Cytotoxic CX3CR1+ T cells drive vascular inflammation in giant cell arteritis but not in Takayasu's arteritis

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Abstract

Objective

To compare the involvement of cytotoxic CX3CR1+ T cell subsets between giant cell arteritis (GCA) and Takayasu's arteritis (TAK).

Methods

We examined the proportions of CX3CR1+ CD4+ and CD8+ T cells in whole blood freshly obtained from 30 treatment-naive patients with active large-vessel vasculitis (GCA, n=22 and TAK, n=8) and 16 healthy controls (HC). Infiltration of CX3CR1+ T cells into the affected arteries was assessed using immunohistochemical staining. Furthermore, CX3CR1+ CD4+ and CD8+ T cells were followed up after glucocorticoid treatment for longitudinal assessment of both diseases.

Results

The proportion of CX3CR1+ CD4+ T cells was significantly higher in GCA than in HC but not in TAK. No differences were observed in the proportions of CX3CR1+ CD8+ T cells among the GCA, TAK, and HC groups. The increased proportion of CX3CR1+ CD4+ T cells in GCA strongly correlated with the severity of systemic inflammation, whereas no significant correlation was found in TAK. Compared to TAK, CX3CR1+ CD4+ T cells from GCA patients showed significantly higher expression of granzyme B and perforin. The inflamed temporal arterial tissues of the GCA were infiltrated by numerous CX3CR1+ T cells, contributing to inflammation, disruption of the elastic lamina, and intimal hyperplasia. In contrast, no infiltration of CX3CR1+ T cells was observed in the aortitis lesions of TAK. Longitudinal analysis of post-glucocorticoid treatment showed a reduction in CX3CR1+ T cells in GCA, whereas no significant change was observed in TAK.

Conclusion

Differences in immune mechanisms between GCA and TAK highlight cytotoxic CX3CR1+ T cells as potential drivers for GCA-related inflammation and vessel damage but not for TAK.

Key words

large-vessel vasculitis, giant cell arteritis, Takayasu's arteritis, CX3CR1, T cells

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Received on March 22, 2024; accepted in revised form on May 27, 2024.

Competing interests: M. Akiyama has received speaker fees from Asahikasei, Astellas, Boehringer Ingelheim, Chugai, Eisai, Eli Lilly, Gilead Sciences, Jansen, Novartis, Pfizer, Taisho and UCB. Y. Kaneko has received grants or speaker fees from AbbVie, Asahikasei, Astellas, Ayumi, Boehringer Ingelheim, Bristol-Myers Squibb, Chugai, Eisai, Eli Lilly, Gilead Sciences, Hisamitsu, Jansen, Kissei, Novartis, Pfizer, Sanofi, Takeda, Tanabe-Mitsubishi, Taisho and UCB. The other authors have declared no competing interests.

Introduction

Large-vessel vasculitis (LVV) encompasses two distinct conditions, giant cell arteritis (GCA) and Takayasu's arteritis (TAK), differentiated by the age of onset (1, 2). Although these two diseases share clinical features involving large-vessel vasculitis, the relevant genetics and comorbidities are different, indicating potential variations in their underlying immunopathophysiology (3-6). Recently, attention has been paid to the role of cytotoxic T cells in both diseases. Separate studies have reported that in GCA, granzyme B-expressing cytotoxic $CD4^+$ T cells are clonally expanded in single-cell analysis (3), whereas in TAK, granzyme B-expressing cytotoxic $CD8^+$ T cells are increased (6). However, a direct comparison between GCA and TAK is lacking, and the difference in cytotoxic T cell involvement in both diseases remains unclear.

We and other research groups have identified CX3CR1 as a specific marker for cytotoxic T cells that produce granzyme B (7, 8). Fractalkine, a ligand expressed by vascular endothelial cells, is known for its role in vascular inflammation (9), raising the hypothesis that CX3CR1+ cytotoxic T cells invade the vascular walls and cause destructive inflammation through the release of cytotoxic molecules. In this study, we aimed to clarify and compare the involvement of CX3CR1+ cytotoxic T-cell subsets in the pathogenesis of GCA and TAK.

