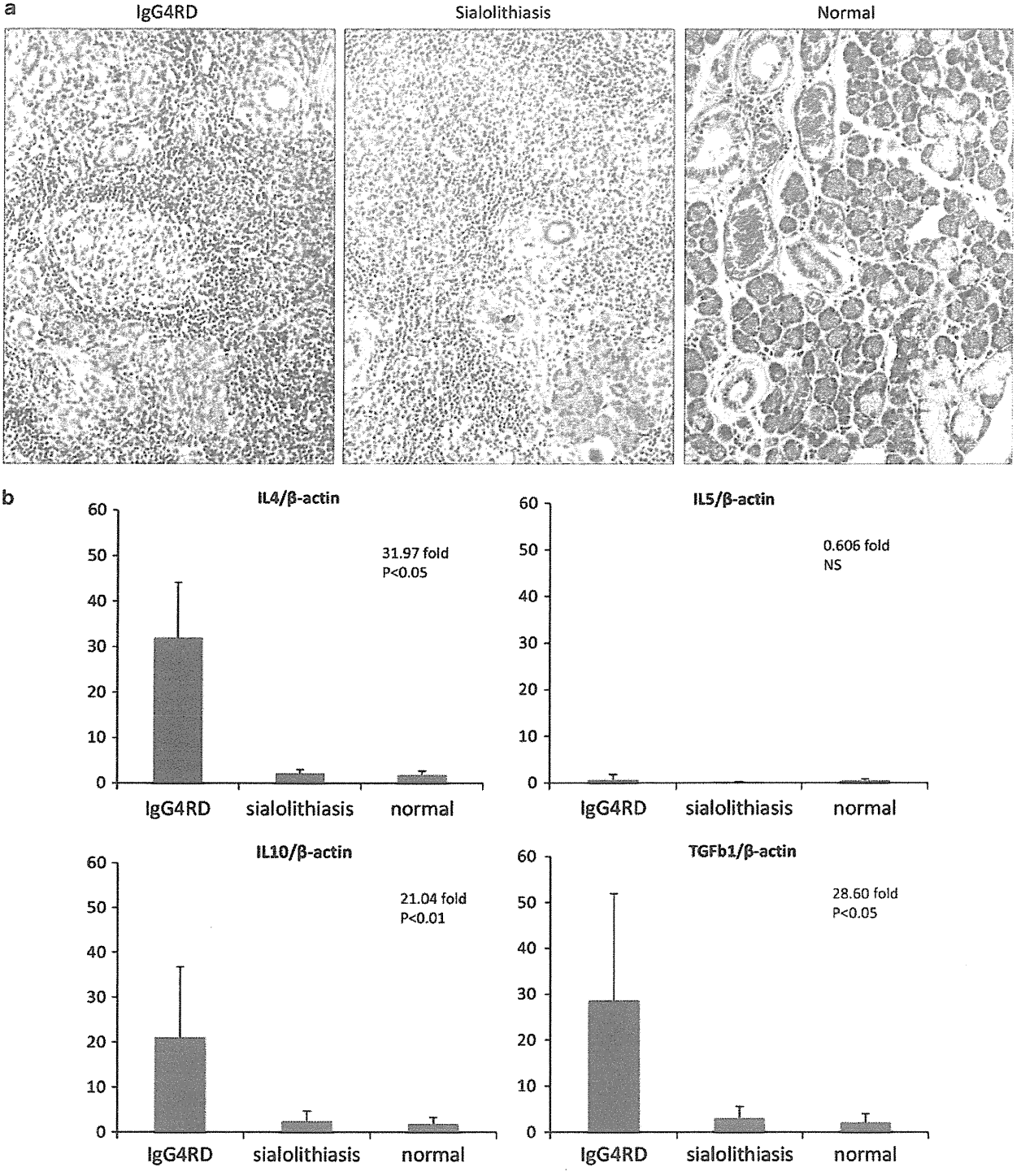

Figure 1 Histological findings and real-time PCR analysis of cytokine expression in IgG4-related disease and control groups. (**a**) IgG4-related submandibular disease showed dense lymphoplasmacytic infiltration with lymphoid follicle (hematoxylin and eosin, H&E, left). Sialolithiasis revealed moderate to severe lymphoplasmacytic infiltration with lymphoid follicle and calculus (H&E, center). Normal submandibular gland (H&E, right). (**b**) The histograms show the relative quantity of mRNA of the cytokines. The expression ratio of interleukin 4 (IL4)/β-actin, IL10/β-actin, and transforming growth factor beta 1 (TGFβ1(/β-actin were significantly higher in IgG4-related submandibular gland disease than in submandibular sialolithiasis and normal submandibular gland. The expression ratio of IL5/β-actin was low in all tissues and was not significantly different between the IgG4-related disease and control groups. (IgG4RD, submandibular IgG4-related disease; sialolithiasis, submandibular sialolithiasis; normal; normal submandibular gland).

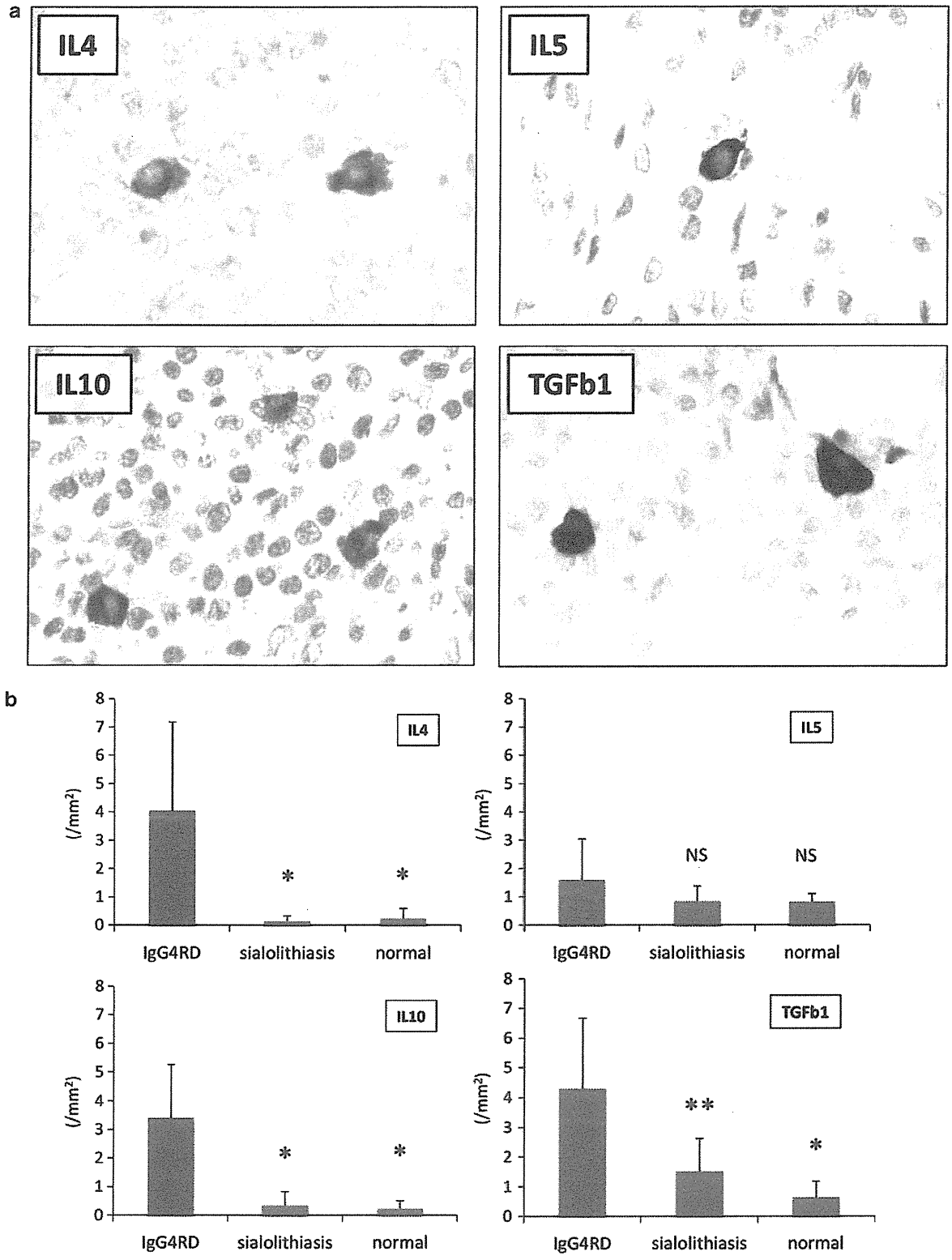

Figure 2 Immunohistochemical analysis of cytokine-expressing cells in IgG4-related disease and control groups. (**a**) Cells stained with antibodies against the indicated cytokines. (**b**) The number of cells positive for interleukin 4 (IL4), IL10, and transforming growth factor beta 1 (TGFβ1) were counted per mm^2 and significantly larger in IgG4-related disease than in the control groups. Consistent with the results of real-time PCR, the number of IL5-positive cells in IgG4-related disease was lesser than those of other cytokines, and no significant difference was observed between the IgG4-related disease and control groups ($*P<0.01$, $**P<0.05$). (IgG4RD, submandibular IgG4-related disease; sialolithiasis, submandibular sialolithiasis; normal; normal submandibular gland).

The number of FOXP3-positive lymphocytes was significantly greater in the submandibular gland IgG4-related disease (834 ± 284 cells/mm^2) than in submandibular sialolithiasis (435 ± 330 cells/mm^2; $P<0.05$) and normal submandibular gland (51.2 ± 33.5 cells/mm^2; $P<0.01$) (Figure 5). However, the distribution of FOXP3-positive cells was different from that of cytokine-positive cells, and no FOXP3/cytokine double-positive cells were observed in dual immunostaining assays (Figure 6). Additionally, we observed that the distribution of CD4-positive lymphocytes was similar to that of FOXP3-positive

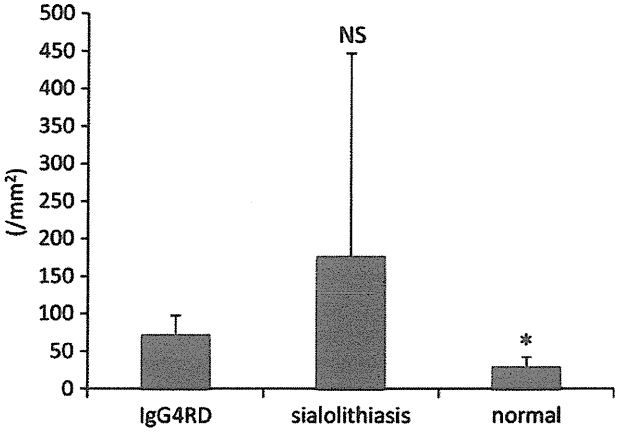

Figure 3 The number of KIT-positive mast cells in IgG4-related disease and control groups. Positive cells were counted per mm^2. (*$P<0.01$; IgG4RD, submandibular IgG4-related disease; sialolithiasis, submandibular sialolithiasis; normal; normal submandibular gland).

