CD14⁺ follicular dendritic cells in lymphoid follicles may play a role in the pathogenesis of IgG4-related disease

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ABSTRACT

Proliferated IgG4⁺ plasma cells are polyclonal, suggesting that the pathogenesis of IgG4-related disease (IgG4-RD) involves upstream events related to the regulation of IgG4 expansion. We hypothesized that lymphoid follicle formation may play an important role in the pathogenesis of IgG4-RD. Using various antibodies, especially against monocyte, macrophage, and follicular dendritic cell markers, we immunohistochemically assessed the distribution of immune cells in lymphoid follicles. Pathological findings of tissue samples from patients with IgG4-RD (n = 22), reactive hyperplasia (n = 3), multicentric Castleman’s disease (n = 3), and Sjögren’s syndrome (n = 13) were analyzed. CD14-positive lymphoid follicles were observed only in patients with IgG4-RD, and CD14-positive cells were identified as follicular dendritic cells by multicolor immunohistochemistry. There were few differences in the distributions of other cell types between the IgG4-RD and control groups. The presence of CD14⁺ follicular dendritic cells in lymphoid follicles may play a pathophysiological role in IgG4-RD.

IgG4-related disease (IgG4-RD) is a newly established clinical entity with a relatively high incidence rate in Japan (7). Patients with IgG4-RD show organ enlargement or nodular/hyperplastic lesions in various organs concurrently or metachronously because of marked infiltration of lymphocytes and IgG4⁺ plasma cells, as well as fibrosis of unknown etiology. Symptoms are dependent on the organs involved and may include occlusion or compression due to organ enlargement or hypertrophy and organ function failure due to fibrosis, and some patients may develop severe complications (17, 33, 36). The diagnosis of IgG4-RD is based on the presence of hyper-IgG4-gammaglobulinemia (≥135 mg/dL) and an increased number of IgG4⁺ plasma cells, defined as an IgG4⁺/IgG⁺ cell ratio >40%, and >10 IgG4⁺ plasma cells per high power field. Some patients with other disorders such as cancer, lymphoma, vasculitis, sarcoidosis, and multicentric Castleman’s disease also have hyper-IgG4-gammaglobulinemia and/or elevated IgG4⁺ cell numbers in the affected tissues, making it necessary to distinguish among these disorders that differ in their clinical course and prognosis. Because IgG4-RD shows a good glucocorticoid responsiveness (2, 7, 10–13, 16–20, 28, 33, 36, 37, 40), diagnosis is directly related to therapeutic strategy.

The histopathological characteristics of IgG4-RD are as follows (2, 16, 19, 28): 1) massive lymphocyte and IgG4⁺ plasma cell infiltration forming lymphoid follicles is a typical feature, whereas duct destruction due to lymphoid cell infiltration into the duct epithelium (lymphoepithelial lesions) is very rare, and therefore organ swelling occurs without impaired secretion or dryness; 2) sclerotic changes or fibrosis
This study immunohistochemically analyzed the distribution of cell types involved in lymphoid follicle formation using various antibodies, especially against monocyte, macrophage, and follicular dendritic cell (FDC) markers rather than those against lymphocytes. Our finding of CD14+ FDCs in the lymphoid follicles, suggests that these cells play an important pathophysiological role in IgG4-RD.

**MATERIALS AND METHODS**

**Materials.** Twenty-two patients with IgG4-RD (seven females and 15 males), referred to the Department of Hematology and Immunology in Kanazawa Medical University, were included in this study (Table 1). IgG4-RD was diagnosed according to the Comprehensive Diagnostic Criteria for IgG4-related Disease (IgG4-RD), 2011 (36). The diagnosis of IgG4-RD was defined as both raised serum IgG4 level (≥135 mg/dL) and histopathological features, including lymphocyte and IgG4+ plasma cell infiltration (IgG4+ plasma cells/IgG+ plasma cells > 40%) with typical (storiform fibrosis) composed of undulating fibers is another typical feature; 3) obliterating phlebitis is observed in some tissues; 4) some cases show eosinophilia that is correlated with allergic reactions and increased serum IgE levels; and 5) necrosis, granuloma formation, and massive neutrophil infiltration are usually not found in IgG4-RD.

