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Abundant a proliferation-inducing ligand (APRIL)-producing macrophages contribute to plasma cell accumulation in immunoglobulin G4-related disease

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ABSTRACT

Background. This study aimed to investigate the contribution of a proliferation-inducing ligand (APRIL), a member of the tumor necrosis factor (TNF) superfamily implicated in plasma cell survival, to the development of plasma cell–rich lesions in immunoglobulin G4–related disease (IgG4-RD).

Methods. We performed immunohistochemical staining for APRIL with Stalk-1 and Aprily-8 antibodies specifically recognizing APRIL-producing cells and secreted APRIL, respectively, in renal and submandibular lesions of IgG4-RD in comparison with those of Sjögren's syndrome and sialolithiasis.

Results. Numerous Stalk-1-positive APRIL-producing cells were detectable in lesions of IgG4-RD. These cells, identified as CD163-positive M2 macrophages, secreted APRIL that distributed close to and even on infiltrating plasma cells. In contrast, APRIL-producing cells and the secreted form of APRIL were rarely detectable in lesions of Sjögren's syndrome or sialolithiasis. Notably, APRIL expression decreased concomitantly with the level of plasma cell infiltration after successful glucocorticoid treatment.

Conclusions. Abundant infiltration into tissue lesions of APRIL-producing M2 macrophages and retention of secreted APRIL in plasma–cell–rich areas support a role for APRIL in the pathogenesis of plasma cell–rich lesions in IgG4-RD.

Keywords: APRIL, IgG4–related disease, macrophages

INTRODUCTION

Immunoglobulin G4–related disease (IgG4-RD) is a recently recognized systemic inflammatory disease with multi-organ involvement [1, 2]. Several relatively large-scale studies [3, 4] have revealed clinical and pathological features of IgG4-related kidney disease (IgG4-RKD). A representative manifestation of IgG4-RKD is tubulointerstitial nephritis (TIN), referred to as IgG4-related TIN. In addition, studies investigating the clinical course and histological changes occurring after glucocorticoid therapy have shown that delayed treatment results in residual renal insufficiency associated with radiologically detectable atrophy and histological fibrosis in IgG4-related TIN [5–7]. Past studies have similarly disclosed the clinical and pathological features of IgG4-related sialoadenitis (IgG4-RS). These include symmetrical, persistent and painless swelling of major salivary glands, in particular the submandibular gland, relatively preserved salivary gland function and improvement of saliva secretion after glucocorticoid therapy, in addition to serological and histopathologic findings common to other IgG4-RDs [8, 9]. However, the underlying pathophysiology of IgG4-RD has not yet been completely clarified, although involvement of not only T-cell subsets [10–12] but also plasmablasts [13], B cells [14], macrophages [15], eosinophils [1] and mast cells [16] in this disease has been demonstrated in past studies. Notably, plasma cell–rich infiltrates in the affected lesions are a pathological feature common to all affected organs. However, the cellular and molecular mechanisms responsible for plasma cell accumulation in tissue lesions of IgG4-RD are unknown.

A proliferation-inducing ligand (APRIL), a member of the tumor necrosis factor (TNF) superfamily (TNFSF13), is produced mostly by cells from the myeloid lineage, including monocytes/macrophages, neutrophils and eosinophils [17]. It is secreted as a soluble factor after cleavage in the Golgi apparatus by furin convertase [18]. APRIL has two signaling receptors from the TNF receptor superfamily, the transmembrane activator and calcium-modulating ligand interactor (TACI, TNFSFR13b) and B-cell maturation antigen (BCMA, TNFSFR17) [19]. These receptors are expressed by cells from the B lineage once B cells have encountered their antigen [20].

In addition, APRIL requires the coreceptor activity of heparan sulfate proteoglycan (HSPG) to efficiently signal into TACI/BCMA-positive target cells [21, 22]. Plasma cells ubiquitously express the HSPG CD138 [23]. Such a pattern of receptor expression explains the late role of APRIL in humoral immunity at the level of immunoglobulin class-switch and plasma cell generation/survival [24–26]. Regarding plasma cell survival, several studies have already established the contribution of APRIL in healthy tissues such as bone marrow and mucosal tissues [27–31]. Intense inflammation in tissues is usually associated with *de novo* plasma cell generation in ectopic germinal center formation and/or plasma cell infiltration. In these latter cases, the role of APRIL is less clear, with plasma cells having been described as localizing in areas rich and scarce for APRIL-producing cells in lesions from rheumatoid arthritis and Sjögren's syndrome, respectively [32, 33]. In IgG4-RD, Kiyama *et al.* [34] recently reported that serum levels of APRIL were elevated in patients with IgG4-RD. These findings prompted us to evaluate the contribution of APRIL to the plasma cell–enriched pathological conditions of IgG4-RD.

MATERIALS AND METHODS

Patients and materials

In this study we included 16 patients diagnosed with IgG-RD between 1 September 2005 and 31 December 2013. In these patients we retrospectively analyzed clinical features, including age, sex and affected organs, and laboratory data, including serum IgG4, IgG, IgE and creatinine (Cr) levels during the clinical course.

Among the 16 patients, we identified 11 with biopsy-proven IgG4-related TIN according to established diagnostic criteria [35]. All biopsied specimens showed abundant lymphoplasmacytic infiltration with copious IgG4-positive plasma cells into the renal interstitium [IgG4-positive plasma cells/IgG-positive plasma cells >40% and/or IgG4-positive plasma cells >10 per high-power field (HPF)] and various degrees of fibrosis, but no obliterative phlebitis. Six of the 11 patients had been included in our earlier studies [5, 36, 37] and 7 patients in a report by Saeki *et al.* [3, 6]. In the present study we compared the histological and immunohistochemical findings of renal specimens from 11 patients with IgG4-related TIN with those from 3 patients displaying TIN associated with Sjögren's syndrome. In addition, we included three patients with drug-induced eosinophil-rich TIN as controls since APRIL is known to be produced by eosinophils [30]. Seven of the 11 IgG4-related TIN patients with initial renal biopsy underwent rebiopsy after starting glucocorticoid therapy. One patient underwent rebiopsy 14 months after the beginning of treatment, one 7 months later, one 5 months later, three 4 months later and one only 1 month later.