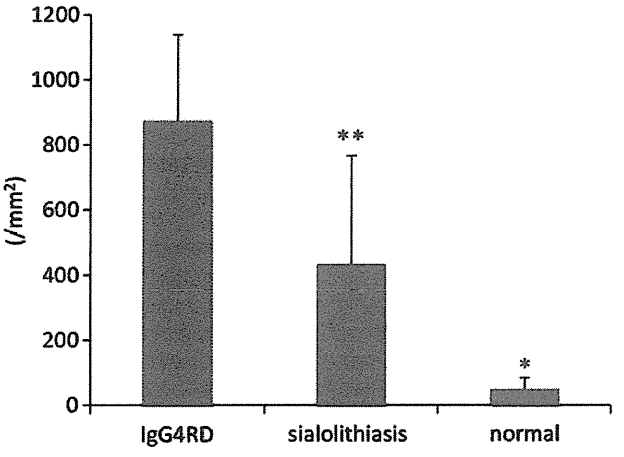

Figure 5 The number of FOXP3-positive lymphocytes. Positive cells were counted per mm^2. (*$P<0.01$, **$P<0.05$; gG4RD, submandibular IgG4-related disease; sialolithiasis, submandibular sialolithiasis; normal; normal submandibular gland).

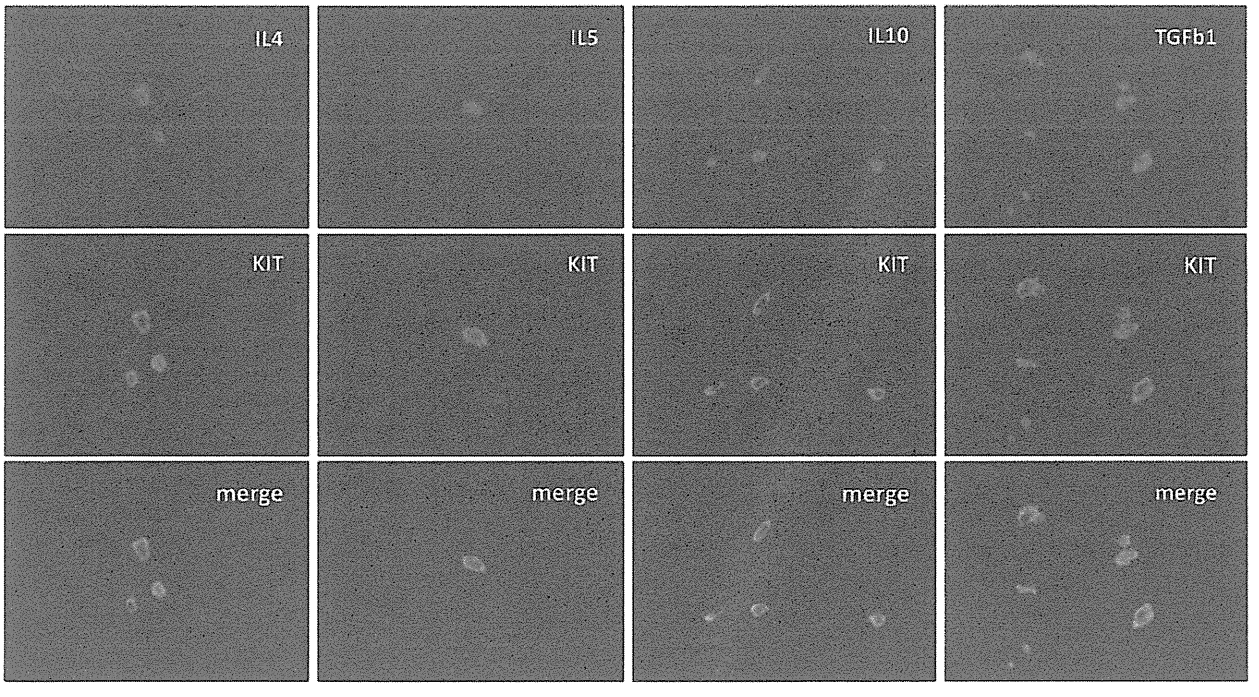

Figure 4 Dual fluorescent analysis of KIT-positive mast cells and cytokine-positive cells in IgG4-related submandibular gland disease. Compared with the results for normal submandibular gland, the number of KIT-positive cells (middle row) was significantly higher in IgG4-related disease and sialolithiasis, and there was no significant difference between these two groups in this regard. Immunostaining for each cytokine (top row) revealed strong cytoplasmic positivity. Positive cells were morphologically similar to mast cells. Dual fluorescent immunostaining detected many positive cells for each cytokine and KIT. The merged image (bottom row) demonstrated double-positive cells for KIT and interleukin 4 (IL4), IL5, IL10, and TGFβ1.

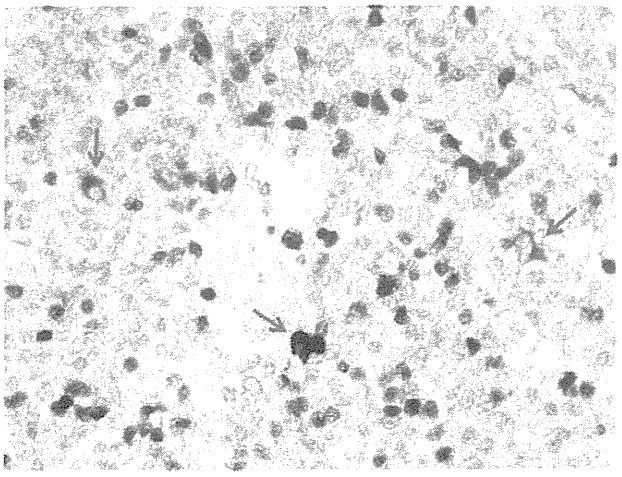

Figure 6 Dual immunostaining of transforming growth factor beta 1 (TGFβ1) and FOXP3. The distribution of TGFβ1- and FOXP3-positive cells were different (TGFβ1/brown, FOXP3/red).

cells, and CD4-positive cell distribution did not correlate with cytokine-positive cell distribution. Moreover, no double-positive cells were observed upon double-immunostaining for each of the cytokines with CD4.

Staining Pattern of IgE in Mast Cells of IgG4-Related Disease

In IgG4-related disease and control groups tested, a high number of mast cells were positive for IgE with varying density and intensity. As is typical for IgE immunostaining, IgE was observed to be weakly-to-moderately expressed on cell surface membranes (Figure 7a). No significant differences were observed in the number of surface membrane IgE-positive cells among the IgG4-related disease and control groups. However, the IgG4-related disease contained a large number of mast cells that were strongly positive for cytoplasmic IgE (Figure 7a). The distribution of the strongly cytoplasmic IgE-positive cells correlated with KIT expression, and the membranous colocalization of IgE and KIT was confirmed by dual immunofluorescence (Figure 7b).

The number of strongly cytoplasmic IgE-positive cells was significantly greater in IgG4-related disease (7.19 ± 5.11 cells/mm^2) than in sialolithiasis (0.068 ± 0.15 cells/mm^2; $P<0.01$) and normal (0.45 ± 0.51 cells/mm^2; $P<0.01$) control tissues (Figure 7c).

Discussion

In this study, we confirmed the upregulation of T helper 2 (IL4) and regulatory T-cell (IL10 and TGFβ1) cytokines in the paraffin-embedded tissue of the submandibular gland IgG4-related disease by real-time PCR. Upregulation of IL5 was not observed in this study, which is similar to previously published data on real-time PCR for paraffin-embedded tissue samples for IgG4-related disease.[11] Interestingly, our immunohistochemical studies detected a large number of mast cells expressing T helper 2 (IL4) and regulatory T-cell (IL10 and TGFβ1) cytokines in the submandibular gland IgG4-related disease, whereas this was not the case for the control groups. Notably, although the overall number of mast cells was similar among the samples of submandibular gland IgG4-related disease, submandibular sialolithiasis, and normal submandibular gland, the IgG4-related disease contained significantly more cytokine-expressing mast cells. Furthermore, interestingly, our immunohistochemical experiments provided no evidence that T cells were producing the upregulated cytokines. In our study, we considered FOXP3-positive cells to be regulatory T cells, as FOXP3 is considered to be a specific marker of regulatory T cells, although a minor amount of effector T cells can exhibit transient expression of FOXP3.[12–14] It has been suggested that T helper 2 and regulatory T cells are most important in the pathogenesis of IgG4-related disease. Although we did find that the number of FOXP3-positive regulatory T cells was significantly increased in IgG4-related disease in our study, we did not find any evidence that the FOXP3-positive regulatory T cells produced regulatory cytokines. Thus, our results do not support the hypothesis that T cells express the cytokines associated with IgG4-related disease; rather, our data indicate that mast cells are the source of these upregulated cytokines. However, a close relationship between regulatory T cells and mast cells has been reported, and TGFβ1 has been shown to promote differentiation of naive T cells to regulatory T cells.[15] Therefore, we hypothesized that the upregulated mast cells produce TGFβ1, which secondarily promotes the differentiation of naïve T cells to regulatory T cells in the tissue affected.

We observed that mast cells in IgG4-related disease were strongly positive for IgE. This result suggests that IgE is associated with the pathogenesis of IgG4-related disease. Consistent with this notion, patients with IgG4-related disease frequently have an allergic background, such as allergic rhinitis, bronchial asthma, and atopic dermatitis. In addition, patients with this disease typically show elevated serum IgE levels as well as elevated IgG4 levels.[16] IgE is a key stimulator of mast cells, and the mechanism underlying IgE stimulation of mast cells to induce various immunological cascades has been extensively studied. Notably, chronic elevation of antigen-independent, non-specific IgE upregulates the high-affinity IgE receptor, FcεRI, on mast cells, which inhibits mast cell apoptosis and promotes cytokine production.[17] Our observation that mast cells in IgG4-related disease were strongly positive for IgE supports the idea that IgE-mediated stimulation of mast cells may have a role in the

