CCR4+Tfh2 cells specifically produce IL-4 driving the pathological reaction in IgG4-related disease

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Abstract

Objective

Human T follicular helper (Tfh) cells are classified into three subsets: Tfh1, Tfh2, and Tfh17 cells. Among them, Tfh2 cells are defined as CXCR3-negative and CCR6-negative, and may contain diverse cell populations. We examined whether CCR4 serves as a marker for identifying Tfh2 cells that produce interleukin (IL)-4 and its involvement in IgG4-related disease (IgG4-RD).

Methods

Single cell analysis of IL-4-producing Tfh subset was performed using multi-colour flow cytometry and t-SNE method. Blood samples were obtained from 23 treatment-naïve patients with active IgG4-RD. CCR4+Tfh2 cells were also assessed in affected tissues of IgG4-RD by flow cytometry and immunohistochemical staining.

Results

Tfh2 cells expressing CCR4 were identified as Tfh cells that specifically produce IL-4. CCR4+Tfh2 cells showed higher expression of GATA-3 and ICOS than CCR4-Tfh2 cells, while there was no difference in the expression of BCL-6 and FOXP3. The proportion of CCR4+Tfh2 cells in peripheral blood was increased in IgG4-RD compared to healthy controls, and even more CCR4+Tfh2 cells infiltrated into the affected lesions. CCR4+GATA-3+Tfh2 cells diffusely infiltrated tertiary lymphoid tissues and storiform fibrosis lesions. The proportion of CCR4+Tfh2 cells showed a significant correlation specifically with serum IgG4 levels among clinical indicators. Glucocorticoid therapy did not correct the increased proportion of CCR4+Tfh2 cells.

Conclusion

CCR4 serves as a marker for identifying Tfh2 cells that specifically produce IL-4. CCR4+Tfh2 cells are a widely present T cell population that infiltrates tertiary lymphoid tissues and storiform fibrosis of IgG4-RD. Glucocorticoid fails to effectively target CCR4+Tfh2 cells that may contribute to a high relapse rate during glucocorticoid tapering in this disease.

Key words

CC chemokine receptor 4, interleukin-4, T follicular helper cells, IgG4-related disease, IgG4

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Competing interests: see page 442.

Introduction

T follicular helper (Tfh) cells are a subset of CD4+helper T cells that are involved in humoral immune responses (1-4). Tfh cells contribute to germinal centre formation in secondary lymphoid tissues, such as lymph nodes, and to the selection of high-affinity B cells, as well as the induction of differentiation into memory B cells and plasma cells. As Tfh cells express CXCR5, they migrate to germinal centres and play an important role in T cell-dependent antibody production. It is also known that there are three subsets of human Tfh cells: Tfh1 (CXCR3+CCR6-), Tfh2 (CXCR3-CCR6-), and Tfh17 (CXCR3-CCR6+) cells with distinct cytokine production and B-cell helper ability (5, 6).

IgG4-related disease (IgG4-RD) is a systemic chronic inflammatory disease characterised by significant infiltration of IgG4+plasma cells in affected organs and often accompanied with high blood levels of IgG4 (7-10). In the pathogenesis of this disease, an increase in peripheral blood Tfh2 cells correlates with serum IgG4 levels and the number of tissue-infiltrating IgG4+plasma cells (11-15). Furthermore, Tfh2 cells are the only CD4+helper T cell subset that can functionally induce the differentiation of IgG4+plasma cells from naive B cells (11). Notably, IgG4 is unique to humans, and IL-4 has been shown to induce class-switching to IgG4 (16, 17). Therefore, it is assumed that there is a group of cells in Tfh2 cells that produce IL-4 directly related to the pathophysiology. However, given that Tfh2 cells are characterised by being CXCR3-negative and CCR6-negative, it raises the possibility that this population might be heterogeneous. An appropriate surface marker to identify IL-4-producing Tfh2 cells has not yet been clarified.

CC chemokine receptor 4 (CCR4) is one of the receptors for chemokines that are involved in leukocyte migration. In human T cells, CCR4-expressing cells have been reported to produce IL-4 but not IFNγ (18, 19). In addition, CCR4 was reported to be expressed in Th2, Th17, and Tregs in human CD4+T cells (16, 17). However, there have been no reports on CCR4 expression in human Tfh cells, and its association with Tfh2 cells are completely unknown.

In this study, we investigated whether CCR4 could be a surface marker for identifying IL-4-producing Tfh2 cells. Furthermore, we aimed to examine the involvement of CCR4+Tfh2 cells in the pathogenesis of IgG4-RD.

