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REVIEW

Current concept and diagnosis of IgG4-related disease in the hepato-bilio-pancreatic system

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Abstract Recently, IgG4-related disease (IgG4-RD) has been recognized as a novel clinical entity with multiorgan involvement and unknown origin, associated with abundant infiltration of IgG4-positive cells. The Japanese research committee, supported by the Ministry of Health, Labor and Welfare of Japan, unified many synonyms for these conditions to the term "IgG4-RD" in 2009. The international symposium on IgG4-RD endorsed the comprehensive nomenclature as IgG4-RD, and proposed the individual nomenclatures for each organ system manifestations in 2011. Although the criteria for diagnosing IgG4-RD have not yet been established, proposals include the International Pathological Consensus (IPC) and the Comprehensive Diagnostic Criteria (CDC) for IgG4-RD for general use, and several organ-specific criteria for organ-specialized physicians, e.g., the International Consensus Diagnostic Criteria (ICDC) and the revised clinical diagnostic criteria in 2011 by the Japan Pancreas Society (JPS-2011) for type1 AIP; the Clinical Diagnostic Criteria 2012 for IgG4-sclerosing cholangitis (IgG4-SC-2012); the diagnostic criteria for IgG4-positive Mikulicz's disease by the Japanese Society for Sjogren's syndrome; and diagnostic criteria for IgG4-related kidney disease by the Japanese Society of Nephrology. In cases of probable or possible IgG4-RD diagnosed by the CDC, organ-specific diagnostic criteria should be concurrently used according to a diagnosis algorithm for IgG4-RD, with referral to a specialist.

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Abbreviations

AIP	Autoimmune pancreatitis
CDC	Comprehensive diagnostic criteria
GEL	Granulocytic epithelial lesion
ICDC	International consensus diagnostic criteria
IDCP	Idiopathic duct-centric pancreatitis
IgG4-RD	IgG4-related disease
IgG4-SC	IgG4-related sclerosing cholangitis
IPC	The international pathologic consensus criteria
JPS	Japan Pancreas Society
LPSP	Lymphoplasmacytic sclerosing pancreatitis
MD	Mikulicz's disease
MOLPS	Multiorgan lymphoproliferative disease
OOI	Other organ involvement
SjS	Sjögren's syndrome
PSC	Primary sclerosing cholangitis
RF	Rheumatoid factor
SIPS	Systemic IgG4-related plasmacytic syndrome

The history of IgG4-related disease: before and after discovery of IgG4

Recently, IgG4-related disease (IgG4-RD) has been recognized as a novel clinical entity with multiorgan involvement and an unknown origin, associated with abundant infiltration of IgG4-positive cells [1–8]. IgG4-RD has been found to affect the pancreas [9, 10], bile duct [10, 11], lacrimal glands [10, 12], salivary glands [10, 12], central nervous system [10, 13, 14], thyroid [10, 15, 16],

lungs [10, 17, 18], liver [10, 19, 20], gastrointestinal tract [10, 21–24], kidney [10, 25, 26], prostate [10, 27, 28], retroperitoneum [10, 29], arteries [10, 30], lymph nodes [10, 31], skin [10, 32], and breast [10, 33]. However, before the disease was identified, each organ lesion was described independently.

In 1892, Mikulicz et al. [34] first observed a patient with symmetrical swelling of the lachrymal, parotid and submandibular glands, with massive infiltration of mononuclear cells. The condition was called Mikulicz's disease (MD); however, it has since been classified as an atypical type of Sjögren's syndrome, which also presents with bilateral, painless, and symmetrical swelling of the lachrymal, parotid, and submandibular glands. Küttner [35] reported a tumor-like enlargement of the submandibular gland that was sometimes a result of stones in the Wharton duct, which indicated that the underlying cause had not been identified. In 1961, Sarles et al. [36] first observed a case of particular pancreatitis with hypergammaglobulinemia, a prototype of autoimmune pancreatitis (AIP). The concept of AIP was first proposed by Yoshida et al. [37] in 1995. Following the histopathological description of lymphoplasmacytic sclerosing pancreatitis (LPSP) in 1991, from the resected pancreas of tumor-forming pancreatitis, which are clinically difficult to distinguish from pancreatic cancer, has been regarded as a characteristic histopathological finding of IgG4-related AIP (type 1 AIP) [38]. In 1967, Comings et al. [39] reported the first familiar case of multifocal fibrosclerosis with retroperitoneal fibrosis, mediastinal fibrosis, sclerosing cholangitis, Riedel's thyroiditis, and pseudotumor of the orbit, which is now regarded as the synonym of IgG4-RD.

Hamano et al. [9] reported increased serum levels of IgG4 in Japanese patients with AIP, an epoch-making discovery in the history of IgG4-RD. Thereafter, many studies of AIP have been reported, mainly by Japanese investigators. The histopathological findings of LPSP are characterized by the periductal localization of predominantly CD4 positive T-cells, IgG4-positive plasma cells, storiform fibrosis with acinar cell atrophy frequently resulting in stenosis of the main pancreatic duct, and obliterative fibrosis [10]. About 60–80 percent of patients with AIP show obstructive jaundice with sclerosing cholangitis (IgG4-related sclerosing cholangitis; IgG4-SC) and other organ involvement (OOI), in which cholangiographic features are similar to those of primary sclerosing cholangitis (PSC), pancreatic cancer, and cholangiocarcinoma. The steroid responses and the prognoses of sclerosing cholangitis associated with AIP differ from patients with PSC, which suggests different pathological conditions. In 2003, Kamisawa et al. [12] suggested that AIP is a systemic sclerosing disease. This was based on findings that the pancreas and other involved organs have fibrosis with abundant infiltration of IgG4-positive plasma cells. This is similar to the concept of multifocal fibrosclerosis proposed by Comings et al. [39]. Further histological and clinical profiling of patients with "AIP" reveals two distinct subtypes, type 1 and type 2 [40, 41]. Type 1 AIP is classified as a pancreatic manifestation of IgG4-RD, and is probably a systemic disease with an abnormal immunological process. Type 2 AIP is thought to be a specific pancreatic disease with granulocytic epithelial lesion (GEL) [42, 43] and occasional coexistence with ulcerative colitis [41, 44].

Conversely, most patients with MD show elevated serum levels of IgG4, negative anti-SS-A/Ro or anti-SS-B/La antibodies, infiltration of IgG4-positive plasma cells into the glands, and recovery of secretion with steroid treatment. The patients with MD often show steroid responsive OOIs such as AIP, sclerosing cholangitis, retroperitoneal fibrosis, enlarged celiac and hilar lymph nodes, chronic thyroiditis, or interstitial nephritis [1–5, 10]. MD has been considered to be completely different from Sjögren's syndrome because of this, and because of its responsiveness to steroid treatment [1–5, 10].

In addition to the original concept of multifocal idiopathic fibrosclerosis, recent studies led us to develop a novel concept of a systemic disease such as IgG4-related systemic sclerosing disease [1], systemic IgG4-related plasmacytic syndrome (SIPS) [2], or IgG4-positive multiorgan lymphoproliferative syndrome (IgG4-MOLPS) [3], all of which may refer to the same conditions. Based on these findings, the members of the Japanese Research Committees for "Systemic IgG4-related Sclerosing Disease" (chaired by Professor Okazaki) and "IgG4-MOLPS" (chaired by Professor Umehara), both of which were supported by the Research for Intractable Disease Program from the Ministry of Health, Labor and Welfare of Japan, have agreed that the comprehensive term "IgG4-related disease IgG4-RD)" includes these conditions at a minimum, although pathogenesis and pathophysiology remain unclear [4, 5]. The first International Symposium on IgG4-RD held in Boston (chaired by Professor Stone of Massachusetts General Hospital) endorsed the Japanese concept and proposed nomenclatures and pathological criteria for individual organ lesions [6, 7] (Tables 1, 2, 3).