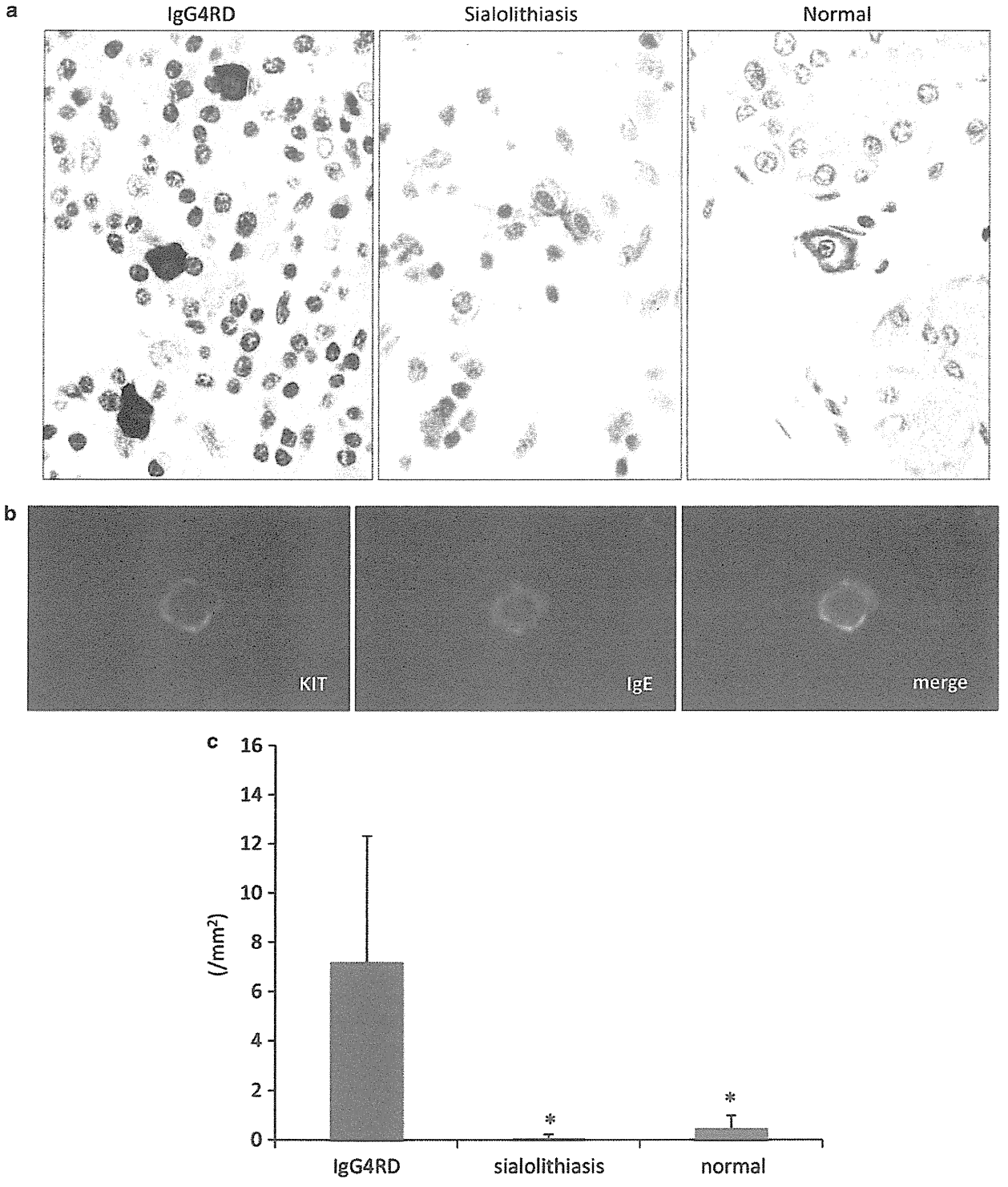

Figure 7 IgE expression in mast cells. (**a**) In IgG4-related disease, mast cells showed strong cytoplasmic positivity for IgE. In contrast, mast cells in sialolithiasis and normal submandibular gland showed only moderate-to-weak IgE positivity and membranous localization. (**b**) Colocalization of IgE- and KIT-positive cells was confirmed by dual immunofluorescence (IgE/red, KIT/green). (**c**) The number of strongly cytoplasmic IgE-positive cells was counted per mm^2 and was significantly different between the IgG4-related disease and control groups. (*$P<0.01$) (IgG4RD, submandibular IgG4-related disease; sialolithiasis, submandibular sialolithiasis; normal; normal submandibular gland).

pathogenesis of IgG4-related disease. However, it remains to be determined why the IgE in these cells was localized to the cytoplasm. Previous studies have shown that the number of FcεRI receptors on mast cells greatly increases in the presence of elevated serum IgE levels.[18] Thus, it is possible that what appeared to be cytoplasmic localization of IgE in our immunostaining experiments may have actually been the result of very high levels of membrane-associated IgE. However, a few reports have suggested that antigen cross-linking could lead to the aggregation and internalization of FcεRI. In particular, IgE was detected in the cytoplasm of mast cells as a result of the internalization and endocytosis of FcεRI.[19] These data suggest that the continuous upregulation of mast cells might result in endocytosis-mediated cytoplasmic accumulation of IgE. However, this hypothesis should be based on the existence of certain antigens, and the role of internalization of FcεRI remains controversial.

Steroid therapy has been the first choice of medication for IgG4-related disease. However, a recent study reported a case of IgG4-related disease that regressed after treatment with an antihistamine agent (epinastine hydrochloride) alone, which indicates an allergic background to the disease and may indirectly suggest the involvement of mast cells.[20] The *in vitro* effects of antihistamines on the inhibition of mediator release from mast cells have been reported.[21] Moreover, an *in vitro* study of human conjunctival mast cells revealed that epinastine inhibited mast cell secretion of cytokines, including IL10.[22] However, it remains unclear why antihistamines inhibit cytokine secretion, although H1 antagonism seems unrelated.

Rituximab is another treatment currently being used for refractory cases of IgG4-related disease. Rituximab therapy has been reported to produce rapid regression in refractory patients.[23] Additionally, rituximab therapy was found to induce regression of symptoms with a decrease in serum IgE levels,[24] and the concentration of IgE showed a steeper decline than serum IgG4 levels. These results provide additional evidence that the allergic reaction mediated by IgE is an important factor in the regulation of IgG4-related disease.

Hyper-IgE syndrome is a complex immunodeficiency characterized by atopic dermatitis associated with extremely high serum IgE levels and susceptibility to infections with extracellular bacteria.[25] Despite the continuously elevated serum IgE level, hyper-IgE syndrome shows clinical features that are quite different from those of IgG4-related disease. Furthermore, hyper-IgE syndrome has been linked to mutations in signal transducer and activator of transcription 3 (STAT3) and tyrosine kinase 2 (TYK2), and to defective signal transduction pathways involving multiple cytokines. In contrast to this downregulation of cytokine signaling, IgG4-related disease is associated with the upregulation of multiple cytokine signals.

To our knowledge, this study provides the first evidence relating mast cells to IgG4-related disease. However, as mast cells are currently known as powerful mediators of the immune response and are closely related with allergic responses, the involvement of mast cells in IgG4-related disease seems logical. Moreover, mast cells have been implicated in other inflammatory diseases. For instance, it was reported that a large number of

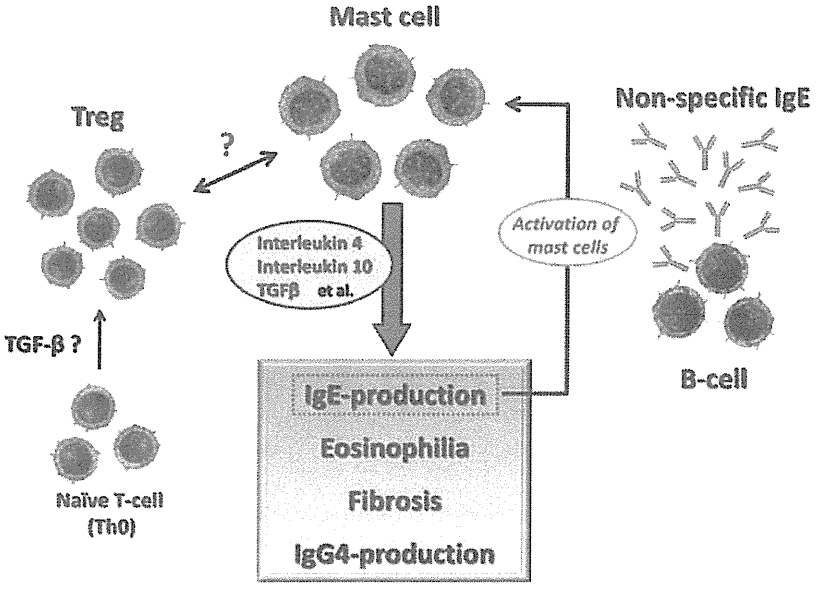

Figure 8 Model for the pathogenesis of IgG4-related disease. Because of an allergic background with the elevation of non-specific IgE levels, activated mast cells produce interleukin 4 (IL4), IL10, and transforming growth factor beta 1 (TGFβ1), which induce the distinctive features of IgG4-related disease. In addition, IL4 and IL10 themselves induce IgE production to upregulate mast cells, and TGFβ1 induces the transformation of naive T cells to regulatory T cells. The relationship between regulatory T cells and mast cells remains unclear.

mast cells was observed in periductal and ductal fibrosis in primary sclerosing cholangitis (PSC) and chronic sclerosing sialadenitis, indicating that mast cells might contribute to fibrosis.[26,27] Mast cell expression of IL10, as observed in our immunohistochemical experiments, may affect mast cell involvement in IgG4-related disease. IL10 is known to be an anti-inflammatory cytokine that can suppress acquired or innate immune responses. Mast cell-derived IL10 has been reported to limit contact dermatitis and chronic irradiation with ultraviolet B.[28] Similarly, in IgG4-related disease, mast cell production of IL10 may prevent allergic inflammation in the affected organs.

On the basis of results of our study and those of previously published reports, we established a hypothesis for the pathogenesis of IgG4-related disease, which is summarized in Figure 8. Patients with IgG4-related disease usually have an allergic background with elevation of non-specific IgE levels. This antigen-independent non-specific IgE binds to FcεRI on mast cells to promote the production of T helper 2 (IL4) and regulatory T-cell (IL10 and TGFβ1) cytokines, which induce the distinctive features of IgG4-related disease such as lymphoplasmacytic infiltration, interstitial fibrosis, and IgG4 production. In addition, IL4 and IL10 induce IgE production, which promotes the upregulation of mast cells. Although it is possible that regulatory T cells interact with mast cells, their involvement in this process is uncertain and they might be secondarily recruited to the affected tissue. According to this scenario, rituximab might exert its effects through the inhibition of IgE-production of B-cells (Figure 8). This model is based on the major finding of this study, which indicates that mast cells have a key role in IgG4-related disease.

Acknowledgments

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Author contributions

Conceived and designed the experiments: YS. Performed the experiments: MT and YS. Analyzed the data: YS, MT, ST, KO, KT, YG, and TI. Contributed materials: YO and TT. Wrote the paper: MT, YS, and TY. All authors read and approved the final manuscript.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Epstein-Barr Virus–infected Cells in IgG4-related Lymphadenopathy With Comparison With Extranodal IgG4-related Disease

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Abstract: IgG4-related lymphadenopathy with increased numbers of Epstein-Barr virus (EBV)-infected cells has been reported but not fully described. We analyzed 31 cases of IgG4-related lymphadenopathy and 24 cases of extranodal IgG4-related diseases for their possible relationship with EBV. Other types of reactive lymph nodes (22) and angioimmunoblastic T-cell lymphoma (AITL) (10) were also studied for comparison. EBV-encoded RNA (EBER) in situ hybridization revealed EBER$^+$ cells in 18 of 31 cases (58%) of IgG4-related lymphadenopathy. Increased EBER$^+$ cells were found in only 4 of 22 (18.1%) non–IgG4-related reactive lymphoid hyperplasia in patients of a similar age ($P = 0.002$) and in only 5 of 24 (21%) extranodal IgG4-related biopsies ($P = 0.006$). Interestingly, all patients with EBER$^+$ progressively transformed germinal center–type IgG4-related lymphadenopathy had systemic lymphadenopathy and/or extranodal involvement. AITL also is associated with EBV, and IgG4-related lymphadenopathy sometimes mimics the morphology of AITL; however, the number of IgG4$^+$ cells in AITL was significantly less than that in IgG4-related lymphadenopathy ($P < 0.001$). Increased numbers of regulatory T cells are seen in IgG4-related disease; however, there was not a significant difference between the EBER$^+$ and EBER$^-$ cases. In conclusion, the presence of increased numbers of EBV-infected cells in IgG4-related lymphadenopathy, compared with other reactive lymphadenopathy or extranodal IgG4-related disease, suggests that there may be a relationship at least between nodal IgG4-related disease and EBV. It is important to avoid overdiagnosing these cases as malignant lymphomas or EBV-related lymphoproliferative disorders.