Materials and methods

Patients and controls

In this study, 23 consecutive patients with active, untreated IgG4-RD (11 males and 12 females; median age, 64 years (36-86), as shown in Supplementary Table S1) and 21 healthy controls (11 males and 10 females; median age, 31 years (22-81)) were included. It is noteworthy that the cohort in this study exhibits a higher proportion of female patients (52%) and a greater prevalence of the 'head and neck' phenotype compared to the 'pancreas and bile ducts' phenotype. All patients with IgG4-RD fulfilled the 2011 comprehensive IgG4-RD diagnostic criteria (20) or the 2019 American College of Rheumatology/European League Against Rheumatism classification criteria (21). Disease activity was assessed based on the IgG4-RD responder index score (22). Healthy controls were confirmed to have no autoimmune diseases, allergic disorders, malignancies, or infections. This study was approved by the ethics committee of Keio University School of Medicine and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all the patients and healthy controls.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were purified using gradient centrifugation with a Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). For detecting CCR4+Tfh2 cells (CD3+CD4+CD45RA-CXCR5 +CXCR3-CCR6-cells), enriched PBMCs were stained for 30 minutes with the fluorescent-conjugated antibodies (Suppl. Table S2). For intracellular IL-4 staining, CD4+T cells were enriched from PBMCs using a CD4 T cell isolation kit (Miltenyi Biotec; Bergisch Gladbach, Germany) according to the manufacturer's instructions. Those cells (2 ×

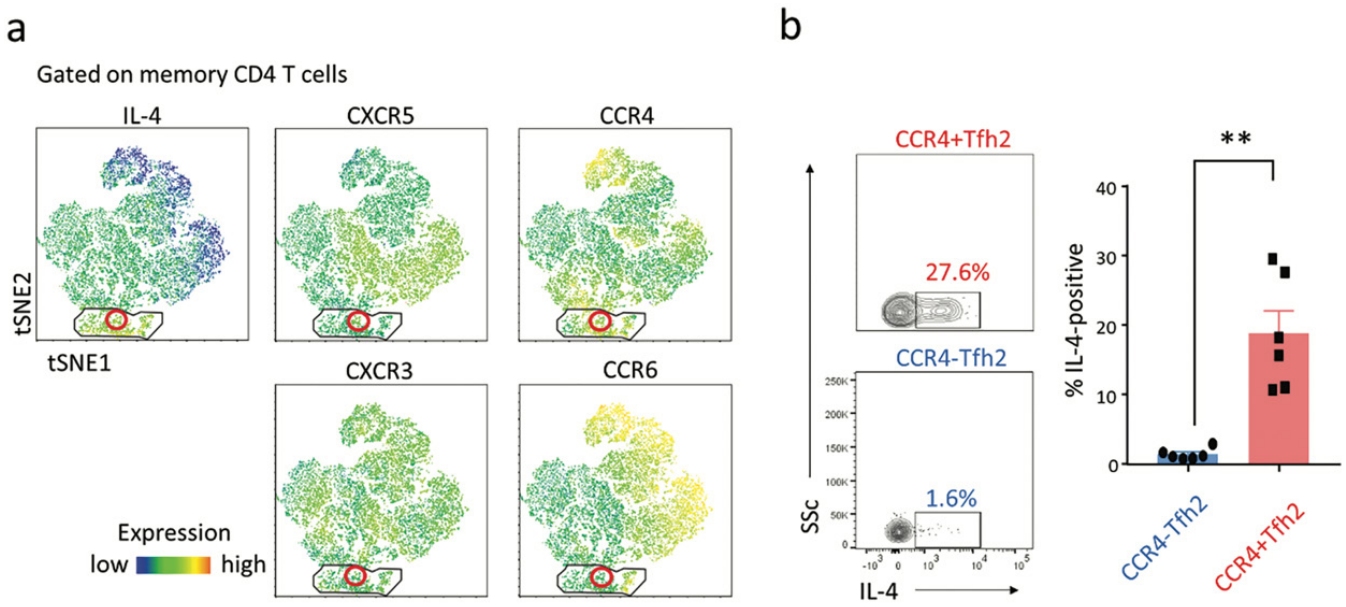

Fig. 1. Identification of CCR4 as a marker for IL-4-producing Tfh2 cells.
a: Memory CD4 T cells (CD3+CD4+CD45RA- cells) were mapped at the single cell level using the t-SNE method, and a cell population producing IL-4 was highlighted by a black box, while IL-4-producing CXCR5+Tfh cells were enclosed in a red circle. IL-4-producing Tfh cells were identified as CCR4+Tfh2 cells (CXCR5+CXCR3-CCR6-cells).
b: The percentage of IL-4-producing cells was compared between CCR4+Tfh2 cells and CCR4-Tfh2 cells in multiple samples (n=6). Paired t test. $^{**}p<0.01$.

10^5/200 µL/well) were stimulated for 4 hours with phorbol myristoyl acetate (PMA) [50 ng/ml] and ionomycin (Sigma, St. Louis, MO, USA) [1 µg/ml] along with Golgistop (BD biosciences, San Jose, CA, USA) [4 µl of BD GolgiStop™ for every 6 mL of cell culture] in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum in 96-well round-bottom plates in humidified atmosphere at 37°C with 5% CO_2. Following stimulation, cells were stained for 30 minutes for the cell-surface marker with the fluorescent-conjugated antibodies (Suppl. Table S2). Cells were then fixed and permeabilised with an Intracellular Fixation & Permeabilization Buffer Set (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol and incubated with PerCP-Cy5.5-conjugated anti-human IL-4 (Clone MP4-25D2; BioLegend) for 40 minutes. Cells were then washed and acquired on a LSR-Fortessa X-20 (BD Biosciences) and data were analysed by FlowJo v. 7.6.4 software (Tree Star, Stanford University, CA, USA). We performed single cell mapping using a t-Distributed Stochastic Neighbor Embedding (tSNE) algorithm (23). The longitudinal analysis was performed for 7 out of the 23 IgG4-RD patients. The time interval between the initial blood collection before the initiation of glucocorticoid therapy and the subsequent blood collection after therapy was 3 months. The lesion tissue used for flow cytometry analysis was pericardial effusion obtained from a patient with IgG4-RD who had developed pericarditis. The accumulated pericardial effusion was collected using pericardiocentesis into a sterile tube. After centrifugation of the pericardial effusion, the supernatant was aspirated and removed. The remaining cells were then resuspended in the same amount of buffer, stained, and subsequently analysed by flow cytometry.