Current concepts of IgG4-RD

General concept of IgG4-RD

Patients with IgG4-RD show diffuse or focal organ enlargement and mass-forming or nodular/thickened lesions in various organs, either synchronously or metachronously. This is due to the prominent infiltration of lymphocytes and plasmacytes with fibrosis [4]. The causes

Table 1 History of IgG4-related disease

Year	Authors	References	Evidences/contents
1892	Mikulicz et al.	[34]	Mikulicz's disease (*Z. Chir. Fesrschr*)
1961	Sarles et al.	[36]	Hyper-gammaglobulinemia in CP (*Am J Dig Di*)
1967	Comings et al.	[39]	Familial multifocal fibrosclerosis. (*Ann Intern Med*)
1972	Küttner	[35]	Küttner tumor (*Acta Otolaryngol*)
1991	Kawaguchi et al.	[38]	Lymphoplasmacytic sclerosing pancreatitis(*Human Pathol*)
1995	Yoshida et al.	[37]	Autoimmune pancreatitis (*Dig Dis Sci*)
2001	Hamano et al.	[9]	High IgG4 levels in sclerosing pancreatitis (*N Eng J Med*)
2002	Japan Pancreas Society	[57]	Clinical diagnostic criteria for AIP 200 (*Suizo*)
2006	Okazaki et al.	[56]	Clinical diagnostic criteria for AIP 2006 (*J Gastroenterol*)
2006	Chari et al.	[58]	Mayo criteria (*Clin Gastroenterol Hepatol*)
2006	Kamisawa et al.	[12]	IgG4-related sclerosing disease (*J Gastroenterol*)
2006	Yamamoto et al.	[2]	IgG4-related plasmacytic disease (*Mod Rheumatol*)
2008	Masaki et al.	[3]	IgG4-multiorgan lymphoproliferative syndrome (MOLPS) (*Ann Rheum Dis*)
2011	Shimosegawa et al.	[41]	International Consensus Diagnostic Criteria (ICDC) for AIP (*Pancreas*)
2012	Umehara, Okazaki, et al.	[4, 5]	Concept and comprehensive Diagnostic Criteria for IgG4-related disease (*Mod Rheumatol*)
2012	Deshpande et al.	[6]	International Pathological Consensus for IgG4-RD (*Mod Pathol*)
2012	Stone et al.	[7]	Nomenclatures of individual organ manifestation of IgG4-RD (*Arthritis Rheum*)

Table 2 Preferred nomenclature for individual organ system manifestations of IgG4-related disease (from [7], with permission)

Organ system/tissue	Preferred name
Pancreas	Type 1 autoimmune pancreatitis (IgG4-related pancreatitis)
Eye	IgG4-related ophthalmic disease is the general term for the peri-ocular manifestations of this disease. There are several subsets, outlined below.
Lacrimal glands	IgG4-related dacryoadenitis
Orbital soft tissue (orbital inflammatory pseudotumor)	IgG4-related orbital inflammation
Extra-ocular muscle disease	IgG4-related orbital myositis
Orbit with involvement of multiple anatomic structures	IgG4-related pan-orbital inflammation (includes lacrimal gland disease, extra-ocular muscle involvement, and other potential intra-orbital complications)
Salivary glands (parotid and submandibular glands)	IgG4-related sialadenitis or, more specifically, IgG4-related parotitis or IgG4-related submandibular gland disease
Pachymeninges	IgG4-related pachymeningitis
Hypophysis	IgG4-related hypophysitis
Thyroid (Riedel's thyroiditis)	IgG4-related thyroid disease
Aorta	IgG4-related aortitis/peri-aortitis
Arteries	IgG4-related periarteritis
Mediastinum	IgG4-related mediastinitis
Retroperitoneum	IgG4-related retroperitoneal fibrosis
Mesentery	IgG4-related mesenteritis
Skin	IgG4-related skin disease
Lymph node	IgG4-related lymphadenopathy
Bile ducts	IgG4-related sclerosing cholangitis
Gallbladder	IgG4-related cholecystitis
Liver	IgG4-related hepatopathy (refers to liver involvement that is distinct from biliary tract involvement)
Lung	IgG4-related lung disease
Pleura	IgG4-related pleuritis
Pericardium	IgG4-related pericarditis
Kidney	IgG4-related kidney disease. The specific renal pattern should be termed IgG4-related tubulointerstitial nephritis and membranous glomerulonephritis secondary to IgG4-RD. Involvement of the renal pelvis should be termed IgG4-related renal pyelitis.
Breast	IgG4-related mastitis
Prostate	IgG4-related prostatitis

of the disease are still not clear; however, some abnormal immunological mechanisms are involved. The organs known to be affected include the pancreas, biliary duct, lacrimal/salivary glands, retroperitoneum, central nervous system, thyroid gland, lungs, liver, gastrointestinal tracts, kidneys, prostate gland, and lymph nodes [21–41]. IgG4-RD mainly affects middle-aged to elderly men, and clinical symptoms vary depending on the organ in which the lesions are located. Many cases are treated effectively by steroid therapy [1–10]; however, the prognosis is not clear. Some patients develop serious complications such as obstructive jaundice due to hepatic, gallbladder, or

Table 3 The three major histopathological features associated with IgG4-RD and the minimal criteria in a new organ/site in the international pathological consensus (from [6])

The three major histopathological features associated with IgG4-RD
1. Dense lymphoplasmacytic infiltrate
2. Fibrosis, arranged at least focally in a storiform pattern
3. Obliterative phlebitis
Other histopathological features associated with IgG4-RD are:
1. Phlebitis without obliteration of the lumen
2. Increased numbers of eosinophils
Minimal Criteria for IgG4-RD in a new organ/Site
(1) characteristic histopathological findings with an elevated IgG4t plasma cells and IgG4-to-IgG ratio
(2) high serum IgG4 concentrations
(3) effective response to glucocorticoid therapy
(4) reports of other organ involvement that is consistent with IgG4-related disease

pancreatic lesions; hydronephrosis due to retroperitoneal fibrosis; or respiratory symptoms due to pulmonary lesions [1–10, 25, 26, 29, 45–47]. Although the infiltration of IgG4-positive cells and increased serum levels of IgG4 are characteristic in IgG4-RD, the severity of fibrosis seems to be different among the individual organs involved. These conditions are quite similar to multifocal idiopathic fibrosclerosis [39]. Storiform fibrosis and obliterative phlebitis are characteristic in pancreatic and biliary tract lesions, but the degree varies depending on the individual organs. For example, very seldom do lesions appear in the lachrymal/salivary gland or lymph node. The previous nomenclature of "IgG4-related sclerosing disease" is mainly based on the fibrous swollen organs, whereas those of "IgG4-SIPS" and "IgG4+ MOLPS" have been based on lymphoplasmacytic proliferation and swollen lymph nodes without fibrosis.

Although most patients have multiorgan lesions synchronously or metachronously, about 10–20 percent of the patients do not have confirmed OOI. Therefore, it is unclear whether the pathogenetic mechanism is same among individual organs or not.

Concept of IgG4-RD in the hepato-bilio-pancreatic system

Type 1 AIP (IgG4-related pancreatitis)

Recent studies have suggested that AIP manifests as two distinct subtypes, type 1 and type 2 [40, 41]. In type 1 AIP, the histologic description is called lymphoplasmacytic sclerosing pancreatitis (LPSP) and the pancreatic histopathology shows the following characteristic features: (i) abundant infiltration of plasma cells (greater than 10 IgG4+ cells per high power field (hpf) and an IgG4 to IgG ratio greater than 40 percent) and lymphocytes; (ii) peculiar storiform or swirling fibrosis; and (iii) perivenular infiltration with lymphocytes and plasma cells often leading to obliterative phlebitis.

Type 2 AIP was proposed by American and European pathologists based on histological examination of the resected pancreases from patients with chronic non-alcoholic pancreatitis. These pathologists reported another histopathological pattern, which they named idiopathic duct-centric pancreatitis (IDCP) or AIP with granulocytic epithelial lesion (GEL) [41–43]. The most characteristic feature of type 2 AIP is the granulocytic epithelial lesion (GEL), which often presents with destruction and obliteration of the pancreatic duct. Type 2 AIP includes swelling of the pancreas, but no or very few IgG4-positive plasma cells. Type 2 AIP clinical features show a distinctly different profile associated with no serum IgG4, IgG elevation, presence of autoantibodies, or other organ involvement.

IgG4-related sclerosing cholangitis (IgG4-SC)

IgG4-SC is a characteristic type of sclerosing cholangitis with dense infiltration of IgG4-positive plasma cells and extensive fibrosis in the bile duct wall [48]. Circular and symmetrical thickening of the bile duct wall is observed not only in the stenotic areas but also in the areas without stenosis that appear normal in the cholangiogram [49]. IgG4-SC related OOI such as IgG4-related dacryoadenitis or sialadenitis or IgG4-related retroperitoneal fibrosis is frequently associated with type 1 AIP [50–54] The differential diagnosis of IgG4-SC from PSC and cholangiocarcinoma is very important. It is also necessary to rule out secondary sclerosing cholangitis caused by diseases with obvious pathogenesis.

Nomenclatures of individual organ manifestation of IgG4-RD

Because multiorgan involvements may occur in IgG4-RD as described above, IgG4-RD includes a wide variety of diseases, including MD, AIP, hypophysitis, Riedel thyroiditis, interstitial pneumonitis, interstitial nephritis, prostatitis, lymphadenopathy, retroperitoneal fibrosis, inflammatory aortic aneurysm, and inflammatory pseudotumor [1–10]. In the International Symposium on IgG4-RD, the nomenclature of individual organ manifestations of IgG4-RD were proposed (Table 2) using "IgG4-related" as a modifier, except for the pancreatic manifestation [7]. The pancreatic manifestation of IgG4-RD was termed "type 1 autoimmune pancreatitis (IgG4-related pancreatitis)." The term "type 1 AIP" is now widely accepted among gastroenterologists and pancreatic surgeons. It also serves to discriminate between type 1 and type 2 AIP, which is not a

part of the IgG4-RD spectrum. When the pathogenesis of type 2 AIP is clarified, the term "type 1 AIP" might be replaced by "IgG4-related pancreatitis." In the biliary manifestation, the nomenclature for IgG4-related biliary tract (but not gall bladder) disease includes "sclerosing" to distinguish between the primary and IgG4-related forms of sclerosing cholangitis.