Key Words: IgG4-related lymphadenopathy, IgG4-related disease, Epstein-Barr virus

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IgG4-related disease is a recently recognized systemic syndrome with unique clinicopathologic features, which frequently include lymphadenopathy.[1] Five histologic patterns are recognized in lymph nodes. The type with progressively transformed germinal centers (PTGCs) has distinct clinical and pathologic features.[2] Although PTGC-type IgG4-related lymphadenopathy is often localized in submandibular lymph nodes and usually asymptomatic, as we previously reported, it usually persists and relapses, and some cases can progress to involve extranodal sites and/or have systemic lymphadenopathy. In other cases, prominent interfollicular expansion with increased immunoblasts and vascular proliferation are observed, which can mimic malignant lymphoma, especially angioimmunoblastic T-cell lymphoma (AITL).[1] Increased forkhead box P3-positive (FOXP3$^+$) regulatory T cells (Tregs) are usually observed in IgG4-related disease. Although the pathogenesis of IgG4-related disease remains to be solved, cytokines produced by Treg and type 2 helper T cells (Th2) are considered to play an important role.[3]

Epstein-Barr virus (EBV) is a common human herpes virus that infects >90% of the adult human population. After primary infection at an early age, the virus persists in a small population of B cells for life. Immunocompetent hosts are asymptomatic, and their lymph nodes show few EBV-encoded RNA–positive (EBER$^+$) cells, whereas those

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M.T. and Y.S. contributed equally.

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with iatrogenic, congenital, or acquired immunodeficiency can reveal an increased number of EBV-infected cells in the lymph nodes.[4] EBV is also associated with various malignant lymphomas and lymphoproliferative disorders.

Only 1 case of IgG4-related lymphadenopathy with EBV-infected cells has been reported, and the relationship between IgG4-related disease and EBV has not been fully addressed.[5] Hence, we analyzed the association of EBV and IgG4-related disease. Lymph node tissues from subjects with various morphologic types of IgG4-related lymphadenopathy were subjected to EBER in situ hybridization (ISH) to examine the presence and localization of EBER$^+$ cells. For comparison with extranodal lesions, IgG4-related lacrimal gland disease, IgG4-related submandibular gland disease, IgG4-related skin disease, and IgG4-related pancreatitis were also examined. Non–IgG4-related benign lymph node hyperplasia and lymph nodes of AITL were also examined for comparison.

MATERIALS AND METHODS

Patients and Materials

Thirty-one Japanese patients with IgG4-related lymphadenopathy, 9 with IgG4-related lacrimal gland disease, 10 with IgG4-related submandibular disease, 2 with IgG4-related skin disease, 3 with IgG4-related pancreatitis, 22 with IgG4$^-$ reactive lymphoid hyperplasia, and 10 with AITL were included. All cases were retrieved from the surgical pathology consultation files of the Department of Pathology, Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University in Okayama, Japan.

The diagnoses of IgG4-related disease were based upon the consensus statement on the pathology of IgG4-related disease published in 2012.[6] The clinical records and pathology materials of all IgG4-related diseases were reviewed, and cases of multicentric Castleman disease, malignant lymphoma, or other lymphoproliferative disorders (including rheumatoid arthritis–related lymphadenopathy and other immune-mediated conditions) were excluded. The number of IgG4$^+$ cells in the cases of IgG4-related lymphadenopathy ranged from 122 to 477 per high-power field (HPF) (mean ± SD, 250 ± 102). The ratio of IgG4$^+$/IgG$^+$ cells was >0.4 in all cases. The specimens of IgG4-related lymphadenopathy were reviewed by the authors Y.S. and M.T. and morphologically categorized into the following subtypes as previously reported[1,7]: Castleman disease–like morphology (type I); reactive follicular hyperplasia (type II); interfollicular expansion and increased immunoblasts (type III); PTGC-type (type IV); and inflammatory pseudotumor-like morphology (type V).

In sections of IgG4-related lacrimal gland disease, IgG4-related submandibular gland disease, IgG4-related skin disease, and IgG4-related pancreatitis, characteristic morphologic features and increased IgG4$^+$ cells were seen. Only in cases of IgG4-related skin diseases, the required numbers of IgG4$^+$ plasma cells were not seen; however, in a previous study of IgG4-related skin disease, we suggested that the cutoff of 200 IgG4$^+$ plasma cells/HPF appeared to be controversial.[8] Two patients with IgG4-related lacrimal gland disease (cases Ex4 and Ex7) were the same patients with IgG4-related lymphadenopathy listed in Table 1 (cases LN6 and LN25, respectively).

To examine whether increased numbers of EBV-infected cells in benign lymph nodes was specifically associated with IgG4-related disease rather than other patient characteristics, EBER detection by ISH was also performed on 22 lymph nodes with IgG4$^-$ lymphoid hyperplasia. The cases of reactive lymphoid hyperplasia did not suggest Castleman disease or any other distinct lymphoproliferative diseases and also did not meet the histologic diagnostic criteria for IgG4-related disease.

To compare with type III IgG4-related lymphadenopathy, immunostaining for IgG4 was performed in 10 lymph nodes with AITL. Cases of AITL were diagnosed on the basis of *World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues* (fourth edition).[9] All cases showed typical histologic features including infiltration of small to medium-sized T cells with clear cytoplasm and proliferation of high endothelial venules. Many EBER$^+$ B cells (from 23 to >1000/0.5 cm^2; mean ± SD 422 ± 399) were observed in 8 of 10 cases (80%), which is similar to the percentage described in the WHO monograph (75%).[9]

Histologic Examination and Immunohistochemistry

Surgically biopsied lymph node specimens were fixed in 10% formaldehyde and embedded in paraffin. Serial sections (4 μm) were cut from each paraffin-embedded tissue block, and stained with hematoxylin and eosin. An automated Bond Max stainer (Leica Biosystems, Melbourne, Vic., Australia) was used for immunohistochemistry. The tissue sections were subjected to standard heating or enzymatic pretreatment for antigen retrieval. The following primary antibodies were used: IgG (polyclonal; 1:20,000; Dako, Carpinteria, CA), IgG4 (HP6025; 1:400; The Binding Site, Birmingham, UK), FOXP3 (236A/E7; 1:100; Abcam, Cambridge, UK), and latent membrane protein-1 (LMP-1) (CS1-CS4; 1:10; Novocastra).

On the basis of the consensus statement on the pathology of IgG4-related disease,[6] 3 different HPFs (eyepiece, ×10; lens, ×40) were examined to calculate the average number of IgG4$^+$ cells per HPF and the IgG4$^+$/IgG$^+$ cell ratio. FOXP3$^+$ cells were also counted at 3 different HPFs with the highest density to calculate the average numbers per HPF.

In Situ Hybridization

ISH of EBER was performed using an automated Bond Max stainer (Leica Biosystems). Three representative fields per case were captured using a ×4 objective lens (covering 0.167 cm^2) to count EBER$^+$ cells per 0.5 cm^2. Increased numbers of EBV$^+$ cells was defined as >10 EBER$^+$ cells/0.5 cm^2.[10] EBER-ISH$^+$ and LMP-1$^-$ cases were classified into EBV latency type I.

TABLE 1. Clinical Features of 31 Patients of IgG4-related Lymphadenopathy

No.	Age/Sex	Affected Lymph Nodes	Extranodal Sites	IgG4 (mg/dL; nl = 4.8-105)	IgG (mg/dL)	IgG4/IgG (%; nl = 3-6)
LN1	58/M	Cervix, supraclavicle, axilla, mediastinum, porta hepatis, lesser omentum		2010	3536	57
LN2	74/M	Cervix, para-aorta, parailiac artery	Submandibular gland	NA	4050	NA
LN3	59/M	Cervix, supraclavicle, axilla, mediastinum, inguen		1140	5257	22
LN4	64/F	Left cervix, mediastinum		446	1989	22
LN5	82/F	Right auricular, supraclavicle	Salivary gland	327	1094	30
LN6*	36/F	Cervix, supraclavicle	Lacrimal gland	561	1961	29
LN7	64/M	Mediastinum, axilla	Right lung	641	3134	20
LN8	77/M	Systemic		1100	5800	19
LN9	78/M	Mediastinum, axilla, inguen		1090	5174	21
LN10	82/M	Cervix, inguen, para-aorta, mediastinum	Parotid gland, lacrimal gland	1050	3447	30
LN11	68/F	Axilla, mediastinum, abdomen, inguen		2120	5057	42
LN12	67/M	Cervix, mediastinum, axilla, para-aorta		583	2468	24
LN13	76/M	Cervix, axilla, para-aorta, inguen		1040	3930	26
LN14	59/F	Supraclavicle, mediastinum, abdomen, inguen	Lacrimal gland, submandibular gland	1500	2524	59
LN15	73/M	Abdomen, inguen	Kidney	505	1600	32
LN16	36/M	Left submandibulla		110	551	20
LN17	69/M	Right submandibulla		693	2315	30
LN18	71/M	Left submandibulla, submentum		275	2144	13
LN19	50/M	Bilateral submandibulla, cervix		183	2719	7
LN20	61/M	Systemic	Skin	1120	3025	37
LN21	51/F	Right submandibulla	Bilateral eyelid, parotid gland	223	NA	NA
LN22	70/M	Cervix	Bilateral parotid gland, right submandibular gland, pancreas	483	1190	41
LN23	67/M	Left submandibulla	Left submandibular gland	141	1543	9
LN24	67/M	Submandibulla	Right lung	389	1619	24
LN25†	68/M	Bilateral submandibulla	Bilateral lacrimal glands	1340	3090	43
LN26	63/M	Left submandibulla	Submandibular gland, kidney, mediastinum, aorta	2240	3007	74
LN27	65M	Right submandibulla, mediastinum	Bilateral lacrimal glands, submandibular gland, parotid gland, kidney	2550	6024	42
LN28	70M		Lung	236	1466	16
LN29	51F	Right submandibulla	Submandibular gland	NA	NA	NA
LN30	76M	Right submandibulla	Lacrimal gland, skin	NA	NA	NA
LN31	49M	Left submandibulla, cervix	Left submandibular gland	NA	NA	NA

*The same patient had IgG4-related lacrimal gland disease listed in Table 4 (Ex4).
†The same patient had IgG4-related lacrimal gland disease listed in Table 4 (Ex7).
F indicates female; LN, lymph node; M, male; NA; not available; nl; normal.