We also identified 11 IgG4-RS patients among 16 IgG4-RD patients according to the diagnostic criteria for IgG4-related dacryoadenitis and sialoadenitis [38] or the comprehensive diagnostic criteria for IgG4-RD [39]. Seven of the 11 patients underwent surgical resection of the affected submandibular gland. These submandibular specimens showed abundant lymphoplasmacytic infiltration with copious IgG4-positive plasma

Table 1. Characteristics of 16 patients with IgG4-RD and 3 patients with Sjögren's syndrome, sialolithiasis or drug-induced TIN

	IgG4-RD ($n = 16$)	TIN associated with SS ($n = 3$)	Sialolithiasis ($n = 3$)	Drug-induced TIN ($n = 3$)
Age (years)	67.2 ± 10.4	57.7 ± 22.2	64.7 ± 7.1	62.0 ± 13.2
M:F, n:n (% male)	13:3 (81.3)	0:3 (0)	1:2 (33.3)	1:2 (33.3)
Cr (mg/dL)	1.50 ± 1.64	1.38 ± 0.55	NA	2.59 ± 1.55
IgG (mg/dL)	2803 ± 1003	3433 ± 1673	NA	1401 ± 476
IgG4 (mg/dL)	755 ± 412	NA	NA	NA
IgG4/IgG (%)	28.3 ± 15.6	NA	NA	NA
IgE (IU/mL)	395 ± 322	NA	NA	NA

Values presented as mean ± SD unless stated otherwise.

M, male; F, female; NA, not assessed; y.o., years old.

cells and various degrees of fibrosis. We compared the histological and immunohistochemical findings of seven submandibular specimens of IgG4-RS with those of three submandibular specimens of sialolithiasis.

This study received institutional ethics board approval and informed consent for use of all data and samples was obtained from each patient. The research was conducted in compliance with the Declaration of Helsinki.

Single immunostaining

Formalin-fixed and paraffin-embedded renal and submandibular gland specimens of patients with IgG4-RD were used for the immunostaining of IgG4 for IgG4-positive cells, CD138 for plasma cells, CD4 and CD8 for T cells, CD20 for B cells, CD68 for macrophages, CD163 for M2 macrophages, a membrane-bound form of APRIL (Stalk-1) for APRIL-producing cells and a soluble form of APRIL (Aprily-8) for secreted APRIL. The immunostaining was performed using a monoclonal antibody against human IgG4 (clone HP6025, fully diluted; Nichirei, Tokyo, Japan), CD138 (clone B-B4, 1:100; AbD Serotec, Oxford, UK), CD4 (clone 1F6, fully diluted; Nichirei), CD8 (clone C8/144B, fully diluted; Nichirei), CD20 (clone L26, fully diluted; Nichirei), CD68 (clone PG-M1, fully diluted; Nichirei) and CD163 (clone 10 D, 1:50; LeicaBiosystems, Nußloch, Germany), respectively. The rabbit anti-human polyclonal antibody (Stalk-1) and the mouse monoclonal antibody (Aprily-8) detecting APRIL-producing cells and secreted APRIL, respectively, were used as described previously [40]. The deparaffinized sections were microwaved in citrate buffer (pH 6.0) for 15 min. Cells positive for Stalk-1, CD138 or CD163 within the areas of most intense inflammatory cell infiltration were counted in five different HPFs; (HPF: a 400-fold magnification achieved by 10 × eyepiece and 40 × lens). Areas positive for secreted APRIL in pixels were quantified in five different HPFs with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Dual fluorescent immunostaining

All renal and submandibular gland specimens of the patients with IgG4-RD, renal specimens of Sjögren's syndrome or drug-induced eosinophil-rich TIN were used for dual fluorescent immunostaining to identify APRIL-producing cells. In addition, two cutaneous specimens of the patients with IgG4-RD were used to verify APRIL production by eosinophils because the renal and submandibular gland specimens evaluated in this study had scarce eosinophil infiltration. The deparaffinized sections were microwaved in citrate buffer (pH 6.0) for 20 min and incubated with normal donkey serum for protein blocking for 30 min. The specimens were incubated with a mouse monoclonal antibody to CD138, CD4, CD8, CD20, CD68, CD163 or human eosinophil major basic protein (MBP) (clone BMK13; Chemicon International, Billerica, MA, USA) as well as Stalk-1 or Aprily-8 overnight at 4°C. Then the specimens were incubated for 1 h at room temperature with Alexa Fluor 488–labeled donkey anti-mouse IgG antibodies and Alexa Fluor 594–labeled donkey anti-rabbit IgG antibodies (Molecular Probes, Carlsbad, CA, USA) and observed under a laser microscope and digitally merged. No positive staining was observed when the primary antibodies were replaced with normal donkey serum in the negative control of the staining procedures.

Statistical analysis

Statistical analysis was performed using SPSS version 19 (Chicago, IL, USA). The significance of differences between groups was determined using Mann–Whitney U test or Wilcoxon signed-rank test for continuous nonnormally distributed data. Significant differences were defined as $P < 0.05$.

