Significant association of CX3CR1+CD8 T cells with aging and distinct clinical features in Sjögren's syndrome and IgG4-related disease

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Abstract

Objective

Recent studies have implicated cytotoxic CD4 and CD8 T cells in primary Sjögren's syndrome (pSS) and IgG4-related disease (IgG4-RD), but their association with immune aging and organ-specific clinical features remain unclear. CX3CR1 is expressed on cytotoxic CD4 and CD8 T cells. The aim of this study was to determine associations of peripheral CX3CR1+CD4 and CX3CR1+CD8 T cells with aging and clinical features.

Methods

Whole blood samples were freshly obtained from consecutive patients with active, treatment-naïve pSS (n=57), IgG4-RD (n=54), and healthy individuals (n=40) and analysed by flow cytometry for CX3CR1+CD4 and CX3CR1+CD8 proportions. Associations of those T cells with aging and clinical features were determined.

Results

CX3CR1+CD4 and CX3CR1+CD8 T cells selectively expressed perforin and granzyme B. Proportions of CX3CR1+CD4 and CX3CR1+CD8 T cells were significantly higher in pSS and IgG4-RD than in healthy individuals. Higher proportions of CX3CR1+CD8 T cells were associated with aging in pSS and IgG4-RD but not in healthy individuals. Sex differences were not associated with proportions of CX3CR1+CD8 T cells. Furthermore, patients with pSS with interstitial lung disease showed higher proportions of CX3CR1+CD8 T cells than those without interstitial lung disease. IgG4-RD patients with retroperitoneal fibrosis and/or aortitis exhibited higher proportions of CX3CR1+CD8 T cells compared with those with Mikulicz's disease. Moreover, proportions of CX3CR1+CD8 T cells were decreased following glucocorticoid treatment in paralleled with clinical improvements in IgG4-RD.

Conclusion

CX3CR1+CD8 T cells might be involved in immune aging and distinct clinical phenotypes of patients with pSS or IgG4-RD.

Key words

CX3CR1, CD8, immune aging, Sjögren's syndrome, IgG4-related disease

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M. Akiyama has received speaker fees from Asahikasei, Astellas, Boehringer Ingelheim, Eli Lilly, Pfizer and UCB.
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Introduction

Primary Sjögren's syndrome (pSS) and IgG4-related disease (IgG4-RD) are the two immune-mediated inflammatory diseases that mainly affect lacrimal and salivary glands but also various extraglandular organs (1-5). For example, interstitial lung disease (ILD) is one of the most serious extraglandular involvements in pSS, leading to a significant morbidity (6, 7). In IgG4-RD, retroperitoneal fibrosis and aortitis are known to be a distinct clinical phenotype which can be life-threatening and show serum C-reactive protein elevation (8-10). Currently, the pathophysiological mechanisms of each clinical phenotype are unknown. A better understanding of the pathogenesis involved in the development of distinct clinical phenotypes of pSS and IgG4-RD should facilitate the discovery of new therapeutic targets based on clinical phenotypes.

The aetiology of pSS and IgG4-RD are also not fully understood. Since pSS and IgG4-RD are more common in the elderly, aging itself is thought to be a risk factor for these two diseases. However, the association of pSS and IgG4-RD with immunosenescence remains unclear.

Recent studies have reported that cytotoxic CD4 and CD8 T cells are involved in both pSS and IgG4-RD (11-18). Those cytotoxic CD4 and CD8 T cells are clonally expanded in peripheral blood and secrete cytotoxic molecules such as perforin and granzyme B, leading to organ damages (17, 18). However, it is currently unknown whether cytotoxic CD4 and CD8 T cell expansions are associated with aging and distinct clinical phenotypes in pSS or IgG4-RD. The fractalkine receptor, CX3C chemokine receptor 1 (CX3CR1), is a surface marker specific for a cytotoxic phenotype of CD4 and CD8 T cells (19, 20). In this study, we examined the association of peripheral CX3CR1+CD4 or CX3CR1+CD8 T cells with aging and clinical features in patients with pSS or IgG4-RD.

Materials and methods

Patients and controls

Fifty-seven patients with pSS, 54 patients with IgG4-RD and 44 healthy individuals were consecutively enrolled in this study. All patients with pSS or IgG4-RD were untreated and in active status at enrolment. Patients with pSS fulfilled the 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria (21, 22). Patients with IgG4-RD fulfilled the 2019 American College of Rheumatology/European League Against Rheumatism classification criteria for IgG4-RD (23, 24) or the 2011 comprehensive IgG4-RD diagnostic criteria (25). Healthy individuals were confirmed to have no autoimmune diseases, malignancies, infections, or allergic disorders. Characteristics of patients and healthy individuals are shown in Supplementary Tables S1-3. The present study was approved by the ethics committee of Keio University School of Medicine and carried out in accordance with the Declaration of Helsinki. We obtained written informed consent from all patients and healthy individuals.

