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REVIEW ARTICLE

A novel clinical entity, IgG4-related disease (IgG4RD): general concept and details

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Abstract IgG4-related disease (IgG4RD) is a novel clinical disease entity characterized by elevated serum IgG4 concentration and tumefaction or tissue infiltration by IgG4-positive plasma cells. IgG4RD may be present in a certain proportion of patients with a wide variety of diseases, including Mikulicz's disease, autoimmune pancreatitis, hypophysitis, Riedel thyroiditis, interstitial pneumonitis, interstitial nephritis, prostatitis, lymphadenopathy, retroperitoneal fibrosis, inflammatory aortic aneurysm, and inflammatory pseudotumor. Although IgG4RD forms a distinct, clinically independent disease category and is attracting strong attention as a new clinical entity, many questions and problems still remain to be elucidated, including its pathogenesis, the establishment of diagnostic criteria, and the role of IgG4. Here we describe the concept of IgG4RD and up-to-date information on this emerging disease entity.

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Abbreviations

IgG4RD	IgG4-related disease
MD	Mikulicz's disease
SS	Sjögren's syndrome
MHLW Japan	Ministry of Health, Labor and Welfare Japan
LPSP	Lymphoplasmacytic sclerosing pancreatitis
AIP	Autoimmune pancreatitis
FMF	Familial multifocal fibrosclerosis
ANA	Anti-nuclear antibody

Introduction

In 1892, Dr. Johann von Mikulicz, also known as Jan Mikulicz-Radecki, published a paper describing a patient with symmetrical swelling of the lachrymal, parotid, and submandibular glands, with massive infiltration of these glands by mononuclear cells [1]. Following reports describing similar patients, this condition was called Mikulicz's disease (MD). In contrast, patients with similar symptoms, but with diseases such as leukemia, malignant lymphoma, and sarcoidosis, were reported to have Mikulicz's syndrome [2]. In 1930, Dr. Henrik Sjögren, an ophthalmologist, published a paper describing a woman with rheumatoid arthritis accompanied by keratoconjunctivitis sicca and severe swelling of the parotid glands, a condition that has been recognized as Sjögren's syndrome (SS) [3]. In 1953, Morgan and Castleman examined 18 patients with MD and concluded that this condition is one manifestation of SS [4]. Since then, MD has attracted very little interest in western countries. In Japan, however, there have been many patients with MD, such that differences between MD and SS have been clarified [5–7]. For example, their gender distribution is quite different, in that MD occurs in both men and women, whereas SS occurs mainly in women. Second, patients with MD have relatively mild xerostomia and xerophthalmia, despite significant enlargement of their lachrymal and salivary glands. Further, MD is accompanied by more complications, such as autoimmune pancreatitis (AIP). Patients with MD show a better response to glucocorticoid therapy than patients with SS. Finally, it has become clear that MD is related to elevated serum IgG4 concentrations and infiltration of IgG4-positive cells [5–9].

Following the description of a patient with chronic pancreatitis due to an autoimmune mechanism [10], lymphoplasmacytic sclerosing pancreatitis (LPSP) was found to be a characteristic histopathological finding in patients with AIP [11]. These findings led to the concept of AIP, which has characteristics similar to those of other autoimmune diseases, such as hypergammaglobulinemia, the

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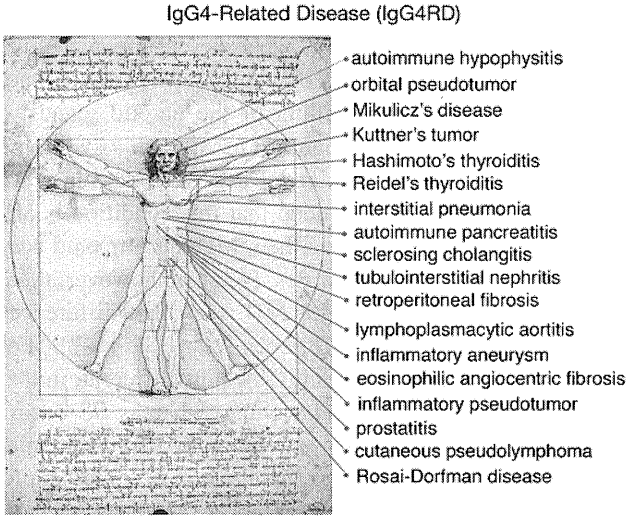


Fig. 1 IgG4-related conditions. Many diseases have been reported to be IgG4-related

Table 1 Nomenclatures of IgG-related conditions

IgG4-related autoimmune disease	Kamisawa [21]
IgG4-associated multifocal systemic fibrosis	van der Vliet [76]
IgG4-related systemic disease	Kamisawa [20]
IgG4-related sclerosing disease	Kamisawa [15]
Hyper-IgG4 disease	Neild [59]
IgG4-related disease (IgG4-RD)	Zen [77]
Systemic IgG4 plasmacytic syndrome (SIPS)	Yamamoto [22]
IgG4-related multi-organ lymphoproliferative syndrome (IgG4-MOLPS)	Masaki [29]
IgG4-associated disease	Geyer [78]

presence of various autoantibodies, lymphocytic infiltration into pancreatic tissue, and good responsiveness to steroids [12]. Following a report showing elevated serum IgG4 concentrations in patients with AIP [13], the pancreatic research team of the Ministry of Health, Labor and Welfare Japan (MHLW Japan) showed that AIP was related to IgG4 [14].

IgG4-positive plasma cell infiltration has also been observed in patients with other conditions, including retroperitoneal and mediastinal fibrosis [15, 16], inflammatory pseudotumor of the lung and liver [17], Küttner tumor [18], and interstitial nephritis [19], indicating that these diseases and conditions collectively constitute a new disease concept, IgG4-related disease (Fig. 1). These findings have led to the organization of two study groups by MHLW Japan to analyze the condition of IgG4-related disease. These groups consist of doctors and researchers in various fields, including rheumatology, hematology, gastroenterology, nephrology, pulmonology, ophthalmology, odontology, pathology, statistics, and basic and molecular immunology, from all over Japan. One of these groups, chaired by Professor Umehara of Kanazawa Medical University, is seeking to establish diagnostic criteria for IgG4-related multi-organ lymphoproliferative syndrome (IgG4-MOLPS), whereas the second group, chaired by Professor Okazaki of Kasai Medical University, is seeking to understand the etiology and pathogenesis of IgG4-related systemic disease.

Unification of different nomenclatures for IgG4-related disease (IgG4RD)

The concept of IgG4RD arose when elevated serum IgG4 concentrations were first reported in patients with sclerosing pancreatitis [13]. Autoimmune pancreatitis (AIP) is also associated with a variety of extrapancreatic lesions, including sclerosing cholangitis, sclerosing sialadenitis, and dacryoadenitis, resulting in the concept of IgG4-related systemic disease [20], also called IgG4-related autoimmune disease [21] or IgG4-related sclerosing disease [15]. The finding of elevated serum IgG4 and IgG4-positive plasma cell infiltration in MD suggested that MD was a systemic disease, which was called systemic IgG4 plasmacytic syndrome (SIPS) [22]. Further, a comparison of patients with MD and those with typical SS resulted in the formulation of a new clinical entity, IgG4+MOLPS [23]. Although many reports from Japan and other countries have described IgG4-related conditions under different names (Table 1), these may refer to the same condition, familial multifocal fibrosclerosis (FMF). Indeed, retroperitoneal fibrosis (RPF), mediastinal fibrosis, sclerosing cholangitis, Riedel's thyroiditis, and pseudotumor of the orbit may all be different manifestations of a single disease [24].

The name "IgG4-related sclerosing disease" is mainly based on the swelling of fibrous organs, such as the pancreas and retroperitoneum, whereas "SIPS" and "IgG4+MOLPS" are based on lymphoplasmacytic proliferation in glands and swollen lymph nodes without fibrosis. Although many patients with this condition (i.e., IgG4-related sclerosing disease, etc.) have lesions in several organs, either synchronously or metachronously, other patients show involvement of only a single organ. At this point, it is unclear whether the pathogenetic mechanism of this disease is systemic or whether it consists of manifestations in individual organs. In addition, several reports have described patients with IgG4-associated conditions concomitant with malignant tumors such as pancreatic [25, 26] and salivary [27] carcinomas, and ocular adnexal lymphoma [28]. Therefore, using the term 'systemic' may lead to an incorrect diagnosis of an IgG4-related condition in a patient with malignant

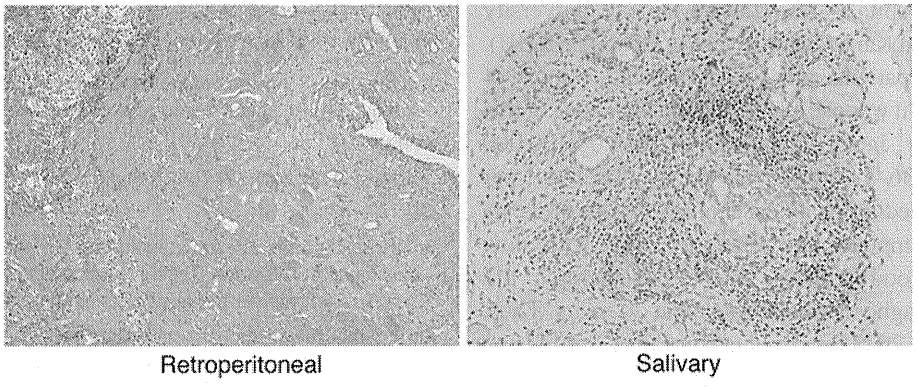


Fig. 2 Histopathology of IgG4-related disease (IgG4RD). IgG4RD is characterized histopathologically by the infiltration of IgG4-positive plasma cells and fibrosis. However, the severity of fibrosis is dependent on the individual organs involved. For example, storiform fibrosis and obliterative phlebitis are characteristic of retroperitoneal lesions, but are very seldom observed in salivary glands (×40)

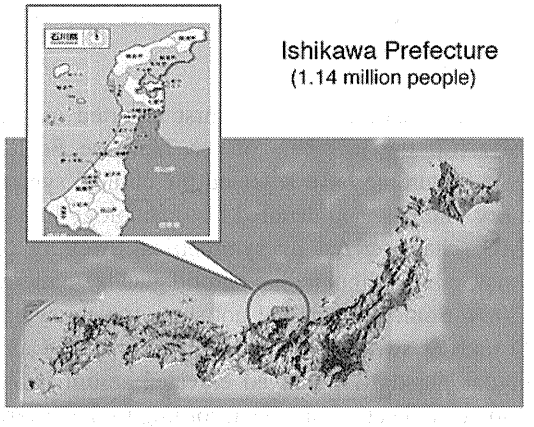
tumors in other organs. Based on these reasons, the members of the two MHLW Japan research teams agreed, at their second meeting in Kanazawa on February 11, 2010, to use the term "IgG4-related disease (IgG4RD)".