RESULTS

Patient profiles

In the present study we analyzed 16 patients with IgG4-RD (Table 1). They were 13 men and 3 women with an average age of 67.2 years (range 44–81). They displayed multiple IgG4-related organ involvement: biopsy-proven renal lesion [11 of 16 (68.8%)], referred to as IgG4-related TIN, sialoadenitis [11 of 16 (68.8%)], referred to as IgG4-RS, multiple lymphadenopathy including cervical, mediastinal and abdominal lymph nodes (62.5%), dacryoadenitis (50.0%), lung lesion (50.0%), periaortic lesion (31.3%), pancreatic lesion (12.5%), prostatic lesion (12.5%), liver (6.3%) and nerve lesion (6.3%). Notably, six patients had both renal and submandibular lesions. Bilateral submandibular gland swelling was noted in all 11 IgG4-RS patients, 4 of whom also had bilateral parotid gland involvement. Seven and one of the IgG4-RS patients had bilateral and right unilateral dacryoadenitis, respectively.

None of the IgG4-RD patients evaluated in the present study had been treated with glucocorticoid or any other

immunosuppressants before the biopsy. After the biopsy (renal biopsy for 11 patients with IgG4-related TIN and submandibular gland biopsy for 7 of the 11 patients with IgG4-RS), all patients were treated with prednisolone at an initial dose of 20–40 mg/day for 2–4 weeks and at a maintenance dose of 2.5–5 mg/day over a period of 2–3 months. Renal lesions of seven patients with IgG4-related TIN were reassessed by biopsy [average period between initial biopsy and rebiopsy 5.6 months (range 1–14)]. As controls, three patients with renal lesions of Sjögren's syndrome, three patients with submandibular lesions of sialolithiasis and three patients with renal lesions of drug-induced TIN were included (Table 1).

Clinical findings

At presentation, all patients with IgG4-RD showed elevated levels of serum IgG4 [average 755 mg/dL (range 136–1520; normal range $<$135 mg/dL); according to the comprehensive diagnostic criteria [39] for IgG4-RD; Table 1]. Increases in serum IgG [average 2803 mg/dL (range 1689–4756; normal range 870–1700)] were noted in 15 patients. Ten patients showed elevated serum IgE levels [average 395 IU/mL (range 8–1226; normal range $<$250)] and four patients had eosinophilia (eosinophils $>$5%). Seven patients (43.8%) had hypocomplementemia. In the IgG4-related TIN group, a serum creatinine (Cr) concentration $>$1.0 mg/dL was observed in eight patients (72.7%) and the average Cr level was 1.87 mg/dL (range 0.54–2.55).

Plasma cell and IgG4-positive plasma cell infiltration in tissue lesions of patients with IgG4-RD, TIN associated with Sjögren's syndrome, drug-induced eosinophil-rich TIN and sialolithiasis

The average and median numbers of CD138-positive plasma cells in the tissue lesions with intense inflammatory cell infiltration were 158.1/HPF and 148.0/HPF ($n = 11$; range 53.2–284.4/HPF), respectively, in IgG4-related TIN, 157.4/HPF and 157.4/HPF ($n = 2$; range 103.2–211.6/HPF) in TIN associated with Sjögren's syndrome, 43.5/HPF and 43.0/HPF ($n = 3$; range 34.0–53.4/HPF) in drug-induced eosinophil-rich TIN, 95.2/HPF and 95.8/HPF ($n = 7$; range 75.6–114.0/HPF) in IgG4-RS and 83.5/HPF and 63.8/HPF ($n = 3$; range 57.0–129.8/HPF) in sialolithiasis (Supplementary data, Figure S1A). The difference in the number of CD138-positive plasma cells was significant only between that in IgG4-related TIN and that in drug-induced eosinophil-rich TIN ($P = 0.016$).

The average and median numbers of IgG4-positive plasma cells were 62.9/HPF and 58.4/HPF ($n = 11$; range 19.3–103.6/HPF), respectively, in IgG4-related TIN, 0/HPF and 0/HPF ($n = 2$; range 0–0/HPF) in TIN associated with Sjögren's syndrome, 0.4/HPF and 0.2/HPF ($n = 3$; range 0–1/HPF) in drug-induced eosinophil-rich TIN, 57.0/HPF and 55.2/HPF ($n = 7$; range 27.6–78.2/HPF) in IgG4-RS and 6.8/HPF and 4.5/HPF ($n = 3$; range 4.0–11.8/HPF) in sialolithiasis (Supplementary data, Figure S1B). The difference in the number of IgG4-positive plasma cells was significant between that in IgG4-related TIN and that in TIN associated with Sjögren's syndrome ($P = 0.030$), between that in IgG4-related TIN and that in drug-

induced eosinophil-rich TIN ($P = 0.010$) and between that in IgG4-RS and that in sialolithiasis ($P = 0.017$).

The average and median ratios of IgG4-positive cells and CD138-positive cells were 0.461 and 0.498 ($n = 11$; range 0.201–0.853), respectively, in IgG4-related TIN, 0 and 0 ($n = 2$; range 0–0) in TIN associated with Sjögren's syndrome, 0.011 and 0.005 ($n = 3$; range 0–0.029) in drug-induced eosinophil-rich TIN, 0.617 and 0.698 ($n = 7$; range 0.242–0.779) in IgG4-RS and 0.077 and 0.079 ($n = 3$; range 0.063–0.091) in sialolithiasis (Supplementary data, Figure S1C). The difference in the ratio of IgG4-positive cells and CD138-positive cells was significant between that in IgG4-related TIN and that in TIN associated with Sjögren's syndrome ($P = 0.030$), between that in IgG4-related TIN and that in drug-induced eosinophil-rich TIN ($P = 0.010$) and between that in IgG4-RS and that in sialolithiasis ($P = 0.017$).