Flow cytometric analysis

FACS analysis of the cells was performed by using whole blood samples from patients and healthy individuals according to the methods recommended by the manufacturers of the antibodies (BioLegend, BD Biosciences Pharmingen and Beckman coulter). In brief, to determine the proportion of CX3CR1+ cells among CD4 T cells or CD8 T cells, 50 μl of whole blood sample was incubated with Pacific Blue-labelled anti-human CD3 (BD Biosciences Pharmingen, San Diego, CA, USA, clone;UCHT1), APC-labelled anti-human CD4 (Beckman coulter, Shizuoka, Japan, clone; 13B8.2) or APC-labelled anti-human CD8 (Beckman coulter, Shizuoka, Japan, clone;B9.11) and PE-labelled anti-human CX3CR1 (BioLegend, San Diego, CA, USA, clone; 2A9-1) for 30min at room temperature. The cells were subsequently lysed and fixed by Lyse/Fix Buffer (BD Biosciences, San Jose, CA, U.S.A.) and applied to MACSQuant Analyzer (MACSQuant Analyzer®, Miltenyi Biotec, Bergisch Gladbach, Germany). For intracellular staining of perforin and granzyme B, cells were permeabilised and fixed

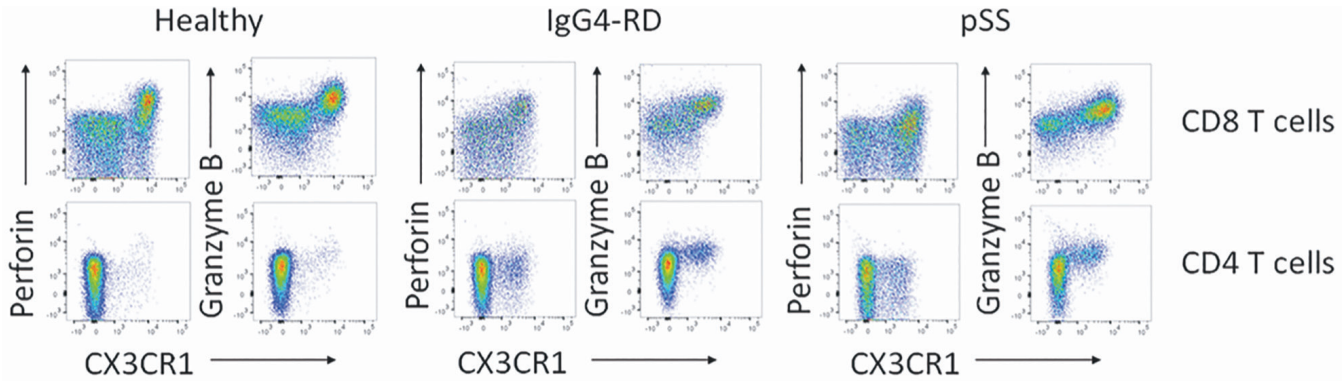

Fig. 1. Perforin and granzyme B production in CX3CR1+CD4 and CX3CR1+CD8 T cells.
We detected the production of perforin and granzyme B within CX3CR1+ T cells using flow cytometry, utilising fresh whole blood obtained from healthy individuals, pSS patients, and IgG4-RD patients.

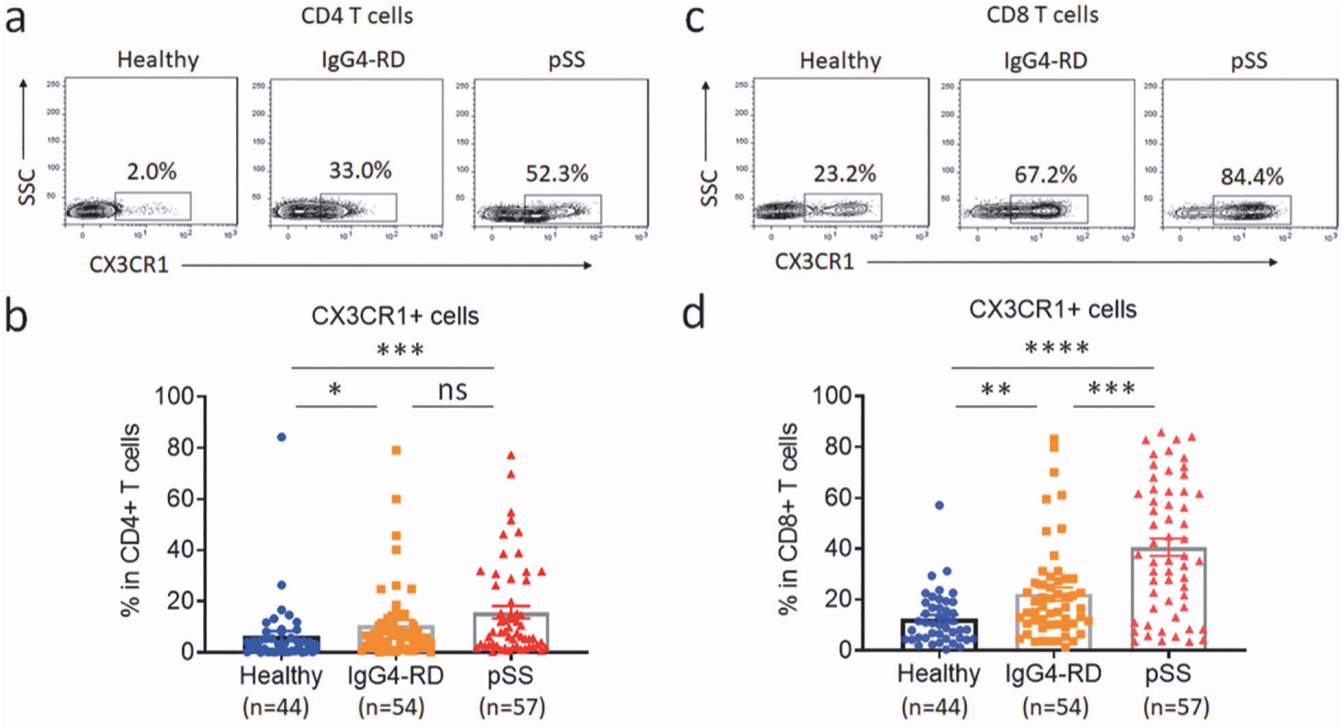

Fig. 2. Comparison of CX3CR1+CD4 and CX3CR1+CD8 T cells between patients and healthy individuals.
Whole blood samples were freshly obtained and immediately stained for detecting CX3CR1+CD4 T cells (**a**) and CX3CR1+CD8 T cells (**c**) by flow cytometry. Multiple comparison results for CX3CR1+CD4 T cells (**b**) and CX3CR1+CD8 T cells (**d**) are shown. Kruskal-Wallis test followed by Dunn's multiple comparisons test.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant.

