflammatory bowel disease	1	0.1	23	48	<0.001
Other (hyperglycaemia, weight loss, etc)	20	3	0	–	
Diabetes management					
Oral medications	99/596	17	6/46	13	0.53
Insulin therapy	136/596	23	4/46	9	0.03
Endoscopic management (for subjects with jaundice)					
Biliary stent placement	351/492	71	10/13	77	0.77

*p Values represent comparison of proportions between patients with type 1 and type 2 AIP using χ^2 and Fisher's exact t test, when appropriate.
†Seventy-seven subjects with type 1 AIP and one subject with type 2 AIP are not displayed in the table due to pending response to treatment at study closure.
AIP, Autoimmune pancreatitis.

Table 2 Distribution of disease relapse episodes according to initial treatment strategies, and location and frequency for those treated with steroids

	Type 1 AIP		Type 2 AIP	
	Relapse, n	%	Relapse, n	%
Initial treatment				
Steroids	245/684	35.8	8/52	15.3
Surgical resection	35/116	30.2	0/25	0
Palliative surgical bypass	11/23	47.8	0/2	0
Conservative	11/57	19.3	0/6	0
Disease relapses following steroid treatment				
Location of relapse	n=245 episodes		n=8 episodes	
Biliary system	124	50.6	–	–
Pancreas	107	42.9	8	100
Salivary	18	7.3	–	–
Lung	11	4.5	–	–
Lymphadenopathy	4	1.6	–	–
Renal	3	1.2	–	–
Other (RPF or NOS)	13	5.3	–	–
Frequency per subject				
One relapse	189	77.1	8	100
Two relapses	39	15.9	–	–
Three relapses	13	5.3	–	–
≥4 relapses	4	1.6	–	–

AIP, Autoimmune pancreatitis; RPF, retroperitoneal fibrosis; NOS, not otherwise specified.

Table 3 Cumulative frequency of malignancies in type 1 AIP subjects

Cancer type	Subjects, n
Gastric	11
Lung	9
Prostate	7
Colon	5
Pancreatic	5
Oesophageal	4
Cholangiocarcinoma	3
Leukaemia	3
Ovarian	2
Renal	2
Other*	6

*Other cancers with only one reported case include: testicular, gastrointestinal stromal tumour, breast, bladder, hepatocellular and adenocarcinoma of unknown primary.
AIP, Autoimmune pancreatitis.

Predictors of relapse

The proportion of subjects having a relapse was similar in those with persistently abnormal IgG4 levels following steroids compared with those with a normal level (32.7% vs 31.4%, respectively, p=0.77). Likewise, the proportion of subjects with at least one relapse was similar regardless of whether they initially had diffuse (42/440, 32.3%) or focal pancreatic parenchymal enlargement (92/285, 32.3%, p=0.99). In contrast, 96/171 (56.1%) subjects with IgG4-related SC had at least one relapse, while only 142/551 (25.7%) subjects without IgG4-related SC had a relapse (p<0.001). The rates of relapse were similar in those with and without distal biliary disease (33.9% vs 31.1%, respectively, p=0.44). Since there were very few relapse episodes in subjects with type 2 AIP, a meaningful comparison of risk factors for relapse could not be completed.

Treatment for disease relapse

Steroids were the most commonly used treatment for managing disease relapse in type 1 AIP, and inducing remission was successful in 201/210 (95%) of subjects. The addition of azathioprine was used for 68 subjects with successful induction in 56 (85%). Medications used in other subjects (n=18) included mycophenolate mofetil (n=8), cyclosporine (n=3), methotrexate, 6-mercaptopurine, cyclophosphamide and rituximab. Successful remission was achieved in 12 (86%) of these subjects with follow-up.

Long-term sequelae in type 1 AIP

Pancreatic duct stones were uncommonly seen occurring in 46/659 (7%) subjects with follow-up imaging permitting evaluation for stone disease. Pancreatic duct stones were more likely to occur in subjects with at least one relapse, compared with those who had never had a relapse (14.4% vs 4.0%, respectively, p<0.001).

The most frequently occurring cancers during follow-up were gastric, lung and prostate (table 3). Importantly, pancreatic cancer was diagnosed in five male patients at a median age of 77 years (range 65–80) at the time of cancer diagnosis. All cancers were diagnosed more than 3 years following AIP diagnosis with the exception of one patient. His cancer was diagnosed 9 months following AIP diagnosis, which was made on the basis of diffuse pancreatic enlargement and elevation of serum IgG4 more than twice the upper limit of normal (definitive histology for type 1 AIP was confirmed on the resected pancreatic specimen). In the two patients with serum IgG4 levels obtained at the time of cancer diagnosis, it was mildly (1–2×upper limits) elevated. Eight (73%) of the subjects with gastric cancers were from study sites located in Japan or Korea, and risk factors for gastric cancer were not reported. No subjects with type 2 AIP developed an incident cancer or pancreatic duct stone during the study period.

DISCUSSION

This study represents the largest, multinational analysis of patients with type 1 and type 2 AIP diagnosed according to ICDC and provides insights into treatment strategies and potential long-term sequelae. Previously noted differences in clinical profiles of type 1 and type 2 AIP, including age and gender differences, were confirmed in this study.[4 11] Type 2 AIP represented a smaller proportion of AIP in Asian countries compared with European and North American countries.

Types 1 and 2 were highly-responsive to steroid treatment; however disease relapses were common in type 1, especially in those with proximal biliary disease (ie, IgG4-related SC). Most patients who required steroid therapy had predominantly pancreatobiliary disease manifestations (jaundice, abdominal pain or abnormal pancreas imaging). Although most subjects with jaundice required biliary intervention prior to steroid therapy, the need for diabetes treatment was unexpectedly low. The remission rate of treating patients following disease relapse remained high. Pancreatic duct stones were relatively uncommon, but occurred more frequently in patients with at least one disease relapse. A number of cancers occurred and further studies are needed to understand whether this represents a true

increased risk for malignancy in subjects with AIP or is due to older age of type 1 patients and ascertainment bias as patients with AIP have extensive diagnostic studies and close follow-up.

Our present compilation of more than 1000 patients is the largest to date, and the number of institutions required to reach this enrolment illustrates the rarity of this disease. Since the landmark discovery that elevated serum IgG4 levels are associated with AIP the number of newly diagnosed cases of AIP has increased dramatically.[2] The disease spectrum of IgG4-related disease, which encompasses AIP and IgG4-related SC, continues to expand also contributing additional diagnoses. It is more likely that the increasing recognition of the diseases is due to its increased awareness rather than true increase in incidence of the disease.

Since its initial description by Yoshida *et al*, type 1 AIP has been recognised as a steroid-responsive disease.[1] The current study shows that both types of disease are characterised by very high remission rates with steroid therapy, suggesting that the diagnosis must be reconsidered in those who do not respond to steroids. Although a noteworthy proportion (55%) of patients initially managed conservatively had spontaneous disease remission, this rate was inferior to that in patients who were treated with steroids or surgery (99% remission rate). Since inflammatory pancreatic and biliary disease can progress to irreversible pancreatic insufficiency and secondary biliary cirrhosis, we feel early treatment is advisable, even in the absence of rigorous evidence-based medicine demonstrating that steroid treatment alters the natural history of AIP. In the absence of a validated induction regimen variation in steroid dosing is inevitable, but despite this remission rates were universally high across all centres. Most patients required treatment for jaundice, abdominal pain or abnormal pancreatic imaging. Interestingly, although most patients with jaundice required endoscopic biliary stenting prior to steroids, less than half of patients required treatment for diabetes. Smaller series have shown an interesting, paradoxical improvement in glycaemic control after steroid therapy, presumably due to recovered pancreatic endocrine function with treatment.[13 14] This finding, experienced anecdotally by many of the authors, led to the withholding of diabetes treatment for some patients with hyperglycaemia. Nonetheless, it is important to monitor blood sugars during steroid treatment to recognise and prevent hyperglycaemia-related morbidity.

Disease relapses in type 1 and type 2 AIP predominantly involve the pancreas and/or biliary system. Cumulative relapse rates could not be accurately calculated since time to event (ie, relapse) data were not available for most patients. However, the relapse rate in this study of 31% falls within the range (15–60%) of that in previous reports. Unfortunately due to the nature of this study, it is not statistically valid to compare relapse rates on the basis of treatment strategies used (eg, with or without maintenance steroids). Due to challenges with study enrolment, a prospective treatment trial to clarify this choice is not expected soon; so the decision must be made on the basis of the provider's familiarity with the treatment strategy, considering the side effect profile, and patients' personal relapse histories and preferences.

For most patients in this study relapses occurred after steroid discontinuation. Patients treated again with steroids continued to respond favourably with a high remission rate. Some patients with relapses were treated with an immunomodulator (most commonly azathioprine or mycophenolate mofetil). These steroid-sparing approaches are attractive to some due to the possibility of avoiding complications from long-term steroid exposure.[6 8 14–16] However, to date no large series have demonstrated either treatment effectiveness or decreased incidence of treatment-related side effects.

Two sequelae identified in other forms of chronic pancreatitis are pancreatic duct stones and pancreatic cancer. So we specifically examined the rates of these complications in this study cohort.[17 18] The occurrence of pancreatic duct stones in this study is low with higher prevalence in those with at least one relapse. Additionally, we report the first systematic collection of malignancies in patients with AIP. Importantly, there were only five cases of pancreatic cancer in this study; however, considering the overall large denominator of AIP patients at risk, limited follow-up and lack of a control population, it is difficult to understand the true clinical significance of this finding. Additional studies with longer follow-up will help refine our understanding of these long-term sequelae.