High expression of APRIL in renal and submandibular gland lesions in patients with IgG4-RD

The number of Stalk-1-positive APRIL-producing cells in the renal lesions with intense inflammatory cell infiltration was significantly higher in patients with IgG4-related TIN [average 34.4/HPF (range 9.8–52.8)] and drug-induced eosinophil-rich TIN [average 32.6/HPF (range 29.8–34.4)] than those with TIN associated with Sjögren's syndrome [average 9.1/HPF (range 6.2–14.8); $P = 0.016$ and 0.050, respectively] (Figure 1A, B and E). Most of the Stalk-1-positive cells were mononuclear cells, which were considered to show in particular the morphological appearance of macrophages (insert of Figure 1A). The ratio of the number of Stalk-1-positive cells and CD138-positive cells was significantly higher in patients with IgG4-related TIN [average 0.292 (range 0.090–0.741)] than in those with TIN associated with Sjögren's syndrome [average 0.065 (range 0.060–0.070); $P = 0.040$]. In addition, high expression of secreted APRIL stained with Aprily-8 was observed in patients with IgG4-related TIN [average 2340 μm^2/HPF (range 307–4727)], while the expression level was close to zero in Sjögren's syndrome [average 273 μm^2/HPF (range 106–567); $P = 0.016$] (Figure 1C, D and F). Notably, we observed an abundance of secreted APRIL in patients with drug-induced eosinophil-rich TIN [average 2279 μm^2/HPF (range 1835–2616)].

The frequency of APRIL-producing cells infiltrating the submandibular gland lesions was also much greater in patients with IgG4-RS [average 68.6/HPF (range 43–83.2)] than those with sialolithiasis [average 3.3/HPF (range 0.6–7.2); $P = 0.017$] (Figure 2A, B and E). Again, the major cell type of APRIL-producing cells was infiltrating mononuclear cells (insert of Figure 2A). Expression of secreted APRIL stained with Aprily-8 was also highly elevated in patients with IgG4-RS [average 4210 μm^2/HPF (range 545–10 035)] as compared with those with sialolithiasis [average 38 μm^2/HPF (range 16–67); $P = 0.017$] (Figure 2C, D and F).

Identification of APRIL-producing cells in tissue lesions of patients with IgG4-RD

Dual fluorescent immunostaining revealed that a subset of CD68-positive macrophages was the major cell type producing
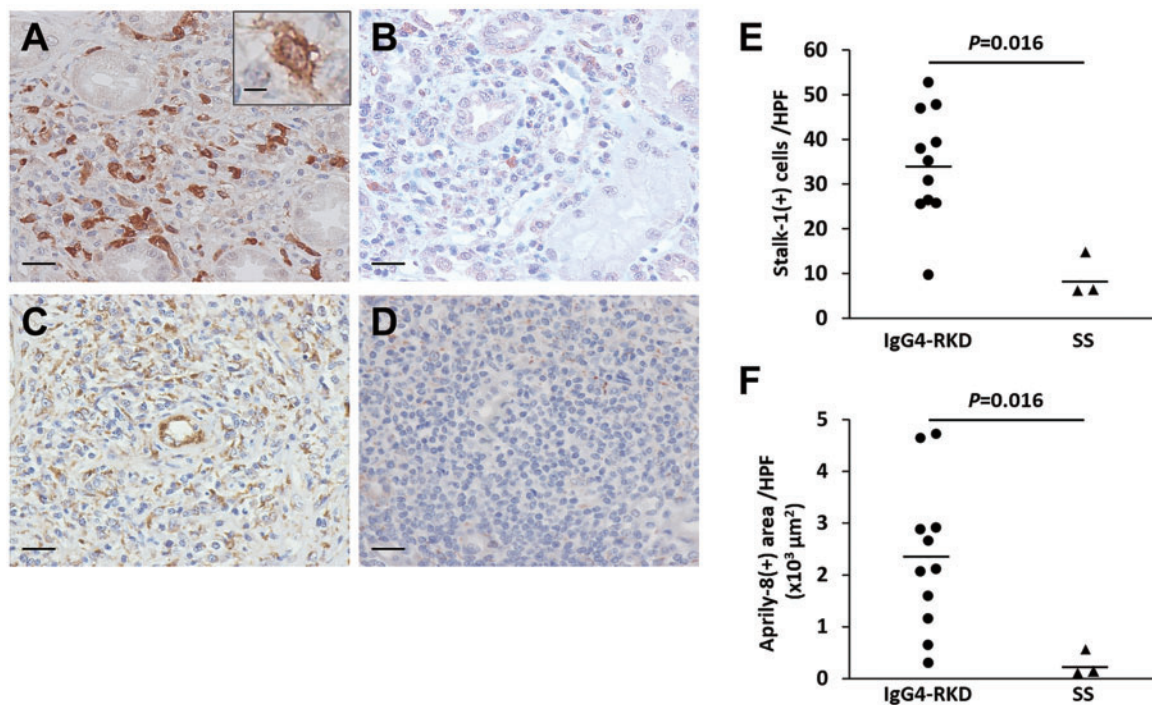
FIGURE 1: Enhanced expression of APRIL in renal lesions of patients with IgG4-related TIN. Immunohistochemical analysis of Stalk-1 staining for the detection of APRIL-producing cells in renal lesions of (**A**) IgG4-related TIN and (**B**) Sjögren's syndrome and of Aprily-8 for the detection of secreted APRIL in renal lesions of (**C**) IgG4-related TIN and (**D**) Sjögren's syndrome. Insert in (**A**) represents a high magnification of Stalk-1-positive mononuclear cells infiltrating in renal lesions. Scale bars represent 20 μm in original magnification and 4 μm in high magnification, respectively. The quantification of (**E**) Stalk-1-positive cells and (**F**) Aprily-8-positive area detectable in lesions of patients with IgG4-related TIN ($n = 11$) and Sjögren's syndrome ($n = 3$) (E and F, respectively) in which results are expressed as the number of Stalk-1-positive cells and Aprily-8-positive area within the areas of most intense inflammatory cell infiltration counted by five different HPFs. Note the marked increases in Stalk-1-positive APRIL-producing cells and secreted APRIL in patients with IgG4-related TIN.