Materials and methods

Patients and controls

Thirty patients with active treatment-naive LVV (GCA, n=22; TAK, n=8) and 16 healthy controls (HC) were enrolled in the study. The characteristics of the patients and HCs are shown in Supplementary Table S1. All patients with GCA and TAK fulfilled the 1990 American College of Rheumatology criteria or 2022 American College of Rheumatology/EULAR Classification Criteria (10, 11). The study was approved by the Ethics Committee of Keio University School of Medicine and was conducted in compliance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all the participants.

Flow cytometry

Flow cytometry was performed using freshly obtained whole blood samples from patients and HCs. To determine the proportion of CX3CR1+ cells within CD4 and CD8 T cells, 50 μL of whole blood was incubated with Pacific Blue-labelled anti-human CD3 (BD Biosciences Pharmingen), APC-labelled anti-human CD4, APC-labelled anti-human CD8 (both from Beckman Coulter), and PE-labelled anti-human CX3CR1 (BioLegend) for 30 min at room temperature. Following incubation, the cells were lysed and fixed using Lyse/Fix Buffer (BP Phosflow Lyse/Fix Buffer 5X) for 7 min. Cell washing was performed, and the pellet was resuspended in 250 μL of cell wash solution (BD Biosciences, CellWASH). Cells were analysed using a MACS Quant Analyzer (Miltenyi Biotec). The gating strategy used for flow cytometry is shown in Figure 1a.

To perform intracellular staining of perforin and granzyme B, frozen PBMCs were thawed and initially stained with BV786-labelled anti-human CD3 (BD Biosciences Pharmingen), BUV737-labelled anti-human CD4 (BD Biosciences Pharmingen), BUV563-labelled anti-human CD8 (BD Biosciences Pharmingen), and PE-labelled anti-human CX3CR1 (BioLegend) antibodies for 30 minutes. Subsequently, the cells were permeabilised and fixed using reagents from the 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). The processed cells were acquired on a Fortessa flow cytometer and analysed using FlowJo.

Immunohistochemistry

Paraffin-embedded temporal artery sections from patients with GCA (n=4) and aortitis sections from TAK (n=1) were used for immunohistochemistry. An automated BOND immunostaining system was used for deparaffinisation, antigen retrieval (20 min in citrate buffer, pH 6.0), primary antibody incubation (30 min), peroxide blocking