Immunohistochemistry

Immunohistochemical staining was performed using paraffin-embedded lacrimal gland tissue sections of patients with IgG4-RD (n=3). The staining process was performed using an automated immunostaining system (BOND), which included deparaffinisation, antigen retrieval (20 minutes in citrate-based pH 6.0), incubation with the primary antibody (30 minutes), peroxide block (5 minutes), DAB staining (two rounds of 5 minutes each), and haematoxylin staining (5 minutes). The primary antibodies (CCR4 Antibody, Novus Biologicals, Cat. no. NBP1-86584; Monoclonal Mouse anti-Human GATA3 Antibody, Sigma-Aldrich, Cat. no. LS-C348458; Human CXCR5 Antibody, R&D systems, Cat. no. MAB190R-SP) were used at a dilution of 1:50.

Statistical analysis

Group-wise comparisons were performed using the Mann-Whitney U-test. Differences between pre- and post-treatment data were assessed using the Wilcoxon signed-rank test or paired t-test. Correlations between two groups were analysed using Spearman's correlation coefficient. Statistical significance was determined using GraphPad Prism software v. 6.0 (GraphPad software; San Diego, CA, USA), with two-sided $p<0.05$ considered significant.

Results

Human CCR4+Tfh2 cells specifically produce IL-4

To identify subsets of human CD4+ helper T cells that produce IL-4 using an unbiased analysis method, we first conducted a comprehensive analysis at the single cell level using the t-SNE method (23). The experimental

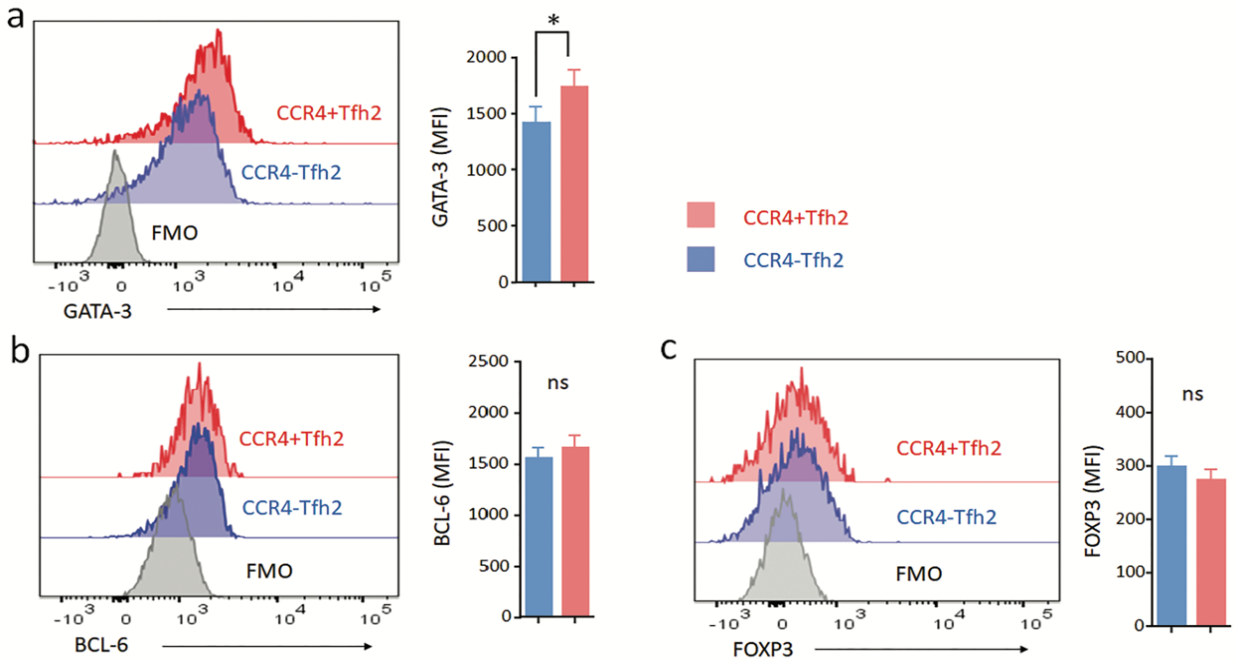

Fig. 2. CCR4+Tfh2 cells exhibit higher expression of GATA-3.
Expression levels of intracellular transcription factors (GATA-3, BCL-6, FOXP3) were compared between CCR4+Tfh2 cells and CCR4-Tfh2 cells by flow cytometry analysis (n=3). Paired t test. *p<0.05, ns: not significant.