Clinical diagnostic criteria for IgG4-RD

International pathological consensus criteria for IgG4-RD

To diagnose IgG4-RD, histopathological findings are critical, and the international histological consensus criteria (IPC) (Table 3) have been proposed [6]. There are three major histopathological features associated with IgG4-RD: (i) dense lymphoplasmacytic infiltration; (ii) fibrosis, arranged at least focally in a storiform pattern; and (iii) obliterative phlebitis. Other minor histopathological features associated with IgG4-RD are phlebitis without obliteration of the lumen and increased numbers of eosinophils. IgG4-positive plasma cells are ubiquitous in diverse localised non-specific chronic inflammatory conditions and need to be distinguished from IgG4-related systemic disorders [55]. Therefore, different numbers of IgG4-positive cells among individual organ lesions were required for diagnosing IgG4-RD (Fig. 1). For examples, more than 100 IgG4-positive cells are required in the lacrimal and salivary gland and lymph node, and more than 200 cells in the skin. On the other hand, in the liver, bile duct and pancreas,

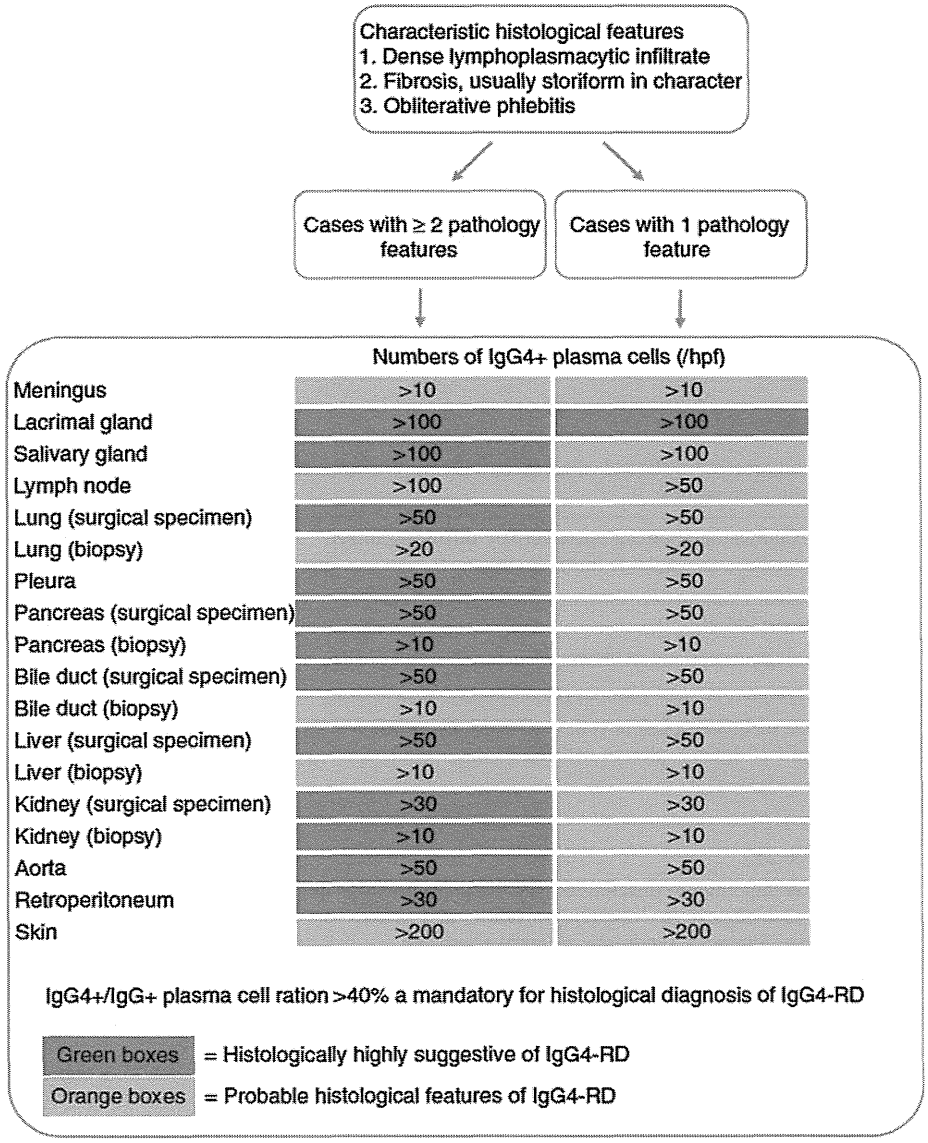

Fig. 1 Histologic diagnostic schema of IgG4-related disease (reproduced from [6])

more than 10 cells by biopsy and 50 cells by surgical specimen are needed. In order to diagnose involvement of a new organ or site, it has been recommended that at least three, but ideally four, of the following criteria for IgG4-RD be met: (i) characteristic histopathological findings of an elevated concentration of IgG4+ plasma cells and elevated IgG4 to IgG ratio; (ii) high serum IgG4 concentrations; (iii) effective response to glucocorticoid therapy; and (iv) other organ involvement that is consistent with IgG4-RD. Appropriate histopathologic findings are essential, but not sufficient, to establish a new manifestation or site of IgG4-RD, so a combination of clinical manifestations, including serum IgG4 levels and histological findings, are necessary.

Japanese comprehensive diagnostic criteria for IgG4-RD

The Japanese comprehensive diagnostic criteria for IgG4-RD [5] (Table 4) are based on two major characteristics of IgG4-RD: increased serum concentrations of IgG4 and infiltration of IgG4+ cells into the affected organ. The cutoff value for serum IgG4 concentration, 135 mg/dl, is the same as that in AIP [9]. Although tissue biopsies are difficult to obtain from some organs, including the pancreas, retroperitoneum, and ocular cavity, histopathological examination is required. Pathological criteria should be rigorous because IgG4+ plasma cell infiltration has been reported in various diseases and clinical conditions, such as rheumatoid synovitis, inflammatory oral and skin lesions, and carcinomas with a peritumoral inflammatory response [55]. Histopathological findings of marked IgG4+ cell infiltration (concentration of greater than 10 cells per hpg) and an IgG4/IgG cell ratio of greater than 40 percent are diagnostic of IgG4-RD.

Table 4 Comprehensive clinical diagnostic criteria for IgG4-RD (from [5], with permission)

1. Clinical examination showing characteristic diffuse/localized swelling or masses in single or multiple organs
2. Hematological examination shows elevated serum IgG4 concentrations(135 mg/dl)
3. Histopathologic examination shows:
(1) Marked lymphocyte and plasmacyte infiltration and fibrosis.
(2) Infiltration of IgG4+ plasma cells: ratio of IgG4+/IgG+ cells >40 % and >10 IgG4+ plasma cells/HPF
Definite: 1) + 2) + 3)
Probable: 1) + 3)
Possible: 1) + 2)
However, it is important to differentiate IgG4-RD from malignant tumors of each organ (e.g., cancer, lymphoma) and similar diseases (e.g. Sjögren's syndrome, primary sclerosing cholangitis, Castleman's disease, secondary retroperitoneal fibrosis, Wegener's granulomatosis, sarcoidosis, Churg–Strauss syndrome) by additional histopathological examination
Even when patients cannot be diagnosed using the CCD criteria, they may be diagnosed using organ-specific diagnostic criteria for IgG4RD.

Diagnostic criteria for individual organ manifestation of IgG4-RD in hepato-bilio-pancreatic organs

Diagnostic criteria for type1 AIP

International consensus of diagnostic criteria (ICDC) for type1 AIP

The ICDC for AIP [41] were developed based on previous criteria, including JPS (2002, 2006) [56, 57], HISORt (2006, 2009) [58, 59] Korean (2007) [60], Asian (2008) [61], Mannheim (2009) [62] and Italian (2003, 2009) [63], and first enabled us to make an independent clinical diagnosis of type 1 or type 2 AIP. The diagnosis of type 1 AIP by ICDC requires a combination of five primary cardinal features (Tables 5,6): (i) imaging features of a) pancreatic parenchyma (on CT/MRI) and b) pancreatic duct (ERCP or MRCP); (ii) serology (IgG4); (iii) other organ involvement; (iv) histopathology of the pancreas; and (v) response to steroid therapy. Each criterion, except for steroid responsiveness, is classified as either level 1 or level 2 collateral criteria. Level 1 collateral is highly suggestive of AIP. Patients with obstructive jaundice and a diffusely enlarged pancreas (especially with a capsule-like rim) without pancreatic ductal dilatation/cutoff or pancreatic low-density mass on CT/MRI are highly likely to have AIP. However, subjects with typical findings of pancreatic cancer (e.g., low-density mass on contrast-enhanced CT, pancreatic ductal dilatation/cutoff with or without pancreatic atrophy) should be considered as having pancreatic cancer. Subjects without features typical of AIP or pancreatic cancer should first be investigated for pancreatic cancer. AIP should be considered only after negative work-up of malignancy. Response to steroids can confirm a strong suspicion of AIP. However, a steroid trial as a means to diagnose AIP is to be used sparingly and should not be used as a substitute for a thorough search for an etiology.