We estimate that this multinational collaboration of many of the academic foci for AIP permitted analysis of a significant proportion of the world's current AIP population. Our utilisation of the recently developed ICDC permitted study of patients with a unified set of diagnostic criteria. Our data must be interpreted with caution recognising this collaboration could inadvertently introduce heterogeneity in disease on the basis of unknown ethnic differences in the natural history of disease, as well as variations in the standards of care regarding disease evaluation and follow-up.

Although the basic clinical profiles and initial treatment strategies for AIP are generally understood, many questions remain. The importance of steroid treatment at disease onset is commonly accepted, however whether or not one could use a lower steroid dose has not been systematically evaluated and may potentially decrease treatment-related morbidity. The prediction of disease relapse remains inadequate. Except for IgG4-related SC, no clinical factor has been consistently demonstrated to predict subsequent relapse. Finally, it remains unclear whether or not maintenance therapy (using either low-dose steroids or an immunomodulator) actually prolongs relapse-free survival, thereby altering the course of disease.

In summary, in this multinational analysis of more than 1000 patients with AIP we have shown that most patients are treated with steroids for predominantly pancreatobiliary manifestations of their disease. Initial and subsequent treatment responses to steroid therapy are exceedingly high, so the diagnosis should be reconsidered if patients do not respond to steroids. Relapses occur in a substantial proportion of patients and typically involve the pancreas and/or biliary system. Pancreatic duct stones and malignancies are two potential long-term sequelae, which require ongoing surveillance to further understand their full clinical significance. We are hopeful that multinational collaborations, such as the present one, will provide opportunities to better understand this disease, and permit a long-awaited randomised treatment trial.

Author affiliations
[1]Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, Minnesota, USA
[2]Department of Internal Medicine, Tokyo Metropolitan Komagome Hospital, Tokyo, Japan
[3]Department of Internal Medicine, GI Unit, Massachusetts General Hospital, Boston, Massachusetts, USA
[4]Department of Internal Medicine, Institute of Gastroenterology, Severance Hospital, Yonsei University College of Medicine, Seoul, Korea
[5]Translational Gastroenterology Unit, John Radcliffe Hospital, Oxford, UK
[6]First Department of Internal Medicine, University of Szeged, Szeged, Hungary
[7]Department of Medicine, Biomedical and Surgical Sciences, University of Verona, Verona, Italy
[8]Center for Excellence in Pancreatic Disease, David Geffen School of Medicine at University of California, Los Angeles, California, USA

[9]Department of Gastroenterology, Endocrinology, Metabolism and Infectiology, Philipps University of Marburg, Marburg, Germany
[10]Department of Internal Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea
[11]Center for Health, Safety and Environmental Management, Shinshu University, Matsumoto, Japan
[12]Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea
[13]Department of Medicine A, University Medicine Greifswald, Greifswald, Germany
[14]Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan
[15]Department of Surgical Gastroenterology, Karolinska Institutet & Karolinska University Hospital, Stockholm, Sweden
[16]Department of Gastroenterology and Hepatology, Kansai Medical University, Osaka, Japan
[17]Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea
[18]Department of Internal Medicine, Aix-Marseille University, Marseille, France
[19]Department of Gastroenterology, Tokyo Women's Medical University, School of Medicine, Tokyo, Japan
[20]Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan
[21]Department of Internal Medicine, Affiliated Stanford University, Palo Alto, California, USA
[22]Department of Gastroenterology, University College Hospital, London, UK
[23]Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh, Pennsylvania, USA
[24]Institute of Liver Studies, King's College Hospital, London, UK

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Contributors Each author was responsible for acquisition of data and critical revision of the manuscript for important intellectual content. TK is the guarantor. PAH, TK, and STC were also involved in the study concept and design, analysis and interpretation of data, and drafting of the initial manuscript. Each author approved the final manuscript prior to submission.

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HEPATOLOGY

Establishment of a serum IgG4 cut-off value for the differential diagnosis of IgG4-related sclerosing cholangitis: A Japanese cohort

Hirotaka Ohara,* Takahiro Nakazawa,† Shigeyuki Kawa,§ Terumi Kamisawa,†† Tooru Shimosegawa,¶¶ Kazushige Uchida,*** Kenji Hirano,‡‡ Takayoshi Nishino,§§ Hideaki Hamano,¶ Atsushi Kanno,¶¶ Kenji Notohara,††† Osamu Hasebe,‡ Takashi Muraki,** Etsuji Ishida,‡‡‡ Itaru Naitoh† and Kazuichi Okazaki***

Departments of *Community-Based Medical Education and †Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, ‡Department of Gastroenterology, Nagano Municipal Hospital, Nagano, §Center for Health, Safety and Environmental Management, Shinshu University, ¶Medical Informatics Division and Internal Medicine, Gastroenterology, Shinshu University Hospital, **Department of Gastroenterology, Shinshu University School of Medicine, Matsumoto, ††Internal Medicine, Tokyo Metropolitan Komagome Hospital, ‡‡Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, §§Department of Gastroenterology, Tokyo Women's Medical University Yachiyo Medical Center, Tokyo, ¶¶Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, ***The Third Department of Internal Medicine, Kansai Medical University, Hirakata, †††Department of Pathology, Kurashiki Central Hospital, and ‡‡‡Department of Gastroenterology, Kurashiki Central Hospital, Kurashiki, Japan

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autoimmune pancreatitis, IgG4-related sclerosing cholangitis, IgG4-SC, primary sclerosing cholangitis.

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Correspondence
Dr Takahiro Nakazawa, Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku Nagoya 467-8601, Japan.
Email: tnakazaw@med.nagoya-cu.ac.jp

Abstract

Background and Aim: IgG4-related sclerosing cholangitis (IgG4-SC) must be precisely distinguished from primary sclerosing cholangitis and cholangiocarcinoma (CC) because the treatments are completely different. However, the pathological diagnosis of IgG4-SC is difficult. Therefore, highly specific non-invasive criteria such as serum IgG4 should be established. This study established a cut-off for serum IgG4 to differentiate IgG4-SC from respective controls using serum IgG4 levels measured in Japanese centers.

Methods: A total of 344 IgG4-SC patients were enrolled in this study. As controls, 245, 110, and 149 patients with pancreatic cancer, primary sclerosing cholangitis, and CC, respectively, were enrolled. IgG4-SC patients were classified into three groups: type 1 (stenosis only in the lower part of the common bile duct), type 2 (stenosis diffusely distributed throughout the intrahepatic and extrahepatic bile ducts), and types 3 and 4 (stenosis in the hilar hepatic region) with 246, 56, and 42 patients, respectively. Serum IgG4 levels were compared, and the cut-offs were established.

Results: The cut-off obtained from receiver operator characteristic curves showed similar sensitivity and specificity to that of 135 mg/dL when all IgG4-SC and controls were compared. However, a new cut-off value was established when subgroups of IgG4-SC and controls were compared. A cut-off of 182 mg/dL can increase the specificity to 96.6% (4.7% increase) for distinguishing types 3 and 4 IgG4-SC from CC. A cut-off of 207 mg/dL might be useful for completely distinguishing types 3 and 4 IgG4-SC from all CC.

Conclusions: Serum IgG4 is useful for the differential diagnosis of IgG4-SC and controls.

Introduction

IgG4-related sclerosing cholangitis (IgG4-SC) is a biliary IgG4-related disease.[1,2] The first Japanese clinical diagnostic criteria of IgG4-SC were published in 2012.[3] Diffuse cholangioraphic abnormalities observed in association with IgG4-SC may resemble those observed in primary sclerosing cholangitis (PSC). Moreover, the presence of segmental stenosis suggests cholangiocarcinoma (CC).[4,5] The coexistence of autoimmune pancreatitis (AIP) is the most useful finding for the diagnosis of IgG4-SC.[6] IgG4-SC is occasionally described as an isolated biliary tract lesion, even in the absence of pancreatic involvement. It is especially difficult to differentiate IgG4-SC without AIP from PSC or CC.[7] IgG4-SC responds well to steroid therapy, whereas liver transplantation is the only effective therapy for PSC, and surgical intervention is needed for CC. Therefore, IgG4-SC must be precisely differentiated from PSC and CC.

Serum IgG4 is a useful marker for discriminating AIP from other pancreatic diseases;[8] a cut-off value of 135 mg/dL is widely used as a diagnostic criterion of AIP. However, twice the upper

limit of the normal value is also recommended to distinguish AIP from pancreatic cancer (PCa). In the international consensus diagnostic criteria for AIP (ICDC), twice the upper limit of the normal value is included in level 1, and 1–2 times the upper limit of the normal value is included in level 2.[9]

There are only a few reports concerning cut-off values in the diagnosis of IgG4-SC. We previously reported that a cut-off value of 135 mg/dL is useful for distinguishing IgG4-SC from PCa and PSC. However, this cut-off shows lower specificity for distinguishing IgG4-SC from CC.[6] Oseini *et al.*[10] evaluated the utility of serum IgG4 for distinguishing IgG4-SC from CC and concluded that some patients with CC, particularly those associated with PSC, have elevated serum IgG4 levels and that the use of a twofold cut-off for serum IgG4 may not reliably distinguish IgG4-SC from CC. At a cut-off of four times the upper limit of the normal range, serum IgG4 is 100% specific for IgG4-SC.