APRIL in both renal (Figure 3A) and submandibular lesions (data not shown) in patients with IgG4-RD, consistent with the morphological appearance of Stalk-1-positive cells (inserts of Figures 1A and 2A). Notably, the majority of APRIL-producing cells were CD163-positive M2 macrophages (Figure 3B). On the other hand, we confirmed the lack of APRIL production by CD138-positive plasma cells, CD20-positive B cells and CD4-positive or CD8-positive T cells (Figure 3C–F). Significantly, dual fluorescent immunostaining revealed that the secreted APRIL stained with Aprily-8 was distributed close to and even onto infiltrating CD138-positive plasma cells (Figure 3G). Notably, the number of CD163-positive M2 macrophages was significantly higher in patients with IgG4-related TIN [average 60.1/HPF (range 42.5–95.0)] than in those with TIN associated with Sjogren's syndrome [average 19.3/HPF (range 15.8–22.8); P = 0.037] and no M2 macrophage in Sjogren's syndrome was positive for Stalk-1 (Supplementary data, Figures S2 and S3).

Eosinophils, the infiltration of which is common in tissue lesions of IgG4-RD [1], are also known to produce APRIL [30]. To evaluate the contribution of eosinophils to the production of APRIL in IgG4-RD, we performed dual immunostaining with an eosinophil marker, MBP, in cutaneous specimens. Although the production of APRIL by infiltrating eosinophils was confirmed, CD163-positive M2 macrophages were the major cell type producing APRIL, as a small proportion amounting to ~10% of APRIL-producing cells were eosinophils (Supplementary data, Figure S4A). Notably, similar results were obtained in renal lesions of patients with drug-induced TIN (Supplementary data, Figure S4B), although only limited numbers of IgG4-positive plasma cells were observed.

Down-regulated expression of APRIL in renal lesions after glucocorticoid therapy in IgG4-related TIN

Swelling of all the affected organs, radiographic abnormalities such as multiple low-density lesions of the renal parenchyma on contrast-enhanced CT and bronchovascular bundle thickening as well as serological findings including Cr and IgG4 levels promptly improved within 1 month after the start of glucocorticoid therapy. In 11 patients with IgG4-related TIN, the average serum Cr levels (1.87 ± 1.89 mg/dL before therapy) were decreased to 1.10 ± 0.41 at 1 month after the start of therapy (P = 0.155) and 1.04 ± 0.34 at 12 months (P = 0.037). Serum IgG4 levels (885 ± 389 mg/dL before therapy) decreased to 317 ± 151 at 1 month after the start of therapy (P = 0.003) and 210 ± 150 at 12 months (P = 0.005). Histologically, as noted in our previous report [5], lymphoplasmacytic and IgG4-positive plasma cell infiltration ameliorated after the therapy (data not shown).
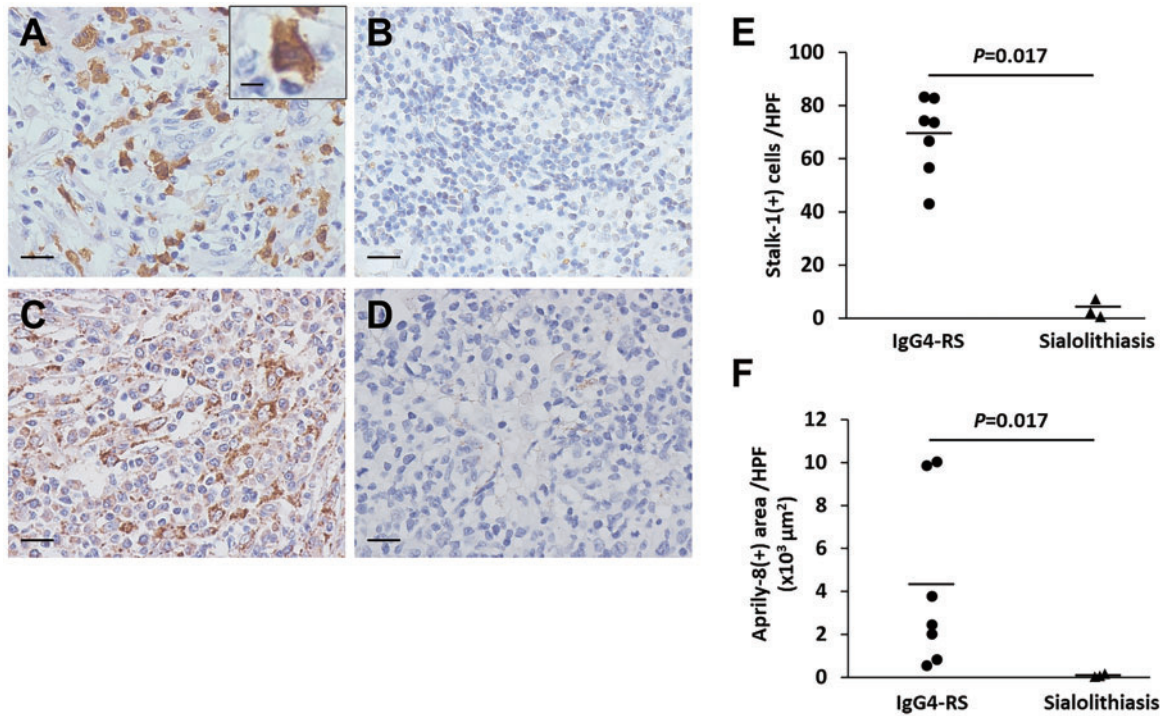
FIGURE 2: Intense expression of APRIL in submandibular gland lesions of patients with IgG4-RS. Immunohistochemical analysis of Stalk-1 and Aprily-8 staining in patient with IgG4-RS (**A** and **C**, respectively) and sialolithiasis (**B** and **D**, respectively). Insert in (A) represents a high magnification of Stalk-1-positive infiltrating mononuclear cells. Scale bars represent 20 µm in original magnification and 4 µm in high magnification, respectively. The quantification of (**E**) Stalk-1-positive cells and (**F**) Aprily-8-positive area detectable in lesions of IgG4-RS ($n = 7$) and sialolithiasis ($n = 3$) in which results are expressed as the number of Stalk-1-positive cells and Aprily-8-positive area within the areas of most intense inflammatory cell infiltration counted in five different HPFs. Note the marked increases in Stalk-1-positive APRIL-producing cells and secreted APRIL in patients with IgG4-RS.