Proliferated IgG4+ plasma cells are polyclonal (39), suggesting that the pathogenesis of this disease involves important upstream events related to the regulation of IgG4 expression. We hypothesized that ectopic lymphoid follicle formation, which is frequently observed in involved organs, may play an important role in the pathogenesis of IgG4-RD. Although the functions of various immune cells, cytokines, and chemokines in the formation of ectopic lymphoid-like structures in secondary lymphoid organs are becoming clearer (26), this is not yet the case for IgG4-RD. Th2-dominant T cell infiltration and upregulated Treg cells have been reported in IgG4-RD (21, 23, 34, 42), but the roles of other immune cells remain unclear.

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We counted the number of all lymphoid follicles and CD14 positive follicles in each sample, and the number of CD14 positive cells in each 5 lymphoid follicles.
fibrosis or sclerosis in the tissue. Tissue biopsy samples were obtained from labial salivary glands (n = 5), submandibular glands (n = 8), bile ducts (n = 2), lymph nodes (n = 2), lacrimal glands (n = 2), retroperitoneum (n = 1), duodenum (n = 1), and prostate (n = 1) of patients with IgG4-RD prior to glucocorticoid treatment.

As controls, tissue samples were obtained from the lymph nodes of patients with reactive hyperplasia (n = 3) and multicentric Castleman’s disease (n = 3) and from labial salivary glands of patients with Sjögren’s syndrome (n = 13) (Table 2). Sjögren’s syndrome was diagnosed according to the criteria of the Research Committee on Sjögren’s Syndrome of the Ministry of Health and Welfare of the Japanese Government (5), the American-European Consensus Group Criteria for Sjögren’s syndrome (38), and the American College of Rheumatology Classification Criteria for Sjögren’s syndrome (Sjögren’s International Collaborative Clinical Alliance; SICCA) (30). Reactive hyperplasia and multicentric Castleman’s disease were diagnosed according to pathological findings.

This study was approved by the Ethics Committee of Kanazawa Medical University, and informed consent was obtained from all subjects prior to enrollment in this study.

**Staining methods.** Formalin-fixed and paraffin-embedded sections of the tissue samples were prepared and stained with hematoxylin and eosin. Tissue samples were also incubated with primary antibodies against human IgG4 (MC011; The Binding Site, Birmingham, UK), CD68 (macrophage marker; Clono KP1 Code Is609; Dako, Glostrup, Denmark), CD163 (M2 macrophage marker; NCL-CD163; Leica, Wetzlar, Germany), CD21 (FDC marker; M7157; Dako), CD14 (marker of monocytes and other cells; NCL-CD14–223; Leica), CD16 (monocyte marker; NCL-CD16; Leica), CD3 (T-cell marker; A0452; Dako), and CD20 (B-cell marker; M0755; Dako). Labeled antigens were visualized by secondary antigen-antibody reactions with diaminobenzidine (iVIEWDAB Detection Kit; Ventana) and analyzed with an automated stainer (BenchMark GX; Ventana). Ratios of IgG4+ to IgG+ plasma cells were determined by counting cells in five high-power fields under light microscope, with all examinations performed by an expert pathologist (N. Kurose).

Because the key difference between the IgG4-RD and control groups was in their reaction with anti-CD14 antibody, the total numbers of lymphoid folli-
cles and CD14-positive follicles were counted in each sample, as were the numbers of CD14-positive cells in each of the five lymphoid follicles per sample.

Samples were simultaneously stained for CD14 using goat anti-human CD14 (ab45870, Abcam) and rabbit anti-human CD21 (ab75985, Abcam) antibodies as primary antibodies, followed by staining for CD21 using Alexa Fluor 488-labeled anti-goat IgG (Abcam) and Alexa Fluor 647-labeled anti-rabbit IgG (Abcam) antibodies as secondary antibodies. Samples were simultaneously stained with 4′,6-diamidino-2-phenylindole. Samples were examined by fluorescence microscopy (BZ-9000, Keyence), and green, red, and blue signals were merged (BZ-2 Analyzer, Keyence).

Statistical analysis. Differences between groups were examined using the Mann-Whitney U test and Spearman’s rank correlations. All analyses were performed using StatMate III (ATOMS, Tokyo, Japan). A P-value of <0.05 was considered statistically significant.