using reagents (BD Pharmingen™ Transcription Factor Buffer Set), followed by a 30-minute incubation with antibodies (APC-labelled anti-human Perforin, BioLegend, San Diego, CA, USA, clone; dG9 and BV421-labelled anti-human Granzyme B, BD Biosciences, San Jose, CA, USA, clone; GB11).

Statistical analysis

The data were analysed using GraphPad Prism software (v. 7; GraphPad Software, La Jolla, CA). Two groups were compared by Mann Whitney test (unpaired samples) or Wilcoxon matched-pairs signed rank test (paired samples). To compare more than two groups, analysis of variance by Kruskal-Wallis test was used followed by Dunn's multiple comparisons test. Spearman's r was used for correlation analysis. A two-sided p<0.05 was considered statistically significant.

Results

Peripheral CX3CR1+CD4 and CX3CR1+CD8 T cells were increased in pSS and IgG4-RD

We first examined the expression of cytotoxic molecules (perforin and granzyme B) within peripheral CX3CR1+T cells. Consequently, we confirmed that CX3CR1+CD4 and CX3CR1+CD8 T cells selectively produced perforin and granzyme B, while the CX3CR1-negative cell population exhibited minimal

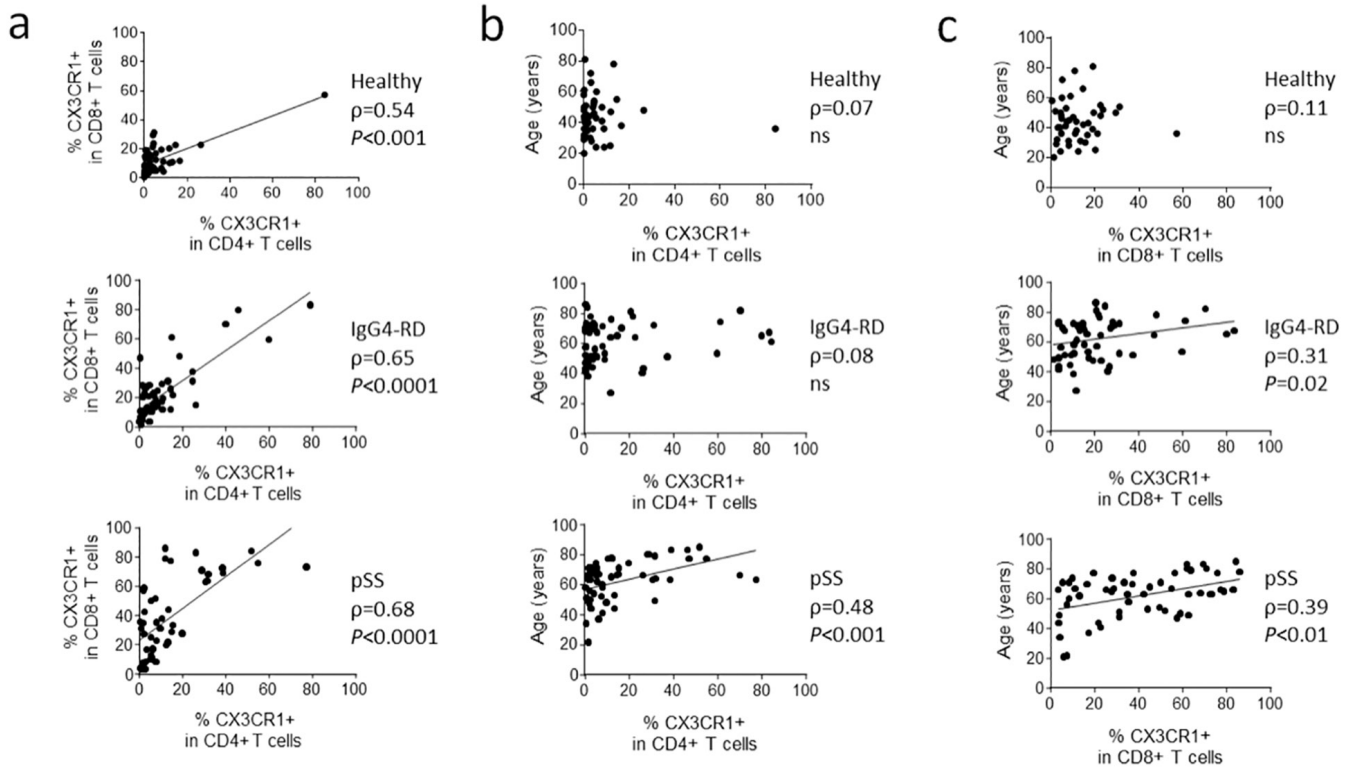

Fig. 3. Correlation of CX3CR1+CD8 T cells with aging in healthy individuals, IgG4-RD and pSS.
(**a**) Correlation was analysed between the proportion of CX3CR1+CD4 and CX3CR1+CD8 T cells in healthy individuals and patients with IgG4-RD or pSS. (**b**) Correlation of CX3CR1+CD4 T cells with aging was analysed in healthy individuals and patients with IgG4-RD or pSS. (**c**) Correlation analysis was performed between CX3CR1+CD8 T cells and aging in healthy individuals and patients with IgG4-RD or pSS. Spearman's correlation analysis.
ns: not significant.