General concept of IgG4RD

After the unification of the disease name as IgG4RD, both MHLW Japan research teams have sought to determine its pathogenesis and to formulate diagnostic criteria. The two teams reached a consensus that IgG4RD can occur in various organs, including the central nervous system, salivary glands, thyroid gland, lungs, pancreas, biliary duct, liver, gastrointestinal tract, kidneys, prostate gland, retroperitoneum, and lymph nodes, but that clinical symptoms depend on the location of the lesion. IgG4RD mainly affects middle-aged to elderly men. Its clinical symptoms are relatively mild, and the condition usually comes to clinical attention due to organ swelling or damage. Many patients with IgG4RD are treated effectively by steroid therapy. Although the infiltration of IgG4-positive cells and increased serum concentrations of IgG4 are characteristic of IgG4RD, the severity of fibrosis is dependent on the individual organs involved. For example, storiform fibrosis and obliterative phlebitis are characteristic of pancreatic, biliary tract, and retroperitoneal lesions, but are very seldom found in salivary glands or lymph nodes (Fig. 2).

Prevalence of IgG4RD

It is difficult to ascertain the number of patients with IgG4RD because its diagnostic criteria have not yet been established, the awareness of this disease is low, and its symptoms vary. An attempt was made to estimate the number of individuals with IgG4RD throughout Japan by using as an example Ishikawa Prefecture, which has a population of 1.14 million people with little population inflow/outflow (Fig. 3). In Ishikawa Prefecture, there are



	KMU	KUH	total
2003	2	2	4
2004	0	1	1
2005	1	3	4
2006	1	3	4
2007	1	4	5
2008	1	3	4
2009	1	6	7
	7	22	29

Fig. 3 Prevalence of patients with IgG4RD. An attempt was made to estimate the number of individuals with IgG4RD throughout Japan by using as an example Ishikawa Prefecture (population 1.14 million people) with little population inflow/outflow. If all new patients with IgG4RD visit Kanazawa Medical University Hospital (*KMU*) or Kanazawa University Hospital (*KUH*), the incidence of this disease throughout Japan would be 0.28–1.08/100,000 population, with 336–1,300 patients newly diagnosed per year. If life expectancy after diagnosis is 20 years, then approximately 6,700–26,000 patients in Japan would have developed IgG4RD over the past 20 years. The *numbers in the table* represent the numbers of patients who visited KMU or KUH each year

two University Hospitals, Kanazawa Medical University Hospital (KMU) and Kanazawa University Hospital (KUH). Assuming that new patients with IgG4RD would visit one of these two hospitals, it was estimated that the incidence of this disease throughout Japan would be 0.28–1.08/100,000 population, with 336–1,300 patients newly diagnosed per year. Because the median age of onset of IgG4RD is 58 years and the clinical symptoms are relatively mild, with slow progression and good response to steroid therapy, life expectancy after diagnosis was estimated at 20 years. Thus, an estimated 6,700–26,000 individuals in Japan would have developed IgG4RD over the past 20 years.

Clinicopathological features of IgG4RD

Differences between IgG4-related MD and Sjögren's syndrome

Since elevated serum IgG4 was first reported in patients with MD [6], the members of the Japanese Society of Sjögren's Syndrome have assessed the clinical symptoms, laboratory findings, and detailed histopathology in patients with MD (characterized by symmetrical swelling of the lachrymal, submandibular, and parotid glands), nationwide, since 2004. Some patients did not show typical symptoms of MD such as swelling of the lachrymal, parotid, or submandibular glands but showed elevated serum IgG4 and other indices indicative of MD according to the criteria for the diagnosis of IgG4-related MD shown in Table 2 [8]. Sixty-four patients with MD or elevated serum IgG4 (>135 mg/dl) and characteristic histological findings were initially diagnosed with IgG4RD (formerly called IgG4+MOLPS) based on proposed guidelines for the diagnosis of IgG4RD (Table 3). A comparison of patients with IgG4RD and those with typical SS showed: (1) compared with SS patients, fewer patients with IgG4RD had symptoms of xerophthalmia, xerostomia, or arthralgia, whereas many had coexisting AIP, interstitial nephritis, allergic rhinitis, and/or bronchial asthma (Fig. 4a); (2) most patients with IgG4RD were negative for anti-SS-A and anti-SS-B antibodies, as well as for rheumatoid factor (RF) and anti-nuclear antibody (ANA) (Fig. 4b); (3) serum IgG4 and IgE concentrations were significantly higher in IgG4RD than in SS patients (Fig. 4c); and (4) steroid therapy was extremely effective in patients with IgG4RD but had limited effect in patients with SS [29].

The histopathological features of IgG4RD are unique, though both IgG4RD and SS show marked lymphocytic infiltration. IgG4RD is characterized by the formation of lymphoid follicles but lower levels of lymphocytic infiltration into the salivary ducts, such that their structure remains intact (Fig. 5a). Therefore, the absence of

Table 2 Diagnostic criteria of IgG4+ Mikulicz's disease [8] (approved by the Japanese Society for Sjögren's Syndrome 2008)

1. Symmetrical swelling of at least 2 pairs of lachrymal, parotid, or submandibular glands for at least 3 months
AND
2. Elevated serum IgG4 (>135 mg/dl)
OR
3. Histopathological features including lymphocyte and IgG4+ plasma cell infiltration (IgG4+ plasma cells/IgG+ plasma cells >50%) with typical tissue fibrosis or sclerosis
Differential diagnosis is necessary to distinguish IgG4+ Mikulicz's disease from other distinct disorders, including sarcoidosis, Castleman's disease, Wegener's granulomatosis, lymphoma, and cancer. The diagnostic criteria for Sjögren's syndrome (SS) may also include some patients with IgG4+ Mikulicz's disease; however, the clinicopathological conditions of patients with typical SS and IgG4+ Mikulicz's disease are different

Table 3 Guidelines for diagnosis of IgG4RD (proposed by the Research Program for Intractable Disease Ministry of Health, Labor and Welfare Japan, G4 team)

Clinical features highly suggestive of IgG4RD
1. Symmetrical swelling of lachrymal, parotid, or submandibular glands
2. Autoimmune pancreatitis
3. Inflammatory pseudotumor
4. Retroperitoneal fibrosis
5. Suspicion of Castleman's disease
Laboratory data highly suggestive of IgG4RD
1. Serum IgG4 >135 mg/dl
2. IgG4+ cells/IgG+ cells >40% in biopsy
Clinical features suggestive of IgG4RD
1. Unilateral swelling of at least one lachrymal, parotid, or submandibular gland
2. Orbital pseudotumor
3. Sclerosing cholangitis
4. Prostatitis
5. Hypertrophic pachymeningitis
6. Interstitial pneumonitis
7. Interstitial nephritis
8. Thyroiditis/hypo-function of thyroid
9. Hypophysitis
10. Inflammatory aneurysm
Laboratory data suggestive of IgG4RD
1. Hypergammaglobulinemia of unknown origin
2. Hypocomplementemia or existence of immune complex
3. Increase of IgE or eosinophils
4. Tumefactive lesions or lymph node swelling detected by gallium scan or fluoro-D-glucose positron emission tomography (FDG-PET)