RESULTS
Histopathological findings of submandibular glands in a patient with IgG4-RD are shown in Fig. 1. Histological samples in 13/22 patients with IgG4-RD and in all control samples contained lymphoid follicles. CD14⁺ lymphoid follicles, however, were observed only in tissue samples from patients with IgG4-RD (Fig. 1, A2; Fig. 2, A3; Fig. 3). CD14 was posi-
tive in all patients with lymphoid follicle-positive IgG4-RD, especially in the germinal center of those follicles, with these cells also being positive for CD21. Most CD21-positive follicles were also positive for CD14, although a few were negative (Fig. 1, A2, A3, arrows). Multicolor immunohistochemistry showed that CD21 was co-expressed in nearly all CD14-negative cells in lymphoid follicles, suggesting that these CD14-negative cells were FDCs (Fig. 4, yellow arrows). CD14-negative CD21-positive follicles were also confirmed by multicolor staining (Fig. 4, white arrow). IgG4-positive plasma cells were scattered in the periphery of the follicles in tissue samples from patients with IgG4-RD (Fig. 1, B9). For other markers, few monocytes expressing CD16 were observed in the whole tissue (Fig. 1, B4), whereas CD16-positive cells were widely distributed in the control group (data not shown). A few M1 macrophages that expressed CD68 were present in and around the follicles (Fig. 1, B5), and many M2 macrophages expressing CD163 were present mainly around the follicles in tissue samples from patients with IgG4-RD (Fig. 1, B6). Analysis of the macrophage subtype showed that CD163 > CD68, indicating M2 dominance in both the IgG4-RD and control groups, with no significant difference between groups. CD14-positive monocytes were present in the intrafibrotic tissues of patients with multicentric Castleman’s disease (Fig. 2, C3) who had severe fibrosis. Few CD14-negative cells were observed in the tissue samples from patients with reactive hyperplasia and Sjögren’s syndrome (Fig. 2, B3, D3).

The IgG4/IgG ratio tended to be higher in CD14-positive tissue than in CD14-negative tissue (Fig. 5A), whereas serum IgG4 levels did not differ in these tissues (Fig. 5B). Serum IgE levels (Fig. 5C) and blood eosinophil proportion (Fig. 5D) tended to be higher in the CD14-positive groups, but these differences were not statistically significant. CD14-positive IgG4-RD, IgG4-related disease; RLH, reactive lymphoid hyperplasia; MCD, multicentric Castleman’s disease; SjS, Sjögren’s syndrome.

![Fig. 2](image1) Comparison of histopathological findings of patients with IgG4-RD and controls. Histopathological findings of submandibular gland biopsy specimens from a patient with IgG4-RD (A1, A2, A3, A4), of lymph nodes from patients with reactive hyperplasia (B1, B2, B3, B4), multicentric Castleman’s disease (C1, C2, C3, C4), and of the labial salivary glands from a patient with Sjögren’s syndrome (D1, D2, D3, D4). HE staining (A1, B1, C1, D1) and IgG4 (A2, B2, C2, D2), CD14 (A3, B3, C3, D3), and CD21 (A4, B4, C4, D4) immunostainings. CD14-negative lymphoid follicles were observed only in tissue samples from patients with IgG4-RD. CD14-positive monocytes were present in the intrafibrotic tissues of patients with MCD (C3) who had severe fibrosis. IgG4-RD, IgG4-related disease; RLH, reactive lymphoid hyperplasia; MCD, multicentric Castleman’s disease; SjS, Sjögren’s syndrome.
tive cells were observed in interfollicular areas as well as in lymphoid follicles of tissue samples from patients with IgG4-RD. However, CD14+ cells in fibrotic areas were negative for CD21, suggesting that these cells were inflammatory monocytes (Fig. 4). Both the number of CD14-positive lymphoid follicles and the total number of CD14-positive cells in lymphoid follicles were correlated with the number of lymphoid follicles (Fig. 6A, B).

