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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)- β 1 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 involved¹. 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 asthma⁴. 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 disease⁶.

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-13³. 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)- β 1, in IgG4-related disease⁷. 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 study⁷. 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 2012⁸, 3 different high-power fields (HPFs) (eyepiece, $\times 10$; lens, $\times 40$) 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, $\times 10$; lens, $\times 20$). 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 \pm 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 reported⁷.

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 therapy¹¹. 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 elucidated¹².

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 controversial¹³. 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-atopic¹⁴. According to the study, non-atopic patients with

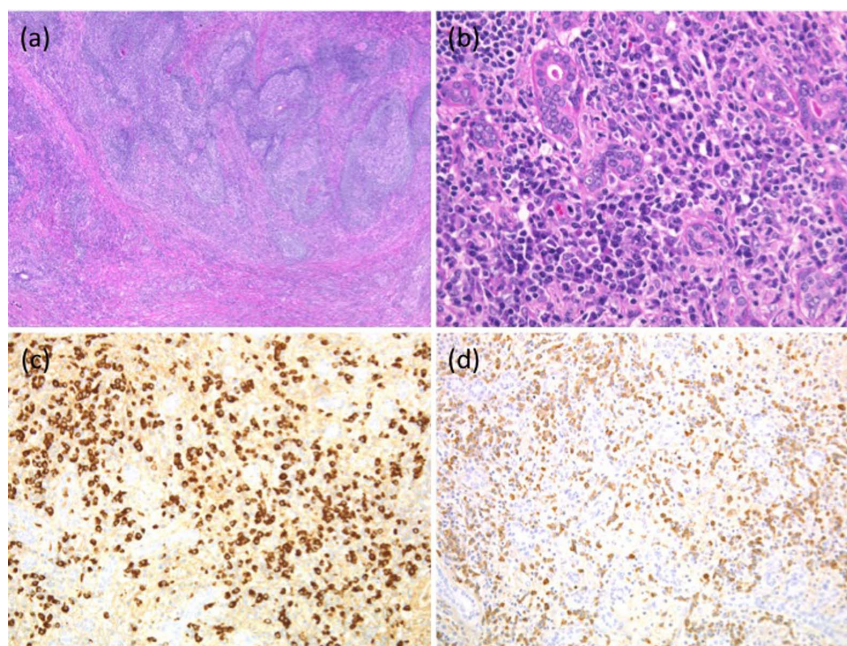


Figure 1 | Immunohistochemical analysis of IgG4-related disease. (a) Tissue samples of patients with IgG4-related submandibular disease showed dense fibrosis with lymphoid follicles (hematoxylin and eosin [H&E] staining; original magnification $\times 40$). (b) Lymphoplasmacytic infiltration was observed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