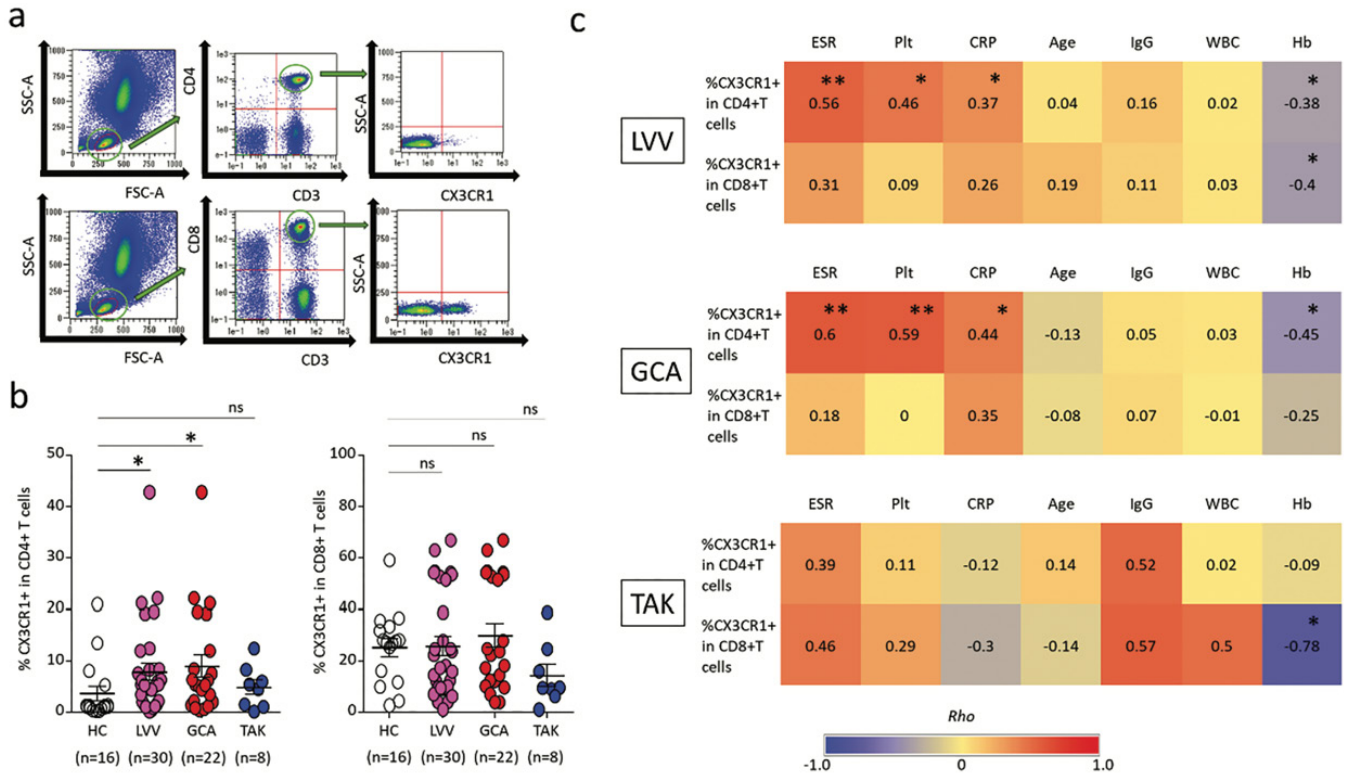

Fig. 1. CX3CR1+ T cells in peripheral blood are increased and correlated with systemic inflammatory markers in patients with GCA but not in TAK. Freshly obtained whole blood samples were collected from untreated, active LVV patients and HC. Subsequently, CX3CR1+ CD4+ T cells and CX3CR1+ CD8+ T cells were analysed using flow cytometry. (**a**) Gating strategy of flow cytometric analysis. (**b**) Comparison of the proportions of CX3CR1+ CD4+ T cells or CX3CR1+ CD8+ T cells among LVV, GCA, TAK and HC. Mann-Whitney U-test. (**c**) Correlation of CX3CR1+ CD4+ T cells or CX3CR1+ CD8+ T cells with clinical indicators in LVV, GCA, and TAK. Spearman's correlation coefficient.
*p<0.05, **p<0.01, ns: not significant.

(5 min), DAB staining (two rounds of 5 min), and haematoxylin staining (5 min). A CX3CR1 Polyclonal antibody (Proteintech Group, Inc., no. 13885-1-AP) was applied at a 1:50 dilution.

Statistical analysis

Data analysis was performed using the GraphPad Prism software (v. 7 GraphPad Software). Group comparisons were conducted using the Mann-Whitney U test for unpaired samples. The Wilcoxon rank-sum test was used for paired analyses after GC treatment. Spearman's correlation analysis was performed between the proportion of CX3CR1+ T cells and the clinical indicators. Statistical significance was defined as a two-sided p-value of <0.05.

Results

Increased CX3CR1+ CD4⁺ T cells in GCA but not in TAK

Initially, we evaluated the cytotoxic T cell population in freshly collected peripheral blood by analysing CX3CR1+ CD4⁺ T cells and CX3CR1+ CD8⁺ T cells. The proportion of CX3CR1+ CD4⁺ T cells in the peripheral blood of patients with GCA was higher than that in HC, whereas no significant change was observed in patients with TAK (Fig. 1b). The proportion of CX3CR1+ CD8⁺ T cells did not differ between the peripheral blood of the GCA, TAK, and HC groups (Fig. 1b). Notably, no correlation was observed between the proportion of CX3CR1+ CD4⁺ T cells and age in the HCs (data not shown).

Relevance of CX3CR1+ CD4⁺ T cells to disease severity

We further explored the relevance of CX3CR1+ CD4⁺ and CD8⁺ T cells in LVV disease activity, as represented by inflammation markers. The proportion of CX3CR1+ CD4⁺ T cells positively correlated with erythrocyte sedimentation rate, platelet count, and serum C-reactive protein levels and negatively correlated with the severity of anaemia, whereas little correlation was observed between CX3CR1+ CD8⁺ T cells and disease activity, confirming a link between CX3CR1+ CD4⁺ T cells and systemic inflammation in GCA (Fig. 1c). Notably, this correlation was specifically observed in patients with GCA, whereas no significant correlation was observed in patients with TAK. Because GCA is sometimes complicated by polymyalgia rheumatica, we further analysed CX3CR1+ T cells in patients with GCA stratified according to the presence or absence of polymyalgia rheumatica. The presence of polymyalgia rheumatica did not affect the proportion of CX3CR1+ T cells (Suppl. Fig. S1). Taken together, the immunological pathogenesis is different between GCA and TAK, with a particular implication for CX3CR1+ CD4⁺ T cells in GCA but not in TAK.