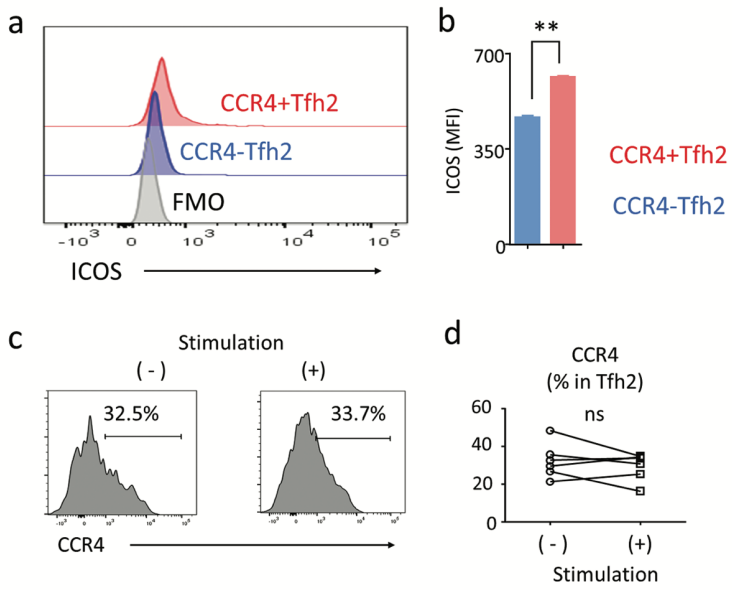

Fig. 3. CCR4+Tfh2 cells express ICOS and were not just the activated phenotype.
We compared the expression of cell surface ICOS between CCR4+Tfh2 cells and CCR4-Tfh2 cells using flow cytometry (**a**), representative figure and (**b**), analysis in multiple cases, n=3). Paired t-test. **p<0.01. We also analysed the changes in CCR4 expression in Tfh2 cells after stimulation with PMA/Ionomycin (**c**), representative figure and (**d**), analysis in multiple cases, n=6). Paired t test. ns: not significant.

results were obtained using CD4+T cells isolated from PBMCs of healthy individuals. As a result, we found that CD4+helper T cells that produce IL-4 were Tfh2 cells (CXCR5+CXCR3-CCR6-), Th2 cells (CXCR5-CXCR3-CCR6- CCR4+), and Th17 cells (CXCR5-CXCR3-CCR6+) (Fig. 1a). Of these T cell subsets, Tfh2 cells are the only CD4+helper T cells that can induce the differentiation of naive B cells into IgG4-producing B cells (11). Importantly, we found that Tfh2 cells producing IL-4 were a subtype limited to CCR4-expressing Tfh2 cells (Fig. 1a). Further verification using multiple samples revealed that CCR4+Tfh2 cells consistently produced higher levels of IL-4 compared to CCR4-Tfh2 cells (p=0.002) (Fig. 1b). These results suggest that CCR4 is a useful surface marker for identifying IL-4-producing Tfh2 cells.

GATA-3 and ICOS are highly expressed in CCR4+Tfh2 cells

Next, we examined the expression of transcription factors in CCR4+Tfh2 cells. We analysed the expression of GATA-3, which has been reported to be expressed in Tfh2 cells (5, 6), BCL-6, known as the master transcription factor of Tfh cells (1-4), and FOXP3, which is reported to be expressed in CCR4+Tregs (19). As a result, we found that the expression level of GATA-3 was higher in CCR4+Tfh2 cells compared to CCR4-Tfh2 cells (p=0.027) (Fig. 2a). On the other hand, there was no significant difference in the expression level of BCL-6 and FOXP3 between CCR4+ and CCR4-Tfh2 cells (p=0.084 and p=0.082) (Fig. 2b-c).

The maintenance of Tfh-cell characteristics is crucial for B-cell helper activity and germinal centre formation, and co-stimulatory signals via ICOS play an essential role in this process (24).