We determined in what way the number of APRIL-producing macrophages in tissue lesions of patients with IgG4-related TIN change in response to glucocorticoid therapy. The rebiopsied renal specimens obtained after glucocorticoid therapy showed that the average number of infiltrating APRIL-producing cells and CD138-positive plasma cells in the renal lesions was significantly decreased as compared with those obtained before the treatment (29.3 to 8.4/HPF, $P = 0.028$ and 157.0 to 35.5/HPF, $P = 0.028$, respectively) (Figure 4A, B, E, F, G and I). In parallel, the expression level of secreted APRIL markedly decreased after glucocorticoid therapy (1723 to 440 µm^2/HPF, $P = 0.028$) (Figure 4C, D and H).

DISCUSSION

We explored a possible overexpression of APRIL in the affected lesions of IgG4-RD. Our results revealed (i) an abundant infiltration of APRIL-producing M2 macrophages and localization of secreted APRIL close to plasma cells within plasma cell-enriched lesions characteristic of patients with IgG4-RD, (ii) a high concentration of soluble APRIL in these lesions with even a binding of soluble APRIL onto target CD138-positive plasma cells and (iii) a marked reduction in the expression of APRIL after successful treatment with glucocorticoids. This contrasted with little or no local production of APRIL in lesions associated with the infiltration of plasma cells of Sjögren's syndrome, as shown by previous study [33] and confirmed by the present study. Collectively these data highlight the role played by APRIL in the survival of plasma cells accumulating in organs affected by IgG4-RD.

Abundant plasma cell infiltration in the affected lesions and copious IgG4 production from these plasma cells are important features of IgG4-RD [41]. Numerous recent studies have focused on the mechanisms underlying the immunoglobulin subclass switch to IgG4. Enhanced expression of interleukin-4 (IL-4) and IL-10 in the affected lesions produced by infiltrating inducible costimulator–positive regulatory T cells [42], M2 macrophages [15] and mast cells [43] can be related to the immunoglobulin class switch to IgG4. APRIL overexpression in the affected lesions may also play some role in the class switching in IgG4-RD since APRIL has been shown to induce IgG4 and IgE class switching in the presence of IL-4 *in vitro* [44]. We showed that numerous APRIL-producing cells and secreted APRIL were present in the lesions of IgG4-RD. The rebiopsied specimens obtained after glucocorticoid therapy showed that APRIL-producing cells and secreted APRIL in the renal lesions with intense inflammatory cell infiltration were considerably reduced in parallel with decreased plasma cell infiltration. These results suggested direct involvement of APRIL in the formation of one of the most characteristic histopathological features of IgG4-RD, the accumulation of plasma cells within the affected organs. On the other hand, because APRIL expression with plasma cell–rich lesions in patients with non-IgG4-RD was reported [32], it should also be stressed that the abundant
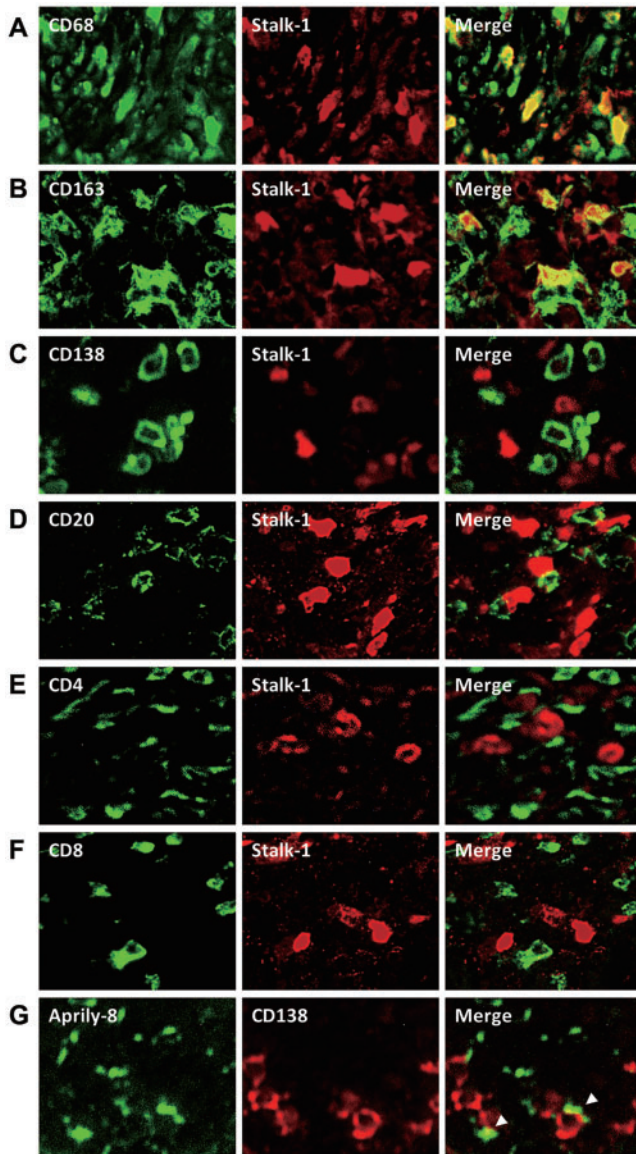
FIGURE 3: Identification of APRIL-producing cells in tissue lesions of IgG4-RD. Renal lesions of IgG4-related TIN were stained with anti-Stalk-1 antibodies and either (**A**) anti-CD68, (**B**) anti-CD163, (**C**) anti-CD138, (**D**) anti-CD20, (**E**) anti-CD4 or (**F**) anti-CD8. In addition, tissues were stained with (**G**) anti-Aprily-8 antibodies and anti-CD138. Note the presence of secreted APRIL stained with Aprily-8 close to and even onto infiltrating plasma cells (G; arrowhead).