production of these molecules (Fig. 1). We observed that the expression levels of perforin and granzyme B were higher in CX3CR1+CD8 T cells compared to CX3CR1+CD4 T cells. This phenomenon was observed consistently across healthy individuals, pSS, and IgG4-RD (Fig. 1). To examine the significance of CX3CR1+ T cells in peripheral blood from patients with pSS or IgG4-RD, we compared proportions of CX3CR1+CD4 and CX3CR1+CD8 T cells between patients and healthy individuals. We discovered a significant increase in CX3CR1+ T cells producing cytotoxic molecules in pSS and IgG4-RD compared with healthy individuals. Specifically, we found a higher proportion of CX3CR1+CD4 T cells in patients with pSS or IgG4-RD compared with healthy individuals (Fig. 2a, b). There was no significant difference in proportions of CX3CR1+CD4 T cells between pSS and IgG4-RD (Figure 2b). Patients with pSS or IgG4-RD also showed a higher proportion of CX3CR1+CD8 T cells than healthy individuals (Fig. 2c, d). Moreover, patients with pSS exhibited a higher proportion of CX3CR1+CD8 T cells compared with patients with IgG4-RD (Fig. 2d).

CX3CR1+CD8 T cells were associated with aging in pSS and IgG4-RD

To examine whether CX3CR1+CD4 and CX3CR1+CD8 T cells are coordinately increased in an identical patient, we next analysed the correlation between proportions of CX3CR1+CD4 T cells and those of CX3CR1+CD8 T cells. We found that proportions of CX3CR1+CD4 T cells showed a strong positive correlation with proportions of CX3CR1+CD8 T cells in both pSS and IgG4-RD as well as in healthy individuals (Fig. 3a). To examine whether the increase of CX3CR1+CD4 and CX3CR1+CD8 T cells are associated with aging, we next performed a correlation analysis of those T cells with age. We found that higher proportions of CX3CR1+CD8 T cells were associated with aging in pSS and IgG4-RD but not in healthy individuals (Fig. 3b, c). Since there is an increasing attention on the sex differences in immune responses, we further compared proportions of CX3CR1+CD4 or CX3CR1+CD8 T cells between female and male. As a result, there were no differences in proportions of those T cells between female and male in pSS and IgG4-RD (Fig. 4). Taken together, CX3CR1+CD8 T cells were increased in pSS and IgG4-RD and were associated with aging.

CX3CR1+CD8 T cells were associated with ILD involvement in pSS

Since we found the increased proportions of CX3CR1+CD4 and CX3CR1+CD8 T cells in patients with pSS, we examined whether CX3CR1+ T cells were associated with their clinical manifestations. There was no difference in proportions of CX3CR1+CD4 or CX3CR1+CD8 T cells regardless of the complication with extraglandular organ involvements (Fig. 5a); however, when analysed based on each organ