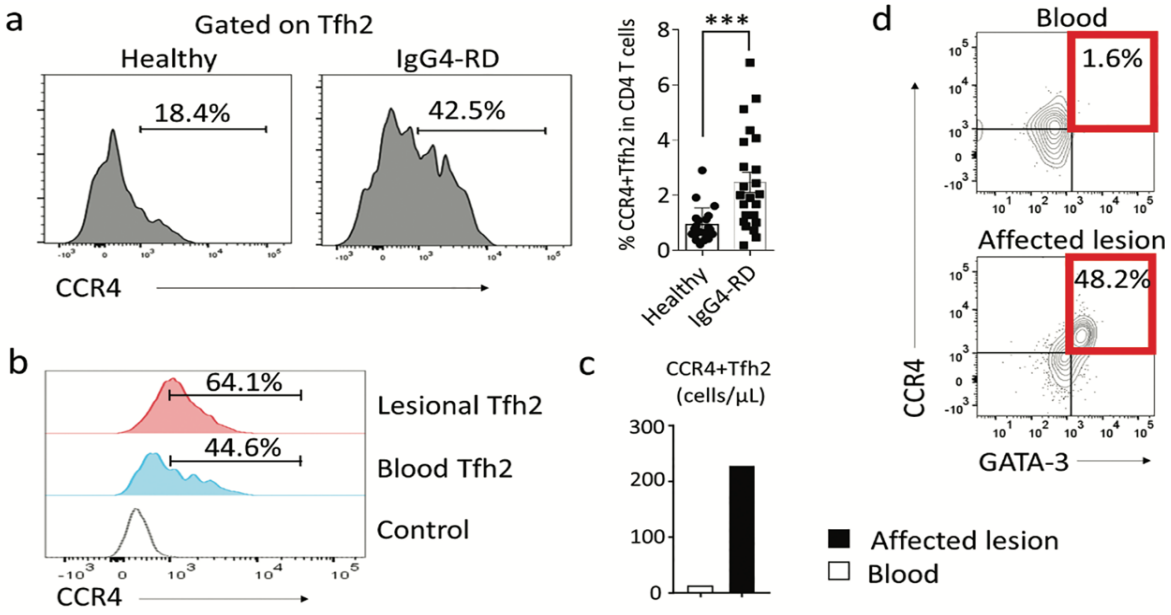

Fig. 4. CCR4+Tfh2 cells are increased in peripheral blood and infiltrated into the affected lesions in IgG4-RD.
We compared the proportion of CCR4+Tfh2 cells in peripheral blood between IgG4-RD patients (n=23) and healthy controls (n=21) using flow cytometry (**a**). Mann-Whitney U-test. ***p<0.001. In an IgG4-RD patient who provided paired samples of peripheral blood and lesion site (pericardial fluid), we analysed the proportion (**b**) and absolute number (**c**) of CCR4+Tfh2 cells and GATA-3 expression (**d**).

Therefore, we examined the expression of ICOS in CCR4+Tfh2 cells. We found that the expression level of ICOS was higher in CCR4+Tfh2 cells than in CCR4-Tfh2 cells (p=0.004) (Fig. 3a-b). We also compared the proportion of CCR4+Tfh2 cells between stimulated and unstimulated cells, but found no increase in the proportion of CCR4+Tfh2 cells (p=0.37) (Fig. 3c-d). Therefore, these data suggest that CCR4+Tfh2 cells are not simply an activated cell population. Taken together, CCR4+Tfh2 cells that produce IL-4 has higher expression of the transcription factor GATA-3 and the co-stimulatory molecule ICOS, and is a distinct cell subset in terms of transcription factor expression and molecular phenotype.

CCR4+Tfh2 cells are increased in blood of IgG4-RD and dominate the tissue infiltrates

IgG4-RD is a representative immunological inflammatory disease in which Tfh2 cells play a role in the pathology (11-15). It has been found that Tfh2 cells in this disease have an enhanced ability to induce differentiation of naive B cells into IgG4-producing B cells compared to healthy individuals' Tfh2 cells (11). In addition, IL-4 produced by Tfh2 cells is involved in this process (16, 17). Therefore, we analysed whether CCR4+Tfh2 cells are increased in IgG4-RD. As a result, we discovered that the proportion of CCR4+Tfh2 cells in peripheral blood is increased in this disease compared to healthy controls (p=0.0002) (Fig. 4a), while total CD4 counts were not different between IgG4-RD and healthy controls (p=0.73) (Suppl. Fig. S1). Furthermore, we successfully compared a paired sample of CCR4+Tfh2 cells in peripheral blood and lesion site (pericardial effusion) in the identical patient. Pericardial effusion obtained from a patient with IgG4-RD who developed pericarditis was collected into a sterile tube via pericardiocentesis. The result revealed that CCR4+Tfh2 cells are further increased and infiltrating in the lesion site of IgG4-RD compared to peripheral blood (Fig. 4b-c). Moreover, those CCR4+Tfh2 cells in the lesion site exhibited higher expression of GATA-3 than CCR4+Tfh2 cells in peripheral blood (Fig. 4d).

Further verification was performed analysing T cell infiltrates at tertiary lymphoid tissues and storiform fibrosis of lacrimal gland lesions from patients with IgG4-RD by immunohistochemical staining. Notably, CCR4+CXCR5+GATA3+Tfh2 cells diffusely infiltrated tertiary lymphoid tissues and storiform fibrosis at the lesion site in all examined cases (Fig. 5). CCR4+Tfh2 cells are a widely present T cell population infiltrating the affected tissues of IgG4-RD, suggesting its involvement in the pathogenesis.

CCR4+Tfh2 cells are specifically correlated with serum IgG4 levels or IgG4 to IgG ratio

Next, we analysed the correlation between the increase of CCR4+Tfh2 cells in peripheral blood and clinical indicators of IgG4-RD. Interestingly, the proportion of CCR4+Tfh2 cells showed a significant positive correlation only with serum IgG4 levels (p=0.026) or IgG4 to IgG ratio (p=0.012), but not with serum IgG levels (p=0.087), serum IgE levels (p=0.21), blood eosinophil counts (p=0.44), disease activity scores (p=0.41), the number of affected organs (p=0.46), or soluble IL-2 receptor levels (p=0.11) (Fig. 6a).