The most important initial modality in the diagnosis of IgG4-SC is cholangiography.[11] IgG4-SC can be classified into four types according to the region of strictures revealed by cholangiography.[12] This classification is intended for the differential diagnosis of IgG4-SC from PCa, PSC, and CC. We recommend that the differential diagnosis of these three intractable diseases should be made according to the cholangiographic classification because IgG4-SC has a variable appearance in cholangiography. Therefore, we also evaluated the cut-off values between each cholangiographic type of IgG4-SC and corresponding controls: PCa, PSC, and CC. However, there are no large multicenter studies regarding this in the literature. Therefore, in this study, we performed a multicenter study in Japan in order to establish a cut-off value to differentiate IgG4-SC from controls.

Methods

Study subjects. A retrospective survey of IgG4-SC focusing on serum IgG4 levels was conducted in the nine centers that participated in this study. The majority of these centers are major referral centers across Japan with established expertise in the diagnosis and management of IgG4-SC.

A total of 344 IgG4-SC patients (273 men and 71 women; age [mean ± standard deviation {SD}], 65.2 ± 10.2 years) diagnosed with IgG4-SC according to the 2012 Japanese clinical diagnostic criteria for IgG4-SC between 1993 and 2012 were enrolled in this study. All 344 patients fulfilled the criteria of imaging. In addition, 329 patients were associated with AIP. Four out of 15 patients with no association of AIP were diagnosed by the patholoIgical findings of resected specimen. The other 11 patients were diagnosed by the effect of steroid therapy after malignancy had been ruled out by the bile duct biopsy.

AIP patients were diagnosed according to the revised Japanese diagnostic criteria[13] or ICDC.[9] All patients with AIP included in this study had type 1 AIP. A total of 245 patients with PCa (90 men, 70 women, and 85 patients with unknown sex and age; mean age, 67.5 ± 11.6 years), 110 with PSC (51 men and 59 women; mean age, 42.8 ± 18.1 years), and 149 with CC (104 men and 45 women; mean age, 70.8 ± 11.4 years) were enrolled as the respective control groups for patients with IgG4-SC, who were classified into three groups. PCa and CC were diagnosed on the basis of histology, standard imaging criteria, or clinical course. PSC was diagnosed using the diagnostic criteria for PSC,[13] including the presence of typical abnormal bile ducts on direct cholangiography, an abnormal clinical course, blood chemistry data, and exclusion of secondary sclerosing cholangitis. Data regarding the serum IgG4 levels of the IgG4-SC and control groups were analyzed. The serum concentrations of IgG4 were measured by automated nephelometry (Behring Nephelometer II; Dade Behring, Newark, DE, USA). This study was approved by the Institutional Human Investigation Committee of Nagoya City University Graduate School of Medical Sciences.

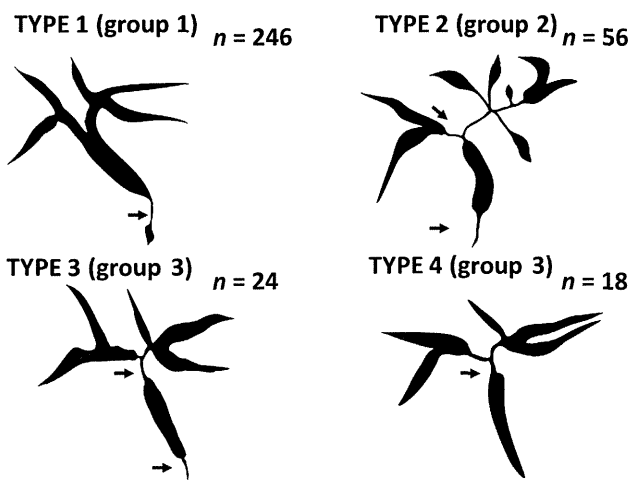

Figure 1 Schematic classification of cholangiographic findings of IgG4-related sclerosing cholangitis (IgG4-SC) (cited from Nakazawa *et al.*[14]). Stenosis is located only in the lower part of the common bile duct in type 1, stenosis is diffusely distributed in the intrahepatic and extrahepatic bile ducts in type 2, stenosis is detected in both the hilar hepatic lesions and the lower part of the common bile duct in type 3, and strictures of the bile duct are detected only in the hilar hepatic lesions in type 4.

Classification of IgG4-SC based on cholangiography. IgG4-SC can be classified into four types on the basis of the regions of stricture revealed by cholangiography (Fig. 1).[14] In the present study, we classified IgG4-SC into three groups for differential diagnosis on the basis of the cholangiographic features. The first group included patients with a type 1 cholangiogram, in which stenosis is located only in the lower part of the common bile duct. The second group included those with type 2 IgG4-SC, in which stenosis is diffusely distributed throughout the intrahepatic and extrahepatic bile ducts. Finally, the third group included those with types 3 and 4, in which stenosis is detected in the hilar hepatic region. Of the 344 IgG4-SC patients, 246, 56, and 42 were classified into the first (type 1, 195 men and 51 women; mean age, 64.6 ± 10.3 years), second (type 2, 46 men and 10 women; mean age, 66.3 ± 9.3 years), and third groups (24 with type 3 and 18 with type 4, 33 men and 9 women; mean age, 68.5 ± 8.9 years), respectively. Types 1, 2, and 3 and 4 cholangiographic findings can often lead to a misdiagnosis of PCa, PSC, and CC, respectively. Therefore, patients with PCa, PSC, and CC were selected as controls for the first, second, and third IgG4-SC groups, respectively.

Statistical analysis. Clinical data are expressed as mean ± SD. The χ^2 test and Mann–Whitney *U*-test for categorical comparisons of data were used to compare histological features, where appropriate. The level of statistical significance was set at $P < 0.05$. Receiver operating characteristic (ROC) curves were used to judge the diagnostic utility of serum IgG4 levels. For ROC curves, the best cut-off values were chosen according to the highest diagnostic accuracy determined using the Youden index: sensitivity – (1 – specificity). Statistical analysis was performed using JMP software (version 8.0.2: SAS Institute, Cary, NC, USA).

Results

Clinical profiles. The clinical profiles are shown in Table 1. All types of IgG4-SC exhibited male preponderance. There were no significant differences between any type of IgG4-SC with respect to age or serum IgG4 levels. The frequency of IgG4-SC without AIP was higher among types 3 and 4 IgG4-SC (type 1 0.8%, type 2 8.9%, type 3 19%, $P < 0.001$). When all IgG4-SC types or type 2 were compared with PSC, PSC did not exhibit any male preponderance ($P < 0.001$) and showed younger age ($P < 0.001$). PSC was significantly more associated with CC than with IgG4-SC (9/101 [8.18%] *vs* 1/343 [0.29%], respectively; $P < 0.001$).

Serum IgG4 values. The serum IgG4 levels of all IgG4-SC groups were significantly higher than those of all control groups (All IgG4-SC groups, 646 ± 662; PCa, 59.3 ± 65.9; PSC, 68.7 ± 86.0; CC, 52.3 ± 46.8; $P < 0.001$) (Table 1, Fig. 2). The serum IgG4 levels of type 1 IgG4-SC were significantly higher than that of PCa (613 ± 618 *vs* 59.3 ± 65.9, $P < 0.001$), that of type 2 IgG4-SC were significantly higher than that of PSC (799 ± 800 *vs* 68.7 ± 86.0, $P < 0.001$), and that of types 3 and 4 IgG4-SC were significantly higher than that of CC (646 ± 711 *vs* 52.3 ± 46.8, $P < 0.001$) (Table 1, Fig. 2). A total of 10.5% of IgG4-SC patients had serum IgG4 levels lower than the cut-off value of 135 mg/dL, whereas 7.7% of the controls had higher levels (6.7%, 11.5%, and 8.1% of PCa, PSC, and CC patients, respectively).

Cut-off values. When all IgG4-SC and controls were compared, the cut-off values of all IgG4-SC patients, from their respective controls obtained from ROC analysis, ranged from 117 to 138 mg/dL (all IgG4-SC *vs* PCa 119 mg/dL, all IgG4-SC *vs* PSC 117 mg/dL, all IgG4-SC *vs* CC 138 mg/dL). Sensitivity ranged from 89.8% to 91.2% (all IgG4-SC *vs* PCa 91.2%, all IgG4-SC *vs* PSC 91.5%, all IgG4-SC *vs* CC 89.8%), and specificity ranged from 87.6% to 93.9% (all IgG4-SC *vs* PCa 93.9%, all IgG4-SC *vs* PSC 87.6%, all IgG4-SC *vs* CC 92.6%). When we used a cut-off value of 135 mg/dL, similar sensitivity and specificity were obtained.