Cytotoxic potential of CX3CR1+ CD4⁺ T cells in untreated GCA patients

To explore the cytotoxic potential of CX3CR1+ CD4⁺ T cells, we examined

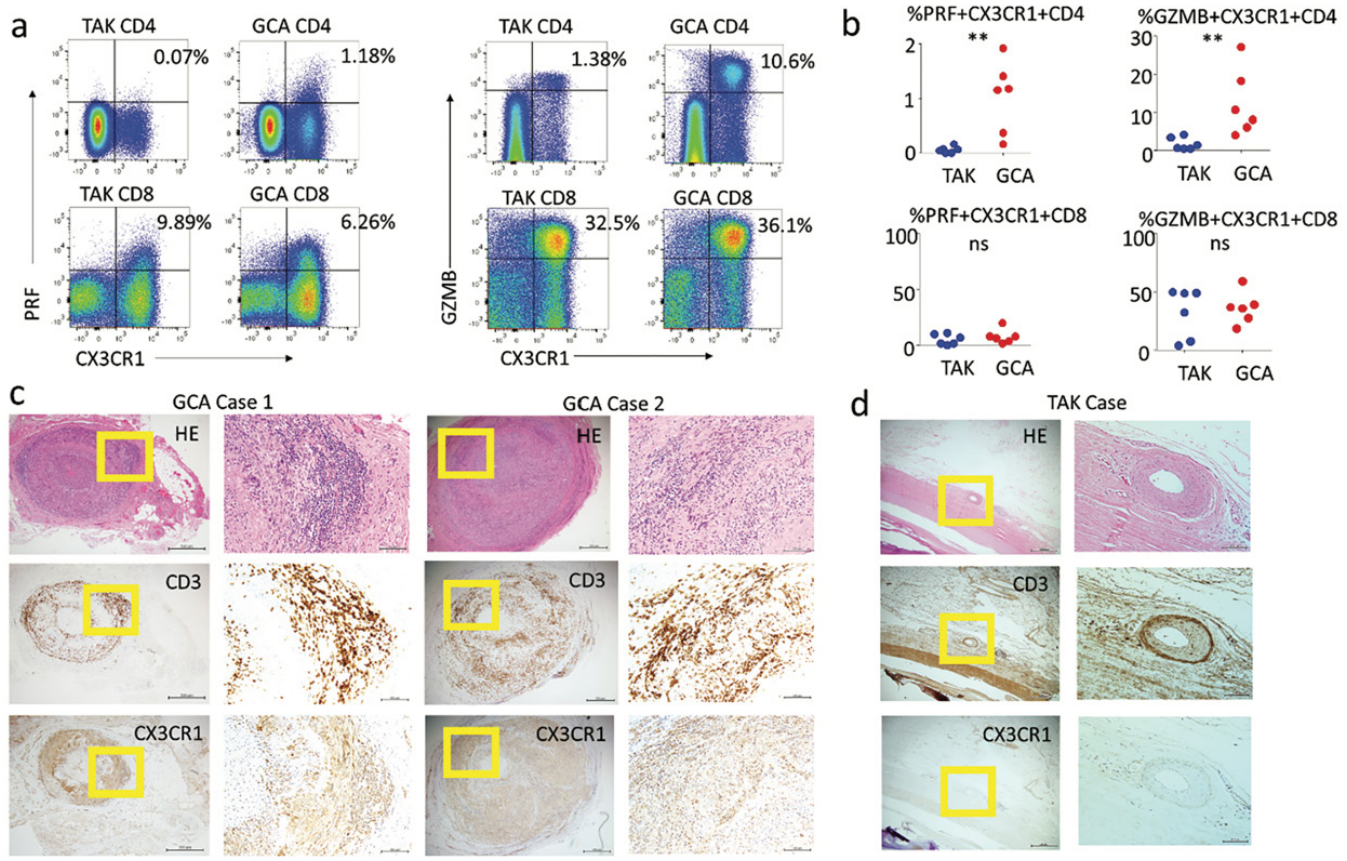

Fig. 2. CX3CR1+cytotoxic T cells infiltrate into the inflamed artery lesions of GCA but not of TAK.
(**a, b**) A comparative analysis was done for the expression levels of cytotoxic molecules (perforin: PRF and granzyme B: GZMB) in CX3CR1+CD4 T cells and CX3CR1+CD8 T cells using PBMCs obtained from GCA (n=6) and TAK (n=6) patients. Mann-Whitney U test. (**c, d**) Immunohistochemical staining was performed to examine the infiltration of CX3CR1+T cells in the temporal arteritis tissues from GCA patients and the aortitis tissues from a TAK patient. HE (haematoxylin and eosin)-stained sections, CD3-stained sections, CX3CR1-stained sections are shown. The scale bar corresponds to 500μm under low magnification and 100μm under high magnification.
***p*<0.01; ns: not significant.

the expression of cytotoxic molecules, granzyme B and perforin, within CX3CR1+ CD4$^+$ T cells and CX3CR1+ CD8$^+$ T cells, using frozen PBMCs from six patients with untreated, active GCA and six patients with untreated, active TAK patients (Fig. 2a-b). Our findings revealed that both CX3CR1+ CD4$^+$ T cells and CX3CR1+ CD8$^+$ T cells exhibit expression of these cytotoxic molecules. Notably, granzyme B and perforin expression was specifically detected in CX3CR1+ cells but not in CX3CR1- cells.