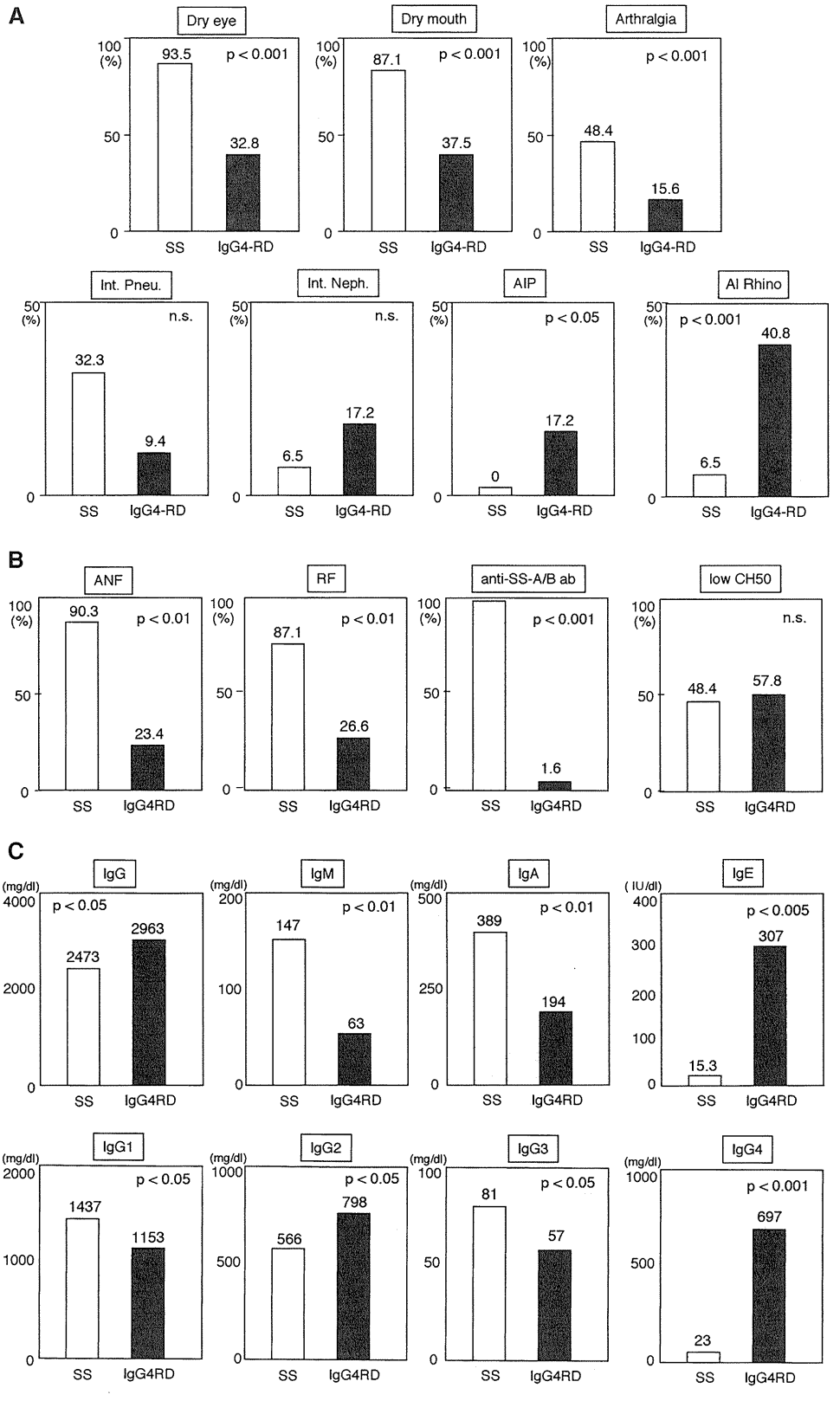


Fig. 4 Comparison of clinical symptoms and laboratory findings in IgG4RD and typical Sjögren's syndrome (*SS*) [29]. **a** Clinical symptoms, **b** immunological findings, and **c** subclasses of immunoglobulins and IgG observed in patients with IgG4RD ($n = 61$) and typical SS ($n = 31$). Data are expressed as percentages. *P* values are for comparisons of IgG4RD with typical SS. Patients with typical SS fulfilled both Japanese and European SS criteria and were positive for both anti-SSA/Ro and anti-SSB/La antibodies

lymphoepithelial lesions in patients with IgG4RD, in contrast to SS, may explain the lower rate of dryness in the former, despite the marked swelling of lachrymal and salivary glands. The most important difference between IgG4RD and SS is that the former is characterized by marked infiltration of IgG4-positive plasma cells, with a

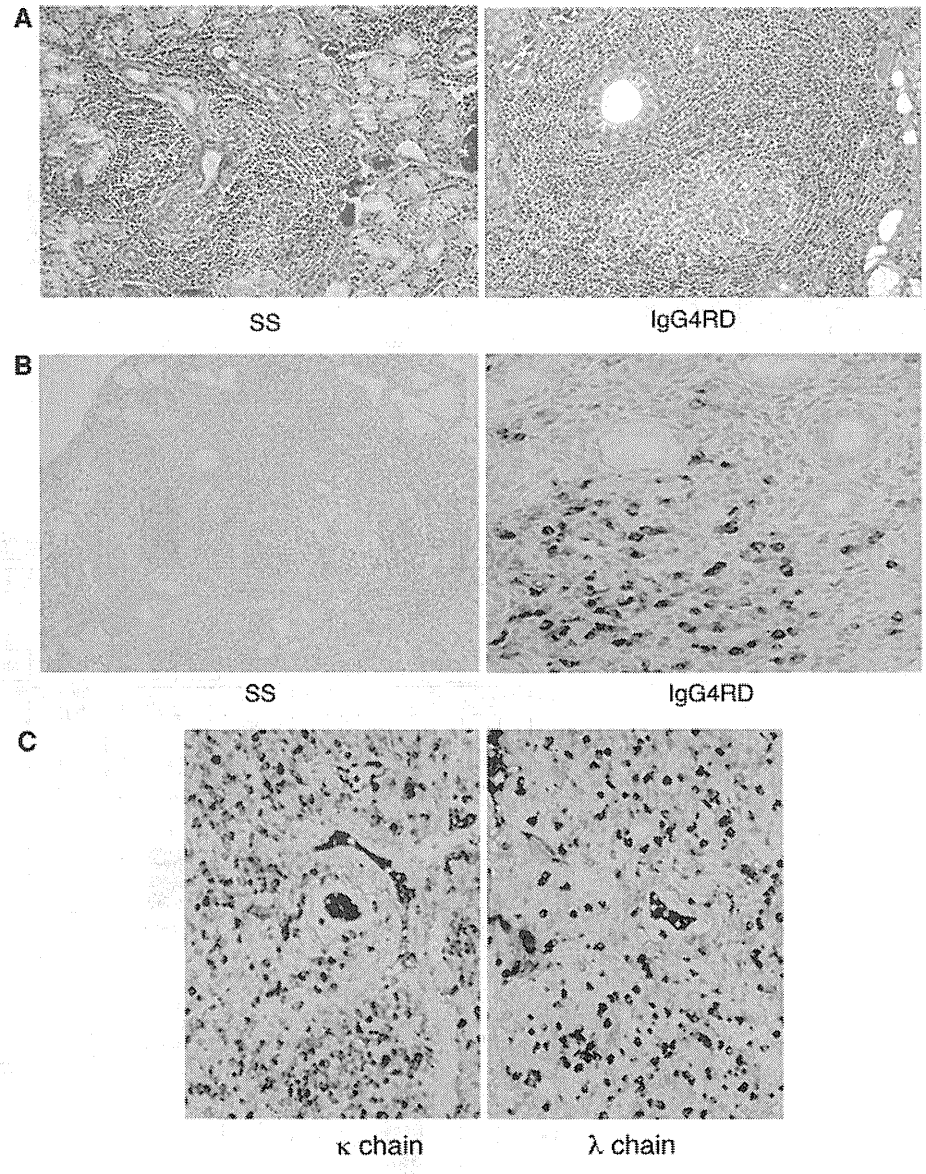


Fig. 5 Histopathological findings of minor labial salivary gland biopsies in patients with IgG4RD and typical SS. **a** Massive infiltration of lymphocytes and plasma cells was observed in patients with IgG4RD and those with typical SS (×200). IgG4RD, however, was characterized by lymphoid follicle formation but ducts were intact without lymphocytic infiltration. H&E staining. **b** IgG4RD showed scattered IgG4+ plasma cells in the periphery of the follicles (×200), whereas typical SS showed few or no IgG4+ cells. IgG4 immunostaining. **c** Staining for immunoglobulin κ- and λ-chains (×200)

ratio of IgG4-positive to IgG-positive cells of >40%, a finding almost never seen in patients with SS (Fig. 5b). Moreover, most patients with IgG4RD show polyclonal B-cell proliferation, with equal staining for immunoglobulin κ- and λ-chains (Fig. 5c). Thus, despite their similarities in organ involvement, IgG4-MD and SS are quite different conditions, with distinct clinical and pathological characteristics [7–9, 22, 29–31].

IgG4-related Küttner tumor

Küttner tumor, a unilateral sclerosing sialadenitis, is an IgG4RD [18]. A common feature of MD and Küttner tumor is that both manifest sialadenitis, as in IgG4RD. Histologically, Küttner tumors are very severe fibrous sclerotic lesions containing IgG4-positive plasma cells [32]. In contrast, fibrosis tends to be less severe in MD, although fibrosis in MD is frequently not examined extensively, because MD is generally diagnosed by the biopsy of minor labial salivary glands. Therefore, at present, it is difficult to set a strict boundary between MD and Küttner tumor.

IgG4-related autoimmune pancreatitis (IgG4-related AIP)

Recent studies have suggested that AIP manifests as two distinct subtypes, called types 1 and 2 (Table 4) [33]. Clinically, type 1 AIP seems to be the pancreatic manifestation of IgG4RD, characterized by: (1) mild abdominal symptoms, usually without acute attacks of pancreatitis; (2) occasional occurrence of obstructive jaundice; (3) increased serum gammaglobulin, IgG, and/or IgG4 concentrations; (4) presence of autoantibodies; (5) diffuse enlargement of the pancreas with a capsule-like low-density rim; (6)

Table 4 Subtypes of autoimmune pancreatitis (AIP) [33]

Subtype of AIP other nomenclatures	Type 1 AIP without GEL IgG4-related, LPSP	Type 2 AIP with GEL IgG4-unrelated IDCP
Prevalence	Asia > USA, Europe	Europe > USA > Asia
Age	High age	Younger
Gender	Male ≫ female	Male = female (NS)
Symptoms	Often obstructive jaundice	Often obstructive jaundice
Jaundice	Rare abdominal pain	Abdominal pain like acute pancreatitis
Pancreas images	Swelling/diffuse	Swelling/diffuse
	Segmental/focal	Segmental/focal
	Mass-forming	Mass-forming
Serology	High serum IgG	Normal IgG
	High serum IgG4	Normal IgG4
	Auto antibodies (+)	Auto antibodies (−)
Other organ involvement (OOI)	Sclerosing cholangitis	Unrelated to OOI
	Sclerosing sialadenitis	
	Retroperitoneal fibrosis	
	Other characteristics	
Ulcerative colitis	Rare	Often
Steroid response	Responsive	Responsive
Relapse	High rate	Rare

GEL, granulocyte epithelial lesion; *LPSP*, lymphoplasmacytic sclerosing pancreatitis; *IDCP*, idiopathic duct-centric chronic pancreatitis; *NS*, not significant

irregular narrowing of the pancreatic duct (sclerosing pancreatitis on endoscopic retrograde cholangiopancreatography [ERCP] images); (7) lymphocyte and IgG4-positive plasmacyte infiltration and fibrosis, and obliterative phlebitis; (8) occasional association with extrapancreatic lesions, such as sclerosing cholangitis similar to primary sclerosing cholangitis (PSC), sclerosing cholecystitis, sclerosing sialoadenitis, RPF, interstitial renal tubular disorders, enlarged celiac and hilar lymph nodes, chronic thyroiditis, and pseudotumor of the pancreas, liver, or lung; and (9) responsiveness to steroid therapy. Older males with IgG-related AIP often have obstructive jaundice, with both pancreatic and extrapancreatic manifestations responding to steroid therapy [12–15, 21, 33, 34].