production of APRIL by infiltrating macrophages is not unique or specific for IgG4-RD. In fact, we have found abundant infiltration of Stalk-1-positive cells (Supplementary data, Figure S5A) as well as CD138-positive plasma cells (Supplementary data, Figure S5B) in the renal interstitium of one renal sample of non-IgG4-RD lesions with many IgG4-positive plasma cells (Supplementary data, Figure S5C) from a case with ANCA-associated glomerulonephritis, although these were only preliminary data from a single sample.

Dual fluorescent immunostaining using Stalk-1 in the renal and submandibular lesions of patients with IgG4-RD

revealed that CD68-positive macrophages were the main cells producing APRIL. In particular, the majority of APRIL-producing cells were identified as CD163-positive M2 macrophages. This is in agreement with a lower production of APRIL in tissue lesions of Sjögren's syndrome displaying a limited infiltration of M2 macrophages. On the other hand, in the renal sample with many IgG4-positive plasma cells from a case with ANCA-associated glomerulonephritis mentioned above, some of the Stalk-1-positive APRIL-producing cells were CD163 positive in dual fluorescent immunostaining (Supplementary data, Figure S5D). It should also be emphasized that an analysis of cutaneous lesions revealed a minor contribution of eosinophils to the production of APRIL in IgG4-RD, which was also the case in renal lesions of drug-induced allergic TIN.

Macrophages are classified into M1 type (classically activated macrophages) and M2 type (alternatively activated macrophages) according to the respective activating pathway [45]. CD163-positive macrophages are generally recognized as M2 macrophages. In IgG4-RD, infiltrating M2 macrophages are thought to play an important role in the generation of the characteristic pathophysiology such as Th2 immune responses and fibrosis through the production of pro-fibrotic cytokines (IL-10, IL-13) and chemokines (CCL18) [15] or through the activation of toll-like receptor 7 [46]. Thus our present data showing M2 macrophage as the major cell type involved in the production of APRIL are consistent with the pathogenic role that has been proposed for this subset of macrophages in IgG4-RD.

Additionally, dual fluorescent immunostaining using Stalk-1 in the skin lesions of patients with IgG4-RD revealed that a small proportion (∼10%) of APRIL-producing cells were eosinophils. Of note, strong Stalk-1 and Aprily-8 positivity was also observed in the specimens of drug-induced TIN as eosinophil-rich controls, and it was revealed by dual fluorescent immunostaining that CD68-positive macrophages and CD163-positive M2 macrophages were the major cell types producing APRIL and only a small proportion (∼<5%) of the APRIL-producing cells were eosinophils. In these specimens, however, a smaller number of CD138-positive plasma cells and IgG4-positive plasma cells were seen to infiltrate, suggesting that other factors such as IL-6 [47], IL-10 [48], IL-21 [49] or CXCL12 [50] in addition to APRIL promote the accumulation of plasma cells in IgG4-RD. In addition, it was also suggested that factors other than APRIL may play a dominant role in plasma cell accumulation in the plasma cell–rich lesions without abundant APRIL expression such as TIN associated with SS and sialolithiasis. Because APRIL contributes to class-switch recombination through TACI as well as plasma cell differentiation and survival, APRIL expressed in drug-induced TIN lesions might play a role in Ig class-switch. Additional factors such as IL-4 are necessary to promote Ig class-switch to IgG4 [44] and the difference in predominance of these factors might account for the difference in IgG4 expression between IgG4-RD and drug-induced TIN.