Moreover, we observed a significantly higher proportion of CX3CR1+ CD4$^+$ T cells expressing perforin or granzyme B in patients with GCA compared to those with TAK (Fig. 2a-b). Conversely, there was no notable difference in the proportion of CX3CR1+ CD8$^+$ T cells expressing perforin or granzyme B between GCA and TAK patients. These results suggest that CX3CR1+ CD4$^+$ T cells with enhanced cytotoxic potential are involved in the pathophysiology of GCA but not of TAK.

Infiltration of CX3CR1+ T cells to the temporal artery lesions of GCA

Given the implication of CX3CR1+ T cells in the pathophysiology of GCA, we examined whether the T cell subset invaded the arterial inflammation site of GCA-affected arteries. Figure 2c shows that in the localised lesions of the temporal arteries in the GCA, haematoxylin and eosin staining revealed dense, full-layer infiltration of lymphocytes from the adventitia to the intima, at the centre of inflammation, disruption of the elastic lamina, and significant intimal hyperplasia. CD3 staining demonstrated notable infiltration of T cells throughout all layers, from the adventitia to the intima. Moreover, a substantial number of T cells involved in these inflammatory and tissue destruction processes expressed CX3CR1 (Fig. 2c). On the other hand, the aortitis tissue of TAK showed thickening of the adventitia and inflammation at the same site, along with infiltration of T cells; however, CX3CR1-expressing T cells were virtually absent (Fig. 2d). These results suggest that cytotoxic CX3CR1+ T cells not only increase in the peripheral blood of patients with GCA but also infiltrate localised arterial lesions, contributing to inflammation and tissue damage. Conversely, in TAK, there is no increase in cytotoxic CX3CR1+ T cells in the peripheral blood and no infiltration into affected vascular tissues.