Histological examination by American and European pathologists of the resected pancreases of patients with chronic non-alcoholic pancreatitis revealed another histopathological pattern, called idiopathic duct-centric pancreatitis (IDCP) or AIP with granulocytic epithelial lesions (GELs), later called type 2 AIP [35, 36]. Type 2 AIP is characterized primarily by these GELs, often accompanied by destruction and obliteration of the pancreatic duct [36]. Patients with type 2 AIP show swelling of the pancreas, but no or very few IgG4-positive plasma cells. Type 2 AIP has different clinicopathological features than type 1 AIP. Type 2 AIP shows no elevations in serum IgG4 or IgG, no autoantibodies, and no involvement of other organs, except for inflammatory bowel disease. Inflammatory bowel disease has been observed in approximately 30% of patients with type 2 AIP. Although type 1, or IgG4-related, AIP (LPSP type) often occurs in older men and is accompanied by a variety of extrapancreatic lesions, type 2, or neutrophil-related pancreatitis (IDCP/GEL type), has no gender bias, younger age at onset (often <40 years), and is frequently associated with inflammatory bowel disease. Thus, after a worldwide debate over the diagnostic criteria for AIP, IgG4-related pancreatitis has been defined as type 1 (LPSP type) and neutrophil-related pancreatitis has been defined as type 2 (IDCP/GEL type) [34].

IgG4-related sclerosing cholangitis (IgG4-related SC)

Extrapancreatic bile duct lesions are frequently associated with AIP. For example, 73% of patients with AIP have shown wall thickening or sclerosing changes in extrapancreatic bile ducts on endoscopic ultrasonography (EUS) and intraductal ultrasonography (IDUS), though only 26% of patients with AIP demonstrated sclerosing changes by ERCP [37]. However, many individuals without AIP have shown IgG4-related SC with isolated biliary tract involvement [38, 39]. In IgG4-related SC, stenosis is usually observed in the lower part of the common bile duct. The cholangiographic appearance of stenosis in the intrahepatic or hilar hepatic bile duct is very similar to that observed in PSC [40], a progressive disease of unknown etiology that ultimately results in liver cirrhosis. IgG4-related SC is associated with older age, male predominance, obstructive jaundice, weight loss, and abdominal discomfort [40]. Although steroid therapy has shown mixed results in patients with PSC, IgG4-related SC

responds dramatically to steroid therapy, as does IgG4RD [41]. The histopathological features of IgG4-related SC are similar to those of AIP and include diffuse plasmacytic infiltration, marked interstitial fibrosis with a focal storiform-like pattern, and obliterative phlebitis.

IgG4-related kidney disease (IgG4-related KD)

The kidney is a frequent target organ in IgG4RD, with tubulointerstitial nephritis (TIN) and fibrosis and abundant IgG4-positive plasma cell infiltration being diagnostically important histopathological features of this disease [42–44]. Recently, the clinicopathological features of 23 patients with IgG4-related TIN were reported to be quite uniform and similar to those observed in patients with IgG4-AIP, including high serum concentrations of IgG4 and IgE, hypocomplementemia, and TIN with infiltration of large numbers of IgG4-positive plasma cells plus fibrosis [45].

Kidney diseases in IgG4RD include conditions other than renal parenchymal lesions, such as hydronephrosis associated with RPF and tumors of the renal pelvis and urethra. However, IgG4-related TIN is considered to be representative of IgG4 renal parenchymal lesions [19]. Compared with other types of interstitial nephritis, IgG4-related TIN is often associated with extrarenal lesions, such as pancreatitis, sialadenitis, and lymphadenitis, and a high incidence of hypocomplementemia [46]. Imaging often shows heterogeneous shadows in the kidneys, such as a mass or multiple nodules (findings that are not observed in other types of interstitial nephritis). Histopathologically, the renal tubulointerstitium shows the infiltration of many lymphocytes and plasmacytes, as well as fibrosis, and IgG4 immunostaining shows a number of IgG4-positive plasma cells [47]. Although many studies have found no significant changes in the glomeruli, others have reported an association with glomerular lesions, including membranous nephropathy [46]. In the near future, the Japanese Kidney Society expects to develop diagnostic criteria for IgG4-related KD.

IgG4-related pulmonary diseases (IgG4-related PD)

IgG4-related PD has been described as inflammatory pseudotumor, interstitial pneumonitis, organizing pneumonia, and lymphomatoid granulomatosis [48]. Most (81%) patients with IgG4-related PD have been reported to be men, with a median age at diagnosis of 69 years [48], features similar to those of IgG4RD. Some patients present initially with respiratory symptoms, such as dry cough or dyspnea, whereas 75% of patients are asymptomatic and the disease is found incidentally by abnormal shadows on chest X-rays. Although IgG4-related PD is associated with a variety of radiologic abnormalities [49], diffuse lymphoplasmacytic infiltration has been observed in all lesions, with irregular fibrosis and obliterative vascular changes being more common in solid areas [48]. Hilar and pancreatic accumulation of gallium-67 has been reported as characteristic of the active stage of AIP when serum IgG4 concentrations are high [50].

Radiographically, IgG4-related PD can be divided into two types, inflammatory pseudotumors and interstitial pneumonitis. Inflammatory pseudotumors have been described as nodular or mass lesions, or infiltration, and are characterized by radiating reticular shadows surrounding the tumor. Interstitial pneumonitis presents in most patients with reticular shadows, ground-glass opacity, and interstitial fibrosis in both lower lung fields [17].

Histopathologically, inflammatory pseudotumor is a plasma cell granuloma, with infiltration mainly by plasma cells and lymphocytes, irregular fibrosis, lymphoid follicle formation, findings of interstitial pneumonitis at the periphery of the nodule, obliterating phlebitis and arteritis, and eosinophilic infiltration [17]. Interstitial pneumonitis is characterized by thickening of the alveolar septa due to infiltration by plasma cells and lymphocytes, and by diffuse fibrosis. Histopathologically, interstitial pneumonitis often shows a pattern previously classified as non-specific interstitial pneumonia (NSIP) [51]. The diagnostic criteria for IgG4-related PD are now under consideration by the Japanese Respiratory Association.

IgG4-related Hashimoto's thyroiditis (IgG4-related HT)

Hashimoto's thyroiditis (HT) has been considered a well-defined clinicopathological entity, characterized by the presence of goiter and serum thyroid autoantibodies. Recently, a unique subtype of HT was described, characterized by the presence of prominent fibrosis such as storiform fibrosis and swirling fibrosis, numerous IgG4-positive plasma cells, and elevated serum IgG4 [52], and called IgG4-related HT [53]. Among 23 patients with HT who underwent total thyroidectomy, 14 cases (60.8%) were IgG4-related HT, but there were no significant differences in positivity for thyroid and microsome tests between IgG4-related HT and non-IgG4 HT [54].

Riedel's thyroiditis was first described in 1896 in two patients with hard goiter and tracheal compressive symptoms. One-third of patients with Riedel's thyroiditis have multifocal fibrosclerosis, including sclerosing cholangitis, salivary gland fibrosis, RPF, or fibrotic orbital pseudotumor. Therefore, despite the lack of immunohistochemical staining for IgG4, certain proportions of Riedel's thyroiditis were considered a type of IgG4RD. Although one patient with IgG4RD showed involvement of the lachrymal gland and pulmonary and biliary tracts as well as Riedel's

thyroiditis [32], it is still unclear whether Riedel's thyroiditis is a type of IgG4RD.

IgG4-related lymphadenopathy and Castleman's disease

Concomitant lymphadenopathy is common in patients with IgG4RD, and there have been several reports dealing with the morphological and immunohistological findings of lymph node lesions [55–57]. Although IgG4-related lymphadenopathy is occasionally characterized by systemic lymphadenopathy, polyclonal hyperimmunoglobulinemia, especially elevated IgG and IgE concentrations, and positivity for various autoantibodies, patients with IgG4RD with generalized lymphadenopathy should only be evaluated for lymphoma, sarcoidosis, multicentric Castleman's disease, and other malignancies.

IgG4-related lymphadenopathy can be characterized into five histological subtypes: Castleman's disease-like morphology (type I), reactive follicular hyperplasia (type II), interfollicular plasmacytosis and immunoblastosis (type III), progressive transformation of germinal center-like (type IV), and inflammatory pseudotumor-like morphology (type V) [57]. In addition, IgG4-related lymphadenopathy can be classified into two types based on the infiltrative patterns of IgG4-positive cells: interfollicular plasmacytosis (types I, II, III, and V) and intragerminal center plasmacytosis (type IV). Patients with systemic IgG4-related lymphadenopathy were significantly older (68.8 vs. 43.3 years) and had significantly lower C-reactive protein (0.29 vs. 8.71 mg/dl) and interleukin (IL)-6 (8.45 vs. 34.82 pg/ml) concentrations than patients with multicentric Castleman's disease [56].

IgG4-related retroperitoneal fibrosis (IgG4-related RPF)

RPF is a chronic inflammatory condition with marked fibrosis in retroperitoneal tissue. In patients with advanced RPF a retroperitoneal mass covers the abdominal aorta and compresses the ureters, leading to urinary obstruction. Its etiology is unknown, but it has many causes, including infection, radiation, drugs, malignant tumor, and trauma. Three patients with RPF and elevated serum IgG4 have been described [58], and the histology of all 12 patients with RPF was reported to be similar to that seen in AIP, including fibrosis, intense inflammatory cell infiltration with plasma cells, venulitis, and obliterative arteritis [59]. Of 17 patients with RPF, 10 had both elevated serum IgG4 and histopathological features typical of IgG4RD, suggesting that RPF could be categorized as IgG4-related [60]. However, in RPF, fibrosis gradually progresses during chronic inflammation, with lymphocyte infiltration predominant during the early stages and a fibroinflammatory process occurring later. Therefore, determining the stage of illness seems important for diagnosis and prediction of response to steroid treatment [61].

