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Concise Report

Circulating PD-1^{+}CXCR5^{-}CD4^{+} T cells underlying the immunological mechanisms of IgG4-related disease

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Abstract

Objective. The aim was to study the pathological role of lymphocytes with a peripheral T helper-cell-like phenotype (PD-1^{+}CXCR5^{-}CD4^{+}) in IgG4-related disease (IgG4-RD).

Methods. PD-1^{+}CXCR5^{-}CD4^{+} T cells in the blood of patients with IgG4-RD ($n = 53$), patients with SS ($n = 16$) and healthy volunteers ($n = 34$) as controls were analysed by flow cytometry. Correlations between results obtained by flow cytometry and clinical parameters relevant to IgG4-RD were also analysed.

Results. The percentage and absolute number of PD-1^{+}CXCR5^{-} cells within total CD4^{+} T cells in IgG4-RD patients were significantly increased compared with those in healthy volunteers. Further analysis showed that there were marked positive correlations of the percentage of PD-1^{+}CXCR5^{-}CD4^{+} T cells with the serum level of IgG4 and the number of organs involved. Interestingly, granzyme A (GZMA)$^{+}$ cells were enriched in PD-1^{+}CXCR5^{-}CD4^{+} T cells, and the percentage and absolute number of GZMA^{+}PD-1^{+}CXCR5^{-}CD4^{+} T cells were significantly elevated in IgG4-RD patients. Although no obvious change was observed in the percentage of total CD4^{+} T cells, the percentage and absolute number of PD-1^{+}CXCR5^{-}CD4^{+} T cells decreased in accordance with a reduction of serum IgG4 level after treatment with glucocorticoids.

Conclusion. In IgG4-RD, circulating CD4^{+} T-cell populations were composed of PD-1^{+}CXCR5^{-} cells, and the ratios of these cells were correlated with clinical manifestations of IgG4-RD. Further analysis of GZMA^{+}PD-1^{+}CXCR5^{-}CD4^{+} T cells might lead to a deeper understanding of the pathogenesis of ectopic lymphoid follicles and the persistent inflammation in IgG4-RD.

Key words: IgG4-related disease, PD-1^{+}CXCR5^{-}CD4^{+} T cells, peripheral T helper cells, T follicular helper cells, granzyme A

CLINICAL SCIENCE

Introduction

IgG4-related disease (IgG4-RD) is characterized by a high level of serum IgG4 and by chronic inflammation accompanied by storiform-type fibrosis mainly occurring in various non-lymphoid tissues [1]. The affected organs include the salivary and lacrimal glands, pancreas, lung and retroperitoneum, and organ involvement results in dysfunctions such as dacryoadenitis and sialadenitis (IgG4-DS, also so-called Mikulicz's disease) or type 1 autoimmune pancreatitis. Although the aetiology of IgG4-RD remains controversial, it is well recognized that the lesions of IgG4-RD preferentially harbour mature lymphocytes and IgG4-producing plasma cells, suggesting a certain immunological anomaly to drive the generation of IgG4 as the underlying mechanism of IgG4-RD. Recent studies on tissue-resident T follicular helper cells (Tfh cells, PD-1^{hi}CXCR5^{+}CD4^{+} T cells) and circulating activated Tfh cells (PD-1^{+}CXCR5^{+}CD4^{+} T cells) have revealed their possible roles as B-cell helpers in the

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particular immune mechanism of IgG4-RD [2, 3]. Given that Tfh cells share CXCR5 with B cells to control germinal centre formation for affinity maturation and class switch recombination of immunoglobulins, Tfh cells were originally identified in germinal centres of lymphoid follicles of the tonsils as a CXCR5-expressing subset of $CD4^+$ T cells [4].

A more recent study on RA has revealed a unique subset of $CD4^+$ T cells named peripheral T helper cells (Tph cells, PD-$1^{hi}CXCR5^-CD4^+$ T cells) that also play a role as B-cell helper cells in extranodal sites of rheumatoid joints [5]. Like Tfh cells, Tph cells express CXCL13, which is a ligand of CXCR5. Therefore it is considered that Tph cells possess the capacity to recruit B cells and Tfh cells. $CD4^+$ T cells with a Tph cell-like phenotype have also been found in breast cancer tissues, and their possible regulatory function in immune responses against tumour cells has been reported [6]. However, the biological significance of $CD4^+$ T cells possessing the phenotype related to Tph cells in IgG4-RD remains unknown.