When the subgroups of IgG4-SC and controls were compared, the cut-off values for distinguishing specific types of IgG4-SC from their respective controls ranged from 119 to 182 mg/dL (type 1 IgG4-SC *vs* PCa 119 mg/dL, type 2 IgG4-SC *vs* PSC 125 mg/dL, types 3 and 4 IgG4-SC *vs* CC 182 mg/dL). Sensitivity ranged from

Table 1 Clinical profile

	IgG4-SC all	Type 1	Type 2	Types 3 and 4	Pancreatic cancer	PSC	Cholangiocarcinoma
Number	344	246	56	42 type 3 $n = 24$ type 4 $n = 18$	245	110	149
Male : female (male% to total)	273 : 71 (79.3%)	195 : 51 (79.3%)	46 : 10 (82.1%)	33 : 9 (78.6%)	90 : 70 (unknown 85) (56.2%)	51 : 59 (46.3%)	104 : 45 (69.8%)
IgG4 mg/dL (mean ± SD)	646 ± 662	613 ± 618	799 ± 800	646 ± 711 (type 3 534 ± 429) (type 4 919 ± 871)	59.3 ± 65.9	68.7 ± 86.0	52.3 ± 46.8
% of IgG4 value above 135 mg/dL	89.8	88.6	94.5	88.1	6.7	11.5	8.1
% of IgG4 value above 270 mg/dL	68.4	67.1	70.9	69.0	1.6	3.5	0
% of IgG4 value above 540 mg/dL	39.8	39.4	43.6	35.7	0.4	0.9	0

IgG4-SC, IgG4-related sclerosing cholangitis; PSC, primary sclerosing cholangitis; SD, standard deviation.

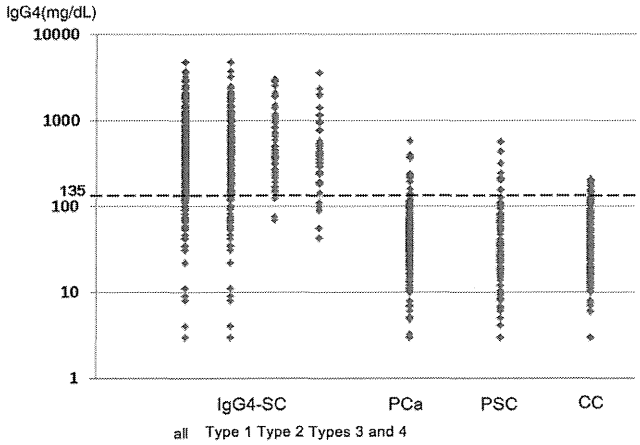

Figure 2 IgG4 value of IgG4-related sclerosing cholangitis (IgG4-SC) and respective controls. CC, cholangiocarcinoma; PCa, pancreatic cancer; PSC, primary sclerosing cholangitis.

85.7% to 96.4% (type 1 IgG4-SC *vs* PCa 90.2%, type 2 IgG4-SC *vs* PSC 96.4%, types 3 and 4 IgG4-SC *vs* CC 85.7%), and specificity ranged from 85.7% to 96.6% (type 1 IgG4-SC *vs* PCa 93.9%, type 2 IgG4-SC *vs* PSC 87.6%, types 3 and 4 IgG4-SC *vs* CC 96.6%). When we used a cut-off value of 135 mg/dL, types 1 and 2 showed similar sensitivity and specificity for distinguishing between PCa and PSC, respectively. However, the specificity for distinguishing types 3 and 4 from CC was lower. In order to rule out almost all control groups, a cut-off of 540 mg/dL, which is fourfold higher than the upper limit of normal, might be useful for distinguishing type 1 and 2 IgG4-SC. However, the maximum value of CC was 206 mg/dL, which was relatively lower. Therefore, the specificity is 100% if we set the cut-off value at 207 mg/dL for the differential diagnosis of type 3 and 4 IgG4-SC and CC.

Discussion

The present results revealed that the serum IgG4 levels of IgG4-SC were significantly higher than that of the control groups. The cut-off value obtained from the ROC curve showed sensitivity and specificity that was similar to the cut-off of 135 mg/dL when all IgG4-SC and controls were compared. However, these results indicate a new cut-off value when the subgroups of IgG4-SC and controls were compared after IgG4-SC was divided into three groups on the basis of cholangiographic classification. The cut-off value of 182 mg/dL for types 3 and 4 IgG4-SC and CC increases the specificity to 96.6% (a 4.7% increase). The cut-off of 207 mg/dL, a relative lower value, might be useful for completely distinguishing types 3 and 4 IgG4-SC from CC.

AIP is often associated with systemic extrapancreatic lesions such as sclerosing cholangitis, sclerosing sialadenitis, retroperitoneal fibrosis, mediastinal lymphadenopathy, sclerosing cholecystitis, interstitial pneumonia, and tubulointerstitial nephritis.[15–17] Similar immunohistopathological features such as lymphoplasmacytic infiltration and abundant IgG4-positive plasma cells are also observed in some extrapancreatic lesions.[17–19] The concept of IgG4-related disease includes high serum IgG4 levels as an essential diagnostic criterion. However, the cut-off values of IgG4-related diseases other than AIP have not been investigated or established until now. The pathological diagnosis of IgG4-SC is difficult compared to that of other IgG4-related diseases such as sclerosing sialadenitis because obtaining a sufficient quantity of bile duct sample is difficult.[20] Therefore, non-invasive criteria with high specificity, such as serum IgG4 levels, should be established for the diagnosis of IgG4-SC.

Several diagnostic criteria have been published for the diagnosis of IgG4-SC.[3,6,21] Our previous study demonstrates that association with AIP is a useful parameter for the diagnosis of IgG4-SC. In that study, 59 of 62 patients (95%) with IgG4-SC had associated AIP.[6] Ghazale *et al.*.[21] report a frequency of AIP association of 92% among 53 patients with IgG4-SC, which is a comparatively large sample. However, focal-type AIP sometimes produces imaging findings similar to those of PCa, making it difficult to distinguish between these two diseases.[22] The sensitivity of the diagnostic criteria for AIP is reported to range from 80% to 92%.[23] Therefore, there is a need to establish useful diagnostic criteria for IgG4-SC when it is not associated with AIP or when the diagnosis of AIP is unclear. The present results indicate that caution should be taken when diagnosing types 3 and 4 IgG4-SC because the frequency of IgG4-SC without AIP was higher in types 3 and 4 IgG4-SC.

Serum IgG4 is the most useful modality for distinguishing IgG4-SC from other biliary diseases. When distinguishing AIP from PCa, only PCa should be considered as a differential diagnostic disease. However, IgG4-SC should be distinguished from all three intractable diseases (i.e. PCa, PSC, and CC). Therefore, there is a need to establish diagnostic criteria that account for the differential diagnoses of these three intractable diseases. IgG4-SC can be classified into four types on the basis of the region of strictures revealed by cholangiography.[14] Furthermore, diagnostic criteria based on cholangiographic classification in the differential diagnosis of IgG4-SC have been proposed.[6] The cut-off value of 135 mg/dL is useful for differentiating IgG4-SC from PCa and PSC. However, it exhibits lower sensitivity and specificity for differentiating IgG4-SC from CC.[6] The present Japanese multicenter study revealed that 10.5% of IgG4-SC patients had serum IgG4 levels lower than the cut-off of 135 mg/dL, whereas 7.7% of the controls had levels greater than the cut-off. High specificity is required in order to differentiate the three intractable diseases from IgG4-SC. A cut-off of 135 mg/dL is recommended in cases of types 1 and 2 IgG4-SC, whereas a cut-off of 182 mg/dL is recommended in cases of type 3 IgG4-SC. A cut-off of 182 mg/dL for distinguishing between type 3 IgG4-SC and CC can increase the specificity by 4.7%.

Twice the upper limit of the normal value 270 mg/dL is recommended to distinguish AIP from PCa in order to increase specificity in the ICDC. Twice the upper limit of the normal value is included in level 1, and one to two times the upper limit of the normal value is included in level 2.[9] Oseini *et al.*[10] report that the use of a cut-off twice that of the upper limit of the normal value for serum IgG4 levels may not reliably distinguish IgG4-SC from CC because some patients with CC, particularly those with PSC, have elevated serum IgG4 levels. At a cut-off 4 times the upper limit of the normal value, serum IgG4 is 100% specific for IgG4-SC. In the present study, cut-offs that were two and four times the upper limit of the normal value of 270 and 540 mg/dL, respectively, showed

higher specificity. Among our CC cases, a cut-off of 206 mg/dL showed the highest specificity. Therefore, a cut-off of 270 mg/dL can completely distinguish IgG4-SC from CC. However, a cut-off of 207 mg/dL can completely distinguish IgG4-SC from CC and maintain a comparatively high sensitivity. Cut-offs of 563 and 580 mg/dL were the highest among our PSC and PCa cases, respectively. A cut-off of 540 mg/dL cannot distinguish IgG4-SC from PSC and PCa completely. However, almost all control cases except two were below this cut-off. This suggests that 540 mg/dL is an appropriate cut-off for distinguishing IgG4-SC from almost all controls worldwide. As mentioned earlier, some patients with CC, particularly those with PSC, have elevated serum IgG4 levels.[10] The present study detected nine PSC patients with CC (8.18%); however, all cases had low serum IgG4 levels (8.3, 13.7, 16.8, 18.0, 18.0, 34.0, 35.0, 41, 4, and 94.8). These differences might be due to the lower frequency in the number of PSC patients in Japan than that in the US.

We confirmed that the cut-off of 135 mg/dL that is adopted in Japanese clinical diagnostic criteria of IgG4-SC is appropriate value, and the higher cut-off value of 207 mg/dL might be useful for completely distinguishing types 3 and 4 IgG4-SC from CC in the present Japanese multicenter study. We hope further international study because the Japanese data may not be applicable to the rest of the world.