Clinical diagnostic criteria for AIP by Japan Pancreas Society in 2011 (JPS-2011)

JPS-2011 [64, 65] (Table 7) took basic concepts from both the previous Japanese criteria [56, 57] and the CDC for type 1 AIP [41]. These include ensuring that the criteria are (i) simple for general physicians' use; (ii) rely on diffuse/segmental/focal classification of pancreatic imaging; (iii)

Table 5 Level 1 and Level 2 criteria for type 1 AIP in international consensus of diagnostic criteria (ICDC) (from [41], with permission)

	Criterion	Level 1	Level 2
P	Parenchymal imaging	Typical:	Indeterminate (including Atypical[a]):
		Diffuse enlargement with delayed enhancement (sometimes associated with rim like enhancement)	Segmental/focal enlargement with delayed enhancement
D	Ductal imaging (ERP)	Long (>1/3 length of the mpd) or multiple strictures without marked upstream dilatation	Segmental/focal narrowing without marked upstream dilatation (duct size <5 mm)
S	Serology	IgG4 >2 X upper limit of normal value	IgG4 1-2 X upper limit of normal value
OOI	Other organ involvement	a or b	a or b
		a. Histology of extrapancreatic organs	a. Histology of extrapancreatic organs including endoscopic biopsies of bile duct[b]:
		any three of the following	both of the following
		i. Marked lymphoplasmacytic infiltration with fibrosis and without granulocytic infiltration	i. Marked lymphoplasmacytic infiltration without granulocytic infiltration
		ii. Striform fibrosis	ii. Abundant (>10 cells/hpf) IgG4 positive cells
		iii. Obliterative phlebitis	
		iv. Abundant (>10 cells/hpf) IgG4 positive cells	
		b. Typical radiological evidence	b. Physical or radiological evidence
		at least one	at least one
		i. Segmental/multiple proximal (hilar/intra hepatic) or proximal and distal bile duct stricture	i. Symmetrically enlarged salivary/lacrimal glands
		ii. Retroperitoneal fibrosis	ii. Radiologic evidence of renal involvement described in association with AIP
H	histology of the pancreas	LPSP (core biopsy/resection) At least 3 of the following	LPSP (core biopsy) Any 2 of the following
		i. Periductal lymphoplasmacytic infiltrate without granulocytic infiltration	i. Periductal lymphoplasmacytic infiltrate without granulocytic infiltration
		ii. Obliterative phlebitis	ii. Obliterative phlebitis
		iii. Storiform fibrosis	iii. Storiform fibrosis
		iv. Abundant (>10 cells/hpf) IgG4 positive cells	iv. Abundant (>10 cells/hpf) IgG4 positive cells
Diagnostic steroid trial			
Response to steroid (Rt)[c]		Rapid (≤2 weeks) radiologically demonstrable resolution or marked improvement in pancreatic/extra-pancreatic Manifestations	

[a] Atypical: some AIP cases may show low-density mass, pancreatic ductal dilatation or distal atrophy. Such atypical imaging findings in patients with obstructive jaundice and/or pancreatic mass are highly suggestive of pancreatic cancer. Such patients should be managed as pancreatic cancer unless there is strong collateral evidence for AIP and a thorough work-up for cancer is negative (see algorithm)

[b] Endoscopic biopsy of duodenal papilla is a useful adjunctive method because ampulla is often involved pathologically in AIP

[c] Diagnostic steroid trial should be conducted carefully by pancreatologists with caveats (see text) only after negative work-up for cancer including EUS-FNA

Table 6 Diagnosis of definitive and probable type 1 AIP using international consensus diagnostic criteria (ICDC) (from [41], with permission)

Diagnosis	Primary basis for diagnosis	Imaging evidence	Collateral evidence
Definitive Type 1 AIP	Histology	Typical/indeterminate	Histologically confirmed LPSP (Level 1 H)
	Imaging	Typical	Any non-D Level 1/Level 2
		Indeterminate	Two or more from Level 1 (+Level 2 D[a])
	Response to steroid	Indeterminate	Level 1 S/OOI + Rt or IBD + Level 1 D + Level 2 S/OOI/H + Rt
Probable Type 1 AIP		Indeterminate	Level 2 S/OOI/H + Rt

[a] Level 2 D is counted as Level 1 in this setting

Table 7 Clinical diagnostic criteria for autoimmune pancreatitis in 2011 by Japan Pancreas Society (JPS-2011) (from [64, 65], with permission)

A. Diagnostic criterion
I. Enlargement of the pancreas:
a. Diffuse enlargement
b. Segmental/focal enlargement
II. ERP (endoscopic retrograde pancreatography) shows irregular narrowing of the main pancreatic duct
III. Serological findings
Elevated levels of serum IgG4 (≥135 mg/dl)
IV. Pathological findings: among i)–iv) listed below,
a. Three or more are observed
b. Two are observed
i) Prominent infiltration and fibrosis of lymphocytes and plasmacytes
ii) Ten or more diffuse IgG4-positive plasmacytes per high-power microscope field
iii) Storiform fibrosis
iv) Obliterative phlebitis
V. Other organ involvement (OOI): sclerosing cholangitis, sclerosing dacryoadenitis/sialoadenitis, retroperitoneal fibrosis
a. Clinical lesions
Extra-pancreatic sclerosing cholangitis, sclerosing dacryoadenitis/sialoadenitis (Mikulicz disease), or retroperitoneal fibrosis can be diagnosed with clinical and image findings.
b. Pathological lesions
Pathological examination shows characteristic features of sclerosing cholangitis, sclerosing dacryoadenitis/sialoadenitis, or retroperitoneal fibrosis.
<Option> Effectiveness of steroid therapy
A specialized facility may include in its diagnosis the effectiveness of steroid therapy, once pancreatic or bile duct cancers have been ruled out. When it is difficult to differentiate from malignant conditions, it is desirable to perform cytological examination using an endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). Facile therapeutic diagnosis by steroids should be avoided unless the possibility of malignant tumor has been ruled out by pathological diagnosis.
B. Diagnosis
I. Definite diagnosis
1 Diffuse type
I a + < III/IVb/V(a/b)>
2 Segmental/focal type
I b + II + two or more of <III/IV b/V (a/b)>
I b + II + < III/IV b/V (a/b)> + Option
3 Definite diagnosis by histopathological study
IV a
II. Probable diagnosis
Segmental/focal type: I b + II + < III/IV b/V (a/b)>
III. Possible diagnosis[a]
Diffuse type: I a + II + Option
Segmental/focal type: I b + II + Option

When a patient with a focal/segmental image of AIP on CT/MRI without ERCP findings fulfill more than one of III, IVb and V(a/b) criteria, he/she can be diagnosed as possible AIP only after the negative workup for malignancy by EUS-FNA, and confirmed as probable one by an optional steroid response

[a] Possible diagnosis: a case may be possibly type 2, although it is extremely rare in Japan

"+" refers to "and", and "/" refers to "or"

use IgG4 alone as a serological marker; (iv) identify OOIs such as sclerosing cholangitis; sclerosing sialadenitis and retroperitoneal fibrosis; (v) have no classifications of level1/2 in serum IgG4 and OOI; (vi) apply optional steroid trial only after determining non-malignancy using EUS-FNA. Different from the previous Japanese criteria, with

Table 8 Clinical diagnostic criteria of IgG4-related sclerosing cholangitis 2012 (from [66], with permission)

Diagnostic items
(1) Biliary tract imaging reveals diffuse or segmental narrowing of the intrahepatic and/or extrahepatic bile duct associated with the thickening of bile duct wall
(2) Hematological examination shows elevated serum IgG4 concentrations (≥135 mg/dl)
(3) Coexistence of autoimmune pancreatitis, IgG4-related dacryoadenitis/sialadenitis, or IgG4-related retroperitoneal fibrosis
(4) Histopathological examination shows:
a. Marked lymphocytic and plasmacyte infiltration and fibrosis
b. Infiltration of IgG4-positive plasma cells:[10 IgG4-positive plasma cells/HPF
c. Storiform fibrosis
d. Obliterative phlebitis
<Option> effectiveness of steroid therapy
A specialized facility, in which detailed examinations such as endoscopic biliary biopsy and endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) can be administered, may include in its diagnosis the effectiveness of steroid therapy, once pancreatic or biliary cancers have been ruled out.
Diagnosis
Definite diagnosis
(1) + (3)
(1) + (2) + (4) a, b
(4) a, b, c
(4) a, b, d
Probable diagnosis
(1) + (2) + option
Possible diagnosis
(1) + (2)

It is necessary to exclude PSC, malignant diseases such as pancreatic or biliary cancers, and secondary sclerosing cholangitis caused by the diseases with obvious pathogenesis. When it is difficult to differentiate from malignant conditions, a patient must not be treated with facile steroid therapy, but should be referred to a specialized medical facility

JPS-2011, patients are diagnosed as having definitive, probable, or possible AIP by a combination of the criteria described. This is similar to the concept of the ICDC.