The proportion of CCR4+Tfh2 cells are not corrected by glucocorticoid therapy

Finally, we measured the longitudinal changes in the proportion of CCR4+Tfh2 cells before and after glucocorticoid therapy in patients with

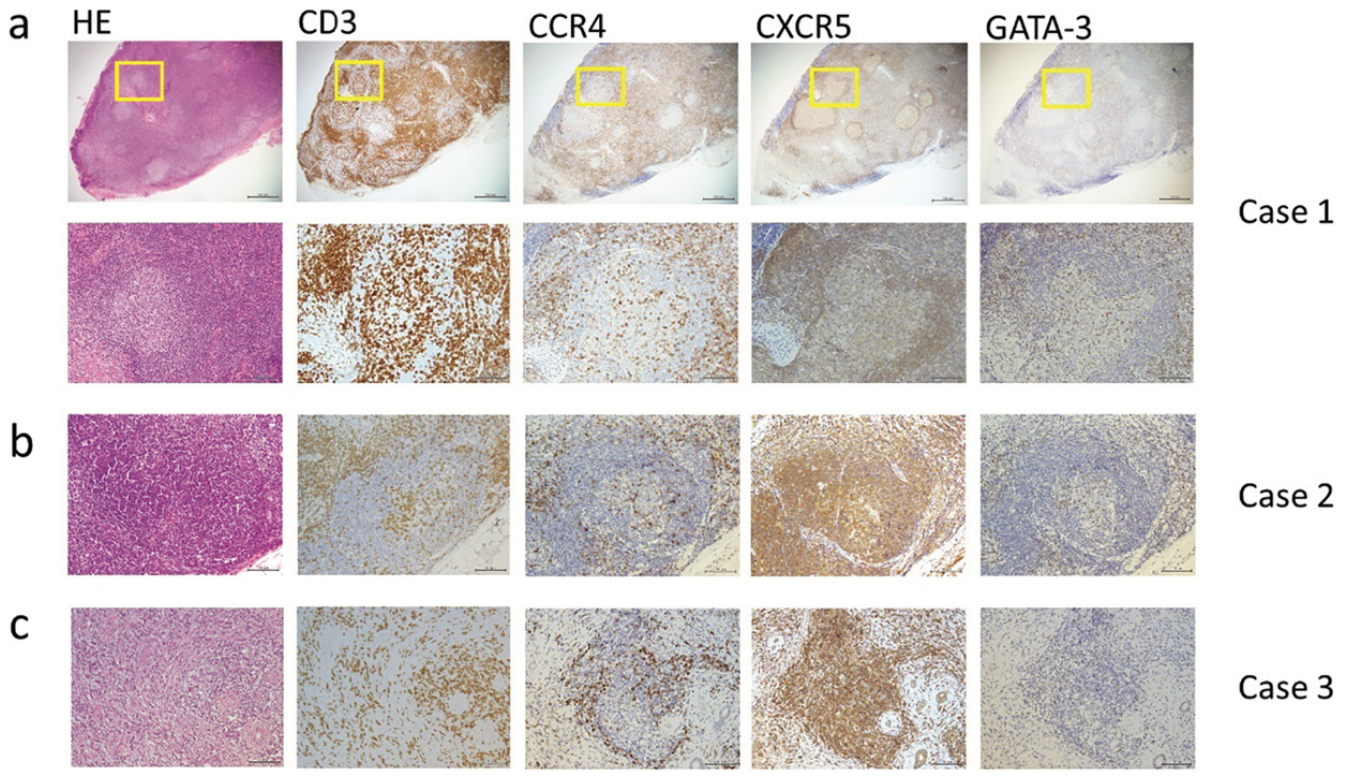

Fig. 5. CCR4+Tfh2 cells in tertiary lymphoid tissues and storiform fibrosis of patients with IgG4-RD.
Tissue biopsies were collected from lacrimal gland lesions of patients with IgG4-RD (n=3). H&E (haematoxylin and eosin)-stained sections, CD3-stained sections, CCR4-stained sections, CXCR5-stained sections, GATA-3-stained sections are shown. CCR4+Tfh2 cell infiltration is observed in tertiary lymphoid tissues (**a**-**c**) as well as storiform fibrosis lesions (**c**). Scale bar shows 500μm at low magnification and 100μm at high magnification.

IgG4-RD (n=7). Three months after therapy, IgG4-RD RI scores (p=0.016) and serum IgG4 levels (p=0.015) significantly improved in all cases, but the proportion of CCR4+Tfh2 cells did not improve (p=0.69) (Figure 6b). These results suggest that while glucocorticoid therapy improves clinical symptoms, it does not correct the increase in CCR4+Tfh2 cells.

Discussion

In this study, CCR4 was identified as a marker for detecting Tfh2 cells that specifically produce IL-4. We found that CCR4+Tfh2 cells were not just the activated phenotype but the functionally meaningful phenotype exclusively producing IL-4. Interestingly, this newly identified CCR4+Tfh2 cells exhibited higher expression of GATA-3 and ICOS. We also found that CCR4+Tfh2 cells were increased in patients with active untreated IgG4-RD in peripheral blood, correlating specifically with their levels of serum IgG4 or IgG4 to IgG ratio. Moreover, CCR4+Tfh2 cells dominated tertiary lymphoid tissues and storiform fibrosis of IgG4-RD. Thus, our findings suggest that CCR4+Tfh2 cells may be involved in IgG4 class switching and fibrosis development in this disease. Glucocorticoid therapy improved clinical symptoms; however, it failed to correct the increase in CCR4+Tfh2 cells. Therefore, the development of novel treatment strategies targeting CCR4+Tfh2 cells holds the potential to improve the management of this condition.