B cells, plasma cells and Igs secreted from those cells have been shown to play an important role in the pathophysiology of IgG4-RD. Pancreatic and salivary gland injuries have been
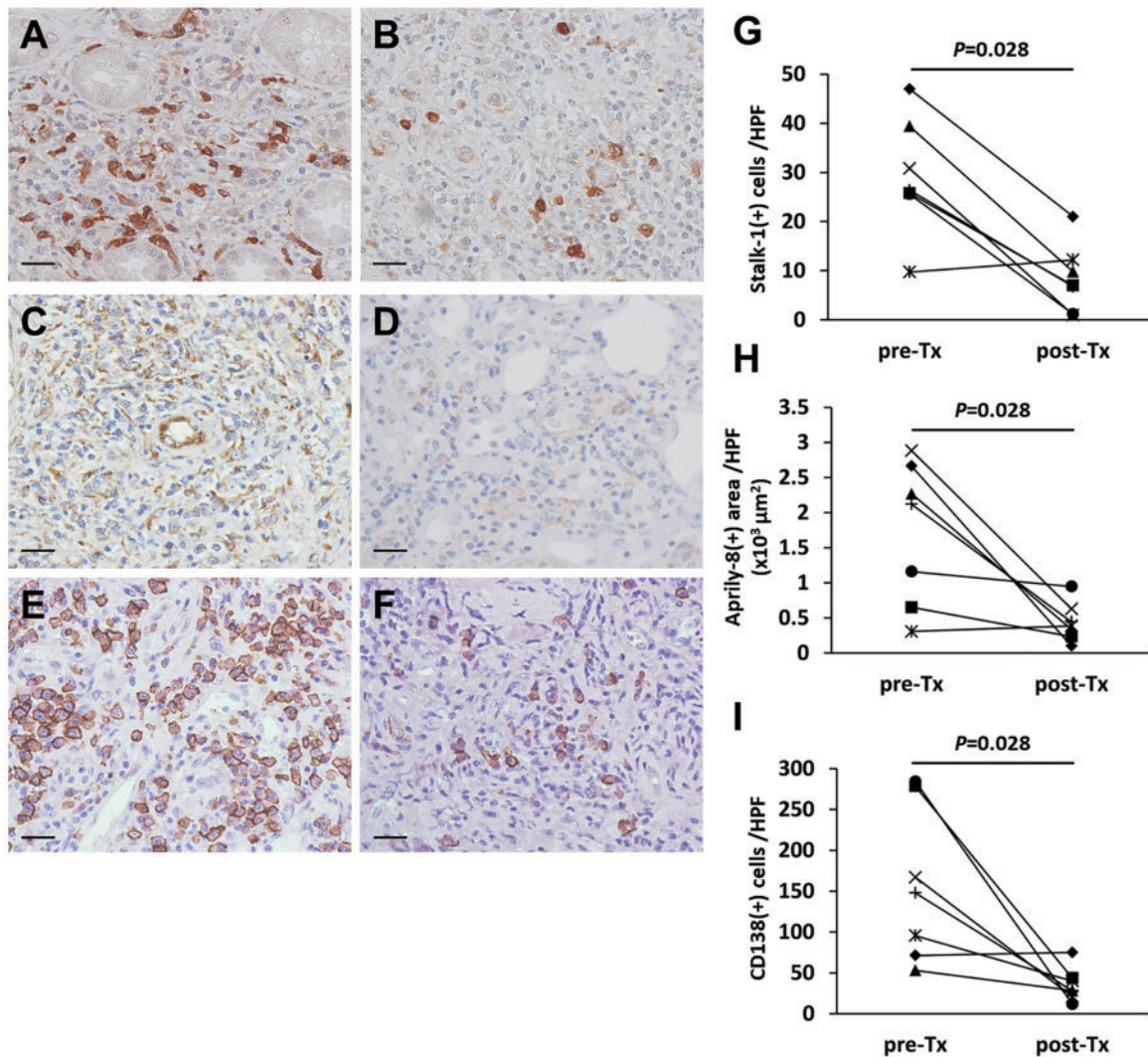
FIGURE 4: Down-regulated expression of APRIL in renal lesions after glucocorticoid therapy in IgG4-related TIN. Immunohistochemical analysis of Stalk-1 staining in seven patients with IgG4-related TIN (**A**) before and (**B**) after glucocorticoid treatment. Immunohistochemical analysis of Aprily-8 staining in patients with IgG4-related TIN (**C**) before and (**D**) after glucocorticoid treatment. Immunohistochemical analysis of CD138 staining in patients with IgG4-related TIN (**E**) before and (**F**) after glucocorticoid treatment. The number of (**G**) Stalk-1-positive cells and (**I**) CD138-positive cells before and after glucocorticoid treatment. (**H**) Aprily-8-positive area before and after glucocorticoid treatment. Results are expressed as the number of Stalk-1-positive cells and CD138-positive cells and Aprily-8-positive area within the areas of most intense inflammatory cell infiltration counted in five different HPFs. Scale bars represent 20 μm. Note the significant decreases in Stalk-1-positive APRIL-producing cells, secreted APRIL and CD138-positive cells after treatment with glucocorticoid.

induced by injecting IgGs from patients with IgG4-RD into neonatal male BALB/c mice [14]. In addition, binding of IgG, especially IgG1 and IgG4, to pancreatic tissue was confirmed in both the mouse model and tissue samples from patients with autoimmune pancreatitis [14], suggesting the pathogenicity of IgGs in this disease. Nowadays, B-cell depletion therapy is widely recognized as an effective treatment option for IgG4-RD [51, 52], as in the case of B cell–mediated autoimmune diseases, such as systemic lupus erythematosus. Moreover, our present findings implicating APRIL in IgG4-RD strongly suggest the possibility of APRIL as a therapeutic target of IgG4-RD.

Maehara *et al.* [53] recently demonstrated that CD4$^+$ cytotoxic T cells (CTLs), but not Th2 cells, were more abundant in the affected organs of IgG4-RD as compared with patients with Sjögren's syndrome as well as healthy controls. They speculated

that reactivated CD4-positive CTLs in tissue sites might mediate fibrosis and inflammation as a result of cytokine (interferon-γ and transforming growth factor-β) secretion or possibly the induction of cell death. At present, the relationship between these CD4-positive CTLs and APRIL-producing macrophages is unclear. Since T cells lack the expression of receptors for APRIL, it is unlikely that increased production of APRIL could affect the reactivation and survival of CD4-positive CTLs within tissue lesions of IgG4-RD. However, it would be of interest to explore the possibility that these CD4-positive CTLs might promote the recruitment of M2 macrophages and hence the production of APRIL.

In conclusion, our study suggested a pathogenic role of APRIL in the development of plasma cell–enriched tissue lesions characteristic of IgG4-RD. Notably, CD163-positive M2

macrophages are the major cell type for the production of APRIL in the affected lesions. In view of the involvement of M2 macrophages in IgG4-RD [15], APRIL secreted by M2 macrophages likely participated in plasma cell accumulation in tissue lesions and may play an additional role in the enhanced production of IgG4 with immunopathological consequences. Further investigations will be required to confirm our findings and better define the pathogenic role of APRIL in the development of IgG4-RD.

SUPPLEMENTARY DATA

Supplementary data are available at ndt online.

AUTHORS' CONTRIBUTIONS

T.K., I.M., K.Y., B.H. and M.K. designed the research. T.K., I.M., K.Y., H.F., K.I. and T.Y. acquired data. I.M., K.Y., H.F., K.I. and M.K. analyzed the data. T.K. and I.M. drafted the manuscript. All authors interpreted the results, revised the drafts and approved the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

None declared.

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