In the present study, we demonstrated, for the first time, that circulating PD-1^+CXCR5^- cells (including PD-$1^{hi}CXCR5^-$ cells, thus collectively termed Tph-like cells here) within $CD4^+$ T cells in IgG4-RD were significantly increased in comparison with those in healthy volunteers. We also showed that their percentage was positively correlated with serum levels of IgG4 and soluble IL-2 receptor and with the number of organs involved in IgG4-RD patients. Additionally, we found that these Tph-like cells frequently expressed granzyme A (GZMA), which is related to a cytotoxic property. Clinical remission achieved by treatment with glucocorticoids clearly led to a numerical reduction of Tph-like cells. Collectively, these findings regarding circulating Tph-like cells provide new insights for an understanding of the pathogenesis of IgG4-RD.

Methods

Study populations

The characteristics of patients with IgG4-RD ($n = 53$), patients with SS ($n = 16$) and healthy volunteers ($n = 34$) are summarized in supplementary Table S1, available at *Rheumatology Advances in Practice* online. Patients in the IgG4-RD and SS groups and subjects in the healthy volunteer group were matched in terms of age. The organs involved in patients with IgG4-RD are summarized in supplementary Table S2, available at *Rheumatology Advances in Practice* online. Diagnosis of IgG4-RD and SS was performed according to the 2011 comprehensive IgG4-RD diagnostic criteria [7] and the revised European criteria [8], respectively. Treatment for IgG4-RD was performed according to the protocol previously described [9]. At the time of initial sampling of peripheral blood, none of the patients had received glucocorticoid therapy. Before entry to the treatment protocols, blood specimens were collected from the subjects and analysed. None of the healthy volunteers had abnormal physical or chest X-ray findings, and the results of all blood tests for the healthy volunteers were negative. Written informed consent was obtained in all cases according to the Declaration of Helsinki. All protocols were approved by the institutional review boards of Sapporo Medical University Hospital.

Antibodies

The following anti-human mAbs and isotype-matched control IgG were purchased from BD Biosciences (San Jose, CA, USA): mouse anti-CD3-APC (UCHT1), anti-CD4-APC-Cy7 (RPA-T4) and anti-PD-1-PE (EH12.1) mAbs, and a rat anti-CXCR5-PerCP-Cy5.5 (RF8B2) mAb. A recombinant anti-GZMA-PE (REA162) mAb was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany).

Flow cytometry and cell sorting

Peripheral blood mononuclear cells were isolated from heparinized blood samples by centrifugation over a discontinuous density gradient (Lympholyte-H, Cedarlane, Burlington, ON, Canada). Cell staining using cell surface markers was performed as previously described [2]. Intracellular staining for GZMA was performed with the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Waltham, MA, USA) as described in the manufacturer's protocol. Samples were then analysed and sorted using a FACSCanto II and FACSAria II, respectively (BD Biosciences). In each experiment using FACS-sorted cells, the purity of cells reached 95% after validation with reanalysis using FACSCanto II. All data were analysed using FACSDiva software (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR, USA).

Quantitative real-time PCR

Quantitative real-time PCR was performed using a TaqMan Gene Expression Assay kit (Life Technologies, Carlsbad, CA, USA) with the Roche LightCycler 480 Real-Time PCR Detection System (Roche Diagnostics GmbH, Mannheim, Germany) as described in the manufacturer's protocol. For TaqMan-based detection, the amount of *GAPDH* mRNA was used to standardize the amounts of target transcripts, including *IL4* (Hs00174122) and *IL21* (Hs00222327). The $\Delta\Delta$CT method was used to calculate the relative levels of transcripts in triplicate specimens.

Statistical analysis

All data are shown as medians. Significant differences between any two groups were determined by using the Mann–Whitney *U* test. Multiple group comparisons were analysed with the Kruskal–Wallis test. Correlations were determined by Spearman's correlation coefficient, and probability values <0.05 were considered statistically significant.