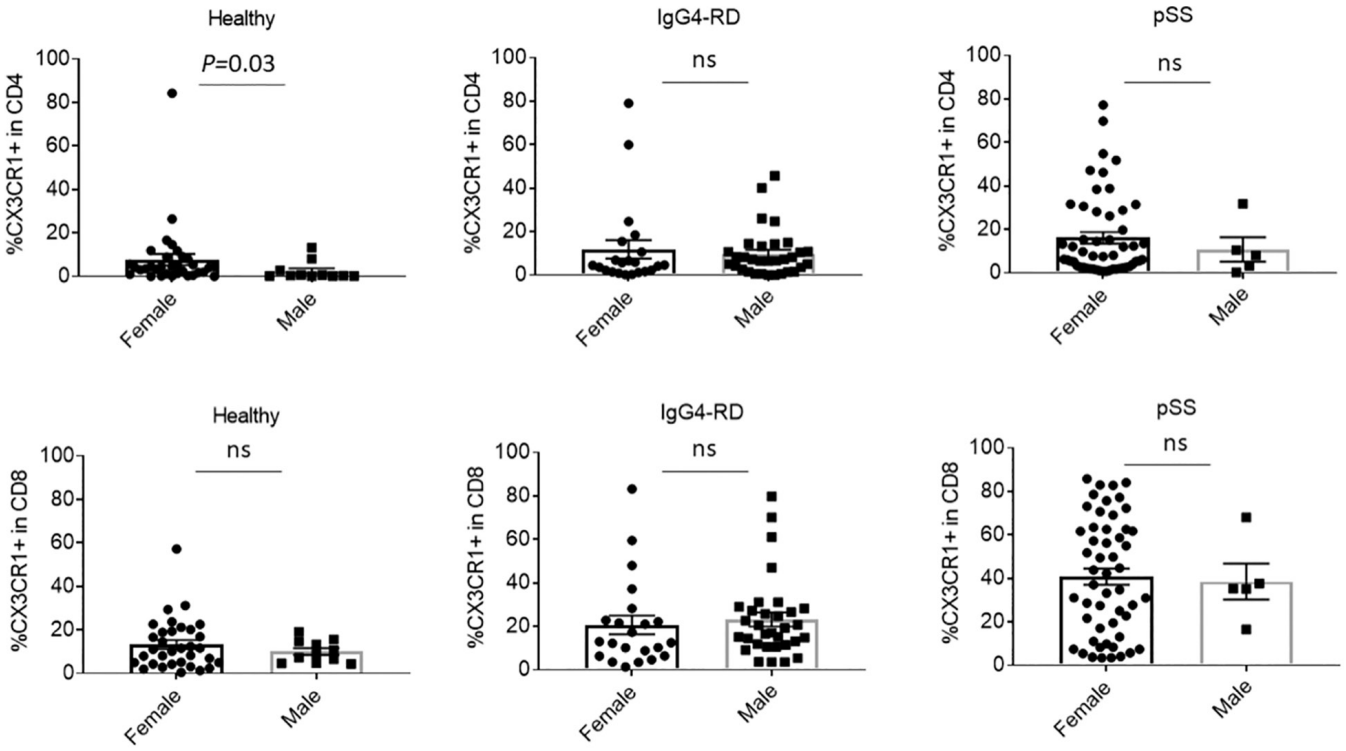

Fig. 4. Association of CX3CR1+T cells with sex differences in healthy individuals, IgG4-RD and pSS.
Proportions of CX3CR1+CD4 or CX3CR1+CD8 T cells were compared between female and male in healthy individuals, IgG4-RD and pSS. Mann-Whitney test. ns: not significant.

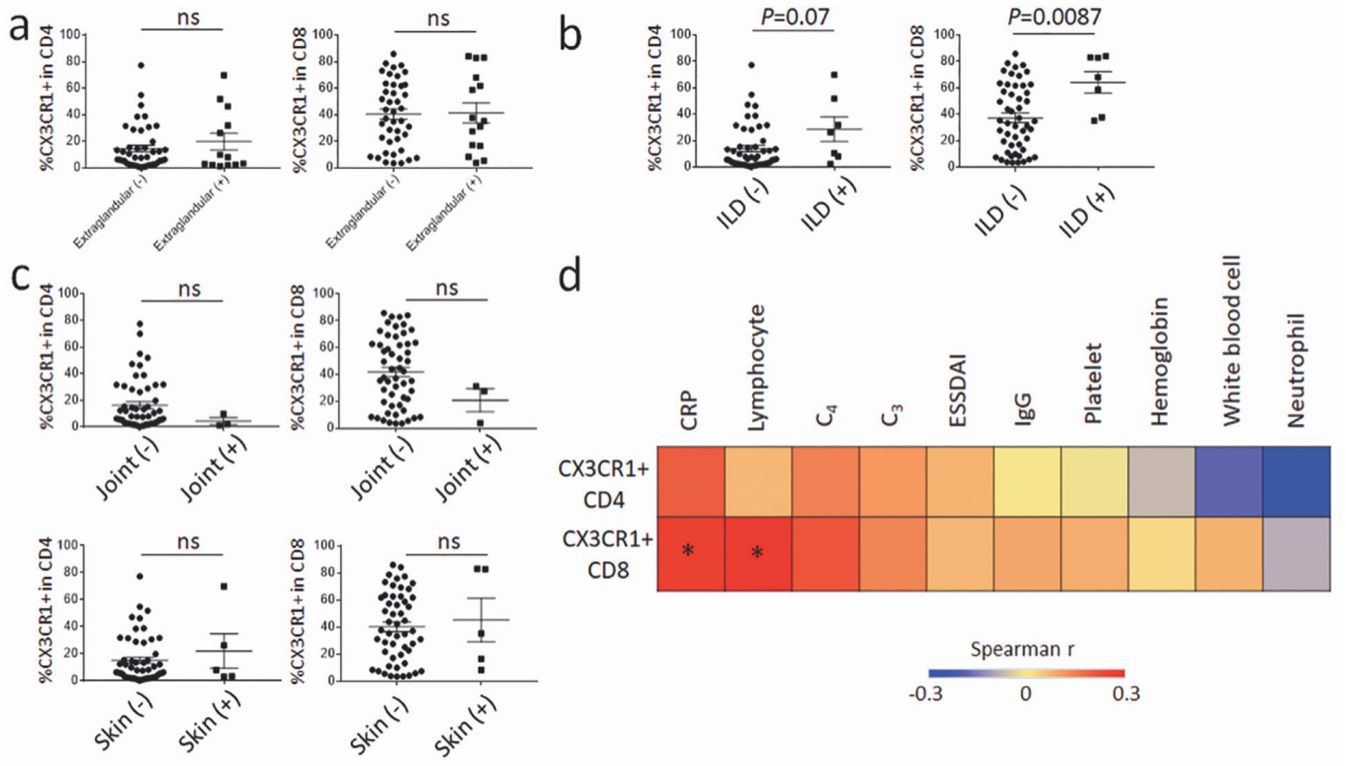

Fig. 5. Association of CX3CR1+CD4 and CX3CR1+CD8 T cells with clinical manifestations in pSS.
Proportions of CX3CR1+CD4 or CX3CR1+CD8 T cells were compared in patients with pSS with and without extraglandular organ involvements (**a**), interstitial lung disease (ILD) (**b**), joint or skin involvements (**c**). Mann-Whitney test. Correlation analysis was performed between the proportion of CX3CR1+CD4 or CX3CR1+CD8 T cells and clinical parameters (**d**). Spearman's correlation analysis.
ns: not significant.