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Review

T helper subsets in Sjögren's syndrome and IgG4-related dacryoadenitis and sialoadenitis: A critical review☆

Masafumi Moriyama [a], Akihiko Tanaka [a], Takashi Maehara [a], Sachiko Furukawa [a], Hitoshi Nakashima [b], Seiji Nakamura [a,*]

[a] Section of Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

[b] Division of Nephrology and Rheumatology, Department of Internal Medicine, Faculty of Medicine, Fukuoka University, Fukuoka, Japan

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ABSTRACT

IgG4-related disease (IgG4-RD) is a systemic disease characterized by the elevation of serum IgG4 and infiltration of IgG4-positive plasma cells in multiple target organs, including the pancreas, kidney, biliary tract and salivary glands. In contrast, Mikulicz's disease (MD) has been considered a subtype of Sjögren's syndrome (SS) based on histopathological similarities. However, it is now recognized that MD is an IgG4-RD distinguishable from SS and called as IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS). Regarding immunological aspects, it is generally accepted that CD4+ T helper (Th) cells play a crucial role in the pathogenesis of SS. Since it is well known that IgG4 is induced by Th2 cytokines such as interleukin (IL)-4 and IL-13, IgG4-DS is speculated to be a unique inflammatory disorder characterized by Th2 immune reactions. However, the involvement of Th cells in the pathogenesis of IgG4-DS remains to be clarified. Exploring the role of Th cell subsets in IgG4-DS is a highly promising field of investigation. In this review, we focus on the selective localization and respective functions of Th cell subsets and discuss the differences between SS and IgG4-DS to clarify the pathogenic mechanisms of these diseases.

Sjögren's syndrome (SS) is an autoimmune disease characterized by lymphocytic infiltration into the salivary and lacrimal glands with concomitant autoantibody production and destruction of the glandular tissue. Patients typically experience symptoms of dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca). Because of its characteristic lymphocytic infiltration and destruction of the salivary and lacrimal glands, SS is considered to be an ideal disease for studying patterns of cytokine production at the site of organ-specific autoimmune damage [1]. SS occurs alone as primary SS, or as secondary SS when underlying other connective tissue diseases [2]. Immunohistochemical studies demonstrated that the salivary glands are predominantly infiltrated by CD4+ T helper (Th) cells at an early stage of SS, and these cells are therefore thought to play a crucial role in the induction and/or maintenance of the disease [3]. In advanced stage, B cells predominate and these infiltration extends to occupy the acinar epithelium and further progress to hypergammaglobulinemia and B cell lymphoma [4]. Recent studies have suggested a central role of the epithelium in orchestrating the immune reaction by expressing HLA antigens, adhesion and costimulatory molecules, cytokines, and chemokines. Therefore, SS has been proposed as an etiological term "autoimmune epithelitis" [4–7], and it is of interest to examine the involvement of interaction between CD4+ Th cells and the epithelium in the initiation and progression of the disease process. Th cell populations comprise functionally distinct subsets characterized by specific patterns of cytokines and transcription factors. At least six Th subsets exist: Th0, Th1, Th2, Th17, regulatory T (Treg), and follicular helper T (Tfh) cells [8], which are suggested to be involved in the pathogenesis of SS [9–12].

On the other hands, Mikulicz's disease (MD) has been considered to be a subtype of SS based on histopathological similarities between the two diseases [13]. However, MD has a number of differences compared with typical SS including: 1) difference of gender distribution (MD occurs in both men and women, while SS occurs mainly in women); 2) persistent enlargement of lacrimal and salivary glands; 3) normal or mild salivary secretion dysfunction; 4) good responsiveness to corticosteroid treatment; 5) hypergammaglobulinemia and low frequency of anti SS-A and SS-B antibodies by serological analyses; and 6) multiple GC formation in

* Corresponding author. Tel.: +81 92 642 6447; fax: +81 92 642 6386.
E-mail address: seiji@dent.kyushu-u.ac.jp (S. Nakamura).

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glandular tissue (Table 1). Previously, we reported that SS was characterized by periductal lymphocytic infiltration with atrophy or severe destruction of the acini, while MD showed non-periductal lymphocytic infiltration with hyperplastic GCs and mild destruction of the acini (Fig. 1) [14]. Fifteen of 66 patients with SS (23%) and 12 of 20 patients with MD (60%) showed ectopic GC formation in labial salivary glands (LSGs). Patients with MD showed a significantly higher frequency, higher number and larger size of GCs compared with SS patients [15]. In addition, Yamamoto et al. [16–18] reported that patients with MD had elevated levels of serum IgG4 and infiltrating IgG4-positive plasma cells in the gland tissues. Similar findings have been observed in autoimmune pancreatitis (AIP) [19], sclerosing cholangitis [20], tubulointerstitial nephritis [21], Ridel's thyroiditis [22] and Küttner's tumor [23]. These diseases are now referred to as IgG4-related disease (IgG4-RD) [24,25]. We recently described the concept of IgG4-RD and provided up-to-date information regarding this emerging disease entity [26]. Recent studies have referred to MD as IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) [15,27] (Table 2).

IgG4 molecules are symmetrical homobivalent antibodies that can exchange half-molecules (heavy and light chain) specific for two different antigens ("Fab-arm exchange"), which results in losing the ability to cross-link antigens and to form immune complexes [28]. In addition, IgG4 also can bind the Fc fragment of other IgG molecule, particularly other IgG4 molecules ("Fc–Fc interactions"). These IgG4 Fc–Fc interactions proceed to Fab-arm exchange reaction and may contribute to the anti-inflammatory activity, which includes a poor ability to induce complement and cell activation caused by low affinity for C1q (Fig. 2) [29]. Another characteristic is that IgG4 is a Th2-dependent immunoglobulin and has low affinity for its target antigen. interleukin (IL)-4 directs naive human B cell immunoglobulin isotype switching to IgG4 and IgE production [30]. We previously reported that peripheral CD4+ Th cells from patients with IgG4-DS revealed a deviation in the Th1/Th2 balance to Th2 and elevated expression of Th2-type cytokines [15,31,32]. Therefore, IgG4-DS is suggested to have a Th2-predominant phenotype. This review article will emphasize recent studies seeking to understand the role of Th cell subsets in primary SS and IgG4-DS.

1. Cytokine profiles of CD4+ Th cells

1.1. Th1/Th2 paradigm

Th1 cells support cell-mediated immunity and produce IL-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, which induce inflammatory responses responsible for killing intracellular parasites and perpetuating autoimmune responses. However, excessive inflammatory responses can lead to uncontrolled tissue damage. Th2 cells produce IL-4, IL-5, and IL-13, which provide help for humoral immunity and promote IgE secretion and eosinophilic responses. Th2 responses can counteract Th1-mediated microbicidal action. Thus, the Th1/Th2 balance plays an important role in immunoregulation. In contrast, Th0 cells are characterized by the production of both Th1 and Th2 cytokines and are considered precursors of Th1 and Th2 cells. Several studies have revealed that autoimmune diseases are caused by disruption to the Th1/Th2 balance [33,34]. The relationship of Th1/Th2 imbalance to the pathogenesis of SS has been widely investigated. Polarized Th1 responses were associated with the immunopathology of SS [9]. High numbers of IFN-γ-positive CD4+ T cells were detected in the salivary glands of SS patients and intracellular cytokine analysis demonstrated the polarization of Th cells to a Th1 phenotype [35]. Furthermore, we reported that IL-2 and IFN-γ were consistently detected in all SS patients, while IL-4 and IL-5 were only detected in patients with high levels of B cell accumulation in the salivary glands [10,36]. Recently, Theander et al. [37] reported that the detection of GC-like structures (B cell accumulation) in LSG biopsy specimens from primary SS patients could be used as a highly predictive and easy-to-obtain marker for B cell lymphoma development. Taken together, these studies suggest that Th1 cytokines are essential for the induction and/or maintenance of SS, whereas Th2 cytokines may be involved in disease progression, especially local B cell activation. Our clinical data was demonstrated that Th1 and Th2 cytokine concentrations were significantly higher in saliva from SS patients than from controls, and the levels of Th2 cytokines were closely associated with increased lymphocytic accumulation in LSGs. Thus, the measurement of cytokines in saliva may be useful for diagnosis and to reveal disease status [12].

IgG4-DS patients frequently have a history of bronchial asthma and allergic rhinitis with severe eosinophilia and elevated serum IgE levels [38]. It is well known that allergic immune responses are induced by allergen-specific Th2 cytokines, such as IL-4 and IL-13, which promote the secretion of IgG4 and IgE by B cells [39]. Recent studies indicated that Th2 immune reactions contributed to IgG4-DS [15,32,40] and IgG4-related tubulointerstitial nephritis [31,41]. The expression profile of cytokines suggested that IgG4-DS was characterized by a deviation of the Th1/Th2 balance to a Th2 phenotype and elevated expression of Th2 cytokines. Contrary to our results, Ohta et al. [42] reported a strong predominance of Th1 and cytotoxic type 1 cells in the salivary glands from IgG4-DS patients. They concluded that disruption of the Th1/Th2 balance might be due to differences in the specimens examined or the severity of the disease.