Results

PD-1$^+$CXCR5$^-$CD4$^+$ T cells were increased in IgG4-RD

We examined the profiles of circulating PD-1$^+$CXCR5$^-$CD4$^+$ T cells in peripheral blood of the patients with IgG4-RD ($n = 53$) and SS ($n = 16$) and the healthy volunteers ($n = 34$) as controls by flow cytometry. As shown in the representative profiles of flow cytometry in Fig. 1A, PD-1$^+$CXCR5$^-$CD4$^+$ T cells (Tph-like cells) included PD-1^{hi}CXCR5$^-$CD4$^+$ T cells (conventional Tph cells). Given that ectopic lymphoid follicles were found in the lesions of SS, as observed in IgG4-RD, we analysed blood specimens from patients with SS as a disease control. Interestingly, the percentage of circulating PD-1$^+$CXCR5$^-$ cells within total CD4$^+$ T cells in patients with IgG4-RD and SS were significantly increased compared with those in healthy volunteers (Fig. 1B). The absolute number of PD-1$^+$CXCR5$^-$CD4$^+$ T cells in patients with IgG4-RD was significantly increased, whereas the absolute number of PD-1$^+$CXCR5$^-$CD4$^+$ T cells in patients with SS was comparable to that in healthy volunteers (Fig. 1C). Given that there was no significant difference in the percentage or absolute number of total CD4$^+$ T cells among the three groups (supplementary Fig. S1, available at *Rheumatology Advances in Practice* online), these results indicate that circulating CD4$^+$ T-cell populations are preferentially composed of PD-1$^+$CXCR5$^-$ cells in the blood of patients with IgG4-RD.

To determine the clinical relevance of PD-1$^+$CXCR5$^-$CD4$^+$ T cells in IgG4-RD patients, we examined the relationship between the percentage of PD-1$^+$CXCR5$^-$CD4$^+$ T cells and the degree of organ involvement in IgG4-RD patients. In the cases of IgG4-RD studied here, lacrimal and salivary glands were the major affected organs (supplementary Table S2, available at *Rheumatology Advances in Practice* online). The results indicated that both the percentage and the absolute number of PD-1$^+$CXCR5$^-$CD4$^+$ T cells in IgG4-RD patients with multiple affected organs (a group with comorbidities) were larger than those in IgG4-RD patients with limited affected organs, such as IgG4-DS patients (a group without comorbidities), who manifested a lesion(s) of the lacrimal and/or salivary gland (Fig. 1D and E). Together with the results showing that the percentages of total CD4$^+$ T cells were comparable in these two cohorts (i.e. groups with and without comorbidities), the results indicate that an increase in PD-1$^+$CXCR5$^-$CD4$^+$ T cells might be related to disease progression of IgG4-RD.