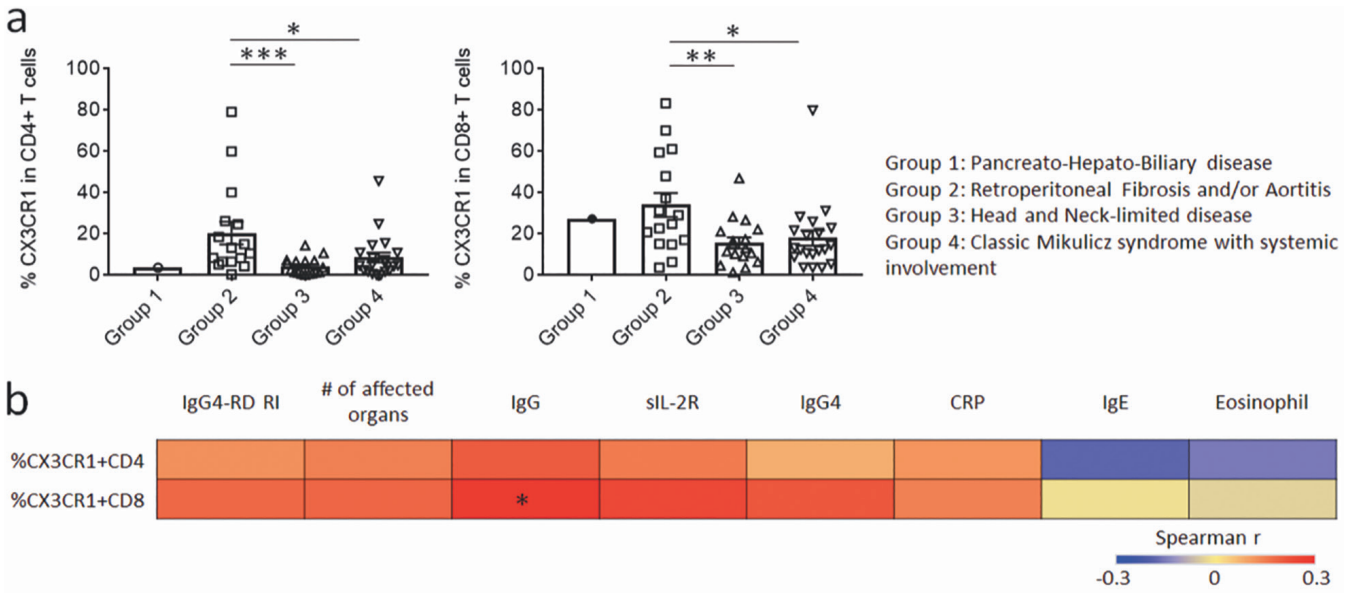

Fig. 6. Association of CX3CR1+CD4 and CX3CR1+CD8 T cells with clinical manifestations in IgG4-RD.
The proportion of CX3CR1+CD4 or CX3CR1+CD8 T cells were compared in patients with IgG4-RD according to the four clinical phenotypes (**a**). Kruskal-Wallis test followed by Dunn's multiple comparisons test. Correlation analysis was performed between the proportion of CX3CR1+CD4 or CX3CR1+CD8 T cells and clinical parameters (**b**). Spearman's correlation analysis.
**p*<0.05, ***p*<0.01, ****p*<0.001.

involvement, we found that patients with ILD showed a significantly higher proportion of CX3CR1+CD8 T cells than those without ILD (Fig. 5b). On the other hand, there was no difference in proportions of CX3CR1+CD4 or CX3CR1+CD8 T cells with and without other extraglandular organ involvements such as joint or skin manifestations (Fig. 5c). In addition, there was no difference in age between patients with ILD and those without ILD.

We next examined the correlation of proportions of CX3CR1+CD4 or CX3CR1+CD8 T cells with clinical parameters. Disease activity was assessed by the European League Against Rheumatism (EULAR) Sjogren Disease Activity Index (ESSDAI) (26). We found that most clinical parameters except for white blood cell and neutrophil counts showed weak positive correlations with proportions of CX3CR1+CD4 or CX3CR1+CD8 T cells, and among them serum C-reactive protein levels or lymphocyte counts showed a significant correlation with proportions of CX3CR1+CD8 T cells (Fig. 5d). Collectively, these results suggest that a higher proportion of CX3CR1+CD8 T cells were associated with ILD involvement and higher disease activity in patients with pSS.

CX3CR1+CD4 and CX3CR1+CD8 T cells were increased in IgG4-RD complicated with retroperitoneal fibrosis and/or aortitis

To determine the clinical significance of increased proportions of CX3CR1+CD4 and CX3CR1+CD8 T cells, we examined whether these differed in IgG4-RD according to their organ involvements. Based on the recent cross-sectional studies in international cohorts, patients with IgG4-RD were divided into 4 distinct clinical phenotypes: group 1, pancreato-hepato-biliary disease; group 2, retroperitoneal fibrosis and/or aortitis; group 3, head and neck-limited disease; and group 4, classic Mikulicz syndrome with systemic involvement (27). We found that CX3CR1+CD4 and CX3CR1+CD8 T cells were significantly increased in group 2 compared with group 3 or 4 (Fig. 6a). Group 1 was not evaluated due to small patient numbers (n=1). On the other hand, the distribution of age was not statistically different among the groups. Next, disease activity was determined based on the IgG4-RD responder index (IgG4-RD RI) score (28) and further analysis demonstrated that the proportion of CX3CR1+CD4 and CX3CR1+CD8 T cells tended to show weak positive correlations with the scores of IgG4-RD RI, number of affected organs, soluble interleukin-2 receptors, and serum levels of IgG, IgG4, and C-reactive protein (Fig. 6b). Atopic parameters (blood eosinophil count or serum IgE level) did not show a positive correlation with the proportion of CX3CR1+CD4 or CX3CR1+CD8 T cells (Fig. 6b). Consistently, the increase of CX3CR1+CD4 and CX3CR1+CD8 T cells did not depend on atopic history (asthma and/or allergic rhinitis) of patients (Supplementary Fig. S1).