Chemokines are important for leukocyte activation and chemotaxis. Interactions between chemokines and chemokine receptors promote the selective local infiltration of specific cells into inflamed areas. Furthermore, chemokines are intimately involved in maintenance of the Th1/Th2 balance and immune responses in cardiac allograft rejection [43], atopic keratoconjunctivitis [44], and cutaneous lupus erythematosus [45]. Chemokines also play a key role in lymphoid neogenesis in target organs [46]. Immunohistochemical staining in our studies indicated that Th2-type chemokines including macrophage-derived chemokine (MDC)/CCL22 and thymus and activation regulated chemokine (TARC)/CCL17, natural ligands for CCR4 on Th2 cells, were detectable in and around the ductal epithelial cells and GCs, while CCR4 was expressed on infiltrating lymphocytes in LSGs in both SS and IgG4-DS patients. Thus, interactions of CCR4 with MDC and TARC may play a critical role in the accumulation of Th2 cells and subsequently, the progression of SS and IgG4-DS [12,32]. In contrast, interferon gamma induced protein 10 (IP-10)/CXCL10, natural ligand for CXCR3 on Th1 cells, was detected in and around the ductal epithelial cells, while CXCR3 was only expressed on infiltrating lymphocytes in LSGs from SS patients [47].

Table 1
Clinical and laboratory findings of Sjögren's syndrome (SS) and IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS). § IgG4 positive plasma cells/IgG positive plasma cells >50%.

	SS	IgG4-DS
Peak age of onset	40's and 50's	60's
Sex	Male ≪ Female	Male ≦ Female
Salivary secretion dysfunction	Moderate or severe	None or mild
Glandular swelling	Recurrent	Persistent
Sialography	Apple-tree sign	Parenchymal defect
$IgG4^+$ plasma cell infiltration§	Positive	Negative
Serum IgG	Often high	High
Serum IgG4	Normal	High
Serum complement	Normal	Often low
Anti SS-A/SS-B antibody (+)	High rate	Rare
Antinuclear antibody (+)	Often	Rare

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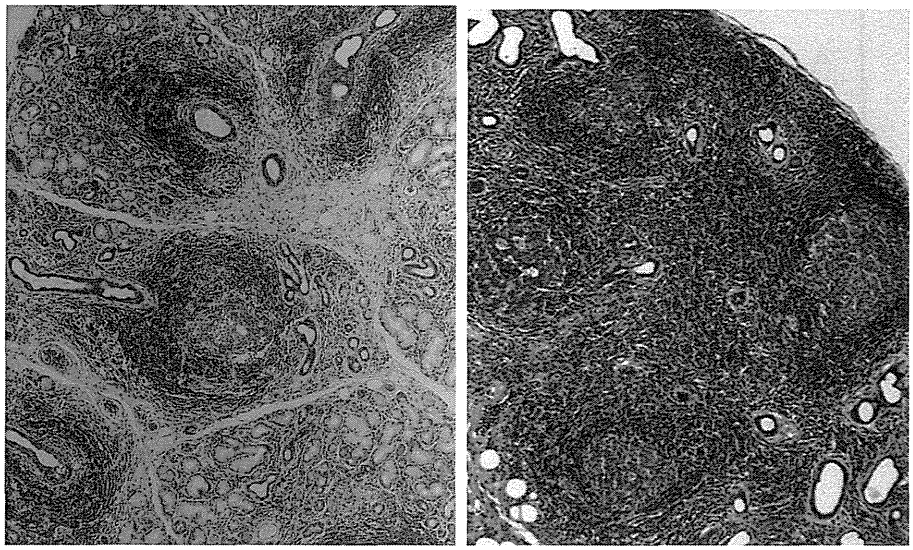

Fig. 1. Histopathological findings in salivary glands from patients with Sjögren's syndrome (SS) and IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS). SS is characterized by periductal lymphocytic infiltration with atrophy or severe destruction of the acini, while IgG4-DS shows non-periductal lymphocytic infiltration with hyperplastic GCs and mild destruction of the acini. Abbreviations: GC, germinal center.

1.2. Th17 cells

The Th1/Th2 paradigm was recently expanded by the identification of Th17 cells, a subset of CD4+ Th cells characterized by their ability to produce IL-17. Several studies have reported that IL-17 was detected in epithelial and infiltrating mononuclear cells in LSGs from patients with SS. In addition, Th17 cells are "tissue seeking" and intimately involved in the initiation of SS [48]. Youinou et al. [49] reported that Th17 cells orchestrate autoreactive GCs. However, Our previous data in selectively extracted lesions from LSGs by laser capture microdissection showed that the expressions of Th17-related molecules in infiltrating lymphocytes outside ectopic GCs were higher than inside ectopic GCs [36]. Interestingly, a subset of Th17/Th1 cells identified in the gut of Crohn's disease patients may co-express IFN-γ and IL-17 [50]. Both Th1 and Th17 cells were involved in the pathogenesis of SS [51], and the early induction of a CD4+ Th1/Th17 pathway caused the systemic release of IL-17 in mice [52]. Our previous data suggest that both Th1 and Th17 cells present around the ductal epithelial cells might be of critical importance in the initiation of SS. Furthermore, the destruction of epithelial by Th1 and Th17 cells are thought to play an important pathogenetic role by the occurrence of infiltrating lesions in various epithelial tissues as well as the increased epithelial expression of various immunoactive molecules. Thus, SS has been described as "autoimmune epithelitis" [6]. In contrast, Th17-related molecules were rarely expressed in patients with IgG4-DS [32,36]. As mentioned above, IgG4-DS showed non-periductal lymphocytic infiltration and mild destruction of the epithelial cells. These findings were speculated that IgG4-DS might be a "non- autoimmune epithelitis".

Table 2
Role of Th subsets in IgG4-related disease (IgG4-RD). Abbreviations: Th, T helper; MD, Mikulicz's disease; AID, activation-induced cytidine deaminase; LSG, labial salivary gland; Tc1, T cytotoxic type 1; Tfh, follicular helper T; NLR, nucleotide-binding oligomerization domain-like receptor; TLR, Toll-like receptor; AIP, autoimmune pancreatitis; BAFF, B-cell activating factor belonging to the tumor necrosis factor family; APRIL, a proliferation-inducing ligand; Treg, regulatory T; TGF-β, transforming growth factor β.

Principal findings	Reference
Overexpression of IL-21 by Th2 cells play a key role in germinal center formation and IgG4 production in IgG4-DS.	[15]
Peripheral CD4+ T cells from the patient with MD reveal the deviation of the Th1/Th2 balance to Th2.	[31]
Th2 and regulatory immune reactions play a key role of IgG4 production in MD.	[32]
The production of IgG4 antibodies appears to be driven in part by Th2 cytokines that mediate allergic responses and IgE production.	[38]
Th2 cells are involved in the pathogenesis of IgG4-related lacrimal gland enlargement.	[39]
Overexpressions of IL-10, TGF-β, and AID in LSGs play important roles in the pathogenesis of IgG4-RD, such as IgG4-specific class-switch recombination and fibrosis.	[81]
IgG4-related tubulointerstitial nephritis shows amplification of IL-10 and TGF-β.	[41]
Th1 and Tc1 cell populations and IL-17 expression are involved in the mechanism of pathogenesis of IgG4-related sclerosing sialadenitis.	[42]
IgG4-related interstitial nephritis shows Tfh cells in enhancing a skewed B-cell terminal maturation and of CD20 + B cells in disease progression.	[66]
Activation of NLR and TLR in monocytes from AIP patients induces IgG4 production by B cells.	[76]
BAFF and APRIL are useful markers for predicting disease activity in IgG4-RD.	[78]
The progression and induction of AIP was supported by increased memory Treg and Th2 immune responses.	[80]

1.3. Regulatory T cells

Treg cells, identified by the expression of Foxp3, are essential for the maintenance of immunological self-tolerance and immune homeostasis to prevent the development of various inflammatory diseases. It achieves this either by direct contact with effector immune cells and/or by secreting anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β. Treg cells exert their effects through the modulation of both T and B cell responses. Two subsets of Treg cells, CD4+ CD25+ Foxp3+ Treg cells [53] and IL-10–producing Tr1 cells [54] are crucial for regulating effector T cell functions. CD4+ CD25+ Foxp3+ Treg cells can prevent

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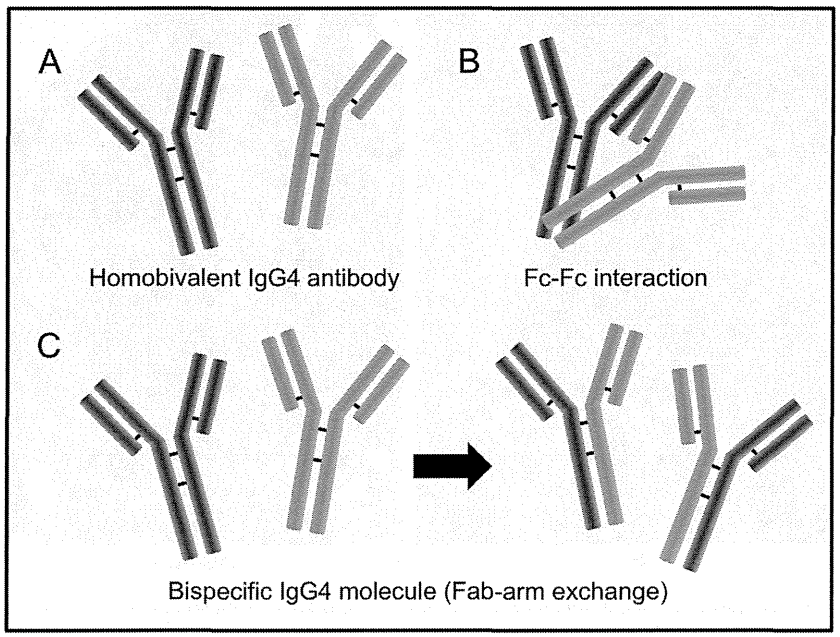