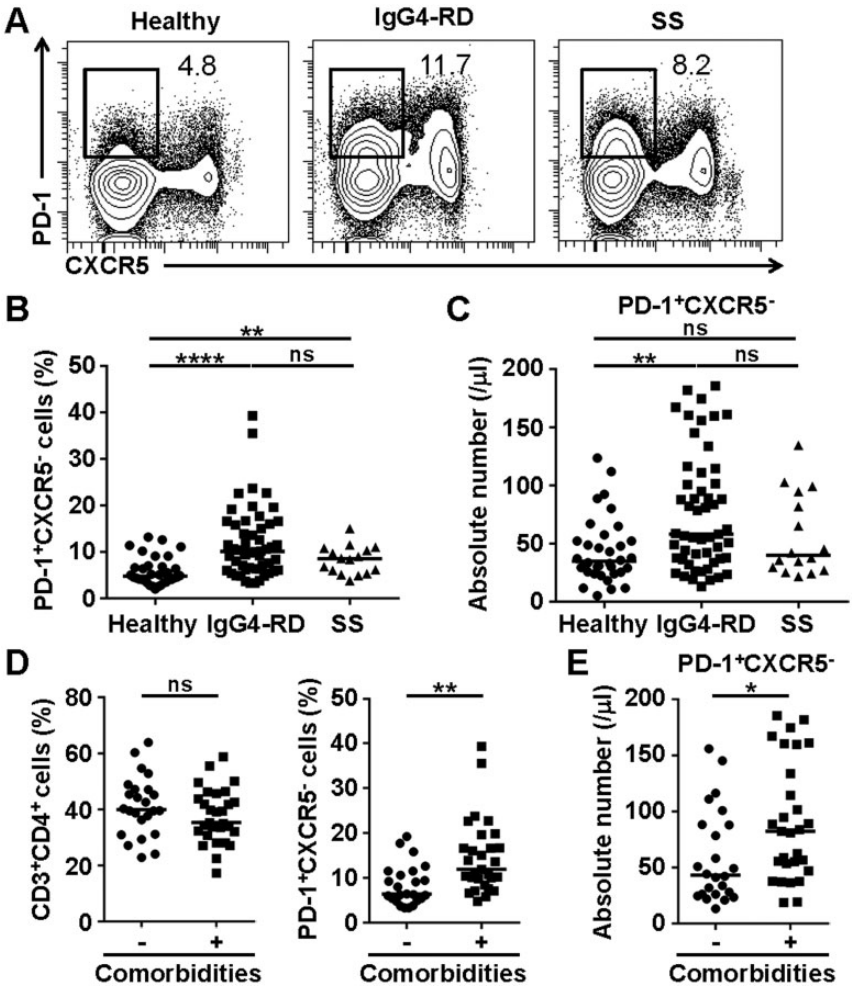
PD-1$^+$CXCR5$^-$CD4$^+$ T cells were positively correlated with clinical parameters of IgG4-RD

We next examined the relationships between the percentage of PD-1$^+$CXCR5$^-$CD4$^+$ T cells and chief clinical findings, including the serum level of IgG4, IgG4/IgG ratio, number of involved organs and soluble IL-2 receptor [10], as diagnostic parameters in IgG4-RD as assessed by scatter plot analyses. The results showed that the percentage of PD-1$^+$CXCR5$^-$CD4$^+$ T cells was weakly or moderately correlated with the serum level of IgG4 ($r = 0.4240$, $P < 0.01$) and IgG4/IgG ratio ($r = 0.3624$, $P < 0.01$), whereas we failed to find any correlation of the percentage of PD-1$^+$CXCR5$^-$CD4$^+$ T cells with the serum levels of IgE ($r = -0.0948$, $P = 0.5039$) and IgA ($r = 0.0745$, $P = 0.5962$), as shown in Fig. 2A and supplementary Fig. S2, available at *Rheumatology Advances in Practice* online. In accordance with the results as shown in Fig. 1D and E, the number of organs involved ($r = 0.5638$, $P < 0.0001$) and the serum level of soluble IL-2 receptor ($r = 0.4780$, $P < 0.001$) were positively associated with the percentage of PD-1$^+$CXCR5$^-$CD4$^+$ T cells in IgG4-RD.

We next addressed the question of whether standard treatment with glucocorticoids affected the proportion of circulating PD-1$^+$CXCR5$^-$CD4$^+$ T cells, because glucocorticoids have been shown to be fairly effective for improving clinical manifestations of patients with IgG4-RD [9, 11]. Of note, after glucocorticoid treatment, the percentage and absolute number of PD-1$^+$CXCR5$^-$CD4$^+$ T cells were markedly decreased in parallel with the reduction of serum IgG4 level (Fig. 2B and C). In contrast to these remarkable changes of PD-1$^+$CXCR5$^-$CD4$^+$ T cells, the treatment itself did not seem to influence the percentage of total CD4$^+$ T cells in patients with IgG4-RD (Fig. 2B).