Tfh cells are classified into three subsets, Tfh1, Tfh2, and Tfh17 cells, based on their expression pattern of CXCR3 and CCR6. These three subsets are known to produce important effector cytokines such as IFNγ, IL-4, and IL-17, respectively (5, 6). However, among these subsets, only Tfh2 cells are defined by a subset in which both CXCR3 and CCR6 are negative, suggesting that it may contain a diverse population of cells. In this study, we identified CCR4 as a cell-type specific surface marker for functional Tfh2 cells that produce IL-4. Importantly, CCR4+Tfh2 cells were found to express higher levels of the transcription factor GATA-3 and the co-stimulatory molecule ICOS, suggesting that they are a molecularly independent subset of cells. In the future, it will be interesting to investigate the involvement of this T cell subset in the pathogenesis of other autoimmune diseases. Furthermore, it is expected that the origin and differentiation mechanism of CCR4+Tfh2 cells will be elucidated in detail at the molecular level in the future.

Maehara *et al*. reported that a large proportion of Tfh cells in the affected tissues and secondary lymphoid organs secreted IL-4 compared to those from healthy tonsillar Tfh cells, suggesting that those IL-4-producing Tfh cells are the pathogenic immune cell subset in IgG4-RD (17). Furthermore, the accumulation of those IL-4-producing Tfh cell subset in affected tissues was tightly associated with serum IgG4 levels and the proportion of IgG4+plasma cells, consistent with our previous study that showed contribution of IL-4 to the shift of balance toward IgG4 class-switching in this disease (16). Interestingly, we