Polymerase Chain Reaction for Immunoglobulin Heavy Chain and T-cell Receptor γ Chain

DNA was extracted from formalin-fixed and paraffin-embedded tissues. T-cell receptor γ chain (TCRG) VgIf and Vg10 primers (tube A) and TCRG Vg9 and Vg11 primers (tube B) were used for TCR gene rearrangement study, and each of the tubes (A and B) also included 2 primers targeting the J segments (Jg1.1/2.1 and Jg1.3/Jg2.3) as previously reported.[11] Polymerase chain reaction (PCR) for immunoglobulin heavy chain (IgH) was also performed using consensus primers directed to VH framework (FR) II of the IgH gene using BIOMED-2 primer sets as previously described.[11] The products of PCR reactions were subsequently analyzed by ABI PRISM 310 Genetic Analyzer and Gene Mapper software version 3.7 (Applied Biosystems, CA).[12]

Statistical Analysis

Differences between groups were determined by the Student t test and χ^2 test with SPSS software (version 14.0; SPSS Inc., Chicago, IL). Values of $P < 0.05$ was considered to be statistically significant.

RESULTS

IgG4-related Lymphadenopathy

The clinical and pathologic findings for 31 cases of IgG4-related lymphadenopathy are summarized

TABLE 2. Pathologic Features of 31 Patients of IgG4-related Lymphadenopathy

No.	Histologic Subtype	EBER-ISH$^+$ Cells (/0.5 cm^2)	Distribution of EBER$^+$ Cells
LN1	Type I	0	
LN2	Type I	48	Interfollicular/scattered
LN3	Type I	27	Interfollicular/scattered
LN4	Type II	0	
LN5	Type II	3	
LN6	Type II	13	Interfollicular/scattered
LN7	Type II	25	Interfollicular/scattered
LN8	Type III	3	
LN9	Type III	0	
LN10	Type III	0	
LN11	Type III	163	Interfollicular/scattered
LN12	Type III	> 1000	Diffuse
LN13	Type III	> 1000	Diffuse
LN14	Type III	133	Interfollicular/scattered
LN15	Type III	52	Interfollicular/scattered
LN16	Type IV	0	
LN17	Type IV	1	
LN18	Type IV	0	
LN19	Type IV	9	
LN20	Type IV	10	
LN21	Type IV	6	
LN22	Type IV	6	
LN23	Type IV	19	Interfollicular/scattered
LN24	Type IV	12	Interfollicular/scattered
LN25	Type IV	248	Intrafollicular/localized
LN26	Type IV	72	Interfollicular/scattered
LN27	Type IV	46	Interfollicular/scattered
LN28	Type IV	27	Interfollicular/scattered
LN29	Type IV	20	Interfollicular/scattered
LN30	Type IV	55	Interfollicular/scattered
LN31	Type IV	27	Interfollicular/scattered

in Tables 1 and 2. There were 24 men and 7 women. The ages ranged from 36 to 82 years (mean ± SD, 64.5 ± 11.6). No patient had a record of congenital, iatrogenic, or acquired immunodeficiency. There was no evidence of acute infection of EBV, and EBV serology indicated past infection in all patients tested. Serum IgG4 levels were elevated in all 27 examined patients and ranged from 110 to 2550 mg/dL (mean ± SD, 869.0 ± 695.3). Twenty-seven patients had systemic or multifocal lymphadenopathy, whereas 4 cases of PTGC-type (type IV) IgG4-related lymphadenopathy were localized in the submandibular lymph nodes (cases LN16 to LN19).

Histologically, cases were classified into type I (n = 3), type II (n = 4), type III (n = 8), and type IV (n = 16) lymphadenopathy. The immunohistochemical and ISH findings are summarized in Table 3. Immunohistochemistry revealed numerous IgG4$^+$ cells ranging from 122 to 477/HPF (mean ± SD, 250 ± 102), and ratios of IgG4$^+$/IgG$^+$ cells were > 0.4 in all cases. Greater than 10 EBER$^+$ cells/0.5 cm^2 were found in 18 of 31 cases (58%) with no significant differences in the rates of positivity between the 4 subgroups (2/3 type I, 2/4 type II, 5/8 type III, 9/16 type IV). EBER latency was classified into latency I in examined EBER$^+$ cases (LN12 and LN13). In 15 of 18 cases, scattered EBER$^+$ lymphocytes were seen in interfollicular areas. Two type III cases (cases LN12 and LN13) had numerous diffusely distributed EBER$^+$ cells, with some in intrafollicular areas as well (Fig. 1). EBER$^+$ cells included small lymphocytes and immunoblasts, as is found in AITL.[13] EBER$^+$ cells were localized in germinal centers in 1 type IV case (case LN25); this case also displayed epithelioid granuloma (Fig. 2). All 9 patients with EBER$^+$ type IV IgG4-related lymphadenopathy had systemic lymphadenopathy and/or extranodal involvement. PCR studies for TCR and IGH rearrangements did not demonstrate evidence of clonal T or B cells in 5/5 tested cases (LNs 2, 11, 12, 13, and 14).

Comparison Between EBER$^+$ and EBER$^-$ Cases

There were no differences in age between EBER$^+$ (51 to 76 y; mean ± SD, 64.0 ± 10.2 y) and EBER$^-$ cases (36

TABLE 3. Summary of Clinicopathologic Features of IgG4-related Lymphadenopathy

	Type I (n = 3)	Type II (n = 4)	Type III (n = 8)	Type IV (n = 16)
Age (y)				
Mean ± SD	63.6 ± 8.9	61.5 ± 19	72.5 ± 6.9	61.5 ± 10.8
> 50 y old (n [%])	3 (100)	3 (75)	8 (100)	13 (81)
> 60 y old (n [%])	1 (33)	3 (75)	7 (88)	11 (69)
Sex (Male/Female)	3/0	1/3	6/2	14/2
The number of IgG4$^+$ cells (/HPF)				
Mean ± SD	345 ± 116	155 ± 13.3	337 ± 47.3	198 ± 81.6
IgG4$^+$/IgG$^+$ plasma cell ratio (%)				
Mean ± SD	84.0 ± 14.3	50.2 ± 10.5	73.2 ± 12.7	72.5 ± 15.4
Disease distribution (n [%])				
Localized lymphadenopathy	0 (0)	0 (0)	0 (0)	4 (25)
Systemic lymphadenopathy	3 (100)	4 (100)	8 (100)	2 (13)
With extranodal involvement	1 (33)	3 (75)	3 (38)	12 (75)
EBER-ISH positivity	2 (75)	2 (50)	5 (63)	9 (56)
Distribution (n [%])				
Interfollicular/scattered	2 (100)	2 (100)	3 (60)	8 (88)
Intrafollicular/localized	0 (0)	0 (0)	0 (0)	1 (12)
Diffuse	0 (0)	0 (0)	2 (40)	0 (0)
Age (n [%])				
> 50 y old	2 (100)	1 (50)	5 (100)	8 (88)
> 60 y old	1 (50)	1 (50)	4 (80)	7 (77)

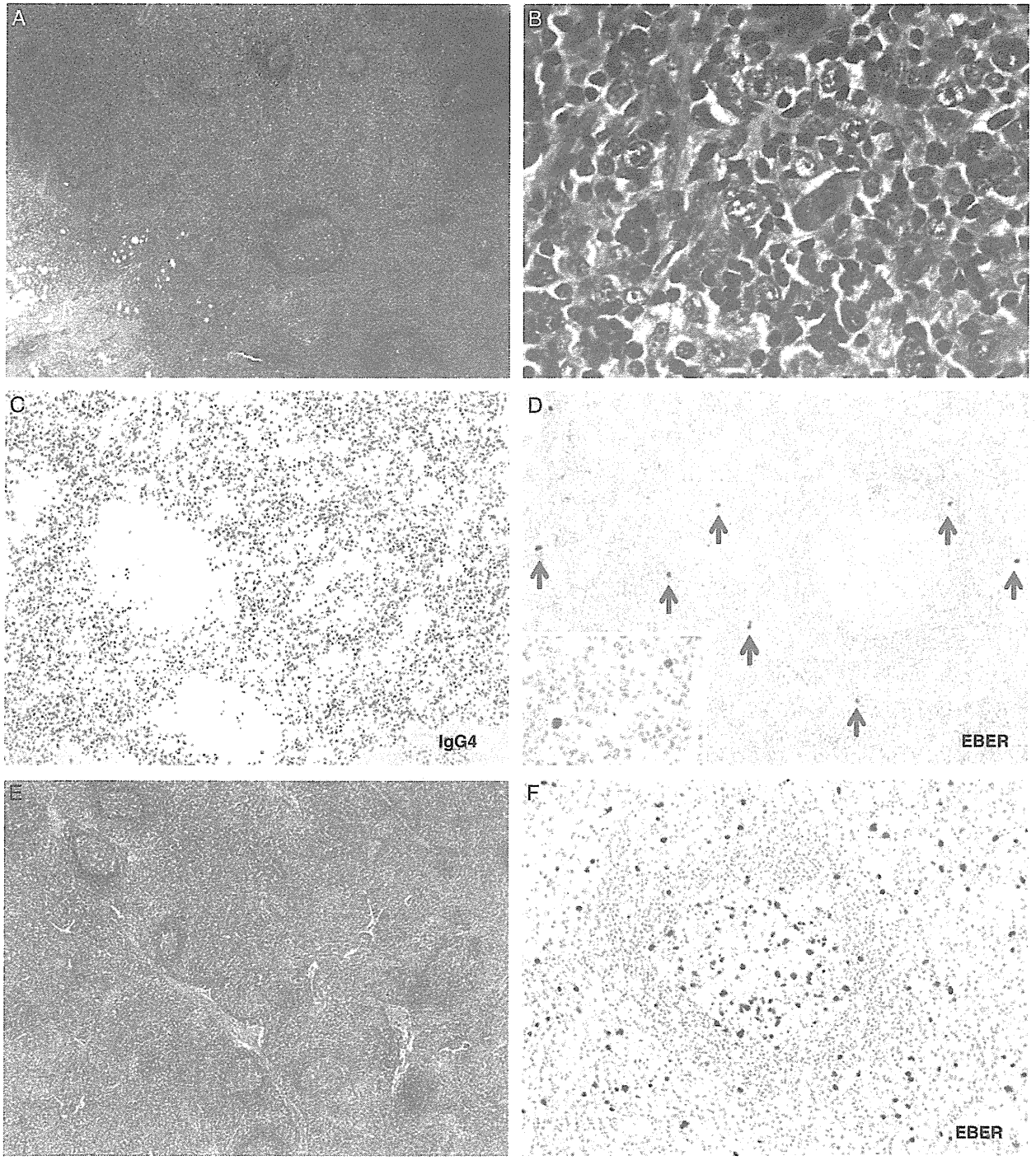

FIGURE 1. Histologic and immunohistochemical studies on type III IgG4-related lymphadenopathy (A–D, LN14; E–F, LN13). A and B, Expanded interfollicular area reveals polymorphous population consisting of mature and immature plasma cells, eosinophils, and numerous immunoblasts with small vessel proliferation (A and B, H&E). C, IgG4$^+$ plasma cells (C, IgG4). D, Small to large EBER-ISH$^+$ cells (133/0.5 cm^2) in the interfollicular area (D, EBER-ISH). E, Expanded interfollicular areas of LN13 (E, H&E). F, Diffuse infiltration of EBER$^+$ cells (F, EBER-ISH). H&E indicates hematoxylin and eosin.