Finally, we would like to address the functional properties of PD-1$^+$CXCR5$^-$CD4$^+$ T cells. Given that lesional CD4$^+$GZMA$^+$ cytotoxic T cells have been shown to play an important role in the pathogenesis of IgG4-RD [12], we were interested in the possible relationship between CD4$^+$GZMA$^+$ cytotoxic T cells and PD-1$^+$CXCR5$^-$CD4$^+$ T cells. When we performed intracellular staining analysis of GZMA in CD4$^+$ T cells, GZMA$^+$ T cells were found to be abundantly enriched in PD-1$^+$ T cells, but not PD-1$^-$ T cells, within CD4$^+$ T cells (data not shown). In both the IgG4-RD and healthy volunteer groups, the percentages and absolute numbers of GZMA$^+$ T cells were increased in PD-1$^+$CXCR5$^-$CD4$^+$ T cells compared with those in PD-1$^+$CXCR5$^+$CD4$^+$ T cells (Tfh cells), as shown in Fig. 2D and E and supplementary Fig. S3, available at *Rheumatology Advances in Practice* online. Moreover, GZMA$^+$ T cells were significantly increased in PD-1$^+$CXCR5$^-$CD4$^+$ T cells from patients with IgG4-RD in comparison with those in PD-1$^+$CXCR5$^-$CD4$^+$ T cells from healthy volunteers (Fig. 2D and E and supplementary Fig. S3, available at *Rheumatology Advances in Practice* online). Given that it has been reported that PD-1$^+$CXCR5$^{-/dim}$ CD4$^+$ T cells and Tph cells also have a functional property as B-cell helpers [5, 13], we performed functional analysis regarding the promotion of B-cell responses. However, we failed to observe significant levels of expression of *IL4* and *IL21* transcripts in Tph-like cells from healthy volunteers and from patients with IgG4-RD compared with those in Tfh cells (supplementary Fig. S4, available at *Rheumatology Advances in Practice* online).

Fig. 1 Circulating PD-1$^+$CXCR5$^-$CD4$^+$ T cells are increased in IgG4-related disease