Fig. 2. Unique structure of IgG4 antibody. A, IgG4 antibody consists of two heavy chains and two light chains. B, Fc fragment of IgG4 can interact with the Fc fragment of another IgG4 molecule. C, Exchange of half-molecules (Fab-arm exchange) results in IgG4 combining two different specificities in a single molecule (bispecific antibody).

autoimmune hepatitis and primary biliary cirrhosis [55]. Mice with defects in Treg cell generation often develop T cell-mediated systemic autoimmune responses that affect multiple organs. Kolkowski et al. [56] demonstrated that salivary glands in SS constitutively expressed IL-10 and TGF-β. Other studies reported a significant reduction of Tregs in LSGs and peripheral blood from SS patients that might be involved in the pathogenesis of salivary gland destruction [57,58]. In contrast, Gottenberg et al. [59] reported increased Treg cell numbers in the peripheral blood of SS patients. Therefore, it is unclear whether Tregs are involved in the pathogenesis of SS. According to recent data, Foxp3+ T-regulatory cell frequency in the salivary glands of SS patients correlates with inflammation grade and certain risk factors for lymphoma development [60]. While in early and moderate infiltrations a compensatory control of Tregs in response to Th17 expansion seems to occur, in advanced SS lesions Tregs may fail to control the immune mediated tissue injury [7,61]. Increased levels of Treg cells in salivary glands from SS patients might suggest negative feedback is more active than in healthy subjects. Therefore, Treg cells might be not involved in the initiation of disease.

Zen et al. [62] reported that significant numbers of CD4+ CD25+ Foxp3+ Tregs infiltrated the affected tissues in cases of autoimmune pancreato-cholangitis (AIPC), which is one of IgG4-RD. Furthermore, another study demonstrated that IL-10 decreased IL-4-induced IgE switching but increased IL-4-induced IgG4 production [63]. We found that IL-4, IL-10, and Foxp3 were positively correlated with the IgG4/IgG ratio in the salivary glands from patients with IgG4-DS [32]. These results suggest that Th2 and regulatory immune reactions might play key roles in IgG4 production.

2. Role of IL-21 in SS and IgG4-DS

2.1. Follicular helper T cells

Tfh cells were recently identified as a unique Th phenotype, expressing high levels of CXCR5, a chemokine receptor [64]. Several studies reported that Tfh cells control the functional activity of effector Th cells and promote ectopic GC formation by IL-21, which contributed to impaired B cell differentiation [65,66]. Once GCs are formed, Tfh cells are required for their maintenance and the regulation of B cell differentiation into plasma cells and memory B cells. Several studies in SS patients demonstrated that IL-21 was increased in serum and high levels of IL-21 receptor were present on the surface of most B cells [67]. Furthermore, IL-4 and IL-21 receptors knockout mice have greatly reduced IgG responses, indicating that IL-21 co-operates with IL-4 to regulate humoral immune responses [68]. We previously observed that Tfh-related molecules, CXCR5 and B-cell lymphoma 6 protein (Bcl-6), were highly expressed on infiltrating lymphocytes in ectopic GCs of LSG lesions from both SS and IgG4-DS patients [15,36]. These results provide strong support for Tfh cells in the progression of disease as a lymphoproliferative disorder, particularly in the growth and activation of ectopic GC formation (Fig. 3).

IL-21 was mainly produced by Th2 and Th17 cells in addition to Tfh cells [68,69]. Interestingly, high IL-21 expression was only detected outside ectopic GCs in patients with IgG4-DS in our immunohistological analyses. The expression patterns of Th2-related molecules (IL-4, CCR4 and c-Maf) in LSGs were similar to that of IL-21 in patients with IgG4-DS. In contrast, Th17-related molecules were rarely expressed in patients with IgG4-DS. Furthermore, IL-21 positively correlated with the number of GCs formed in LSGs from patients with IgG4-DS [15]. Taken together, these findings suggest that excessive IL-21 production by Th2 cells in salivary glands from IgG4-DS patients might induce Bcl-6 expression in B cells resulting in multiple GC formation. Furthermore, IL-21 directly inhibited IL-4-induced IgE production [70], and IgG4 class switching was induced by co-stimulation with IL-4 and IL-21 in humans and mice [71]. In addition, IL-21 induced IL-10 production by mitogen-stimulated peripheral blood mononuclear cells in humans [72]. Therefore, we speculate that IL-21 correlates with IL-4 and IL-10 for IgG4 class switching. In the current study, we found that IL-21 positively correlated with the IgG4/IgG ratio in immunohistochemically positive cells

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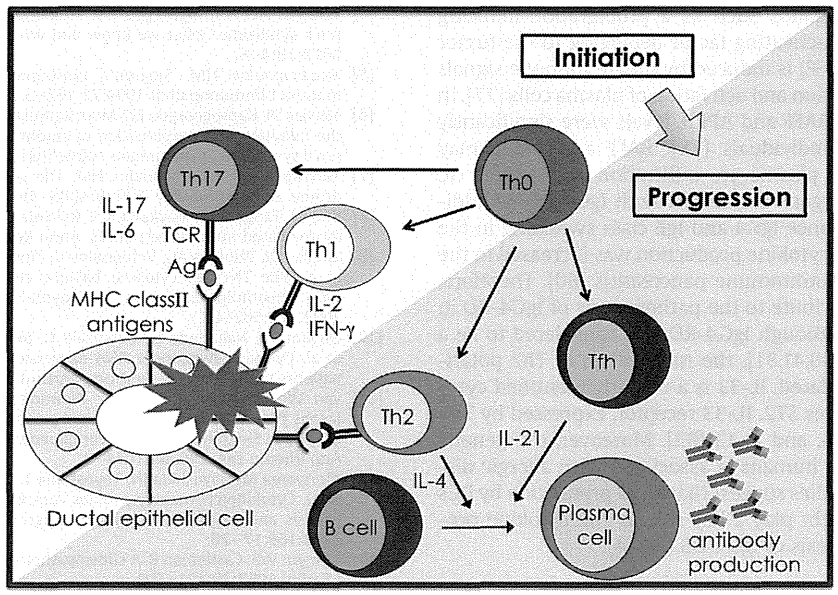

Fig. 3. Schematic model of Th cell network in SS. Th1 and Th17 cells are involved in early stages of disease, while Th2 and Tfh cells are associated with GC formation in the late stage. Abbreviations: Th, T helper; Tfh, follicular helper T.

[15] suggesting that IL-21 might also be involved in the class switching of IgG4 in IgG4-DS [73].

2.2. Innate immunity in IgG4-DS

Macrophages act as cells in the immune response to foreign invaders of the body, by presenting pathogenic antigens to antigen-specific Th cells. Historically, they have been classified into two distinct macrophage phenotypes, "classically activated" proinflammatory (M1) and "alternatively activated" anti-inflammatory (M2) macrophages [74]. M2 macrophages are activated by IL-4, produce high levels of IL-10 and are important for debris scavenging, wound healing and fibrosis. These polarized macrophage populations can also contribute to systemic diseases [75]. Watanabe et al. [76] demonstrated that abnormal innate immune responses induced via Toll-like receptor signaling in macrophages might enhance Th2 immune responses and the immunopathogenesis of IgG4-RD. Our current studies observed that IgG4-DS patients showed predominant infiltration by M2 macrophages that secreted IL-10 and IL-13 in salivary glands.

Dendritic cells (DCs) are professional antigen presenting cells that bridge innate and adaptive immunity. Expression of

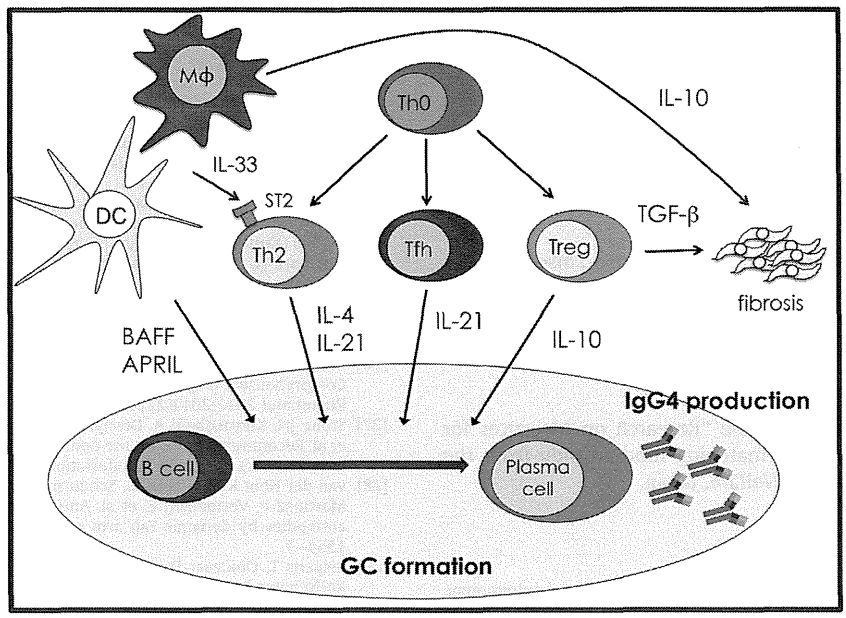

Fig. 4. Schematic model of Th cell and innate immune network in IgG4-DS. Th2, Treg, and Tfh cells play key roles in GC formation and IgG4 production. Dendritic cells and macrophages promote Th2 immune reaction by IL-33 as well as BAFF and APRIL. Abbreviations: Treg, regulatory T; BAFF, B cell activating factor belonging to the tumor necrosis factor family; APRIL, a proliferation-inducing ligand.