to 82 y; mean ± SD, 65.3 ± 13.8 y) of IgG4-related lymphadenopathy ($P = 0.764$). There was also no difference in serum IgG levels between EBER$^+$ (mean ± SD, 3115 ± 1468 mg/dL; n = 15) and EBER$^-$ (mean ± SD, 2748 ± 1581 mg/dL; n = 12) cases ($P = 0.539$). There was no significant difference in the numbers of FOXP3$^+$ cells

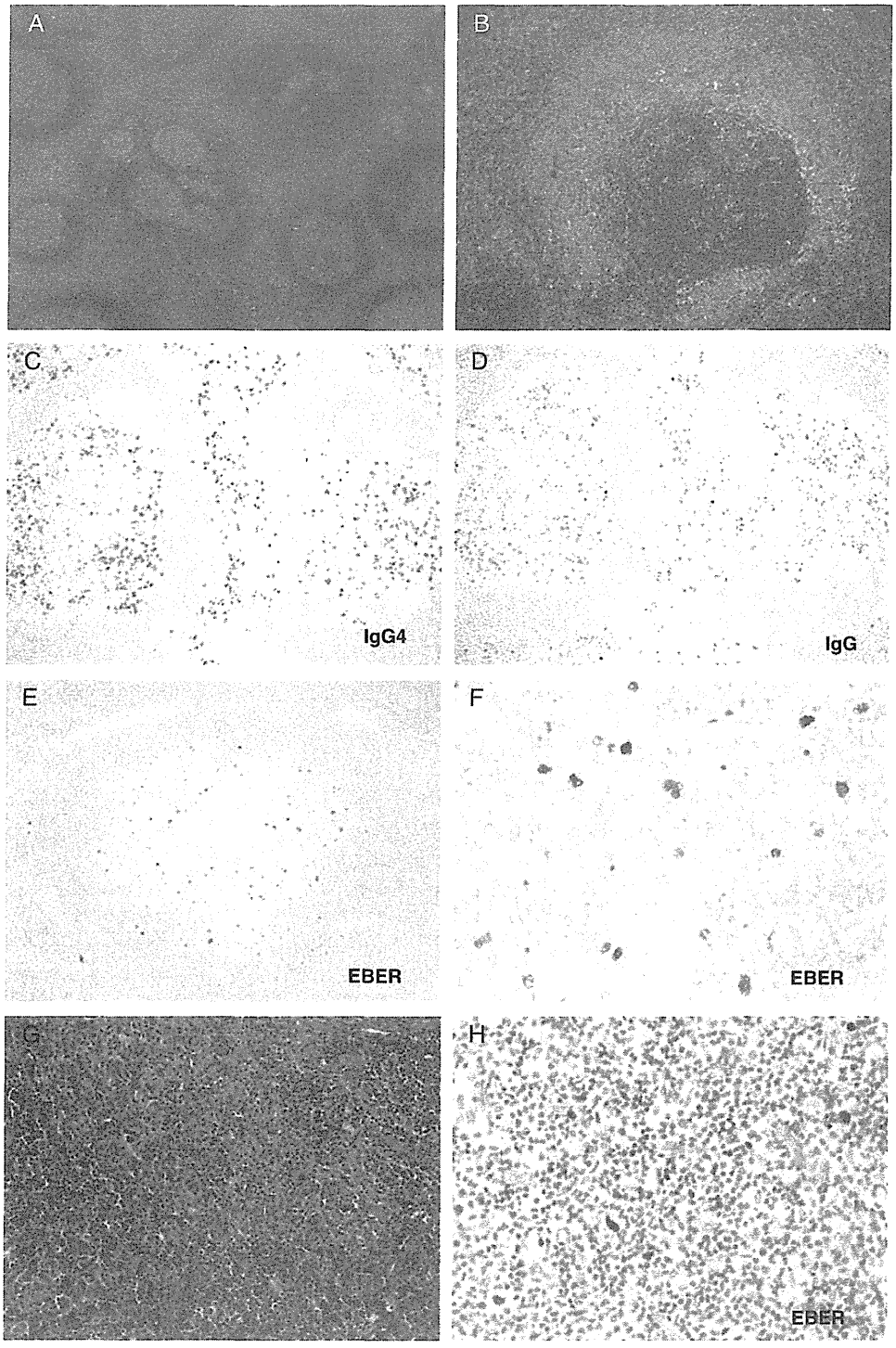

FIGURE 2. Histologic and immunohistochemical features of PTGC-type IgG4-related lymphadenopathy (LN25). A, PTGC-like germinal centers are observed (H&E). B, Epithelioid granuloma (H&E). C and D, Numerous $IgG4^+$ cells are localized in germinal centers. $IgG4^+/IgG^+$ cell ratio is >40% (C, IgG4; D, IgG). E and F, Many EBER-ISH^+ cells localize in a PTGC-like germinal center (E and F, EBER-ISH). G and H, IgG4-related lacrimal gland disease in the same patient (Ex7). G, Fibrosis and lymphoplasmacytic infiltration (H&E). H, Increased $EBER^+$ lymphocytes (56/0.5 cm^2) (EBER-ISH). H&E indicates hematoxylin and eosin.

between EBER$^+$ (mean ± SD, 304 ± 134; n = 11) and EBER$^-$ (mean ± SD, 322 ± 189; n = 8) cases ($P = 0.809$).

IgG4$^-$ Reactive Lymphoid Hyperplasia

Clinicopathologic and demographic characteristics of IgG4$^-$ reactive lymphoid hyperplasia are listed in Table 4. The age distribution was similar to that of the patients with IgG4-related lymphadenopathy and ranged from 50 to 85 years (mean ± SD, 67.1 ± 12.0). There were 18% of cases (4/22) with >10 EBER$^+$ cells/0.5 cm^2 (ranging from 35 to 395/0.5 cm^2; mean ± SD, 155 ± 162), a significantly lower proportion than was seen in cases of IgG4-related lymphadenopathy ($P = 0.002$).

Extranodal IgG4-related Disease

The clinicopathologic findings for cases of extranodal IgG4-related diseases are listed in Tables 5 and 6. Greater than 10 EBER$^+$ cells/0.5 cm^2 were detected in 3 of 9 cases (33%) of IgG4-related lacrimal gland disease, 2 of 10 cases (20%) of IgG4-related submandibular gland disease, 0 of 2 cases (0%) of IgG4-related skin disease, and 0 of 3 cases of IgG4-related pancreatitis (Fig. 3). Although cases Ex4 and Ex7 had EBER$^+$ lymph nodes, only case Ex7 had EBV positivity in the lacrimal gland (Fig. 2G, H). In total, >10 EBER$^+$ cells/0.5 cm^2 were detected in 5 of 24 (21%) extranodal IgG4-related biopsies, which was significantly less frequent than IgG4-related lymphadenopathy ($P = 0.006$).

IgG4$^+$ Cells in AITL

Seven cases showed few IgG4$^+$ cells (0 to 5/HPF). Three cases revealed scattered IgG4$^+$ cells (19, 24, and 47/HPF, respectively); none was in the range of the cases with nodal IgG4-related disease. In addition, overall, there were significantly fewer IgG4$^+$ cells present compared with type III IgG4-related lymphadenopathy (mean ± SD, 10.6 ± 15.3 compared with 337 ± 47.3) ($P < 0.001$).

TABLE 4. Clinicopathologic Features of IgG4$^-$ Reactive Lymphoid Hyperplasia

No.	Age/Sex	Affected Lymph Nodes	EBER-ISH$^+$ Cells (/0.5 cm^2)
RLH1	84/F	Cervix	103
RLH2	69/M	Para-aorta	0
RLH3	79/M	Axilla	0
RLH4	78/F	Inguen	6
RLH5	79/F	Mediastinum	0
RLH6	69/F	Inguen	0
RLH7	63/F	Cervix	0
RLH8	51/F	Inguen	5
RLH9	56/F	Cervix	0
RLH10	50/M	Cervix	395
RLH11	52/M	Cervix	8
RLH12	80/M	Inguen	2
RLH13	78/M	Cervix	1
RLH14	85/F	Axilla	4
RLH15	59/M	Inguen	7
RLH16	72/M	Cervix	0
RLH17	62/M	Inguen	35
RLH18	55/F	Abdomen	0
RLH19	55/F	Axilla	0
RLH20	67/F	Axilla	90
RLH21	53/F	Axilla	4
RLH22	82/M	Cervix	0

F indicates female; M, male; RLH, reactive lymphoid hyperplasia.

DISCUSSION

Very limited previous reports have indicated a possible relationship between IgG4-related disease and EBV. Acute EBV mononucleosis has been reported to be associated with a transient 75% increase in mean serum IgG4 levels.[14] A case of EBV-related lymphadenopathy has been reported that mimicked the clinical features of IgG4-related disease, with an elevated serum IgG4 level but without many IgG4$^+$ cells in the biopsied lymph nodes.[15] EBV$^+$ classical Hodgkin lymphoma was found in a patient with IgG4-related cervical fibrosis.[16] An EBV$^+$ inflammatory pseudotumor-like follicular dendritic cell sarcoma was also reported to have numerous IgG4$^+$ cells.[17] The authors discussed a relationship between EBV-related immune dysfunction and the proliferation of IgG4$^+$ cells. Although these reports mentioned some relationship between EBV and increase of IgG4, EBV infection in IgG4-related disease has not been examined except for a single case report.[5] Hence, this study investigated the relationship between EBV and IgG4-related disease. Increased EBV-infected cells were found in 18 of 31 lymph nodes (58%) with IgG4-related lymphadenopathy. This proportion was significantly higher than that of reactive lymphoid hyperplasia in similarly aged group, which confirmed the clear relationship between IgG4-related lymphadenopathy and EBV. As most Japanese are infected by EBV in childhood, and new onset infection in adulthood is very rare, the increased numbers of EBV$^+$ cells was considered to most likely represent EBV reactivation rather than acute infection.

Type III IgG4-related lymphadenopathy has distinct histologic features, including interfollicular lymphoplasmacytic infiltration, angiogenesis, and many immunoblasts, which mimic AITL.[1] In this study, only a few scattered IgG4$^+$ cells were detected in AITL, which indicated that IgG4$^+$ cell proliferation was not simply induced by EBV reactivation or dysfunction of helper T cells. There is a possibility that both IgG4-related disease and EBV reactivation are induced by other factors.