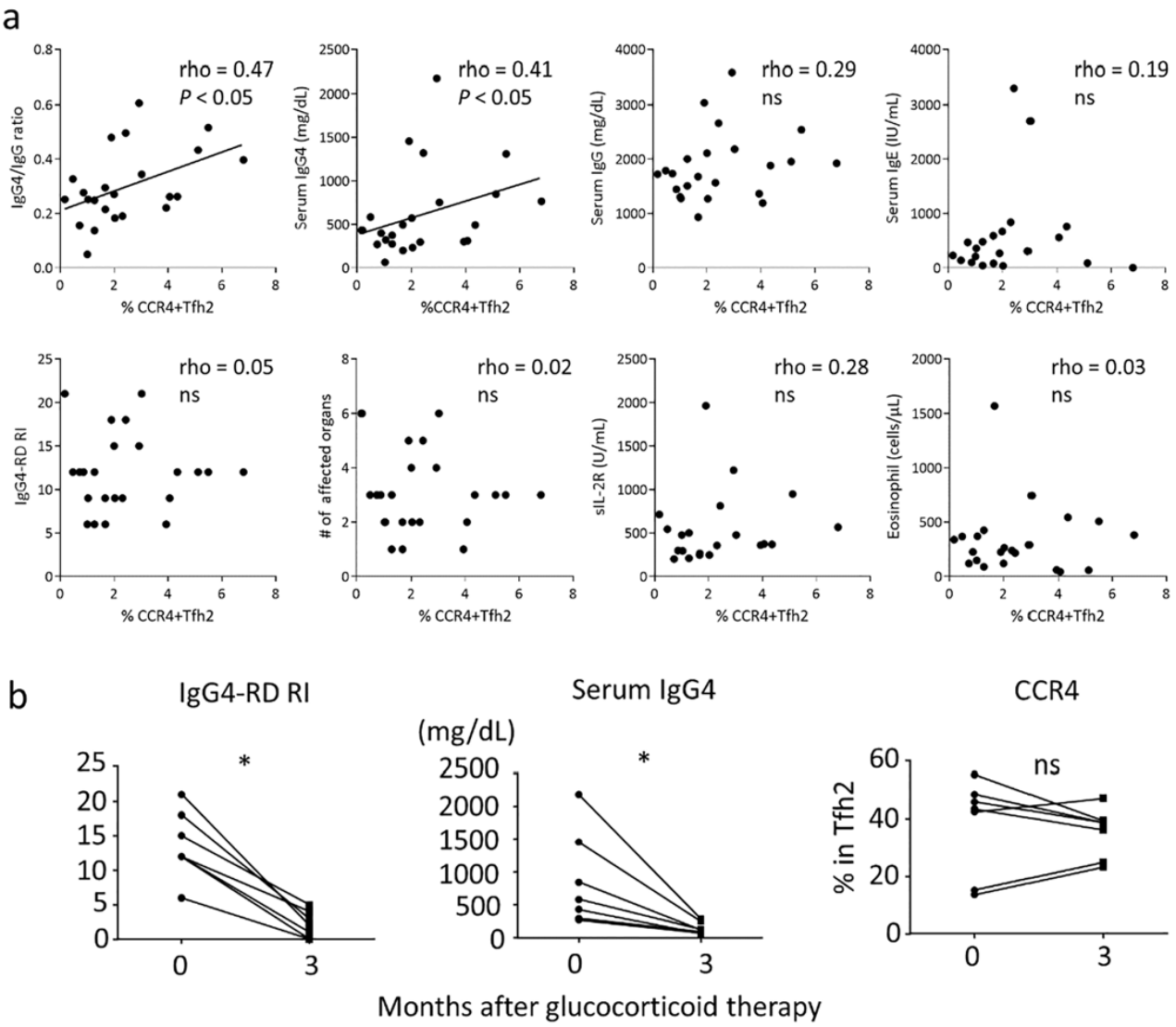

Fig. 6. Correlation of blood CCR4+Tfh2 cells levels with clinical indicators.
a: We conducted a correlation analysis between the proportion of CCR4+Tfh2 cells and clinical parameters (serum IgG4/IgG ratio, IgG4 levels, IgG levels, IgE levels, disease activity scores, number of affected organs, soluble IL-2 receptor levels, and blood eosinophil counts). Spearman's correlation coefficient. **b**: We evaluated the longitudinal changes in disease activity scores, serum IgG4 levels, and the proportion of CCR4+Tfh2 cells, before and three months after starting glucocorticoid therapy. Wilcoxon signed-rank test. *p<0.05, ***p<0.001, ns: not significant.

found that CCR4+Tfh2 cells were increased in peripheral blood of IgG4-RD patients and showed marked infiltration of this immune cell subset in the tertiary lymphoid tissues of lesion sites of lacrimal glands. Importantly, IgG4+B cell differentiation is actively induced at the germinal centre of tertiary lymphoid tissues and Tfh2 cells were an only T cell subset which could induce IgG4+B cell differentiation (11, 15). Furthermore, we found in this study that CCR4+Tfh2 cells were significantly and positively correlated only with serum IgG4 levels or IgG4 to IgG ratio among the clinical parameters. Therefore, CCR4+Tfh2 cells may be involved in the induction of class-switching to IgG4 through IL-4 production in this disease.

In IgG4-RD, it will be an important issue in the future whether CCR4+Tfh2 cells are also involved in the formation of fibrotic lesions. Previous studies have reported that IgG4-producing B cells and M2 macrophages differentiated by IL-4 are involved in fibrosis formation in IgG4-RD (25-27). Furthermore, we found that CCR4+Tfh2 cells infiltrated not only tertiary lymphoid tissues but also storiform fibrosis lesions of IgG4-RD. Thus, CCR4+Tfh2 cells may be indirectly involved in the formation of fibrotic lesions in this disease through IL-4 production.

In this study, the increased CCR4+Tfh2 cells in IgG4-RD patients were not corrected by glucocorticoid therapy. This suggests that glucocorticoid therapy is not a definitive treatment for IgG4-RD, as corroborated by the fact that glucocorticoid tapering frequently causes disease recurrence in this disease (28, 29). On the other hand, in recent years, reports on the efficacy of dupilumab, an anti-IL-4Rα antibody, for IgG4-RD have been increasing (30-33). In some cases, IgG4-RD improved with dupilumab monotherapy, suggesting a deep involvement of IL-4 in the pathology of this disease. It is noteworthy that a marked decrease of Tfh2 cells

was observed in IgG4-RD treated with dupilumab (34).

We have newly discovered in this study that Tfh2 cells express CCR4, which raises the possibility of CCR4 being used as a therapeutic target for immune-inflammatory diseases in the future. Mogamulizumab, an anti-CCR4 monoclonal antibody, is already being clinically used in haematological malignancies as a molecular targeted drug (19). Recently, it has also been reported to have potential as an immune checkpoint inhibitor for solid tumours, as it was able to selectively eliminate Tregs expressing CCR4 in cancer tissues, leading to therapeutic effects (35). Tregs are believed to suppress excessive immune responses such as autoimmune diseases, inflammatory diseases, and allergic diseases. On the other hand, cancer cells are thought to avoid attacks from immune cells by activating these Tregs.

In conclusion, we newly discovered that CCR4 is a useful marker for identifying a subset of Tfh2 cells that produce IL-4. CCR4+Tfh2 cells may be associated with class-switching to IgG4 and fibrosis formation in IgG4-RD. Glucocorticoid therapy is insufficient for the fundamental correction of CCR4+Tfh2 cells associated with this disease. By elucidating the more detailed molecular mechanisms of CCR4+Tfh2 cells in the future, we hope to discover novel therapeutic targets.

Competing interests

M. Akiyama has received speaker fees from Asahikasei, Astellas, Boehringer Ingelheim, Chugai, Eisai, Eli Lilly, Gilead, Janssen, Otsuka, Pfizer, Taisho, and UCB.

H. Yasuoka has received grants from Asahi Kasei Pharma, Mitsubishi-Tanabe Pharma, Chugai, Eisai, Taisho Pharma and speaker fees from Asahi Kasei Pharma, Abbvie, Boehringer Ingelheim, Ono Pharmaceutical, Bayer, Eli Lilly, Eisai, Janssen, Pfizer, Amgen, Astellas, Gilead Sciences, Teijin Pharma, Kissei Pharmaceutical, Nippon Kayaku, Viatris, Ayumi Pharmaceutical, Kyorin Pharmaceutical, Mitsubishi-Tanabe Pharma, Nippon Shinyaku.

S. Takanashi has received speaking fees from AsahiKasei, Astellas, Bristol-Myers Squibb, Eisai, Eli Lilly, Janssen, Mitsubishi-Tanabe, Taisho, and UCB.

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K. Yoshimoto and S. Ishigaki have declared no competing interests.

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