Although the JPS-2011 is focused on type 1 AIP, some patients with type 2 AIP, which is extremely rare in Japan, may be diagnosed as possible AIP using these criteria. As ERCP is more commonly performed to diagnose AIP or pancreatic cancer than EUS-FNA in Japan, ERCP is essentially required in the diagnosis of the focal/segmental type of AIP. However, to follow the concept of the ICDC as much as possible, the following exceptional case can be deemed acceptable only by an expert: when a patient with a focal/segmental image of AIP on CT/MRI without ERCP findings fulfills more than

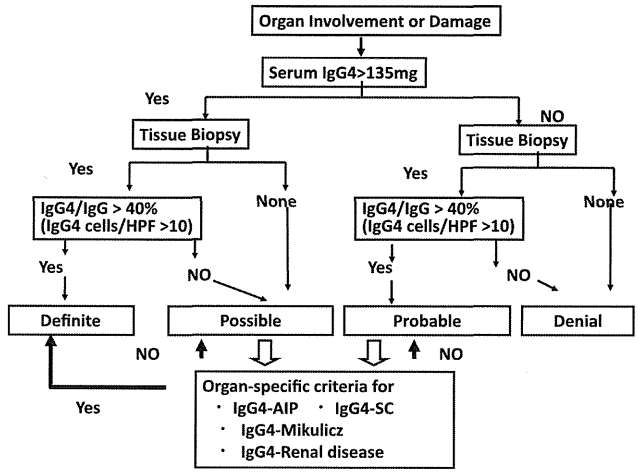

Fig. 2 Algorism for Diagnosis of IgG4RD (reproduced from [5], with permission) Diagnostic algorithm performance for comprehensive diagnostic criteria for IgG4-related disease (IgG4-RD) using comprehensive diagnostic criteria combined with organ-specific criteria. A diagnosis of IgG4-RD is definitive in patients with (1) organ enlargement, mass or nodular lesions, or organ dysfunction, (2) a serum IgG4 concentration >135 mg/dl, and (3) histopathological findings of >10 IgG4+ cells/HPF and an IgG4/IgG cell ratio >40 %

one of III (serum IgG4), IVb (two of pathological findings) and V(a/b) (OOI), he can be diagnosed as possible AIP only after the negative workup for malignancy by EUS-FNA and AIP is confirmed as probable by an optional steroid response.

Diagnostic criteria for IgG4-related sclerosing cholangitis

Primary sclerosing cholangitis and cholangiocarcinoma must be discriminated from IgG4-SC. The diagnosis of IgG4-SC is based on the combination of the following criteria [66] (Table 8): (i) characteristic biliary imaging findings; (ii) elevation of serum IgG4 concentrations; (iii) coexistence of IgG4-RDs except those of the biliary tract; and (iv) characteristic histopathological features. It is difficult to obtain sufficient biliary tract tissue to determine the characteristic histology of IgG4-SC by biopsy [67]. Similar to the ICDC or JPS-2011 diagnostic criteria for AIP, the effectiveness of steroid therapy is an optional additional diagnostic criterion to confirm an accurate diagnosis of IgG4-SC.

Algorithm for diagnosing IgG4-RD in combination with the comprehensive and individual organ diagnostic criteria

A diagnostic algorithm for IgG4-RD [5], using comprehensive diagnostic criteria combined with organ-

Table 9 Comparison of diagnostic criteria among pathological, comprehensive and individual organ manifestation for IgG4-RD

Diagnostic criteria (Ref.)	Clinical findings/images	Serology histology (Serum IgG4)	Histology	OOI	Efficacy of Steroid
Minimal Criteria by the International Pathological Consensus [6]	ND	High	Characteristic histopathological findings with an elevated IgG4t plasma cells and IgG4-to-IgG ratio	Reports of OOI consistent with IgG-RD	Yes
Comprehensive Diagnostic Criteria (CDC) for IgG4-RD [5]	Diffuse/localized swelling or masses in single or multiple organs	≤135 mg/dl	(i) marked lymphocyte and plasmacyte infiltration and fibrosis (ii) ratio of IgG4+/IgG+ cells >40 % and >10 IgG4+ plasma cells/HPF	ND	No
ICDC for typeI AIP [41]	(i) parenchymal image on CT/MRI (ii) duct image on ERP		LPSP (i) Lymphoplasmacyte infiltrate (ii) >10 IgG4+ cells (/hpf) (iii) Storiform fibrosis (iv) Obliterative phlebitis	a. Histology of OOI b. Radiological evidence	Yes
Level 1	Diffuse	>2 × ULN	More than 3 (i-iv)	a. any three (i-iv); b. typical one	
Level 2	Segmental/focal	1–2 × ULN	Any 2	a. two (i + ii); b or physical	
JPS-2011 [64, 65]	Enlarged pancreas on CT/MRI Diffuse enlargement Segmental/focal enlargement Irregular narrowing of the MPD on ERP	≥135 mg/dl	(i) Lymphoplasmacyte infiltrate (ii) >10 IgG4+ cells (/hpf) (iii) Storiform fibrosis (iv) Obliterative phlebitis	Sclerosing cholangitis, Dacryoadenitis/sialoadenitis Retroperitoneal fibrosis (a) Clinical lesions (b) Pathological lesions	
Clinical Diagnostic Criteria for IgG4-related sclerosing cholangitis 2012 [66]	(i) Narrowing of the intrahepatic/extrahepatic bile duct (Diffuse/Segmental) (ii) Thickening of bile duct wall	≥135 mg/dl	(i) Lymphoplasmacyte infiltrate (ii) >10 IgG4+ cells (/hpf) (iii) Storiform fibrosis (iv) Obliterative phlebitis	AIP Dacryoadenitis/sialoadenitis Retroperitoneal fibrosis	Yes

specific criteria, is shown in Fig. 2. Table 9 shows a comparison of minimal pathological criteria, comprehensive diagnostic criteria, and individual specific criteria in the hepato-bilio-pancreatic system for IgG4-RD. A diagnosis of IgG4-RD is definitive in patients with: (i) organ enlargement, mass or nodular lesions, or organ dysfunction; (ii) a serum IgG4 concentration of 135 mg/dl or higher; and (iii) histopathological findings of greater than 10 IgG4 cells per HPF and an IgG4+/IgG+ cell ratio greater than 40 percent. A diagnosis of IgG4-RD is possible in patients who fulfill criteria (i) and (ii), but with negative results on histopathology or without histopathologic examination, whereas a diagnosis of IgG4-RD is probable in patients with organ involvement (i) and fulfilled histopathologic criteria (iii), but without increased serum IgG4 concentration (ii). Patients with organ symptoms but without satisfying serologic or histopathologic criteria are considered unlikely to have IgG4-RD. For possible or probable cases, organ-specific criteria for IgG4-RD could be applied, such as those for AIP [41, 56, 64, 65], MD [68], and KD [69] associated with IgG4. Patients who fulfill the organ-specific criteria for IgG4-RD have a definite diagnosis of this disease.

Conclusion

Current concepts and clinical diagnostic criteria for IgG4-RD and individual organ manifestations are mentioned. The Comprehensive Diagnostic Criteria for IgG4-RD have been proposed for the general and specific criteria for individual organs. It is recommended that in cases of probable or possible IgG4-RD diagnosed by the CDC,

organ specific diagnostic criteria should be concurrently used according to the diagnostic algorithm for IgG4-RD in Japan. When the precise mechanism in the development of IgG4-RD is clarified, establishment of the clinical diagnostic criteria for IgG4-RD should be needed in the near future.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Toll-like receptor activation in basophils contributes to the development of IgG4-related disease

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Abstract
Background IgG4-related disease (IRD) is characterized by systemic IgG4 antibody responses and by infiltration of IgG4-expressing plasma cells into the affected organs. Although T helper type 2 (Th2) cytokines are implicated in enhanced IgG4 responses, molecular mechanisms accounting for the development of IgG4 antibody responses are poorly defined. Since basophils function as antigen-presenting cells for Th2 responses, we tried to clarify the role of basophils in the development of IgG4 responses in this study.
Methods IgG4 and cytokine responses to various nucleotide-binding oligomerization domain-like receptor and Toll-like receptor (TLR) ligands were examined by using basophils isolated from healthy controls and from patients with IgG4-related disease.
Results Activation of TLRs in basophils from healthy controls induced IgG4 production by B cells, which effect was associated with enhanced production of B cell activating factor (BAFF) and IL-13. In addition, activation of TLRs in basophils from patients with IRD induced a large amount of IgG4 by B cells from healthy controls. This enhancement of IgG4 production was again associated with BAFF and IL-13.
Conclusions These data suggest that innate immune responses mediated through TLRs may play a role in the development of IgG4-related disease, in part by production of BAFF from basophils.