Type IV cases are usually localized in submandibular lymph nodes, although approximately 50% of cases have extranodal involvement. Less than 10% develop systemic lymphadenopathy.[2] In contrast, in this study, all the patients with type IV IgG4-related lymphadenopathy with EBV reactivation developed systemic lymphadenopathy and/or extranodal involvement. The lymph node in 1 EBV$^+$ type IV case had epithelioid granulomas, which was similar to a previously reported case of EBV$^+$ IgG4-related lymphadenopathy.[5] Cases of IgG4-related lymphadenopathy with epithelioid granulomas have been reported sporadically.[18] Of interest,

TABLE 5. Clinicopathologic Features of Extranodal IgG4-related Diseases

No.	Age/Sex	Affected Organs	Average IgG4$^+$ Cells (/HPF)	EBER-ISH$^+$ Cells (/0.5 cm^2)	Other Lesions
Ex1	56/F	Lacrimal gland	111	1	
Ex2	60/F	Lacrimal gland	282	1	
Ex3	49/M	Lacrimal gland	377	9	
Ex4*	36/F	Lacrimal gland	239	2	Cervical lymph node
Ex5	61/M	Lacrimal gland	373	0	
Ex6	39/F	Lacrimal gland	301	0	
Ex7†	68/M	Lacrimal gland	204	56	Submandibular lymph node
Ex8	67/F	Lacrimal gland	177	175	
Ex9	72/M	Lacrimal gland	231	542	
Ex10	57/F	Submandibular gland	109	0	
Ex11	57/F	Submandibular gland	144	0	Parotid gland
Ex12	59/M	Submandibular gland	190	1	
Ex13	61/M	Submandibular gland	216	5	
Ex14	69/M	Submandibular gland	435	7	
Ex15	76/M	Submandibular gland	160	3	
Ex16	66/F	Submandibular gland	404	2	
Ex17	59/F	Submandibular gland	264	0	
Ex18	69/M	Submandibular gland	307	66	
Ex19	67/M	Submandibular gland	224	157	
Ex20	65/M	Skin	189	0	
Ex21	63/F	Skin	46	0	Parotid gland
Ex22	69/M	Pancreas	195	0	
Ex23	61/M	Pancreas	437	0	
Ex24	64/M	Pancreas	76	0	

*The same patient had IgG4-related lymphadenopathy listed in Table 1 (LN6).
†The same patient had IgG4-related lymphadenopathy listed in Table 1 (LN25).
Ex indicates extranodal; F, female; M, male.

EBER$^+$ cells were localized in germinal centers in this type IV case with epithelioid granuloma. There are reports of EBV$^+$ nonendemic Burkitt lymphoma associated with epithelioid granulomas.[19,20] The significance of this finding is still unclear.

EBV-infected cells are usually observed and studied in the lymph nodes, where the virus is known to remain in the dormant stage for many years.[10] In this study, increased numbers of EBER$^+$ cells were significantly more frequently observed in IgG4-related lymphadenopathy than in extranodal IgG4-related disease. The proportion of EBV$^+$ cases was similar between extranodal IgG4-related disease and non–IgG4-related reactive lymphoid hyperplasia ($P = 0.559$). Increased numbers of EBV-infected cells may be observed more frequently in lymph nodes than in other sites. Lacrimal glands and salivary glands seemed to have increased numbers of EBV-infected cells more frequently than other extranodal sites; however, the number of extranodal samples was too small for a meaningful statistical analysis.

Although our study clearly suggested a relationship between IgG4-related disease and EBV, the precise mechanism remains unclear. Whether IgG4-related disease directly or indirectly induces expansion of EBV-infected cells, possibly related to EBV reactivation, or is secondary to EBV infection is unknown. As mentioned above, acute EBV infection can lead to an increase in the serum IgG4 level. However, in other situations like AITL, EBV-infected cells do not lead to increased numbers of IgG4$^+$ cells, suggesting that IgG4 lymphadenopathy is not the direct effect of EBV infection and that the increased number of EBV$^+$ cells, present in only a subset of cases, is somehow related to the IgG4 disease.

Because of concern as to whether the expansion of EBV-infected cells in many cases of IgG4-related lymphadenopathy could simply reflect an age-related phenomenon, a group of non–IgG4-related reactive lymph nodes were studied for comparison purposes. This patient cohort did not show a difference in age from the patients with IgG4-related disease but had significantly fewer cases with increased EBV-infected cells. In addition, there was no significant difference in age distribution between EBER$^+$ and EBER$^-$ IgG4-related disease cases. Therefore, age does not seem to be a factor in the association of IgG4-related nodal disease with increased numbers of EBV-infected cells.

Upregulation of Treg and helper T-cell (Th2) activity has been reported in tissues with IgG4-related

TABLE 6. Summary of Clinicopathologic Features of IgG4-related Diseases

	Lymph Node (n = 31)	Lacrimal Gland (n = 9)	Submandibular Gland (n = 10)	Skin (n = 2)	Pancreas (n = 3)
Age (y)	64.5 ± 11.6	56.4 ± 12.7	64.0 ± 6.0	64.0 ± 1.4	64.6 ± 4.0
Sex	M:F = 24:7	M:F = 4:5	M:F = 6:4	M:F = 1:1	M:F = 3:0
EBER$^+$ cases (n [%])	18 (58)	3 (33)	2 (20)	0 (0)	0 (0)

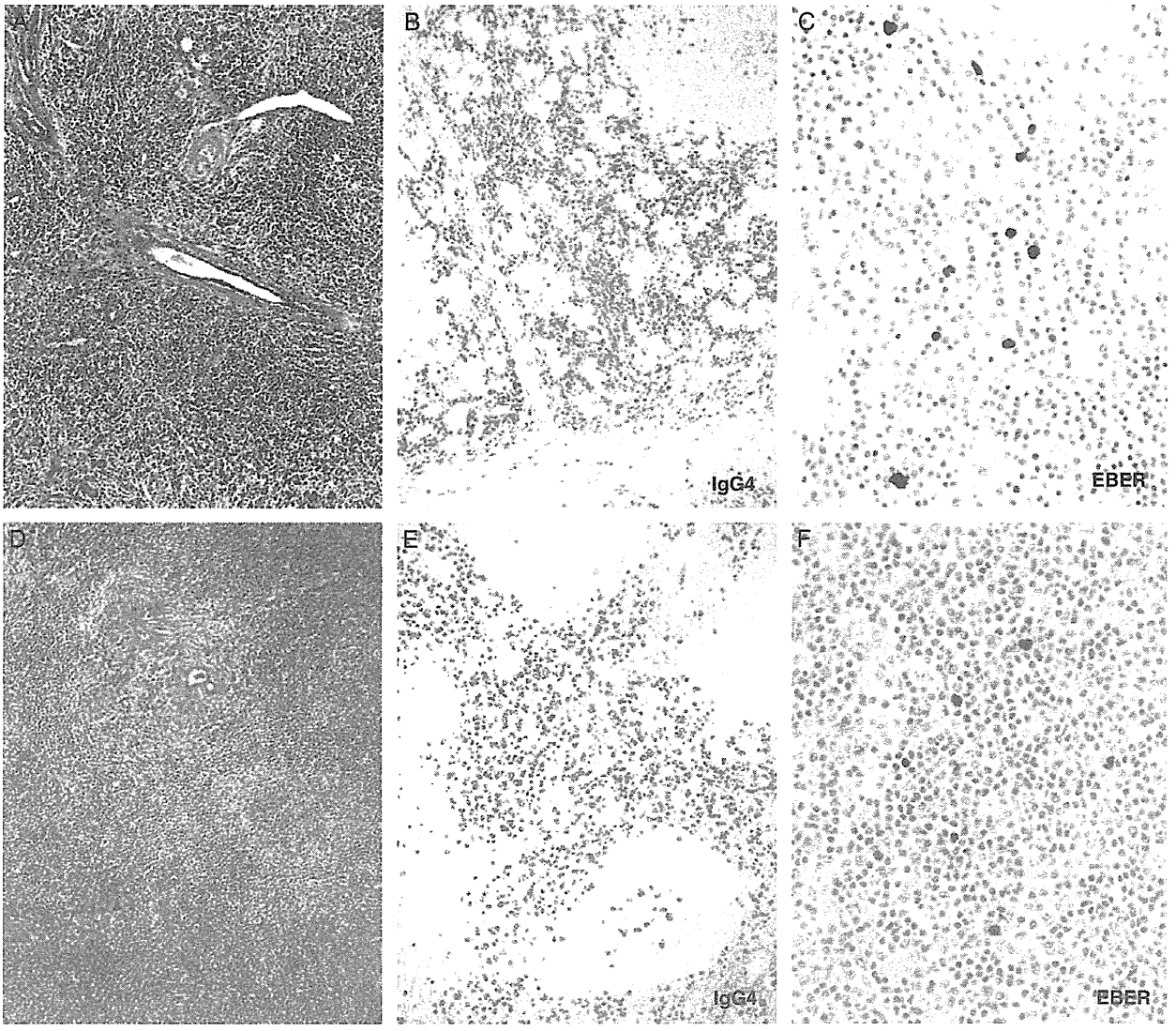

FIGURE 3. Analysis of extranodal IgG4-related disease: IgG4-related submandibular gland disease (Ex19). A, Lymphoplasmacytic infiltration and dense fibrosis. Salivary gland ducts remain intact (H&E). B, Numerous IgG4$^+$ cells (IgG4). C, Increased EBER$^+$ cells (157/0.5 cm^2) (EBER-ISH). D–F, IgG4-related lacrimal gland disease (Ex9). D, Infiltration of small lymphocytes and plasma cells (H&E). E, Significant IgG4$^+$ cells (IgG4). F, Increased EBER$^+$ cells (542/0.5 cm^2) (EBER-ISH). H&E indicates hematoxylin and eosin.

disease.[3] Although the precise mechanism of immune suppression by Treg is not fully understood, secretion of interleukin-10 and transforming growth factor β1 seems to be involved.[21] Although the relationship between Treg and EBV has not been fully investigated, upregulation of Treg activity might contribute to a disruption of immune balance. However, in our study, the number of FOXP3$^+$ Tregs was similar between EBER$^+$ and EBER$^-$ groups. Further study is required to understand the relationship between IgG4-related disease and EBV.

In conclusion, increased numbers of EBV-infected cells are frequently found in IgG4-related lymphadenopathy, including in the type III cases with many immunoblasts that can morphologically mimic AITL, and is not related to patient age. Distinction from AITL, however, was not difficult, in part because AITL never had sufficient IgG4 positivity to fulfill the criteria for IgG4-related lymphadenopathy. IgG4-related disease with increased numbers of EBER$^+$ cells may have some clinical implications as, in contrast to usual expectation, all the patients with EBV$^+$ type IV IgG4-related lymphadenopathy developed systemic lymphadenopathy and/or extranodal involvement. Finally, particularly given that IgG4-related lymphadenopathy often occurs in individuals over 50 years of age, it is important to recognize that it must be included in the differential diagnosis of EBV$^+$ lymphoproliferative disorders, including EBV$^+$ diffuse large B-cell lymphoma of the elderly.