IgG4-related aortitis

There have been several recent reports of inflammatory aneurysms in the abdominal or thoracic aorta [62–64]. For example, 40% of inflammatory abdominal aortic aneurysms (AAAs) were IgG4RD, with elevated IgG4 in serum and abundant infiltration of IgG4+ plasma cells and obliterative phlebitis [62]. These findings suggested that inflammatory AAAs can be classified into 2 groups: IgG4-related and IgG4-unrelated [62]. Although IgG4RD shows good response to steroid therapy, treatment with the anti-CD20 monoclonal antibody, rituximab, may result not only in clinical improvement, but in the tapering or discontinuation of steroids or other drugs [65].

Pathogenesis and pathophysiology of IgG4RD

At present, the pathogenetic mechanism and underlying immunological abnormalities in IgG4RD remain unclear. The elevated serum IgG4 concentration and tissue infiltration of IgG4-positive plasma cells are characteristic features of IgG4RD. Because IgG4 antibodies are dynamic molecules that can exchange Fab arms by swapping a heavy chain and attached light chain, IgG4 can form bi-specific antibodies, as well as functioning as a monovalent molecule [66, 67]. These properties may protect against type I allergy by inhibiting IgE functions, and may prevent type II and III allergy by blocking the Fc-mediated effector functions of IgG1 and inhibiting the formation of large immune complexes. The predominant expression of IgG4 under conditions of chronic antigen exposure is compatible with the clinical features of IgG4RD, including its slow progression and relatively weak immune response.

Some autoantibodies, including those to pancreatic trypsin inhibitor (PSTI), lactoferrin (LF), and carbonic anhydrase (CA), have been detected in patients with IgG4RD, especially in those with IgG4-related AIP [34]. Although IgG4 from the patients was able to bind the normal epithelia of the pancreatic ducts, gallbladder, and salivary gland ducts [68], IgG4-type autoantibodies have not been detected in patients with IgG4RD.

Aberrant immunological findings have been observed in patients with IgG4RD. For example, the Th2-dominant immune response and the production of Th2-type cytokines, such as IL-4, IL-5, IL-10, and IL-13, are increased [69–71]. Furthermore, the numbers of regulatory T cells (Treg) expressing CD4+CD25+Foxp3 are significantly higher in the affected tissues and peripheral blood of

patients with IgG4RD than the numbers in patients with autoimmune and nonautoimmune diseases [72–74]. Overexpression of the regulatory cytokines IL-10 and transforming growth factor β (TGF-β) has also been reported in patients with IgG4RD [74, 75]. IL-10 and TGF-β have potent activities in directing B cells to produce IgG4 and induce fibroplasia, respectively. IL-4, IL-5, and IL-13 are important for class switching to IgE production and eosinophil migration. Therefore, abnormalities in the production of these cytokines may be involved in the pathogenesis of IgG4RD.

Perspectives on IgG4RD

Although IgG4RD is a novel clinical entity, it is not a rare disease. Despite the effectiveness of steroid therapy, for IgG4RD, the condition has often been misdiagnosed as a malignant tumor, lymphoma, Sjögren's syndrome, or other diseases. To date, the clinical diagnostic criteria for IgG4RD have not been established. Because IgG4RD may occur in a variety of organs throughout the body, comprehensive discussions with the cooperation of many clinicians from various specialized fields is needed to establish uniform diagnostic criteria. At present, the diagnostic criteria for IgG4-MD (Table 2) [8] and those for IgG4-AIP type 1 (Table 5) [14] have been established.

Consensus has been reached on two diagnostic criteria for IgG4RD: (1) serum IgG4 concentration >135 mg/dl, and (2) >40% of IgG-positive plasma cells being IgG4-positive. The MHLW Japan team has proposed guidelines for the diagnosis of IgG4RD; these are shown in Table 3. The formulation of organ-specific (i.e., kidney and pulmonary) diagnostic criteria for IgG4RD requires cooperation with the relevant societies. Although IgG4RD responds well to steroid therapy, recurrence and relapse occur following the early reduction or withdrawal of prednisone. Therefore, it is necessary to develop treatment guidelines to establish initial doses of steroids, tapering procedures, and maintenance doses. The MHLW Japan team is currently pursuing a "Prospective study for creating IgG4-related disease treatment guidelines", and unified clinical guidelines are expected in the near future.

Table 5 Clinical diagnostic criteria of autoimmune pancreatitis; revised proposal in Japan (2006) [79]

1. Diffuse or segmental narrowing of the main pancreatic duct with irregular wall and diffuse or localized enlargement of the pancreas on imaging modalities, such as abdominal ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI)
2. High-serum F-globulin, IgG, or IgG4, or the presence of autoantibodies, such as antinuclear antibodies and rheumatoid factor
3. Marked interlobular fibrosis and prominent infiltration of lymphocytes and plasma cells into the periductal area, with occasional lymphoid follicles in the pancreas

For diagnosis, criterion 1 must be present, together with criteria 2 and/or 3

However, it is necessary to exclude malignant diseases such as pancreatic and biliary cancers

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Conflict of interest None.

Appendix

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Article

Deficiency of Rap1-Binding Protein RAPL Causes Lymphoproliferative Disorders through Mislocalization of p27kip1

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SUMMARY

RAPL (an alternative spliced form of *Rassf5*) is a critical Ras-related protein1 (Rap1) effector that regulates lymphocyte adhesion. Here, we have shown that in addition to this previously described function, RAPL also negatively controls lymphocyte proliferation and prevents autoimmunity and lymphoma. RAPL-deficient mice experienced age-related lupus-like glomerulonephritis and developed B cell lymphomas. RAPL-deficient lymphocytes showed hyperproliferation by enhanced S phase entry after antigen receptor ligation. Compared to wild-type cells, RAPL-deficient naive lymphocytes had a 2- to 3-fold increase in Cdk2 kinase activity with a cytoplasmic mislocalization of the cyclin-dependent kinase inhibitor $p27^{kip1}$. RAPL was found to suppress the phosphorylation of $p27^{kip1}$ on serine 10 (S10) and promoted $p27^{kip1}$ nuclear translocation. An S10A mutation in $p27^{kip1}$ corrected its cytoplasmic accumulation, reduced hyperproliferation in RAPL-deficient lymphocytes, and suppressed glomerulonephritis and development of B cell lymphoma. Thus, RAPL serves as a checkpoint for S phase entry to prevent lymphoproliferative disorders through the spatial regulation of $p27^{kip1}$.

INTRODUCTION

The small GTPase Ras-related protein1 (Rap1) regulates multiple functions such as cell proliferation, differentiation, and adhesion (Bos et al., 2001). RAPL was identified as a Rap1-GTP binding protein that mediates Rap1 functions by modulating lymphocyte function-associated antigen-1 (LFA-1) adhesiveness in concert with cell polarization (Katagiri et al., 2003). RAPL is predominantly expressed in immune cells, and RAPL-deficient lymphocytes and dendritic cells have impaired adhesion and motility triggered by chemokines and defective trafficking to peripheral lymph nodes, resulting in hypoplastic lymphoid tissues (Ebisuno et al., 2010; Katagiri et al., 2004). Upon T cell receptor (TCR) ligation, RAPL also mediates important inside-out signal downstream of the Src kinase-associated phosphoprotein 1, SKAP-1 (Raab et al., 2010). In previous studies, we further demonstrated that the Sterile20-like kinase Mst1 is a downstream effector that mediates LFA-1-dependent lymphocyte adhesion upon TCR and chemokine stimulation (Katagiri et al., 2006, 2009).

Rapl (also known as Nore1b) is an alternative spliced form of *Rassf5*, which belongs to a member of the putative tumor-suppressor Rassf family (Avruch et al., 2009). Nore1A, a longer isoform of Rassf5 (Rassf5a) has antiproliferative activity in tumor cell lines (Vos et al., 2003) and epigenetic inactivation of Rassf members including *RASSF5A* (previously also known as *NORE1A*, which has been reported for various types of cancers) (Avruch et al., 2009). Although the Rap1 pathways that affect cell proliferation are thought to be distinct from those that mediate adhesion (Stork and Dillon, 2005), these studies imply that RAPL also controls cell proliferation. However, it is not known whether RAPL regulates cell proliferation in physiological situations or whether RAPL mutations or silencing lead to pathological conditions associated with hyperproliferation and tumor development.

Abnormal cell cycle progression in lymphocytes leads to excessive activation and proliferation and the subsequent loss of self tolerance and development of autoimmunity (Balomenos and Martinez, 2000). Cell cycle progression by cyclins and cyclin-dependent kinases (Cdks) is tightly regulated by Cdk inhibitors (CdkIs) (Sherr and Roberts, 1999). A Cdk2 inhibitor, $p27^{kip1}$ (encoded by the gene *Cdkn1b* and hereafter p27) (Polyak et al., 1994) is a critical regulator of cell cycle progression. p27 forms a complex with Cdk2 and suppresses its kinase activity,

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thereby inhibiting the G1 to S phase transition. Antigen receptor stimulation downregulates p27 through ubiquitination and subsequent degradation (Appleman et al., 2000). Defective accumulation of p27 contributes to the induction of T cell anergy and tolerance (Li et al., 2006a). A deficiency in p27 results in enlarged lymphoid organs (Fero et al., 1996; Nakayama et al., 1996). p27 activity is also regulated by subcellular localization p27 (Ekholm and Reed, 2000). p27 shuttles between the nucleus and cytoplasm. To exert its inhibitory function, p27 needs to be transported into the nucleus (Reynisdottir and Massague, 1997). p27 nuclear export is regulated by the exportin CRM1 and ras-related nuclear protein, Ran GTPase, as well as phosphorylation of p27 on serine 10 (Rodier et al., 2001) by protein kinases such as kinase interacting stathmin (KIS) (Boehm et al., 2002), and cytoplasmic translocation of p27 is thought to prevent the assembly of p27 into cyclin E-Cdk2 complexes and to impair the antiproliferative effects of p27 (Boehm et al., 2002; Rodier et al., 2001). Furthermore, genetically targeted mice bearing a p27 mutant unable to interact with cyclin-Cdks exhibit a dominant increase in spontaneous tumorigenesis in many tissues, including lymphomas, compared with *Cdkn1b*$^{-/-}$ mice, indicating an oncogenic function independent of the cyclin-Cdks (Besson et al., 2007). The cytoplasmic localization of p27 in tumor cells, such as B cell lymphomas, has been identified as a mechanism promoting carcinogenesis in humans and mice (Barnouin et al., 1999; Qi et al., 2006). However, the physiological relevance and mechanisms regulating the subcellular localization of p27 in lymphocytes are unclear.