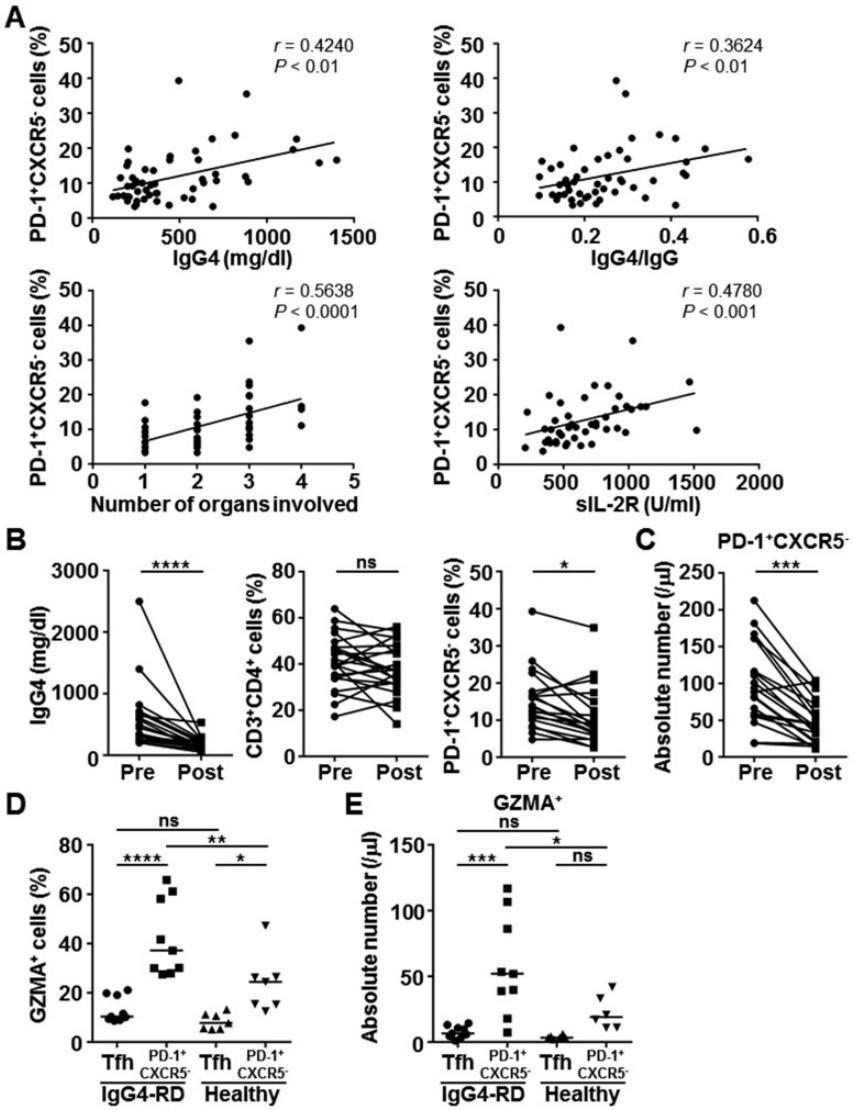


(**A**) Representative FACS profiles indicating PD-1$^+$CXCR5$^-$CD4$^+$ T cells. Plots were pre-gated on CD3$^+$CD4$^+$ cells and examined for the levels of CXCR5 and PD-1. Numbers indicate the percentage of cells in the gate. (**B**) Percentages of PD-1$^+$CXCR5$^-$ cells within CD3$^+$CD4$^+$ cells in healthy volunteers (Healthy) and in patients with IgG4-RD and patients with SS are shown. (**C**) Absolute numbers (per microlitre blood) of PD-1$^+$CXCR5$^-$CD4$^+$ T cells in healthy volunteers (Healthy) and in patients with IgG4-RD and patients with SS are shown. (**D**) Percentages of CD3$^+$CD4$^+$ cells in lymphocytes (left panel) and PD-1$^+$CXCR5$^-$ cells in CD3$^+$CD4$^+$ cells (right panel) from IgG4-RD patients with or without multiple involvement of other organs (Comorbidities) in addition to lacrimal and/or salivary glands are shown. (**E**) Absolute numbers (per microlitre of blood) of PD-1$^+$CXCR5$^-$ cells in CD3$^+$CD4$^+$ cells from IgG4-RD patients with or without multiple involvement of other organs (Comorbidities) in addition to lacrimal and/or salivary glands are shown. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$; ns: not significant. IgG4-RD: IgG4-related disease.

Discussion

In the present study, we comprehensively investigated peripheral blood leucocytes from patients with IgG4-RD and, for the first time, showed the clinical relevance of circulating PD-1$^+$CXCR5$^-$CD4$^+$ T cells (termed Tph-like cells here), which are increased in this disease. PD-1 has been postulated as a common activation molecule for naïve and memory phenotypes of CD4$^+$ T cells in peripheral blood and lymphoid tissues and even in synovia from patients with RA [14, 15]. Thus, Tph-like cells in IgG4-RD would include recently activated and/or memory CD4$^+$ T cells. The precise mechanism by which Tph-like cells in IgG4-RD are activated and then maintained is still unclear, although it has been suggested that B cells, which are abundantly localized in the lesions of IgG4-RD, might act as antigen-presenting cells that are probably related to the pathogenesis of IgG4-RD [16]. As shown in supplementary Fig. S5, available at *Rheumatology Advances in Practice* online, significant correlations were found between circulating PD-1$^+$CXCR5$^+$CD4$^+$ T cells (Tfh cells) and PD-

Fig. 2 Clinical relevance of circulating PD-1$^+$CXCR5$^-$CD4$^+$ T cells in IgG4-related disease



(**A**) Scatter plots demonstrating the relationships of the percentage of PD-1$^+$CXCR5$^-$CD4$^+$ T cells with serum IgG4 (in milligrams per decilitre, $n = 53$), IgG4/IgG ratio ($n = 53$), number of involved organs ($n = 53$) and serum sIL-2R ($n = 46$) in IgG4-RD cases. (**B**) Graphs showing serum IgG4 levels (in milligrams per decilitre, left panel) and percentages of CD3$^+$CD4$^+$ cells (middle panel) and PD-1$^+$CXCR5$^-$CD4$^+$ T cells (right panel) from IgG4-RD patients before (Pre) and after (Post) glucocorticoid treatment. (**C**) Absolute numbers (per microlitre of blood) of PD-1$^+$CXCR5$^-$ cells in CD3$^+$CD4$^+$ cells from IgG4-RD patients before (Pre) and after (Post) glucocorticoid treatment. (**D**) Percentages of GZMA$^+$ cells within PD-1$^+$ Tfh cells and PD-1$^+$CXCR5$^-$CD4$^+$ T cells in healthy volunteers (Healthy) and in patients with IgG4-RD are shown. (**E**) Absolute numbers (per microlitre of blood) of GZMA$^+$PD-1$^+$ Tfh cells and GZMA$^+$PD-1$^+$CXCR5$^-$CD4$^+$ T cells in healthy volunteers (Healthy) and in patients with IgG4-RD are shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$; ns: not significant. GZMA: granzyme A; IgG4-RD: IgG4-related disease; sIL-2R: soluble IL-2 receptor.