Thus, the increased proportion of CX3CR1+CD4 and CX3CR1+CD8 T cells were associated with the presence of retroperitoneal fibrosis and/or aortitis and their disease activity in IgG4-RD.

CX3CR1+CD8 T cells were decreased following glucocorticoid therapy in IgG4-RD

Finally, to evaluate the effects of glucocorticoid therapy on proportions of CX3CR1+CD4 and CX3CR1+CD8 T cells, we performed longitudinal analysis in 15 patients with IgG4-RD before and after 12 weeks of therapy. Proportions of CX3CR1+CD8 T cells significantly decreased after glucocorticoid therapy, while proportions of total CD8 T cells did not (Fig. 7a, b). Proportions of CX3CR1+CD4 T cells tended to de-

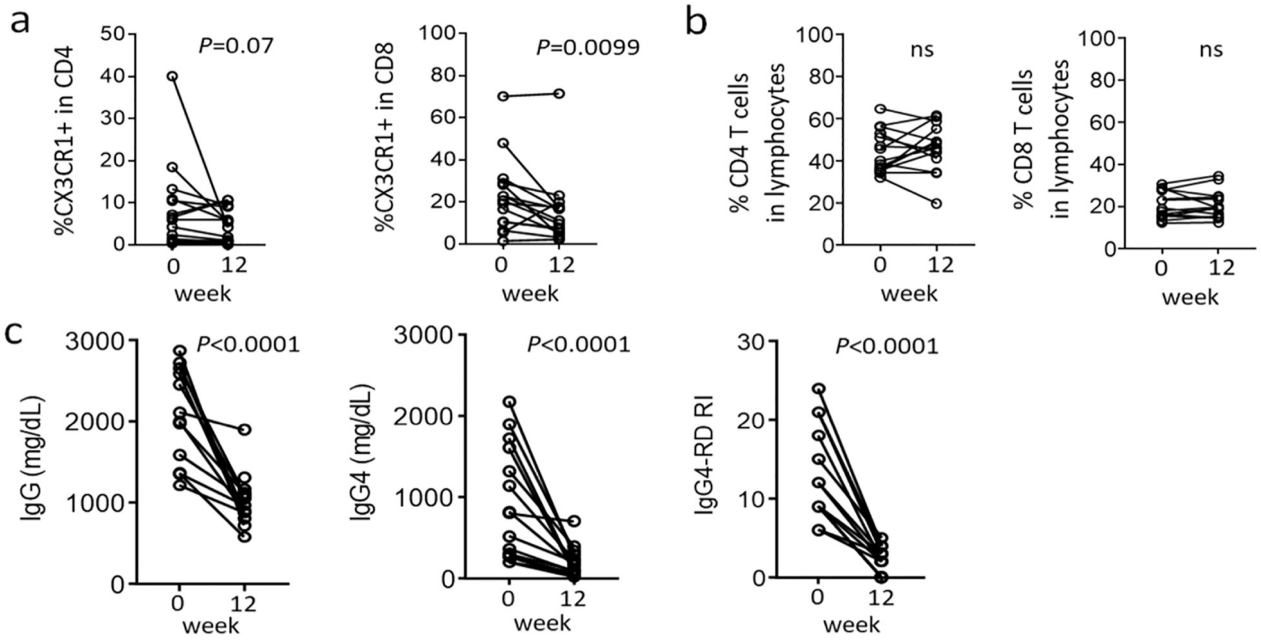

Fig. 7. Longitudinal analysis of CX3CR1+CD4 and CX3CR1+CD8 T cells in IgG4-RD after glucocorticoid therapy.
Serial assessment of the proportion of CX3CR1+CD4 and CX3CR1+CD8 T cells (**a**) and total CD4 and CD8 T cells (**b**) was conducted at baseline and 12 weeks after glucocorticoid therapy (n=15). As well, serum IgG, IgG4 and scores of IgG4-RD RI were analysed (**c**). Wilcoxon matched-pairs signed rank test. ns: not significant.

crease after glucocorticoid therapy but it was not significant (Fig. 7a). Serum levels of IgG and IgG4 and scores of disease activity also decreased (Fig. 7c).

Discussion

In this study we found that CX3CR1+CD4 and CX3CR1+CD8 T cells were increased in peripheral blood from patients with pSS and IgG4-RD. Particularly, the increase of CX3CR1+CD8 T cells were associated with aging and certain organ involvements in both diseases; ILD in pSS and retroperitoneal fibrosis/aortitis in IgG4-RD. Furthermore, proportions of CX3CR1+CD8 T cells were decreased along with the clinical improvements after glucocorticoid therapy. These results suggest that the increase of CX3CR1+CD8 T cells are involved in aging and distinct clinical phenotypes of patients with pSS and IgG4-RD.

Past study reported infiltration of CX3CR1+T cells in the salivary glands of patients with pSS (11). CX3CR1+T cells are specialised immune cell subset that can secrete cytotoxic molecules such as perforin and granzyme B to kill the target cells as shown in the Figure 1, and therefore these cells are involved in the destruction of ductal epithelial cells. Moreover, we found in the present study that pSS patients with ILD showed more intense increase of peripheral CX3CR1+CD8 T cells than those without ILD, suggesting that tissue destruction by those cells is involved in the pathogenesis of pSS-ILD. Future studies are required to examine local infiltration of those immune cells and their mechanistic insights in ILD lesions of patients with pSS.