Here, we report that RAPL is important in p27 nuclear localization in both T and B lymphocytes upon antigen receptor stimulation. As a result, RAPL deficiency caused the mislocalization of p27 in the cytoplasm and hyperproliferation of both T and B cells. RAPL-deficient mice developed lupus-like autoimmunity and B cell lymphomas with high frequencies, suggesting that the regulation of p27 subcellular localization by RAPL serves as a checkpoint for S phase entry to prevent immunoproliferative disorders.

RESULTS

Development with Age of Lupus-like Glomerulonephritis and Lymphoma in RAPL-Deficient Mice

We previously reported that young RAPL-deficient mice (<2 months) had hypoplastic spleens and peripheral lymph nodes resulting from reduced lymphocytes. However, approximately 90% of these mutant female mice, but none of control littermates, developed enlarged lymph nodes and spleens at 10 months of age. Total B and T cell numbers were increased by approximately 50%. CD44$^+$CD62L$^-$ effector-memory T cells and CD138$^+$B220$^+$ plasma cells increased in 10-month-old female RAPL-deficient mice compared to littermate controls (Figure 1A). The aged mutant mice showed a 2-fold increase in serum IgG concentrations relative to wild-type mice and also high titers of antibodies to double-strand DNA (dsDNA) (Figure 1B). Histological examination revealed glomerulonephritis, which was associated with the deposits of IgG and C3 as well as proteinuria (Figures 1B and 1C). Although RAPL-deficient male mice showed similar phenotypes, they were evident at a much later age (15–18 months). There were no significant differences in Foxp3$^+$CD4$^+$ regulatory T cell populations between wild-type and RAPL-deficient mice (Figure S1 available online).

In addition, we often found that spleens and lymph nodes in RAPL-deficient mice were extremely large. Histological and flow cytometric analyses indicated that RAPL-deficient mice developed B220$^+$CD19$^+$, diffuse large B cell lymphomas (Figures 1D and 1E). Approximately 30% of RAPL-deficient mice at 1 yr of age but not in control mice developed B cell lymphomas in the peripheral lymph nodes and spleens. Further examination of these lymphomas by flow cytometry indicated that they were an IgM$^+$, IgD$^+$, CD21$^+$, CD23$^+$, and mature B cell type, and that progressed lymphomas often lost these markers to various extents (Figure 1E). They were monoclonal or oligoclonal in origin (Figure 1F). B cell lymphomas in RAPL-deficient mice were not related to the severity of the autoimmune phenotypes.

RAPL-deficient mice also developed tumors in the liver and lung after 1.5 yr of age, although the frequencies of these tumors were lower than the lymphomas (Figure S1B). Histologically, these tumors were identified as hepatocellular carcinomas and lung adenocarcinomas (Figure S1B).

Enhanced S Phase Entry in RAPL-Deficient Lymphocytes

To examine whether RAPL deficiency affects proliferative responses, primary B or T lymphocytes from 7- to 9-week-old wild-type and RAPL-deficient mice were stimulated by antibody crosslinking the antigen receptors. Compared to wild-type B cells, RAPL-deficient B cells displayed enhanced DNA synthesis with various doses of anti-IgM F(ab′)$_2$ (Figure 2A, left). Similarly, RAPL-deficient T cells also showed increased DNA synthesis in response to crosslinking of the TCR complex in the presence or absence of anti-CD28 (Figure 2A, right). Consistent with the hyperproliferative response to anti-IgM F(ab′)$_2$, RAPL-deficient B cells had greater S phase progression compared to wild-type B cells 48 hr after stimulation (41% versus 62%) (Figure 2B). Compared to wild-type T cells, RAPL-deficient T cells also had accelerated S phase entry 48 hr after anti-CD3 stimulation in the presence or absence of anti-CD28 (8% versus 18%, 20% versus 35%) (Figure 2C). There were no changes in apoptotic responses after antigen receptor ligation (data not shown). Thus, RAPL-deficient lymphocytes hyperproliferate in response to antigen receptor ligation.

To demonstrate whether lymphocyte hyperproliferation occurs in vivo, bromo deoxy uridine (BrdU) was injected intraperitoneally into RAPL-deficient mice. As shown in Figure 2D, B cells and CD4$^+$ T cells as well as CD8$^+$ T cells (data not shown) in lymph nodes of 10-month-old of RAPL-deficient mice bearing no malignancies showed increases in incorporation of BrdU in vivo, compared to those of control lymphocytes, indicating that RAPL deficiency led to lymphocyte hyperproliferation.

Cell Signaling through Antigen Receptors in RAPL-Deficient Lymphocytes

We proceeded to investigate the underlying mechanisms of enhanced lymphocyte proliferation. Because RAPL deficiency does not affect proliferative signaling pathways mediated by mitogen-activated protein (MAP) kinase, nuclear factor-kappa B (NF-κB), or phosphatidyl innositol-3 (PI-3) kinase triggered by the B cell receptor (BCR) or TCR and/or CD28 (Figure S2A),

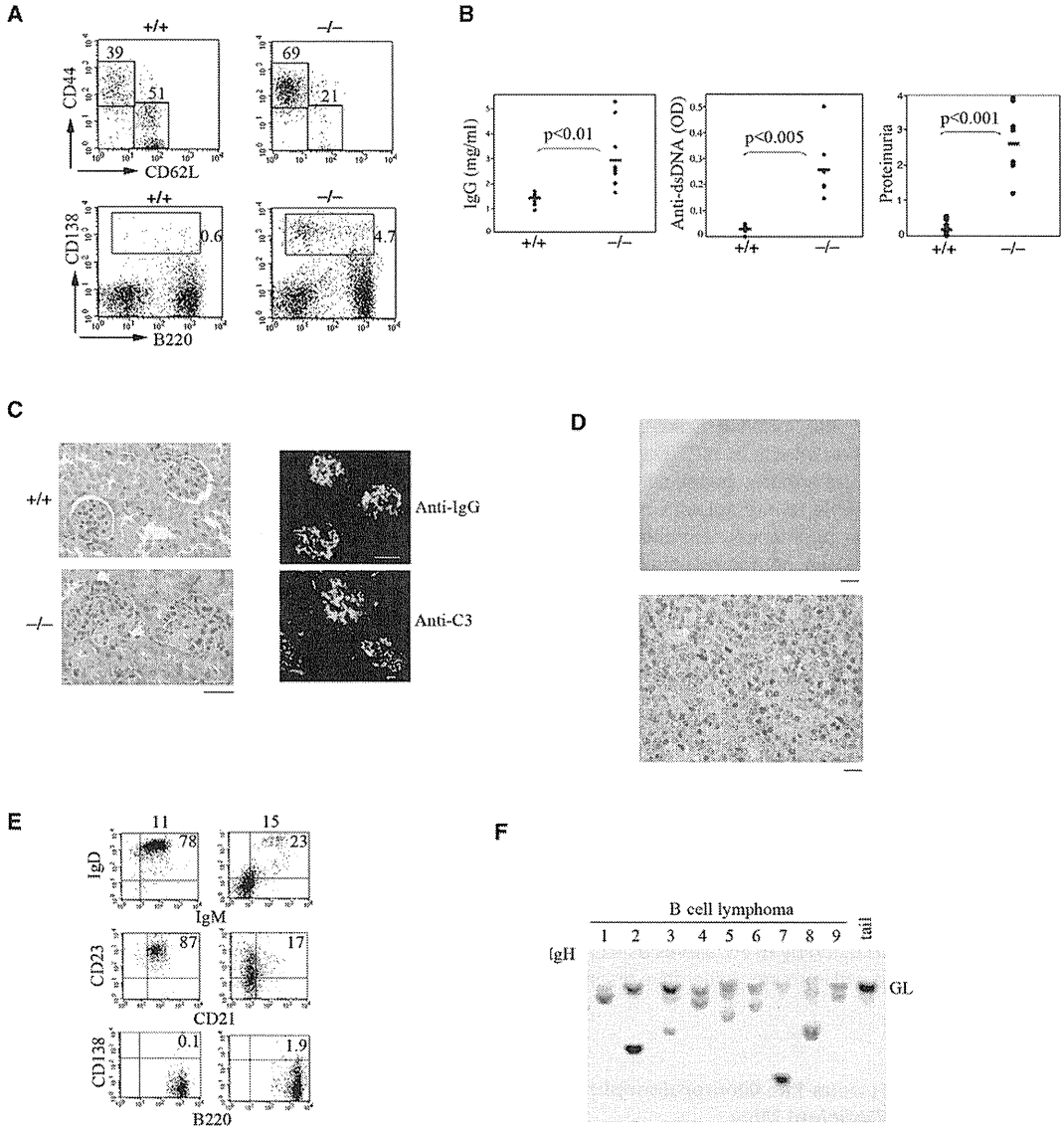

Figure 1. Lupus-like Glomerulonephritis and B Cell Lymphomas in RAPL-Deficient Mice

(A) Flow cytometeric profiles of lymphocytes from spleens of wild-type (+/+) and RAPL-deficient (−/−) mice at 10 months of age. Upper graphs show expression of CD44 and CD62L on $CD4^+$ gated lymphocytes. Lower graphs show expression of CD138 and B220.

(B) Amounts of serum IgG in 12-month-old wild-type (+/+) and RAPL-deficient (−/−) mice. p value for RAPL-deficient mice compared to wild-type control is indicated on the graph (left). Anti-ds DNA titers in 12-month-old wild-type (+/+) and RAPL-deficient (−/−) mice. p value for RAPL-deficient mice compared to wild-type control is indicated on the graph (center). Proteinuria in 12-month-old wild-type (+/+) and RAPL-deficient (−/−) mice. p value for RAPL-deficient mice compared to wild-type control is indicated on the graph (right). Proteinuria was measured with medical color strips dipped in mouse urine and units were defined as follows: 0, no proteinuria; 1, 30 mg/dl; 2, 100 mg/dl; 3, 300 mg/dl; 4, 1000 mg/dl.