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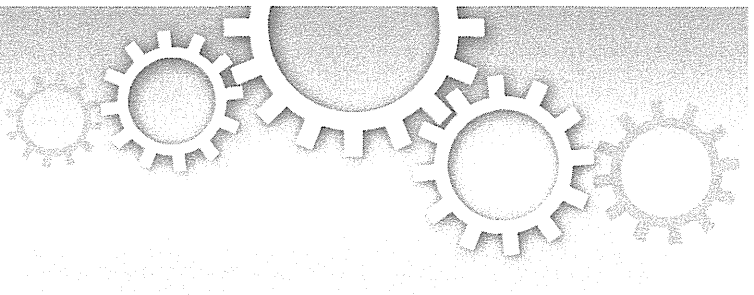

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Interleukin 13-positive mast cells are increased in immunoglobulin G4-related sialadenitis

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Interleukin (IL)-13 is a T helper 2 (Th2) cytokine that plays important roles in the pathogenesis of asthma. IL-13 induces hypersensitivity of the airways, increased mucous production, elevated serum immunoglobulin (Ig) E levels, and increased numbers of eosinophils. Many patients with IgG4-related disease have allergic backgrounds and show elevated serum IgE levels and an increase in the number of eosinophils. Upregulation of Th2/regulatory T (Treg) cytokines, including IL-13, has been detected in affected tissues of patients with IgG4-related disease. We previously reported that mast cells might be responsible for the production of the Th2/Treg cytokines IL-4, IL-10, and transforming growth factor (TGF)-β1 in IgG4-related disease. In this study, immunohistochemical analysis showed increased numbers of IL-13-positive mast cells in IgG4-related disease, which suggests that mast cells also produce IL-13 and contribute to elevation of serum IgE levels and eosinophil infiltration in IgG4-related disease.

Immunoglobulin (Ig)G4-related disease is a recently established systemic disorder with characteristic clinicopathological features that frequently affects the exocrine organs, including the pancreas, salivary glands, and lacrimal glands, although various systemic organs can also be involved[1]. The pathogenesis of IgG4-related disease remains unclear and controversial; however, upregulation of T helper (Th) 2 and regulatory T (Treg) cytokines in diseased areas have been reported[2,3]. To date, these Th2 and Treg reactions have been considered to form characteristic pathological features, including lymphoplasmacytic infiltration, storiform fibrosis, and increased numbers of IgG4-positive plasma cells and eosinophils[2,3].

Interleukin (IL)-13 is one of such Th2 cytokines and is closely related to the pathogenesis of asthma[4]. IL-13 provokes hyperreactivity of the airways, increases in goblet cell numbers and mucous production, activation of fibroblasts, class switching of B-cell antibody from IgM to IgE, and increased numbers of eosinophils in the blood[4,5]. IL-13 is also considered to be associated with elevated serum IgE levels and increased numbers of eosinophils in IgG4-related disease[6].

Upregulation of IL-13 in tissues of patients with IgG4-related disease has been previously demonstrated, and Th2 cells are the most likely candidates for the production of IL-13[2]. However, it has not been confirmed whether Th2/Treg cells directly produce these important cytokines. We recently reported that mast cells can produce Th2 and Treg cytokines, including IL-4, IL-10, and transforming growth factor (TGF)-β1, in IgG4-related disease[7]. Hence, the potential of mast cells to produce IL-13 was examined in this study.

Methods

Samples. Tissue samples from 9 cases of submandibular gland IgG4-related disease were obtained. Samples from 5 cases of submandibular sialolithiasis and 6 normal submandibular glands were also obtained and used as disease and healthy controls, respectively. These samples were also used in our previous study[7]. Serum IgG4 levels were elevated in all cases of IgG4-related disease. Samples from formalin-fixed, paraffin-embedded specimens were used for immunohistochemistry and dual immunofluorescence analyses. Informed consent for the use of their samples in research was obtained from all patients.

Methods. The following methods were carried out in accordance with the approved guidelines. All experimental protocols were approved by the Institutional Review Board at Okayama University.

Histological examination and immunohistochemistry. All of the diseased and normal tissue samples used in this study were surgically resected specimens of the submandibular glands. The specimens were fixed in 10% formaldehyde and embedded in paraffin. Serial 4-μm-thick sections were cut from the blocks of paraffin-embedded tissues and stained with hematoxylin and eosin (H&E). The sections were immunohistochemically stained using an automated Bond Max stainer (Leica Biosystems; Wetzlar, Germany). The following primary antibodies were used: IL-13 (2B5; 1 : 300; Abnova; Taipei City, Taiwan), c-kit/CD117 (YR145; 1 : 100; EPITOMICS; Burlingame, CA, USA), IgG (polyclonal; 1 : 20,000; Dako; Glostrup, Denmark), and IgG4 (HP6025; 1 : 400; The Binding Site; Birmingham, UK).

Confirmation of histological diagnosis of IgG4-related disease. All samples from patients with IgG4-related disease showed typical histological features, including lymphoplasmacytic infiltration and dense fibrosis (Fig 1a, 1b). In accordance with the consensus statement on the pathological features of IgG4-related disease published in 2012[8], 3 different high-power fields (HPFs) (eyepiece, ×10; lens, ×40) were examined to calculate the average number of IgG4-positive cells per HPF and the IgG4-/IgG-positive cell ratio. In all patients with IgG4-related disease, the average number of IgG4-positive plasma cells was >100 cells/HPF, and the ratio of IgG4-/IgG-positive cells was >40% (Fig. 1c, 1d).

Calculation of IL-13- and c-kit-positive cells. Cells that were positive for IL-13 and c-kit were counted in 5 and 3 different fields, respectively, that showed the highest density of positive cells (eye piece, ×10; lens, ×20). Two pathologists (M.T. and Y.S.) counted the positive cells, and the numbers were averaged. The average number of positive cells per square millimeter was calculated.

Dual immunofluorescence assays. For indirect dual immunofluorescence assays, paraffin sections were stained with the primary antibodies for c-kit and IL-13. Fluorescein isothiocyanate-conjugated secondary antibodies (Alexa Fluor anti-mouse 555 and Alexa Fluor anti-rabbit 488; both from Life Technologies, Carlsbad, CA, USA) were used at a dilution of 1 : 400. The stained specimens were examined with a conventional immunofluorescence microscope (IX71; Olympus; Tokyo, Japan).

Statistical analysis. Data are presented as mean ± standard deviation (SD) values. All statistical analyses were performed using the Mann–Whitney *U*-test with SPSS software (version 14.0; SPSS Inc.; Chicago, IL, USA). A probability value of <0.05 was considered to be statistically significant.

Results

Many IL-13-positive cells were observed in tissues from patients with IgG4-related disease (Fig. 2a). The number of IL-13-positive cells was significantly increased in IgG4-related disease samples (4.00 ± 2.21 cells/mm^2) compared to sialolithiasis samples (0.30 ± 0.48 cells/mm^2) and normal submandibular glands (0.10 ± 0.11 cells/mm^2; Fig. 2b). We used c-kit antibody as a marker of mast cells. The numbers of c-kit-positive cells were significantly higher in IgG4-related disease samples (72.2 ± 24.5 cells/mm^2) than in the normal submandibular glands (30.0 ± 11.9 cells/mm^2; $p < 0.01$), whereas no significant difference was observed between the number of mast cells in the IgG4-related disease and submandibular sialolithiasis samples (177 ± 269 cells/mm^2; $p = 0.73$), as previously reported[7].

The morphological features and distribution of the c-kit-positive mast cells were similar to those of IL-13-positive cells. Dual immunofluorescence assays revealed coexpression of IL-13 and c-kit (Fig. 3).

Discussion

Even though Miklicz disease, now recognized as a member of the IgG4-related disease family, was first reported in the late 19th century, the concept of IgG4-related disease has only become well established in the 21st century[9,10]. IgG4-related disease was first described in the pancreas. It was initially considered an autoimmune disease, and was therefore termed "autoimmune pancreatitis" because of concomitant extra-pancreatic autoimmune diseases, e.g., Sjögren syndrome, with features including hypergammaglobulinemia, an elevated titer of anti-nuclear antigens, and good response to steroid therapy[11]. Subsequent research showed that these extra-pancreatic lesions were, in fact, features of multifocal IgG4-related disease; however, the relationship between IgG4-related disease and autoimmunity has not yet been elucidated[12].

Although the pathogenesis of IgG4-related disease remains unclear, Th2/Treg immune reactions seem to contribute to disease formation[2,3]. Enhanced Th2/Treg reaction is important for the establishment of allergic disorders in general, and IgG4-related disease patients often have allergic backgrounds. However, the relationship between allergic backgrounds and pathogenesis of IgG4-related disease is controversial[13]. A recent study showed that the prevalence of atopy in patients with IgG4-related disease did not differ from that of the general population and the majority of the patients were non-atopic[14]. According to the study, non-atopic patients with

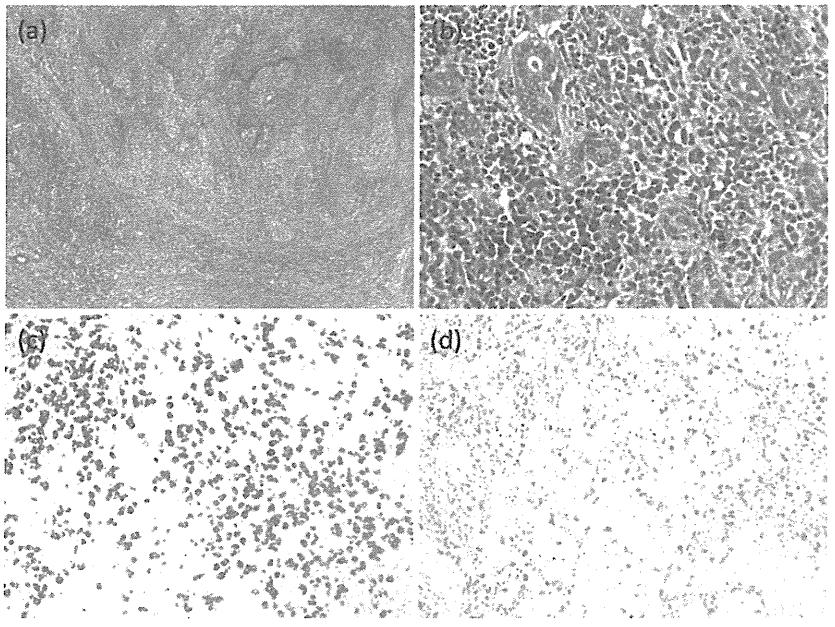

Figure 1 | Immunohistochemical analysis of IgG4-related disease. (a) Tissue samples of patients with IgG4-related submandibular disease showed dense fibrosis with lymphoid follicles (hematoxylin and eosin [H&E] staining; original magnification × 40). (b) Lymphoplasmacytic infiltration was observed in the interfollicular areas (H&E, original magnification × 400). (c) Numerous IgG4-positive cells were detected (IgG4, original magnification × 200). (d) The IgG4/IgG-positive cell ratio was >0.4 (IgG, original magnification × 200).