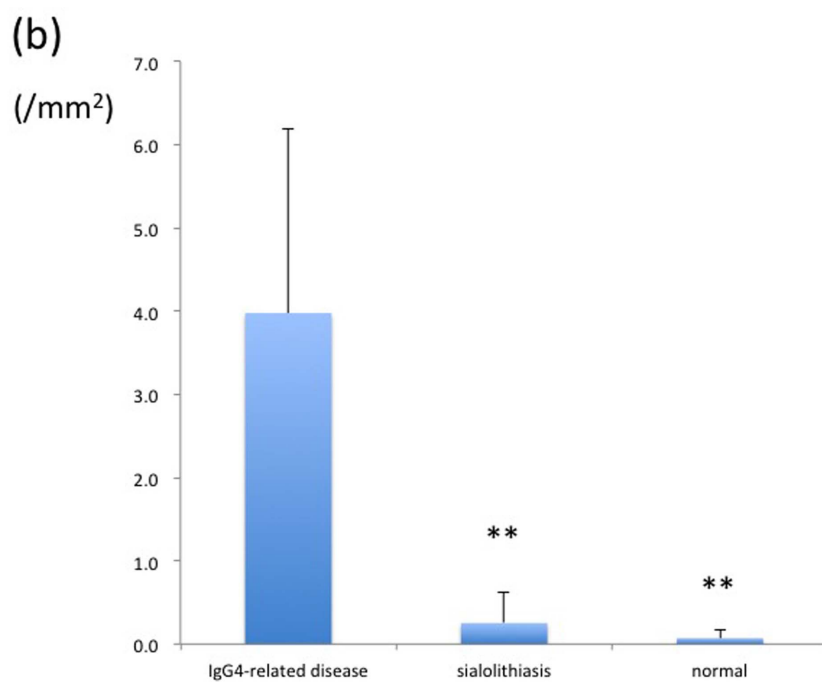
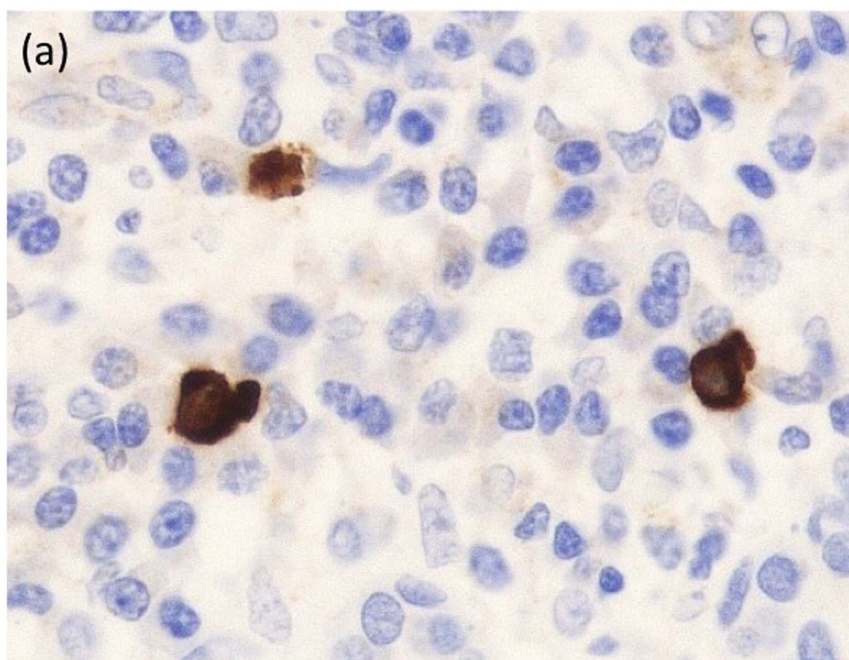


Figure 2 | Counts of IL-13-expressing cells by immunostaining. (a) Immunostaining for IL-13 revealed strong cytoplasmic positivity. Positive cells were morphologically similar to mast cells (IL-13, original magnification $\times 400$). (b) Significantly greater numbers of IL-13-positive cells were observed in the IgG4-related disease group than in the control group ($p < 0.01$).



Figure 3 | Dual fluorescent immunostaining for IL-13 and c-kit. Dual fluorescent immunostaining detected positive cells for IL-13 and c-kit. The merged image demonstrates double positive-stained cells for IL-13 and c-kit.



IgG4-related disease also showed elevated IgE and peripheral blood eosinophilia, which suggested that an enhanced Th2/Treg response is not related to allergic background but rather to IgG4-related disease itself⁴.

On the other hand, some reports have suggested the importance of allergic reactions in the pathogenesis of IgG4-related disease. IgG4 is a unique antibody with a poor ability to activate complements and cells because of its low affinity for C1q and Fc receptors¹⁵. Unlike other IgG subclasses, IgG4 has anti-inflammatory activity and seems to inhibit IgE-mediated type I allergic responses by competing with IgE^{15,16}. For example, some cases of autoimmune pancreatitis following bronchial asthma have been reported¹⁷. Recently, a case of IgG4-related disease was found to have regressed with only treatment of an anti-histamine agent and no systemic steroid therapy¹⁸. Based on these findings, IgG4-related disease may be related to an aberrant anti-inflammatory activity against the allergic reaction.

Mast cells are well known to play important roles in the immediate immune response and release of histamine granules upon binding to IgE. However, previous studies have also shown that mast cells release various cytokines and chemokines and participate in multiple immune reactions^{19,20}. We previously reported the possible role of mast cells in the production of Th2/Treg cytokines (IL-4, IL-10, and TGF- β 1) in IgG4-related disease⁷. In this study, we found that mast cells might also produce IL-13, suggesting that it is a key factor in the elevation of serum IgE levels and number of eosinophils associated with IgG4-related disease. As mast cells are closely related to allergic reaction and IgE stimulation, these results indirectly suggest that a background of an allergic disorder and elevated serum IgE levels can be a trigger for the upregulation of mast cell-derived Th2/Treg cytokines. Further studies on the role of mast cells in IgG4-related disease and their interaction with Th2/Treg cells are required.

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Author contributions

Conceived and designed the experiments: Y.S. Performed the experiments: M.T. and Y.S. Analyzed the data: Y.S., M.T., K.O., K.T. and Y.G. Contributed materials: Y.O. and T.T. Wrote the paper: M.T., Y.S. and T.Y. All authors read and approved the final manuscript.

Additional information

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