1$^+$CXCR5$^-$CD4$^+$ T cells (Tph-like cells) in the IgG4-RD, SS and healthy volunteer groups. Therefore, we presume that various subsets of CD4$^+$ T cells, including Tfh cells and Tph-like cells, are persistently activated in the unique environment of IgG4-RD. This possibility is also supported by recent evidence showing that CD4$^+$ T cells are massively accumulated in affected submandibular glands in patients with IgG4-DS [2].

Given that CXCR5 is preferentially expressed on Tfh cells and B cells and that CXCL13, the ligand of CXCR5, is produced by follicular dendritic cells and by high endothelial venule cells in lymphoid follicles, the pathological background associated with humoral immune responses in IgG4-RD has been focused on and analysed [16]. In light of this anatomical distribution of CXCR5 and CXCL13, attention should be paid to

the fact that the level of circulating Tfh cells (PD-1^{+}CXCR5^{+}CD4^{+} T cells) is significantly elevated in IgG4-RD, which is an important fact for study of the immunological mechanisms in the pathogenesis of IgG4-RD [2, 3]. In contrast to Tfh cells, Tph cells (PD-1^{hi}CXCR5^{-}CD4^{+} T cells), primarily found in synovia of RA, abundantly express chemokine receptors, including CCR2 and CX3CR1, and have a high capacity to secrete CXCL13 [5, 6]. Therefore, conventional Tph cells can recruit immune cells expressing CXCR5, such as Tfh cells and B cells. It remains unknown whether Tph-like cells (PD-1^{+}CXCR5^{-}CD4^{+} T cells) can make B cells become IgG4 producers, although it has been shown that Tfh cells, but not Th2 cells, in IgG4-RD have the capacity to help B cells differentiate into plasmablasts to produce IgG4 [2, 17]. Once Tph-like cells are recruited to affected lesions, it is possible that the interaction of Tfh cells with B cells that subsequently accumulate in the lesions provides an immune microenvironment in which production of IgG4 is induced. However, it needs to be clarified whether Tph-like cells in IgG4-RD can secrete CXCL13. To find out more about the pathological role of Tph-like cells in IgG4-RD, the expression profile of chemokine receptors, such as CCR2 and CX3CR1, expressed in Tph cells should be analysed [5]. Given that fractalkine, which is a ligand of CX3CR1, is expressed by activated endothelial cells within inflamed tissues, Tph cells might have a potential role in the initiation of inflammation and maintenance of the chronic fibroinflammation of IgG4-RD by influencing vascularity [18]. Destructive inflammation is observed in IgG4-RD, and our experimental evidence indicates that Tph-like cells that preferentially contained a cytotoxic granule of GZMA are responsible for these pathological changes in IgG4-RD. In the present study, we could not see abundant expression of *IL4* and *IL21* mRNAs in Tph-like cells compared with that in Tfh cells from patients with IgG4-RD (supplementary Fig. S4, available at *Rheumatology Advances in Practice* online). Taken together, Tph-like cells in IgG4-RD would play a pathological role as CD4^{+} cytotoxic T cells rather than as B cell helpers, such as Tfh cells.

In conclusion, circulating Tph-like cells seem to have pathological importance in IgG4-RD. Diagnosis of IgG4-RD remains difficult owing to its extensive spectrum of clinical manifestations. Moreover, cellular and molecular parameters, such as IgG4^{+} plasma cells in affected tissues and serum IgG4 level, are neither sensitive nor specific for the diagnosis of IgG4-RD. Thus, the increased level of Tph-like cells in patients with IgG4-RD could support the diagnosis and monitoring of the efficacy of standard glucocorticoid therapy.

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Supplementary data

Supplementary data are available at *Rheumatology Advances in Practice* online.

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