Keywords IgG4-related disease · Basophil · TLR

Abbreviations

Ab	Antibody
Ag	Antigen
AIP	Autoimmune pancreatitis
APC	Antigen-presenting cell
BAFF	B cell activating factor
ELISA	Enzyme-linked immunosorbent assay
IRD	IgG4-related disease
LPS	Lipopolysaccharide
MDP	Muramyl dipeptide
NOD2	Nucleotide-binding oligomerization domain 2
NLR	NOD-like receptor
PAM	Pam_3CSK4
PBMC	Peripheral blood mononuclear cell
PGN	Peptidoglycan
TLR	Toll-like receptor
TSLP	Thymic stromal lymphopoietin

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Introduction

IgG4-related disease (IRD) is a newly proposed disease entity that is diagnosed based on enhanced systemic IgG4 antibody (Ab) responses [1, 2]. Patients with IRD exhibit a variety of symptoms associated with pancreatitis, cholangitis, sialoadenitis, nephritis, retroperitoneal fibrosis and inflammatory psuedotumors [1]. Thus, a variety of clinical manifestations related to involvement of multiple organs is one of the characteristic findings in patients with IRD. Importantly, infiltration of IgG4-expressing plasma cells is seen in the affected organs of IRD, which suggests possible involvement of systemic and local IgG4 responses in the pathophysiology of IRD [1].

Although autoimmune responses are considered to be involved in the development of IRD, the pathogenic antigens (Ags) have not been identified in this disorder. Given the fact that a significant population of patients with autoimmune pancreatitis (AIP), pancreatic manifestation of IRD, have a diagnosis of inflammatory bowel disease (IBD), the development and progression of which requires excessive immune reactions to intestinal microflora [3, 4], it is possible that enhanced immune responses toward microbial Ags underlie the immuno-pathogenesis of IRD. Compatible to this idea, we have previously shown that activation of toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) in monocytes by microbial Ags induce IgG4 production through activation of B cell-activation factor (BAFF)-mediated signaling pathways [5]. Thus, our previous data show the involvement of activation of TLRs and NLRs in the immunopathogeneis of IRD.

IgG4 production is regulated by T helper type 2 (Th2) cytokines such as IL-4, IL-10, and IL-13 [6]. Antigen-presenting cells (APCs) and T cells produce these Th2 cytokines to induce IgG4 secretion by B cells. Although basophils have been considered as Th2 effector cells, recent studies have now defined the role of basophils in the initiation of Th2 responses [7, 8]. Exposure of basophils to microbial Ags and protease allergen induce production of Th2 cytokines and BAFF, both of which are necessary for IgG production [9–11]. Therefore, it is possible that activation of basophils via microbial Ags contributes to the development of IRD via production of Th2 cytokines and BAFF.

In this study, we addressed the role of basophils in the development of IRD. Here we show evidence that basophil activation of TLRs enhances production of IgG4 from B cells in a BAFF-dependent manner. In addition, we found that basophils from patients with IRD induce IgG4 production from B cells more efficiently than those from healthy controls upon stimulation with TLR ligands. Thus, we propose that activation of TLRs in basophils contributes to the development of IRD.

Materials and methods

Stimulation of cells with microbial Ags

Basophils and B cells were isolated from peripheral blood mononuclear cells (PBMCs) by using a human basophil isolation kit (Miltenyi Biotech, Auburn, CA) and anti-CD19 microbead (Miltenyi Biotech), respectively. Basophils (1×10^6/mL) and $CD19^+$ B cells (1×10^6/mL) were stimulated with FK156 (Astellas, Tokyo, Japan, 20 μg/mL), muramyl dipeptide (MDP; 20 μg/mL, InvivoGen, San Diego, CA), Pam_3CSK4 (PAM; 10 μg/mL, InvivoGen), and lipopolysaccharide (LPS; 1 μg/mL, Sigma-Aldrich, St. Louis, MO) as described previously [5]. In some experiments, isolated cell populations were cultured with the indicated doses of a neutralizing anti-BAFF monoclonal Ab (R&D Systems, Minneapolis, MN), or anti-IL-13 monoclonal Ab (Pharmingen, San Jose, CA) or mouse IgG control Ab (eBioscience, San Diego, CA). Culture supernatants were collected at the indicated time points.

Enzyme-linked immunosorbent assays

Culture supernatants were assayed for the measurement of IL-8, IL-13, BAFF, and thymic stromal lymphopoietin (TSLP) by using enzyme linked immno-sorbent assay (ELISA) kits (Pharmingen, eBioscience) [5]. The production of IgG1, IgG4, IgE was determined by ELISAs as described previously [5].

Fluorescence-activated cell sorter analysis

Cells were pre-incubated with Fc-blocking solution (Miltenyi Biotech), stained with fluorescein isothiocyanate (FITC)-conjugated anti-human CD123 (Biolegend, San Diego, CA) and phycoerythrin-labeled anti-human CD203c, TLR2, and TLR4 (eBioscience). Stained cells were analyzed on an Accuri C6 cytometer (Accuri Cytometers, Ann Arbor, MI).

Studies using peripheral blood cells from patients

Ethical permission for this study was granted by the review board of Kyoto University. Healthy controls ($n = 8$) and treatment-naïve patients with IRD ($n = 5$) were enrolled in this study after informed consent was obtained. Basophils and $CD19^+$ B cells were isolated from the patients and stimulated with microbial Ags as described above. In some experiments, a neutralizing anti-BAFF monoclonal antibody (R&D systems) or mouse IgG control antibody (eBioscience) was used as described previously [5].

Statistical analysis

The Student's t test was used to evaluate statistical significance. A value of $p < 0.05$ was regarded as statistically significant.

Results

Activation of TLRs in basophils induces IgG4 production by B cells

Th2 responses are implicated in the development of IRD since Th2 cytokines such as IL-4, IL-10, and IL-13 enhance IgG4 production from B cells [6]. Although basophils have recently been identified as APCs necessary for optimal Th2 responses [7, 8], the role played by basophils in the development of IRD remains largely unknown. Our previous studies suggest possible involvement of TLR and NLR ligands in the immuno-pathogenesis of IRD. Given the fact that human primary basophils express functional TLRs [11], we investigated whether innate immune responses mediated by activation of TLRs and NLRs in basophils are involved in the development of IgG4 responses. To this end, basophils isolated from PBMCs from healthy controls were co-cultured with $CD19^+$ B cells in the presence of various TLR and NLR ligands (FK156, NOD1 ligand; MDP, NOD2 ligand; Pam_3CSK4, TLR2 ligand; LPS, TLR4 ligand) for 7 days. The purity of basophils, which are positive for CD203c and CD123 (IL-3Rα), are more than 90 % (Fig. 1a). As shown in Fig. 1b, stimulation of basophils with TLR2 and TLR4 ligands induced the production of IgG4, but not IgG1, by B cells in the absence of T cells. In contrast, the activation of NOD1 and NOD2 in basophils did not induce the production of IgG1 or IgG4. Thus, these data suggest that activation of TLR2 and TLR4 enhances the IgG4 response rather than the IgG1 response in a T cell-independent manner.

Activation of TLRs enhances production of BAFF and IL-13 by basophils

We next examined the effects of TLRs activation on mediator release and cytokine production by basophils. As shown in Fig. 2, basophils secret significant amounts of IL-8 upon stimulation with NOD1, TLR2, and TLR4 ligands. Th2 cytokines and BAFF are involved in the production of IgG4 [5, 6]. Consequently, we investigated whether NLR and TLR ligands induce these cytokines associated with IgG4 responses. TLR4 activation by LPS induced production of IL-13 and BAFF by basophils (Fig. 2a). In contrast, stimulation of basophil by LPS did not lead to the secretion of other Th2 cytokines such as IL-4 and TSLP. Similar results were obtained in basophils stimulated with PAM although not statistically significant. Thus, these data suggest that TLR4 activation in basophils induces production of BAFF and IL-13.

Activation of TLRs in basophils enhances IgG4 production via BAFF signaling pathways

We next addressed whether IgG4 production induced by basophil activation of TLRs depends upon signaling pathways via BAFF and/or IL-13. To this end, neutralizing Abs against BAFF and/or IL-13 were added to the co-culture containing $CD19^+$ B cells and basophils. Blockade of BAFF-signaling, but not IL-13 signaling, reduced IgG4 production induced by TLR4 activation in basophils (Fig. 2b). In contrast, neutralization of BAFF or IL-13 signaling did not change IgG1 production (data not shown). These data suggest that TLR4 activation of basophils induces IgG4 production by B cells via BAFF signaling pathways.