DISCUSSION

Since the first report of high serum IgG4 levels in patients with sclerosing pancreatitis (type 1, autoimmune pancreatitis) in 2001 (7), various disorders have been reported as being IgG4-RD (2, 7, 10–12, 16–20, 28, 33, 36, 37, 40). Typical clinical features of patients with IgG4-RD include polyclonal hypergammaglobulinemia with high serum IgG4 levels, increased number of IgG4-producing plasma cells in the affected organs, storiform fibrosis, obliterating phlebitis, eosinophilia, and lymphoid follicle formation. Increased IgG4-producing plasma cells have also been observed in lacrimal and salivary gland lesions in patients with Mikulicz’s disease and have been categorized as manifestations of Sjögren’s syndrome (22). However, Mikulicz’s disease has also been reported to be an IgG4-RD (40). Thus, IgG4-related Mikulicz’s disease differs from Sjögren’s syndrome, which is an autoimmune condition. Histopathological analysis in patients with Mikulicz’s disease showed marked lymphoid follicle formation, causing swelling of the lacrimal and salivary glands, but duct destruction due to lymphocytic infiltration (lymphoepithelial lesions) is rare (2, 17–20, 28). Most patients with Mikulicz’s disease therefore have markedly swollen glands without dryness. Thus, although the organs that are affected in patients with IgG4-related Mikulicz’s disease and Sjögren’s syndrome are similar, they differ in their pathophysiology. Therefore, recent diagnostic criteria for IgG4-related Mikulicz’s disease and Sjögren’s syndrome include the exclusion of the other disease during differential diagnosis (20, 30, 33).

Although elevated IgG4 levels were the first indicator of IgG4-RD, IgG4 itself has been confirmed as polyclonal (39), suggesting that the pathogenesis of IgG4-RD involves upstream events. A Th2-domi-
To investigate the upstream etiology of IgG4-RD, we focused on the pathological features of the disease. Lymphoid follicle formation is one of the most important pathological features of IgG4-RD because it causes swelling, hypertrophy, and nodular lesions. The frequency, number, and size of ectopic germinal center structures were found to be significantly higher in patients with IgG4-related dacryoadenitis and sialoadenitis than in those with Sjögren’s syndrome (16). Furthermore, overexpression of IL-21 by Th2 cells may play a role in germinal center formation and IgG4 production in IgG4-RD (16). Focusing on immunocompetent cells other than lymphocytes, we assessed the distribution of macrophages, monocytes, and FDCs in IgG4-RD tissue samples. Monocytes are circulating leukocytes that can differentiate into macrophages and FDCs after migration to peripheral tissues (3, 6). Differential expression of CD14 and CD16 allowed monocytes to be divided into two subsets: CD14+ (inflammatory) and CD16+ (resident) monocytes. Macrophages are also heterogeneous in phenotype and function and can be divided into two subtypes: M1 and M2 macrophages. FDCs have not yet been subclassified. To the best of

**Fig. 4** Multicolor staining of CD14+ and CD21+ cells in a submandibular gland biopsy specimen from a patient with IgG4-RD. Cells positive for both CD14+ and CD21+ were observed in the germinal center of a lymphoid follicle (yellow arrows). A CD14−CD21+ lymphoid follicle was also confirmed by multicolor staining (white arrow).
mined, the CD14 protein may activate bacteria- or TLR-associated stimulation, resulting in lymphoid follicle formation in patients with IgG4-RD. CD163+ M2 macrophage (CD163 > CD68) expansion has been observed both in patients with IgG4-RD and with other disorders, suggesting that this expansion is a universal phenomenon in active tissues rather than in lymphoid follicle formation. Subsets of monocytes and macrophages express different surface markers (6). Macrophages can be differentiated from monocytes, but the relationship among these cell subsets remains unclear. FDCs secrete CCL2 (MCP-1), a ligand for monocytes expressing CCR2 (9). Furthermore, FDCs attract Th2 cells by secreting TARC (14). M2 macrophages produce CCL18, which attracts Th2 cells and induces differentiation of monocytes into M2 macrophages (29). In addition, FDCs can be differentiated from monocytes in vitro (8). Thus, FDCs attract monocytes, M2 macrophages, and Th2 cells and correlate with the differentiation of these cells. Alternatively, this phenomenon may act together with Th2 cell-derived IL-21 on germinal centers (16), which may then play a pathophysiological role in IgG4-RD.

Interestingly, both CD14+ and CD14- follicles were present in the same tissue in some samples, but showed no particular difference in histopathological findings on hematoxylin and eosin staining. Further
examination of cytokine and chemokine expressions in CD14+ and CD14− follicles may provide a better insight into the detailed pathophysiology of IgG4-RD. In conclusion, the presence of CD14+ FDCs in lymphoid follicles is a specific feature of IgG4-RD. This phenomenon may play a key role in the pathophysiology of IgG4-RD, thus warranting further analyses.

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