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DC-derived TNF-family ligands such as a proliferation-inducing ligand (APRIL) and B cell activating factor belonging to the tumor necrosis factor family (BAFF) is induced by innate immune signals to promote the differentiation and activation of plasma cells [77]. In IgG4-RD patients, serum BAFF and APRIL levels were significantly higher than in healthy individuals [78]. BAFF and APRIL may contribute to progressive plasmacyte infiltration and ectopic GC formation in the target organs of patients with IgG4-RD. In addition, BAFF and APRIL enhance IgG4 and IgE class switching in the presence of IL-4 [79]. Th2 cytokine production was increased in the tissues of patients with autoimmune pancreatitis [80]. Therefore, BAFF and APRIL may contribute to the pathogenesis of IgG4-RD in concert with Th2 cells. Although IgG4-RD was considered to be a Th2-dependent disease [40,41,81], the mechanism of Th2 polarization has yet to be elucidated. IL-33 is a recently identified cytokine that directly stimulates ST2, IL-33 receptor, expressed by Th2 cells to produce IL-4, IL-5, and IL-13 [82]. Moreover, the genetic polymorphism of IL-33 in humans is associated with allergic diseases [83]. Our current studies suggest that IL-33 production by DCs and M2 macrophages might play a key role in Th2 cytokine production and the pathogenesis of IgG4-DS (Fig. 4).

3. Conclusions

Research accumulated in recent years makes it increasingly clear that the immunological backgrounds are entirely different between SS and IgG4-DS. However, additional research is required to elucidate further the pathogenesis of IgG4-DS, especially the development of a mouse model of IgG4-DS. Although Glucocorticoids are the standard treatment for IgG4-RD, Yamamoto et al. [84] reported that the relapse rate of IgG4-DS during steroid therapy is 26.8%. A more thorough understanding of the complex mechanisms of IgG4-DS, especially the role of Th subset-related cytokines, could lead to the development of novel pharmacological strategies aimed at disrupting the cytokine network and inhibiting the initiation and/or progression of IgG4-DS. Finally, it should be noted that while this thesis focuses primarily on T cells, that there have recently been other extensive reviews and hypotheses published on Sjogren's syndrome, reflecting its increased interest not only to basic immunologists, but also to rheumatologists [4,85–116].

Competing interests

The authors declare no competing interests.

Author contributions

All authors provided substantial contributions to discussions of content, and to reviewing and editing the manuscript before submission. M Moriyama researched the data and wrote the article.

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LETTER

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Genetics of rheumatoid arthritis contributes to biology and drug discovery

A list of authors and their affiliations appears at the end of the paper

A major challenge in human genetics is to devise a systematic strategy to integrate disease-associated variants with diverse genomic and biological data sets to provide insight into disease pathogenesis and guide drug discovery for complex traits such as rheumatoid arthritis (RA)[1]. Here we performed a genome-wide association study meta-analysis in a total of >100,000 subjects of European and Asian ancestries (29,880 RA cases and 73,758 controls), by evaluating ~10 million single-nucleotide polymorphisms. We discovered 42 novel RA risk loci at a genome-wide level of significance, bringing the total to 101 (refs 2–4). We devised an *in silico* pipeline using established bioinformatics methods based on functional annotation[5], *cis*-acting expression quantitative trait loci[6] and pathway analyses[7–9]—as well as novel methods based on genetic overlap with human primary immunodeficiency, haematological cancer somatic mutations and knockout mouse phenotypes—to identify 98 biological candidate genes at these 101 risk loci. We demonstrate that these genes are the targets of approved therapies for RA, and further suggest that drugs approved for other indications may be repurposed for the treatment of RA. Together, this comprehensive genetic study sheds light on fundamental genes, pathways and cell types that contribute to RA pathogenesis, and provides empirical evidence that the genetics of RA can provide important information for drug discovery.

We conducted a three-stage trans-ethnic meta-analysis (Extended Data Fig. 1). On the basis of the polygenic architecture of RA[10] and shared genetic risk among different ancestry[3,4], we proposed that combining a genome-wide association study (GWAS) of European and Asian ancestry would increase power to detect novel risk loci. In stage 1, we combined 22 GWAS for 19,234 cases and 61,565 controls of European and Asian ancestry[2–4]. We performed trans-ethnic, European-specific and Asian-specific GWAS meta-analysis by evaluating ~10 million single-nucleotide polymorphisms (SNPs)[11]. Characteristics of the cohorts, genotyping platforms and quality control criteria are described in Extended Data Table 1 (overall genomic control inflation factor $\lambda_{GC} < 1.075$).

Stage 1 meta-analysis identified 57 loci that satisfied a genome-wide significance threshold of $P < 5.0 \times 10^{-8}$, including 17 novel loci (Extended Data Fig. 2). We then conducted a two-step replication study (stage 2 for *in silico* and stage 3 for *de novo*) in 10,646 RA cases and 12,193 controls for the loci with $P < 5.0 \times 10^{-6}$ in stage 1. In a combined analysis of stages 1–3, we identified 42 novel loci with $P < 5.0 \times 10^{-8}$ in any of the trans-ethnic, European or Asian meta-analyses. This increases the total number of RA risk loci to 101 (Table 1 and Supplementary Table 1).

Comparison of 101 RA risk loci revealed significant correlations of risk allele frequencies (RAFs) and odds ratios (ORs) between Europeans and Asians (Extended Data Fig. 3a–c; Spearman's $\rho = 0.67$ for RAF and 0.76 for OR; $P < 1.0 \times 10^{-13}$), although five loci demonstrated population-specific associations ($P < 5.0 \times 10^{-8}$ in one population but $P > 0.05$ in the other population without overlap of the 95% confidence intervals (95% CIs) of the ORs). In the population-specific genetic risk model, the 100 RA risk loci outside of the major histocompatibility complex (MHC) region[12] explained 5.5% and 4.7% of heritability in Europeans and Asians, respectively, with 1.6% of the heritability explained by the novel loci. The trans-ethnic genetic risk model, based on the RAF from one population but the OR from the other population, could explain the majority (>80%) of the known heritability in each population (4.7% for Europeans and 3.8% for Asians). These observations support our hypothesis that the genetic risk of RA is shared, in general, among Asians and Europeans.

We assessed enrichment of 100 non-MHC RA risk loci in epigenetic chromatin marks[13] (Extended Data Fig. 3d). Of 34 cell types investigated, we observed significant enrichment of RA risk alleles with trimethylation of histone H3 at lysine 4 (H3K4me3) peaks in primary $CD4^+$ regulatory T cells (T_{reg} cells; $P < 1.0 \times 10^{-5}$). For the RA risk loci enriched with T_{reg} H3K4me3 peaks, we incorporated the epigenetic annotations along with trans-ethnic differences in patterns of linkage disequilibrium to fine-map putative causal risk alleles (Extended Data Fig. 3e, f).

We found that approximately two-thirds of RA risk loci demonstrated pleiotropy with other human phenotypes (Extended Data Fig. 4), including immune-related diseases (for example, vitiligo, primary biliary cirrhosis), inflammation-related or haematological biomarkers (for example, fibrinogen, neutrophil counts) and other complex traits (for example, cardiovascular diseases).

Each of 100 non-MHC RA risk loci contains on average ~4 genes in the region of linkage disequilibrium (in total 377 genes). To prioritize systematically the most likely biological candidate gene, we devised an *in silico* bioinformatics pipeline. In addition to the published methods that integrate data across associated loci[7,8], we evaluated several biological data sets to test for enrichment of RA risk genes, which helps to pinpoint a specific gene in each loci (Extended Data Figs 5, 6 and Supplementary Tables 2–4).

We first conducted functional annotation of RA risk SNPs. Sixteen per cent of SNPs were in linkage disequilibrium with missense SNPs ($r^2 > 0.80$; Extended Data Fig. 5a, b). The proportion of missense RA risk SNPs was higher compared with a set of genome-wide common SNPs (8.0%), and relatively much higher in the explained heritability (~26.8%). Using *cis*-acting expression quantitative trait loci (*cis*-eQTL) data obtained from peripheral blood mononuclear cells (5,311 individuals)[6] and from $CD4^+$ T cells and $CD14^+CD16^-$ monocytes (212 individuals), we found that RA risk SNPs in 44 loci showed *cis*-eQTL effects (false discovery rate (FDR) q or permutation $P < 0.05$; Extended Data Table 2).

Second, we evaluated whether genes from RA risk loci overlapped with human primary immunodeficiency (PID) genes[14], and observed significant overlap (14/194 = 7.2%, $P = 1.2 \times 10^{-4}$; Fig. 1a and Extended Data Fig. 5c). Classification categories of PID genes showed different patterns of overlap: the highest proportion of overlap was in 'immune dysregulation' (4/21 = 19.0%, $P = 0.0033$) but there was no overlap in 'innate immunity'.

Third, we evaluated overlap with cancer somatic mutation genes[15], under the hypothesis that genes with cell growth advantages may contribute to RA development. Among 444 genes with registered cancer somatic mutations[15], we observed significant overlap with genes implicated in haematological cancers (17/251 = 6.8%, $P = 1.2 \times 10^{-4}$; Fig. 1b and Extended Data Fig. 5d), but not with genes implicated in non-haematological cancers (6/221 = 2.7%, $P = 0.56$).