Longitudinal changes of CX3CR1+ T cells following glucocorticoid therapy

Finally, to assess whether the pathological increase in CX3CR1+ T cells

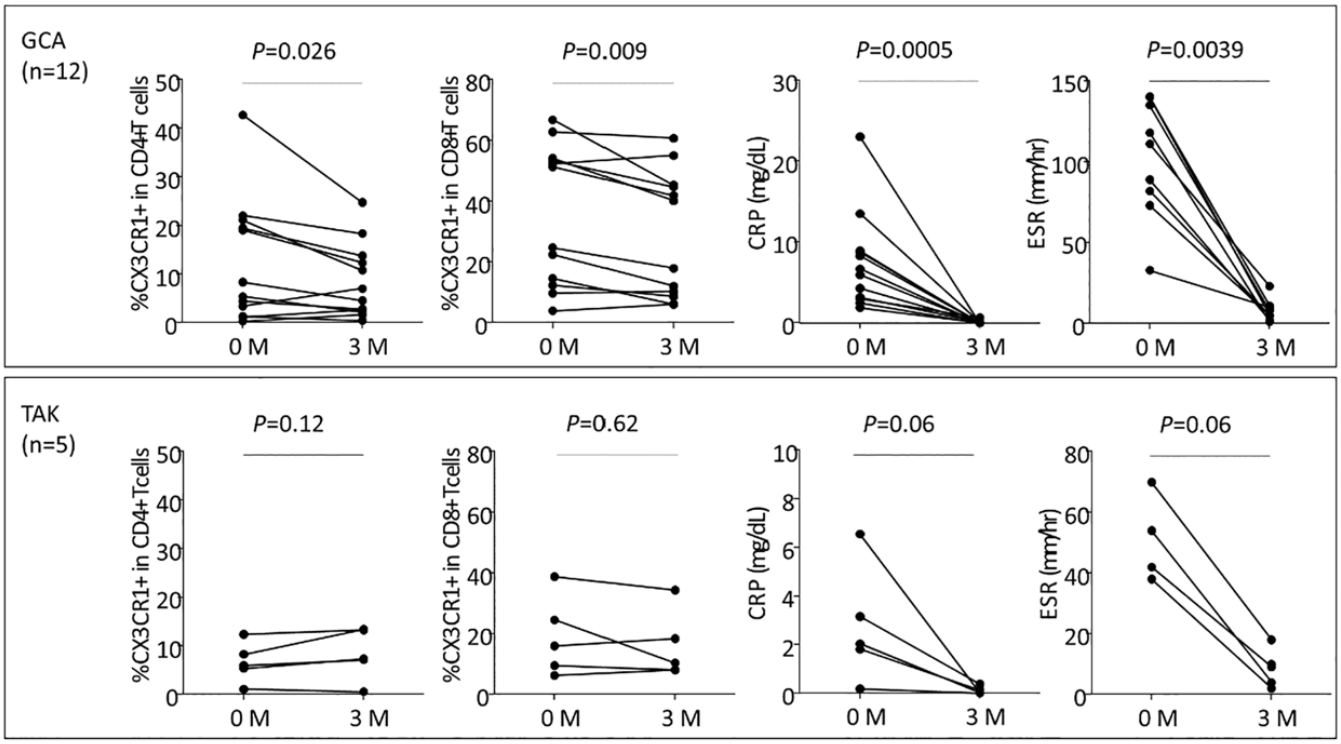

Fig. 3. CX3CR1+ T cells in peripheral blood of GCA decrease after glucocorticoid treatment.
Longitudinal analysis of CX3CR1+ CD4+ T cells, CX3CR1+ CD8+ T cells, CRP levels, and ESR were conducted in patients with GCA or TAK, before and three months after the initiation of glucocorticoid treatment. Wilcoxon signed-rank test.

decreased alongside the amelioration of disease activity with glucocorticoid therapy, we conducted a longitudinal analysis before and after 3 months of glucocorticoid treatment in 12 patients with GCA and five with TAK. We have included and analysed all the patients from whom we were able to obtain samples after the initiation of therapy. Of the 12 GCA patients, 9 (75%) exhibited a fever, 9 (75%) had swelling or tenderness of the temporal artery, 8 (67%) experienced jaw claudication, and 2 (17%) showed polymyalgia rheumatica symptoms. These symptoms rapidly improved following glucocorticoid therapy. On the other hand, among the 5 TAK patients, 3 (60%) presented with a fever, 3 (60%) had a discrepancy in blood pressure between arms, and 2 (40%) experienced syncope. Notably, all TAK patients had stenosis of the left subclavian artery, which persisted even after glucocorticoid therapy. Figure 3 shows improvements in serum C-reactive protein levels and erythrocyte sedimentation rates in all patients after treatment initiation. Importantly, the proportion of CX3CR1+ CD4+ T cells in the peripheral blood significantly decreased with improvement in disease activity in patients with GCA (Figure 3). In contrast, no significant changes were observed in the proportion of CX3CR1+ T cells in patients with TAK.

Discussion

Our study revealed that cytotoxic CX3CR1+ T cells in the peripheral blood increased in correlation with systemic inflammation in patients with LVV and decreased with glucocorticoid treatment, along with an improvement in disease activity. CX3CR1+ T cells infiltrate localised arterial inflammatory lesions. These observations were specific to patients with GCA but not with TAK, suggesting distinct immunopathogenic mechanisms between GCA and TAK.