The involvement of cytotoxic CD4 and CD8 T cells in IgG4-RD has been reported (16-18). Interestingly, cytotoxic CX3CR1+CD4 T cells in IgG4-RD also exhibited B-cell helper functions (16. 29). In our present study, we revealed for the first time that CX3CR1+CD4 and CX3CR1+CD8 T cells were associated with distinct clinical phenotype, specifically with retroperitoneal fibrosis and/or aortitis involvement. Therefore, CX3CR1+CD4 and CX3CR1+CD8 T cells may induce tissue destruction in those organ involvements, leading to the severe complications such as ureteral obstruction, aortic dissection and aneurysmal rupture (8, 9). On the other hand, we previously reported the involvement of T follicular helper (Tfh) cells in IgG4-RD, particularly in lacrimal and salivary gland involvements (so-called Mikulicz syndrome) (30-33). In fact, the inflamed lesions of lacrimal and salivary glands in IgG4-RD frequently show excessive formation of tertiary lymphoid organs, in which Tfh cells play a critical role in differentiation and proliferation of IgG4+B cells (34-38). Therefore, we assume that distinct clinical phenotypes are associated with distinct immune cell subsets in pathogenesis of IgG4-RD.

The ontogeny of CX3CR1+CD4 and CX3CR1+CD8 T cells remains to be elucidated. We observed a positive correlation of an expansion of CX3CR1+CD4 T cells with that of CX3CR1+CD8 T cells, suggesting that CX3CR1+CD4 and CX3CR1+CD8 T cells are coordinately expanded. Both CX3CR1+CD4 and CX3CR1+CD8 T cells are known to be clonally expanded populations in an antigen-driven manner (19). Therefore, disease-triggering antigens may contribute to the expansion of those T cells. Identification of disease-triggering antigens in each clinical phenotype of pSS or IgG4-RD would facilitate more precise understanding of the pathogenesis in future (33, 39). Alternatively, peripheral cytotoxic CD4 and CD8 T cells are reported to be increased in aging, particularly

in supercentenarians (40). Considering that pSS and IgG4-RD frequently develop in the elderly, immune aging may be associated with the expansion of cytotoxic CD4 and CD8 T cells. In our present study, we found that higher proportions of CX3CR1+CD8 T cells were associated with aging in pSS and IgG4-RD but not in healthy individuals, suggesting that immune aging is associated with the expansion of CX3CR1+CD8 T cells in these two diseases.

In the process of immune aging, the thymus undergoes involution, resulting in a decrease in the number of naive CD8 T cells, while there is an increase in senescent CD8 T cells (41). These senescent CD8 T cells are known to acquire CD57 expression and concomitantly lose CD27 and CD28, and they are characterised by their distinctive production of perforin and granzyme B (41). In this study, we demonstrated the utility of CX3CR1 as another marker to identify a subset of CD8 T cells that selectively produce perforin and granzyme B. Moreover, we discovered that CX3CR1+CD8 T cells increase with age in two immune-mediated disorders, pSS and IgG4-RD but not in healthy individuals. These findings suggest the potential of CX3CR1 as an immunosenescence-associated cell surface marker in human CD8 T cells and its involvement in immune-mediated disorders. Based on these findings, further investigations would be conducted to determine whether CX3CR1+CD8 T cells also increase with aging and contribute to the pathogenesis of other immune-mediated disorders.

All T cells express glucocorticoid receptors, and glucocorticoids enter the cells by passing through the cell membrane. Glucocorticoids then bind to glucocorticoid receptors, transduce signals in the nucleus, and exert their effects including apoptosis of T cells and inhibition of cytokine production. Importantly, while the detailed mechanisms are still unclear, it is known that the sensitivity to glucocorticoids varies among different subsets of T cells. For example, Th1 cells are more susceptible to apoptosis induced by glucocorticoid effects compared to Th17 and Th2 cells (42). Additionally, cytomegalovirus-specific cytotoxic CD8 T cells are more prone to apoptosis under the influence of glucocorticoids compared to other CD8 T cells (43, 44). This finding is consistent with the phenomenon of cytomegalovirus reactivation during glucocorticoid therapy, thereby supporting the idea. In our current study, we observed a decrease in the proportion of CX3CR1+CD8 and CX3CR1+CD4 T cells after glucocorticoid treatment, while the overall proportion of CD8 and CD4 T cells remained unchanged. This suggests that CX3CR1+CD8 and CX3CR1+CD4 T cells are relatively more sensitive to effects of glucocorticoids.

Activated vascular endothelial cells produce a chemokine ligand of CX3CR1, so-called fractalkine, and recruit CX3CR1+ T cells into the affected organs (44). Preclinical studies in animal models have demonstrated that targeting CX3CR1-fractalkine axis improves pulmonary fibrosis and cardiovascular involvements (45). Therefore, ILD of pSS and retroperitoneal fibrosis/aortitis of IgG4-RD may be the potential targets for treatment with inhibition of CX3CR1-fractalkine axis.

In conclusion, CX3CR1+CD8 T cells are involved in the pathogenesis of pSS and IgG4-RD and associated with aging and distinct organ involvements. More thorough understanding of molecular mechanisms responsible for the differentiation and function of CX3CR1+CD8 T cells would facilitate the discovery of new therapeutic targets and the development of new treatment strategy based on the specific organ involvements.

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