(C) Glomerulonephritis with immune complex deposits in RAPL-deficient mice. Hematoxlin-Eosin staining (left) and immunostaining with IgG and C3 complement antibodies (right) of kidney sections from 11-month-old RAPL-deficient mice. Scale bar represents 50 μm.

(D) Low (top) and high (bottom) magnifications of the enlarged lymph nodes from 11-month-old RAPL-deficient mice. Scale bars represent 100 (top) and 10 (bottom) μm, respectively.

(E) Flow cytometric analysis of B cell lymphoma surface phenotypes ($B220^+$, $CD19^+$) developed in RAPL-deficient mice (11 and 15 months old).

(F) Southern blot analysis of *Igh* locus rearrangement in B cell lymphomas. Approximately 30% of RAPL-deficient mice at 1 yr of age developed B cell lymphoma in the peripheral lymph nodes or spleens as shown in Figure 7D. Representative B cell lymphomas independently developed in nine RAPL-deficient mice are shown with tail DNA to indicate the germline (GL) *Igh*.

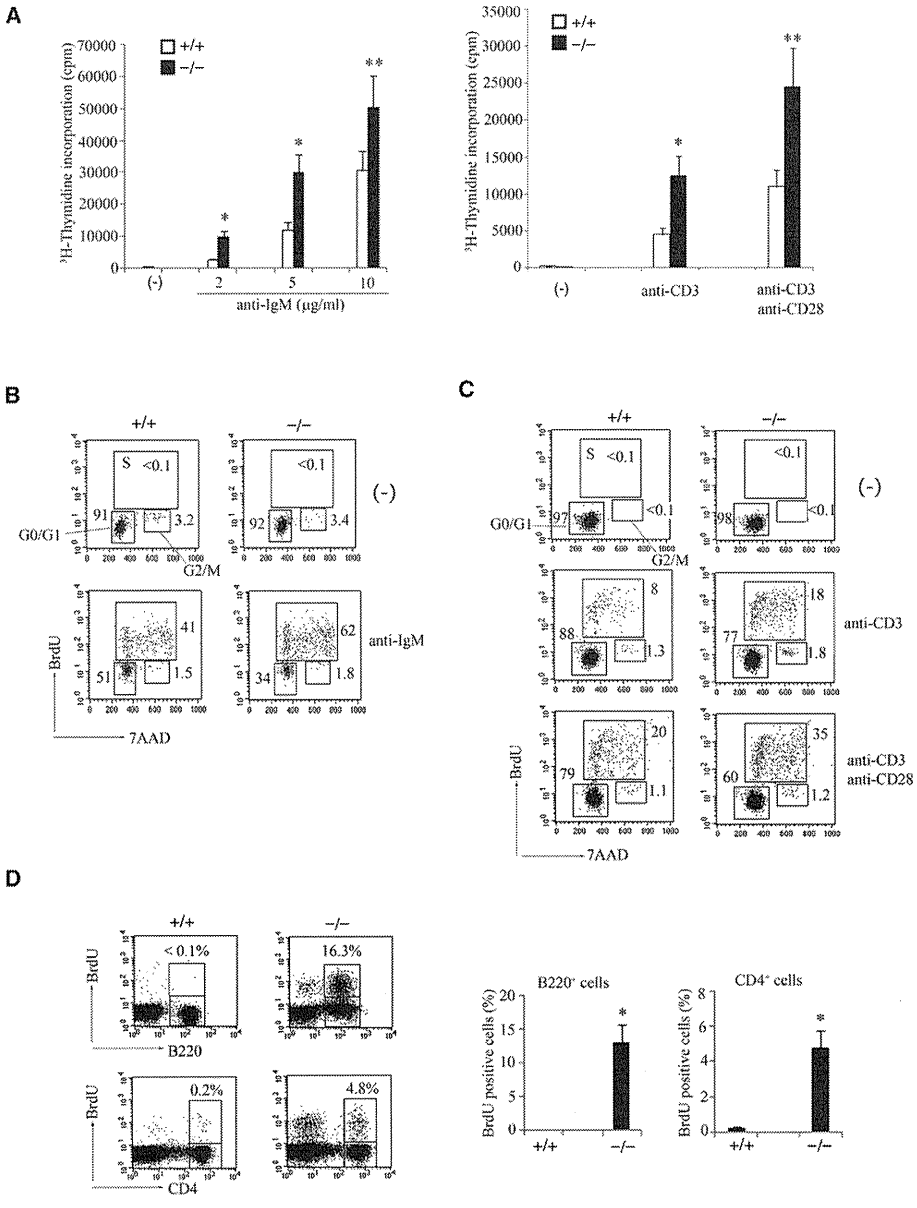

Figure 2. Enhanced Proliferation of RAPL-Deficient Lymphocytes

(A) ^{3}[H]-thymidine uptake by B cells (left) and T cells (right). Primary B and T cells from wild-type (open bar) and RAPL-deficient (closed bar) mice were unstimulated (−) or stimulated with antigen receptor ligation by anti-IgM F(ab′)$_2$ at the indicated concentrations, or by anti-CD3 (5 μg/ml) with or without anti-CD28 (2 μg/ml). ^{3}[H]-thymidine uptake was measured 48 hr after stimulation in triplicate experiments. The mean values and standard errors are shown. *$p < 0.002$, **$p < 0.01$, compared with corresponding wild-type littermate control.

(B and C) Cell cycle profiles of wild-type (+/+) and RAPL-deficient (−/−) B cells (left) or T cells (right) measured at 48 hr without stimulation (−) or with antigen receptor ligation as indicated. The cell populations in each cell cycle phase (G0+G1, S, G2+M) measured by 7AAD and BrdU staining are shown with percentages relative to viable cells in each panel. Data are representative of three independent experiments.

(D) BrdU uptake in vivo. Left: Flow cytometric profiles of BrdU-positive cells in B220$^+$ cells and CD4$^+$ cells of 10-month-old wild-type (+/+) and RAPL-deficient (−/−) mice. Right: Average percentages and standard errors of BrdU incorporated cells for three wild-type (+/+) and RAPL-deficient (−/−) mice are shown. *$p < 0.001$, compared with wild-type littermate control.

we examined cell cycle regulators. Cyclin D and cyclin-dependent kinase 4 (Cdk4) were upregulated and peaked 48 hr after stimulation with anti-BCR or anti-CD3 with or without anti-CD28 (Figure 3A). Cdk4 activity and retinoblastoma (Rb) protein phosphorylation were increased in stimulated B cells and T cells. RAPL-deficient lymphocytes displayed essentially the same amounts of upregulation and activation of cyclin D, Cdk4, and Rb expression and phosphorylation. Similarly, there

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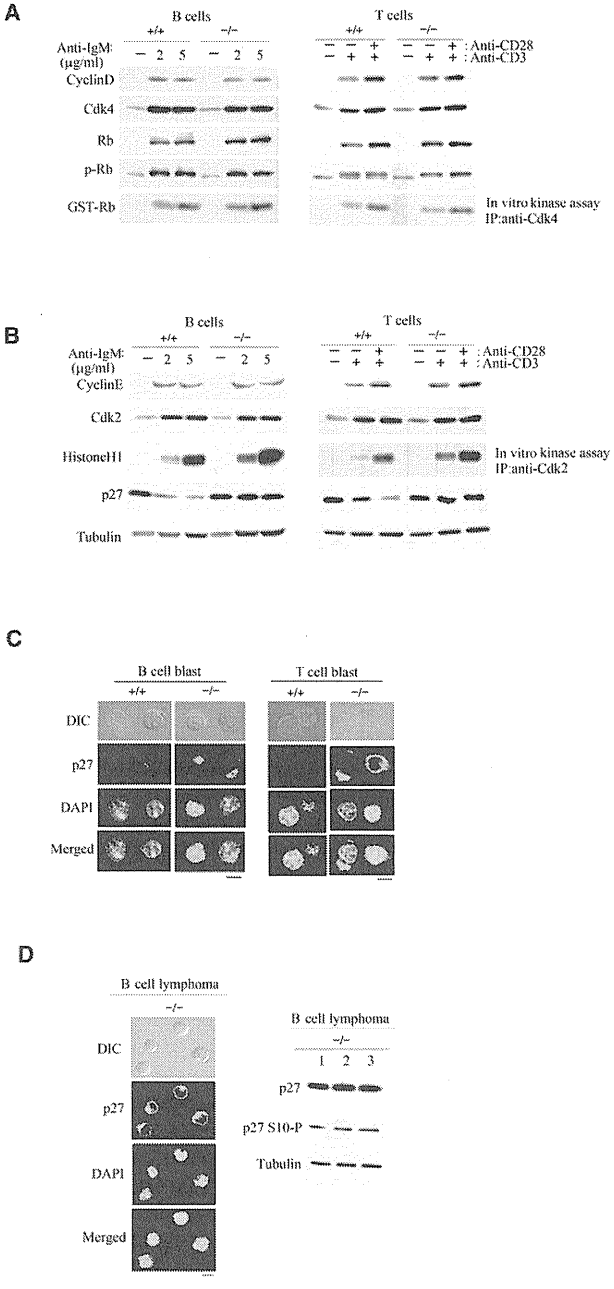

Figure 3. Effects of RAPL Deficiency on Signal Transduction and Cell Cycle Regulators Downstream of Antigen Receptor Signaling

(A) Expression of cyclin D, Cdk4, Rb, and phospho-Rb measured 48 hr after antigen receptor ligation in B (left) and T (right) cells from wild-type (+/+) and RAPL-deficient (−/−) mice. Total lysates derived from 1×10^5 cells were applied in each lane. In vitro Cdk4 kinase activities were measured with GST-Rb as a substrate.

(B) Expression of cyclin E, Cdk2, and its kinase activity measured in vitro via phosphorylation of histone H1 as a substrate. Expression of p27 and tubulin in B cells (left) and T cells (right) from wild-type (+/+) and RAPL-deficient (−/−) mice 48 hr after antigen receptor ligation. Total lysates from 1×10^5 cells were applied in each lane.