We investigated cytotoxic T cells in the largest number of treatment-naïve, active patients with GCA and TAK and identified the involvement of cytotoxic CD4+ T cells in the pathogenesis of GCA. Recently, Carmona *et al*. showed an expansion of blood cytotoxic CD4+ T cells expressing higher levels of granzyme B genes in Spanish patients with active GCA than in remission and HC with single-cell RNA sequencing (3). Our study corroborates these findings, showing a parallel change in cytotoxic CD4+ T cells with disease activity and infiltration of the cells at the inflamed and destroyed sites of the involved temporal arteries. It is also important to investigate Asian populations and show that this pathogenic phenomenon can be commonly observed, irrespective of race. Furthermore, we showed the infiltration of CX3CR1+ T cells into temporal artery lesions that penetrated through the adventitia, media, and intima, suggesting a T cell subset in vessel wall inflammation and destruction.

Fractalkine, a ligand of CX3CR1, is expressed in vascular endothelial cells (9). We found that CX3CR1+ CD4+ T cells selectively express perforin and granzyme B, and noted a reduction in the number of CX3CR1+ CD4+ T cells in the blood after treatment, correlating with improved disease activity. Samson *et al*. reported elevated serum granzyme B levels in patients with GCA, which decreased with glucocorticoid therapy (5). These findings suggest that CX3CR1+ CD4+ T cells may circulate in the bloodstream, be recruited to the inflammation sites of temporal arter-

ies via vascular endothelial cells of the vasa vasorum, and exert their cytotoxicity with granzyme B, leading to inflammation and destruction of vessel walls. Our study implies that the proportion of CX3CR1+ $CD4^+$ T cells could not only serve as a potential indicator of disease activity in GCA but also serve as a therapeutic target. Indeed, in animal models, targeting the CX3CR1-fractalkine axis improves cardiovascular inflammatory diseases (9).

Van Sleen *et al*. reported the presence of CX3CR1+ macrophage infiltration in the temporal arteries affected by GCA (12) Significantly, their study also revealed the expression of fractalkine, a CX3CR1 ligand, in vascular smooth muscle cells within the affected temporal arteries. Fractalkine was detected throughout all layers of the temporal artery (adventitia, media, and intima), with the highest expression levels observed in the media layer. Consequently, in conjunction with the findings of our current study, it is plausible that the CX3CR1-fractalkine axis plays a critical role in GCA pathogenesis. Fractalkine released not only by vascular endothelial cells at the lesion site but also by vascular smooth muscle cells may contribute to the recruitment of CX3CR1+ T cells and macrophages, thereby promoting arterial wall destruction and inflammation in GCA.

Our study highlighted the involvement of CX3CR1+ cytotoxic $CD4^+$ T cells in the pathogenesis of GCA, but not TAK, suggesting distinct immunopathological mechanisms between the two diseases. Previous studies have indicated a predominant role for $CD4^+$ T cells in the pathogenesis of GCA, whereas $CD8^+$ T, gamma-delta T, and NK cells are predominant in the pathogenesis of TAK (13, 14). Although we did not observe a clear involvement of CX3CR1+ $CD8^+$ T cells in the pathogenesis of TAK, $CD8^+$ T cells encompass diverse subsets within a heterogeneous cell population, necessitating more detailed investigations, including single-cell analyses, in the future.

A limitation of our study is the relatively small number of TAK cases (eight cases) compared to GCA cases, although our study is a valuable report using treatment-naïve active TAK samples. This may contribute to the conflicting results with a previous study showing that granzyme B-producing $CD8^+$ T cells are increased in the blood of patients with TAK and infiltrate into aortitis lesions (6). Second, a difference in the average age between the patients with LVV and HCs was observed, which could have influenced the results. However, in this study, no correlation was observed between age and the proportion of CX3CR1+ $CD4^+$ T cells in either the vasculitis or HCs groups. Finally, a more detailed understanding of the molecular mechanisms governing the differentiation, proliferation, and function of CX3CR1+ $CD4^+$ T cells is required.

In conclusion, our results highlight the implications of CX3CR1+ $CD4^+$ T cells in GCA but not in TAK. Further studies are needed to elucidate the distinct pathophysiologies of GCA and TAK in the context of the role of CX3CR1+ $CD4^+$ T cells and their potential as therapeutic targets in the treatment of GCA.

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