Basophils from patients with IgG4-related disease induces IgG4 production by B cells upon stimulation with TLR ligands

As mentioned above, we identified a novel pathway of TLR-mediated IgG4 production by utilizing peripheral blood basophils and B cells from healthy controls. To establish the significance of this finding in the immuno-pathogenesis of IRD, we compared the immune responses of basophils from patients with IgG4-related AIP with those from healthy controls. Three patients enrolled in this study met the criteria for the diagnosis of AIP [1] and were diagnosed with lymphoplasmacytic sclerosing pancreatitis type. These patients enrolled in this study show increased serum levels of IgG4 (948, 469, 262 mg/dL, normal range 4.8–105 mg/dL). For this purpose, basophils isolated from four healthy controls and three patients were co-cultured with $CD19^+$ B cells from healthy controls in the presence of MDP, PAM, and LPS. As shown in Fig. 3a, basophils from patients with IgG4-related AIP enhanced production of IgG1 and IgG4 by healthy $CD19^+$ B cells upon stimulation with TLR2 and TLR4 ligands as compared with basophils from healthy controls. It should be noted that induction of IgG4 production by patients' basophils was much greater than that of IgG1 production. This enhanced IgG4 production by patients' basophils was associated with increased production of BAFF and IL-13 (Fig. 3b). We also measured serum levels of BAFF and IL-13 in these patients. Although serum levels of IL-13 were below the detection limit in both patients and healthy controls, serum levels of BAFF were higher in patients as compared with those in healthy controls (patients versus controls,

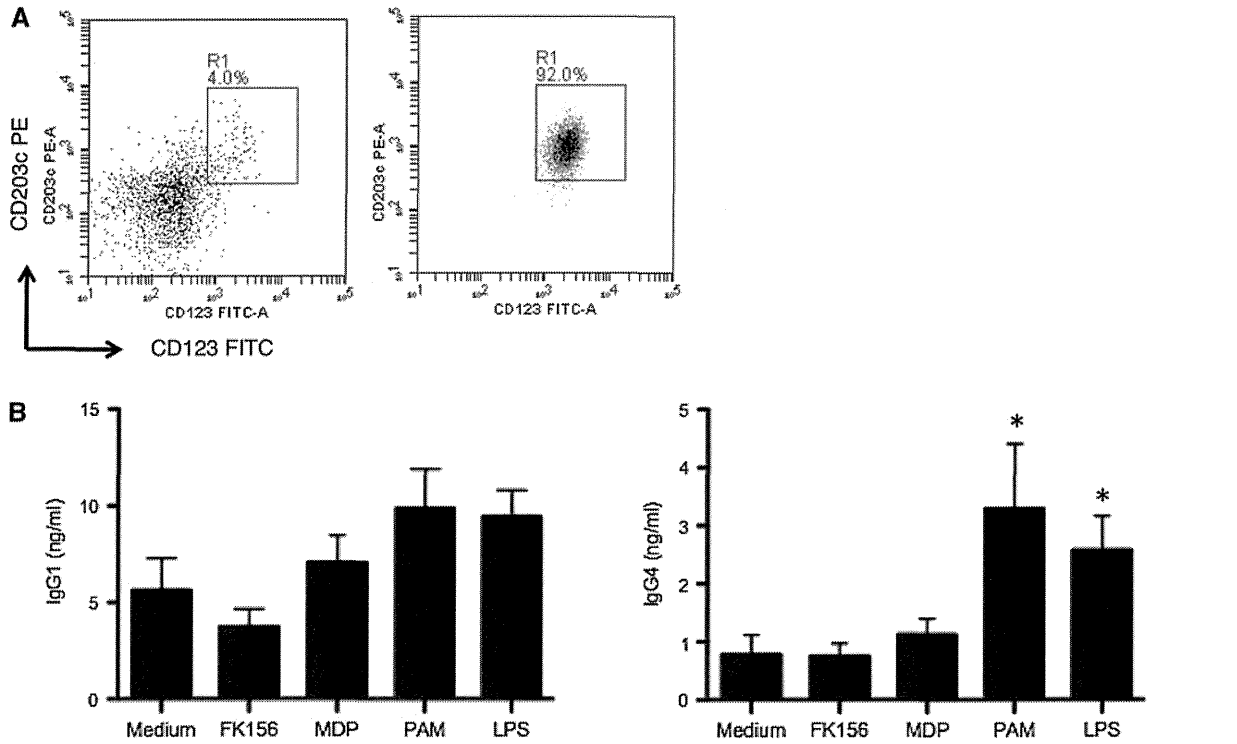


Fig. 1 Activation of TLRs in basophils enhances IgG4 production by the B cells. **a** Basophils were isolated from PBMCs from the healthy controls. The percentages of the basophils expressing CD203c and CD123 are shown in the plots (*left* before separation; *right* after separation). **b** The $CD19^+$ B cells (1×10^6/mL) from the healthy controls ($n = 4$) were co-cultured for 7 days with the basophils (1×10^6/mL) in the presence of FK156 (20 μg/mL), MDP (20 μg/mL), PAM (10 μg/mL), and LPS (1 μg/mL). The culture supernatants were analyzed for the production of IgG1 and IgG4. The results are expressed as mean ± SE. $^*p < 0.05$, as compared with the medium alone

mean + standard deviation, 2.274 + 1.119 vs. 0.751 + 0.535 ng/mL).

BAFF-mediated signaling pathways are involved in IgG4 production induced by basophils from IRD patients

Finally, we addressed whether induction of IgG4 production by baosphils from IRD patients depends upon BAFF-mediated signaling pathways. As shown in Fig. 4, abrogation of BAFF signaling by its neutralizing Ab inhibited the production of IgG4. Thus, these data suggest that activation of TLRs in patients' basophils triggers IgG4 production by healthy $CD19^+$ B cells through signaling pathways mediated by BAFF.

Discussion

In this study we have reported that activation of TLRs in basophils induces IgG4 production by B cells through signaling pathways mediated by BAFF. More importantly, a marked increase in IgG4 production by healthy B cells is seen when B cells are co-cultured with basophils isolated from patients with IRD in the presence of TLR ligands as compared with those from healthy controls. Thus, these data suggest possible involvement of TLRs activation in basophils in the development of IRD. In our previous study, we showed that activation of NOD2 and TLRs in monocytes is involved in enhanced IgG4 responses in patients with IRD [5]. Our previous and present studies suggest that ligation of TLRs play roles in the development of enhanced IgG4 responses through activation of two different innate immune cells; monocytes and basophils. Interestingly, activation of these two different innate immune cells leads to T cell-independent IgG4 responses via BAFF-mediated signaling pathways, which highlights the importance of BAFF in the development of IRD. Therefore, two distinct innate immune cells utilize BAFF-mediated signaling pathways to achieve enhanced IgG4 production.

We previously showed that NOD2 activation in monocytes leads to a T cell-independent IgG4 production via BAFF-mediated signaling pathways [5]. Thus, BAFF-mediated signaling pathways induce T cell-independent IgG4 production either by activation of TLRs in basophils or by that of NOD2 in monocytes. Although serum levels of BAFF are higher in patients with IRD [12],

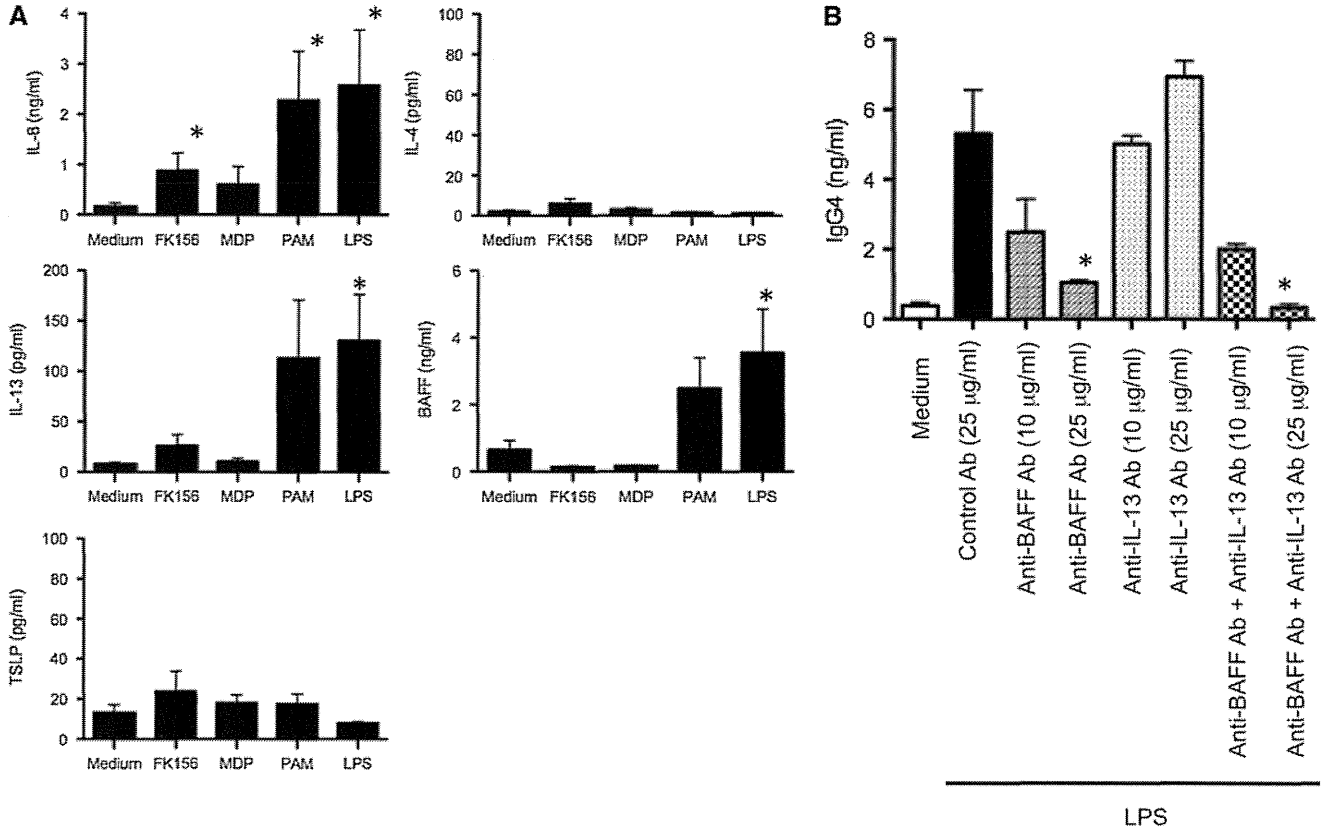
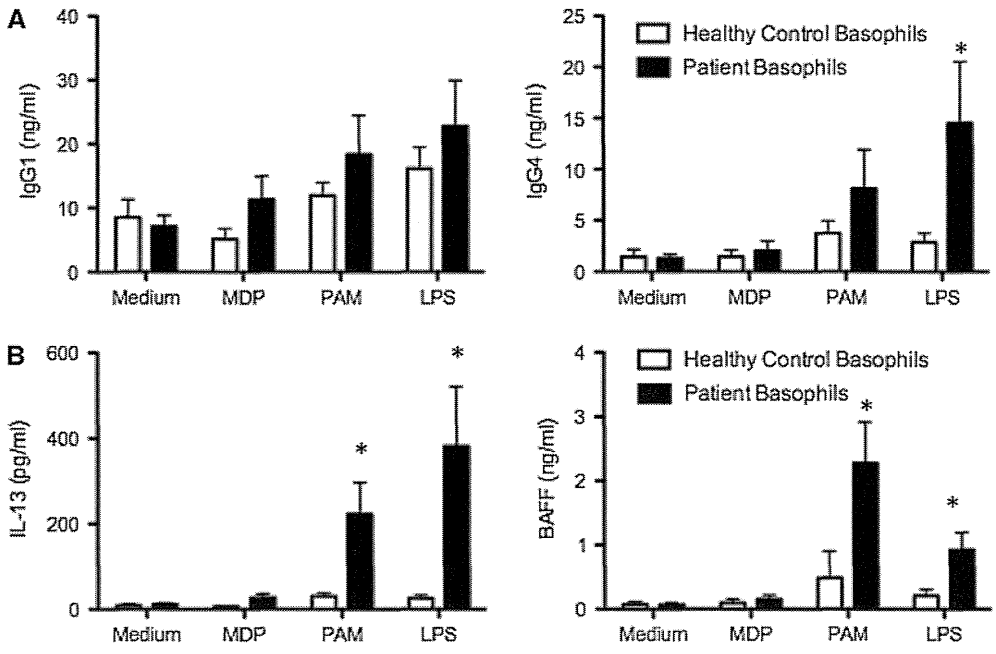