(C) Immunostaining of p27 in wild-type (+/+) and RAPL-deficient (−/−) B and T cell blasts after stimulation with antigen receptor ligation for 2 days. Representative distribution of p27 in lymphocytes is shown. Differential interference contrast (DIC), p27, and DAPI-stained nuclei are indicated. Scale bars represent 5 μm.

(D) Subcellular localization and phosphorylation at serine 10 of p27 in B cell lymphomas. A minimum of 10 independent B cell lymphomas from 10- to 12-month-old RAPL-deficient (−/−) mice were immunostained with anti-p27 or DAPI (nuclei). All B cell lymphomas showed cytoplasmic localization of p27, and a representative example is shown (left). Scale bar represents 10 μm. Immunoblot of total and S10 phosphorylated p27 and tubulin in three independent B cell lymphomas (right).

were no differences in cyclin E and Cdk2 upregulation in both wild-type and RAPL-deficient T and B cells (Figure 3B). However, Cdk2 enzymatic activities in RAPL-deficient B and T cells were augmented at 48 hr compared to wild-type cells after stimulation with anti-BCR (2.6-fold, 2.4-fold) or anti-TCR without (2.5-fold) or with (2.8-fold) anti-CD28 (Figure 3B). These proliferative responses were almost completely inhibited by low doses of a specific Cdk2 inhibitor, CVT-313 (Bhattacharjee et al., 2001), and RAPL-deficient cells tended to be more sensitive to the inhibitor (Figure S2B). These results suggest that Cdk2 drives lymphocyte proliferation and that the enhanced proliferation by RAPL deficiency is largely dependent on Cdk2 activity.

p27, the major Cdk inhibitor for Cdk2, is elevated in G_0 but then downregulated by ubiquitination and proteasomal degradation during the G_0 to S phase transition. In B cells stimulated with anti-BCR, p27 was diminished at 48 hr (Figure 3B). Surprisingly, p27 was not downregulated in RAPL-deficient B cells after 48 hr of stimulation with higher anti-BCR concentrations (5.2-fold compared to wild-type B cells), despite augmented Cdk2 kinase activities (Figure 3B). In wild-type T cells, p27 was downregulated by anti-CD3 and more efficiently by a combination of anti-CD3 and anti-CD28 (Figure 3B), and the degradation was further promoted by the addition of excess amounts of IL-2 (Figure S2C). However, as was the case with RAPL-deficient B cells, there was little to no decrease in p27 by either anti-CD3 or anti-CD3+anti-CD28 (4.1-fold compared to wild-type T cells) at 48 hr, although Cdk2 activities were clearly augmented (Figure 3B). Even excess IL-2 did not promote the degradation of p27 in RAPL-deficient T cells (Figure S2C), although it promoted proliferation of wild-type and RAPL-deficient T cells similarly (data not shown). The amounts of IL-2 in the supernatant of RAPL-deficient T cells were comparable to those in wild-type (Figure S2C), ruling out the possibility that reduced p27 degradation in RAPL-deficient T cells is due to a shortage of IL-2.

Immunostaining of lymphoblasts generated 48 hr after stimulation with antigen receptor ligation revealed that the nuclear p27 was at very low amounts in both WT and RAPL-deficient cells (Figure 3C). Line-profile analysis showed that 4.4% ± 3.5% and 12% ± 8.7% of p27 were present in the nucleus of wild-type B cells and T cells (n = 50), and 5.9% ± 4.7% and 12.6% ± 9.3% in RAPL-deficient B and T lymphoblasts (n = 50) (Figure S3). p27 accumulated predominantly in the cytoplasm of RAPL-deficient T and B cells, although a small amount of p27 remained in the cytoplasm of wild-type cells (Figure 3C; Figure S3). There were no cells with nuclear dominant patterns observed in both wild-type or RAPL-deficient lymphoblasts. Because p27 must be transported into the nucleus to exert its inhibitory action, the cytoplasmic localization of p27 probably

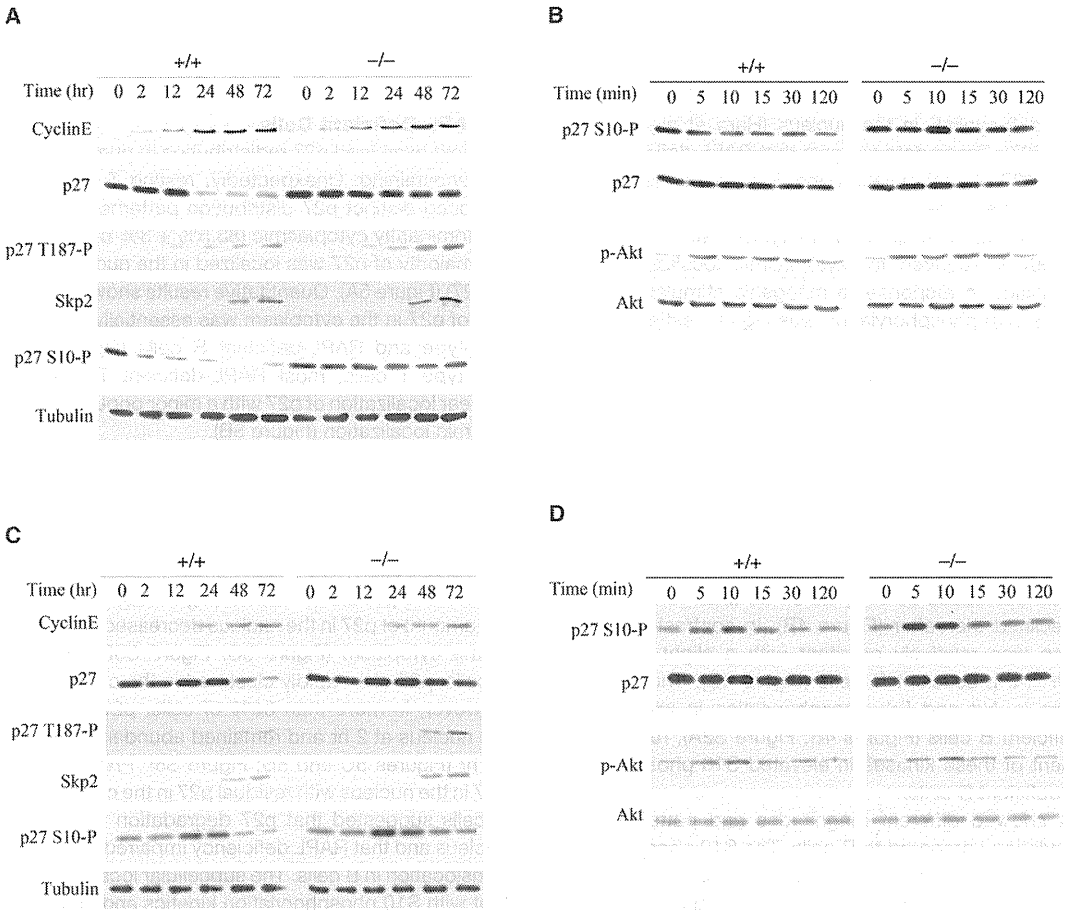

Figure 4. Deregulation of p27 in RAPL-Deficient Lymphocytes

(A) Defective p27 downregulation in RAPL-deficient B cells. Wild-type (+/+) and RAPL-deficient (−/−) B cells were stimulated with anti-IgM F(ab′)$_2$ for the indicated times, followed by immunoblot analysis of cyclin E, p27, phosphorylated p27 at threonine 187, Skp2, phosphorylated p27 at serine 10, and tubulin. Total lysates from 1 × 10^5 cells were applied in each lane.

(B) Kinetics of p27 phosphorylation at serine 10 (S10) in wild-type (+/+) and RAPL-deficient (−/−) B cells after anti-IgM stimulation. Total and S10 phosphorylated p27 and total and phosphorylated Akt are shown. Total lysates from 1 × 10^5 cells were applied.

(C) Defective p27 downregulation in RAPL-deficient T cells. Wild-type (+/+) and RAPL-deficient (−/−) T cells were stimulated with anti-CD3 and anti-CD28 for the indicated times, followed by immunoblot analysis as in (A).

(D) Kinetics of p27 phosphorylation at serine 10 (S10) in wild-type (+/+) and RAPL-deficient T cells (−/−) after anti-CD3 and anti-CD28 stimulation. Total and S10 phosphorylated p27 and total and phosphorylated Akt are shown. Total lysates from 1 × 10^5 cells were applied.

renders it inaccessible to a complex of cyclin E and Cdk2, leading to enhanced Cdk2 activities and resistance to degradation.

In all B cell lymphoma generated in RAPL-deficient mice, p27 was abundantly present in the cytoplasm (Figure 3D), as observed in BCR-stimulated RAPL-deficient B blasts (Figure 3C). Line-profile analysis showed that 87.6% ± 3.7% of p27 was present in the cytoplasm. This suggests that the mislocalization of p27 in the cytoplasm underlies lymphoma development.

Kinetics of Phosphorylation and Degradation of p27 in RAPL-Deficient Cells

To investigate whether the mislocalization of p27 in the cytoplasm underlies lymphoproliferative disorders, we examined p27 degradation in more detail. In wild-type B and T cells, p27 declined sharply between 24 and 48 hr after antigen receptor stimulation (Figures 4A and 4C). Cyclin E increased after 12–24 hr and was maintained at high amounts at 24–72 hr. In contrast, RAPL-deficient cells maintained high p27 even at 72 hr poststimulation, whereas cyclin E upregulation was comparable to that in wild-type cells. T187 phosphorylation of p27, a trigger of p27 downregulation, was detected in wild-type cells at 24–72 hr, which coincided with the kinetics of upregulation of cyclin E and Skp2, a substrate-targeting subunit of the SCF ubiquitin ligase complex that regulates entry into S phase (Bashir et al., 2004). RAPL-deficient B and T cells exhibited comparable Skp2 upregulation and essentially the same time course of T187 phosphorylation (Figures 4A and 4C). The ratios of p27 phosphorylated on T187 to total p27 were lower in RAPL-deficient B cells (0.42 at 48 hr) and T cells (0.18 at 48 hr), compared to those of wild-type B cells (0.9 at