Fig. 2 Basophils produce IL-13 and BAFF upon stimulation with TLR ligands. **a** The basophils isolated from the PBMCs of the healthy controls ($n = 4$, 1×10^6/mL) were stimulated with the NLR and TLR ligands for 24 h as shown in Fig. 1. The culture supernatants were analyzed for the production of IL-8, IL-4, IL-13, BAFF, and TSLP. The results are expressed as mean ± SE. $^*p < 0.05$, as compared with the medium alone. **b** The $CD19^+$ B cells (1×10^6/mL) from the healthy controls ($n = 2$) were co-cultured for 7 days with basophils (1×10^6/mL) in the presence of LPS (1 μg/mL). The neutralizing Abs against BAFF and/or IL-13 were added to the co-culture. The culture supernatants were analyzed for the production of IgG4. The results are expressed as mean ± SE. $^*p < 0.05$, as compared with the control Ab

Fig. 3 Basophils isolated from the patients with IgG4-related AIP induce IgG4 production upon stimulation with TLR ligands by the B cells from the healthy controls. The $CD19^+$ B cells (1×10^6/mL) from the healthy controls ($n = 4$) were co-cultured with the basophils (1×10^6/mL) from the controls ($n = 4$) or the patients ($n = 3$) with IgG4-related AIP in the presence of MDP (20 μg/mL), PAM (10 μg/mL) or LPS (1 μg/mL) for 7 days. The culture supernatants obtained on day 7 were assayed for the presence of IgG1 (**a**), IgG4 (**a**), IL-13 (**b**), and BAFF (**b**). The results are expressed as means ± SE. $^*p < 0.05$, as compared with the culture conditions using the basophils from the healthy controls



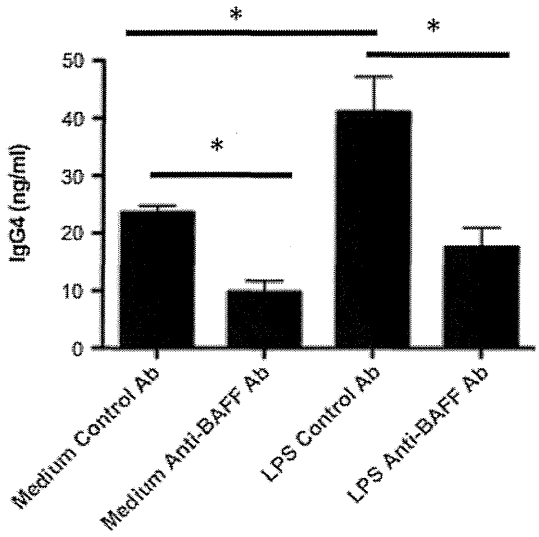


Fig. 4 BAFF-mediated IgG4 production by basophils from patients with IgG4-realted disease. The $CD19^+$ B cells (1×10^6/mL) from the healthy controls ($n = 2$) were co-cultured with the basophils (1×10^6/mL) from the patients ($n = 2$) with IgG4-related disease in the presence of LPS (1 μg/mL) for 7 days. The culture supernatants obtained on day 7 were assayed for the presence of IgG4. The neutralizing Ab against BAFF (25 μg/mL) was added to the co-culture. The results are expressed as means ± SE. $^*p < 0.05$

the mechanisms accounting for enhanced BAFF production have been poorly understood. Our data suggest that activation of TLRs and NLRs in monocytes and basophils induces BAFF production and thereby causes systemic IgG4 responses. As for the mechanisms for enhanced BAFF production by monocytes, our previous study clearly shows that activation of NOD2 induces BAFF production via NF-κB-dependent signaling pathways [5]. It remains unknown whether BAFF production by basophils from IRD patients also requires activation of NF-κB as in the case of monocytes. Alternatively, circulating IgD may enhance BAFF production by basophils since binding of IgD to basophils induces the secretion of pro-inflammatory cytokines such as BAFF and IL-4 [9]. Further studies are necessary to elucidate the molecular mechanisms accounting for enhanced BAFF production by basophils isolated from IRD patients.

Although basophils have been considered as effectors cells for allergic Th2-IgE responses, recent studies show evidence that basophils with the ability to produce Th2 cytokines and to present Ags are inducers for these allergic reactions [7]. Thus, generation of allergic Th2-IgE responses depends upon activation of basophils not only at the effector phase but also at the induction phase. Patients with IRD often exhibit enhanced serum levels of IgE associated Th2 responses [2]. In this study, we clearly showed that activation of TLRs in basophils from IRD patients induce IgG4 production by B cells from healthy controls. Therefore, it is possible that microbial Ags trigger production of IgG4 and IgE through TLR activation in baosphils. However, we failed to detect IgE production (data not shown). This preferential induction of IgG4 responses rather than IgE responses by TLRs-activated basophils can be partially explained by the lack of IL-4 and TSLP production, both of which are necessary for IgE production [13, 14].

One question arising from the present study is the sites where basohil activation of TLRs occur. Given the fact that the mucosa of the gastrointestinal (GI) tract is always exposed to intestinal microflora [4, 15], it is possible that TLR ligands derived from intestinal microflora activate basophils in GI tract. Thus, the GI tract is one possible induction site for the development of systemic IgG4 responses. However, clinical analysis of patients with AIP show poor association between IgG4-related AIP and IBD [16], the latter of which requires excessive innate immune responses against intestinal microflora for the development of inflammation. In this regard, several mechanisms for preventing hyper-responsiveness to TLR ligands operate in the gut [4, 15]. Therefore, one possible explanation is that pathogenic immune reactions causing inflammation are suppressed in the gut by regulatory mechanisms whereas such reactions cause tissue injury in the other sterile organs such as pancreas and bile duct due to the lack of regulatory mechanisms. Confirmation of this idea awaits further investigation of immune responses occurring in the GI tract of patients with IRD.

In conclusion, it is important to note that the findings shown here have certain implications both with respect to possible mechanisms of IRD and with respect to a possible new approach to the treatment of this disease. Activation of TLR2 and TLR4 in basophils enhances IgG4 Ab responses by B cells from healthy controls and IRD, which suggests that abnormal innate immune responses through TLRs may be involved in the development of IRD. With respect to treatment, a link between BAFF and IgG4 implies that patients with IRD may be treated with the inhibition of BAFF signaling.

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Conflict of interest The authors have declared no conflicts of interest.

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