Chemokine Receptor 8 Can Distinguish Antineutrophil Cytoplasmic Antibody–Associated Vasculitis From Infectious Complications

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Introduction: Diagnosing vasculitis is frequently difficult because its clinical symptoms are similar to those of common infectious diseases and other inflammatory disorders. This study focused on chemokine receptor 8 (CCR8) in peripheral blood mononuclear cells to find a new biomarker that distinguishes vasculitis from infectious complications.

Methods: A cross-sectional study was conducted among 113 patients with systemic vasculitis who were referred to Japan Health Care Organization Sendai Hospital from 2014 to 2016, including those with antineutrophil cytoplasmic antibody (ANCA)−associated vasculitis, anti−glomerular basement membrane disease, lupus nephritis, and Henoch-Schonlein purpura. Peripheral blood mononuclear cells were extracted from blood, and CCR8 expression was examined by real time polymerase chain reaction and flow cytometry.

Results: CCR8 gene expression was significantly higher in patients with ANCA-associated vasculitis, which was confirmed by upregulated CCR8 protein expression in flow cytometry ($P < 0.001$ and $P = 0.01$, respectively). Neither lupus nephritis nor Henoch-Schonlein purpura showed upregulated CCR8. Elevated CCR8 in the active phase decreased significantly in remission ($P = 0.002$), which was correlated with decreased serum inflammatory markers. Despite elevated serological inflammatory markers, the CCR8 levels at the time of infection, including bacterial, viral, and fungal, did not increase, indicating that infectious complications did not affect CCR8 expression ($P = 0.02$).

Conclusion: CCR8 in peripheral blood mononuclear cells may be a useful diagnostic marker for ANCA-associated vasculitis to differentiate between active vasculitis and infectious inflammation.

I nfection is a major cause of death in ANCA−associated vasculitis; avoiding infectious complications remains a challenge.[1] Similar clinical manifestations between vasculitis and infection may cause diagnostic difficulty, despite totally opposite treatments in 2 situations: immunosuppression in vasculitis, and antibiotics in infection. Accurate diagnosis is required to prevent unnecessary immunosuppression; however, common inflammatory markers are unable to differentiate between vasculitis and infection because both conditions are characterized by inflammation. Previous studies have tried to find new biomarkers that correlate with vasculitis activity.[2,3] Among them, serum ANCA is a useful tool for assessing

disease activity,[4] but ANCA titer does not fully correlate with disease activity.[5,6] Brix *et al.* found that CC chemokine ligand 18 (CCL18) enhanced activity of ANCA-associated vasculitis; serum CCL18 was elevated in active disease and relapse, then decreased in remission.[7] Other than vasculitis, CCL18 has been shown to be involved in the disease activity of cystic fibrosis, rheumatoid arthritis, and atopic dermatitis.[8–10] Although its essential role has not been fully elucidated, CCL18 could be different from nonspecific inflammatory markers such as the erythrocyte sedimentation rate, C-reactive protein (CRP), and monocyte chemotactic protein 1,[2] implying that CCL18 has a potential to differentiate disease activity and infectious complications.

In general, chemokines induce chemotaxis through the activation of their receptors on which are mainly expressed lymphocytes and monocytes, then stimulate a variety of biological responses. As an agonistic receptor of CCL18, CCR8 found in 2013,[11] is expressed

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mainly on Th2 subset of the T cells.[12] In this study, we focused on CCR8 expression in peripheral blood mononuclear cells (PBMCs) in various types of vasculitis, to identify a new biomarker that can distinguish vasculitis activity from infectious inflammation.

METHODS

Study Design and Participants
We performed a cross-sectional study at Japan Health Care Organization Sendai Hospital in Sendai, Japan. A total of 113 patients who were referred to the hospital with vasculitis, including ANCA-associated vasculitis, anti−glomerular basement membrane disease, lupus nephritis, and Henoch-Schonlein purpura were enrolled in this study from 2014 to 2016. A total of 25 healthy subjects were enrolled as normal controls. Diagnosis was made by kidney biopsy and/or laboratory measurements. ANCA-associated vasculitis was classified as myeloperoxidase (MPO), proteinase 3 (PR3), and ANCA negative serologically. Patients who had overlapping features of vasculitis were excluded from this study (MPO+PR3; n = 3, MPO+GBM; n = 1, MPO+Lupus; n = 2). Patients with cryoglobulinemic vasculitis were excluded because of limited sample availability (n = 2). Blood samples were obtained at several points in time during the course of treatment: before starting the treatment, and at the time of remission, relapse, and occurrence of infectious complications. The study protocol was approved by the Ethic Committee of Japan Health Care Organization Sendai Hospital, and written informed consent was obtained from all participants. This study was conducted with adherence to the Declaration of Helsinki.

Laboratory Analysis
Blood was collected in Vacutainer CPT mononuclear cell preparation tubes (Becton Dickinson, Franklin Lakes, NJ) and PBMCs were extracted by centrifugation. The total RNA was isolated from PBMCs using NucleoSpin RNA Blood (TaKaRa, Shiga, Japan). The integrity and purity of the extracted RNA was evaluated with the 2100 Agilent Bioanalyzer and RNA 6000 Nano kits (Agilent Technologies, Santa Clara, CA) following the manufacturer's instructions. The RNA integrity number of more than 8.5 was used for the following experiments. After reverse transcription with PrimeScript RT reagent (TaKaRa, Shiga, Japan), real-time polymerase chain reaction was carried out using SYBR Premix Ex Taq (TaKaRa, Shiga, Japan). CCR8 gene expression was calculated relative to ribosomal protein, large, P0 (RPLP0). All samples were measured in duplicate. Sequences of CCR8 forward and reverse primers were TCTGGGTCCCATTCAACGTG and GGGTGGCATAAGTCAGCTGT, respectively, and those

of RPLP0 were AATCTCCAGGGGCACCATTG and GAACACCTGCTGGATGACCA, respectively.

Serum CCL1 and CCL18 levels were examined by enzyme-linked immunosorbent assay according to the manufacturer's instructions (KA1725 and KA1757, Abnova, Walnut, CA). The detection ranges were 4.12 to 3000 pg/ml and 2.74 to 2000 pg/ml, respectively.

Flow Cytometry
Isolated PBMCs were incubated with fluorochrome-labeled CCR8 primary antibody (360604, Biolegend, San Diego, CA) or isotype control, then fixed using a fixation kit (Becton Dickinson, Franklin Lakes, NJ) after washes with phosphate-buffered saline solution. Cells were analyzed using a BD flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Immunohistochemical Staining
Paraffin-embedded kidney sections were incubated with CCR8 antibody (PAB26136, Abnova, Walnut, CA), then incubated with biotinylated secondary antibody and with avidin−biotin peroxidase complex. Peroxidase was visualized by incubation in 3,3′-diaminobenzidine (DAB) solution.

Statistical Analysis
CCR8 gene expression levels between patients and control subjects and among MPO patients were compared using the Mann−Whitney U test and Friedman test with post hoc Wilcoxon test. Serum CCL18 and CCL1 levels between patients and control subjects were compared using the Bonferroni test. Receiver operating characteristic curves were constructed to evaluate the diagnostic accuracy of CCR8. Correlation analyses between CCR8 and erethrocyte sedimentation rate, C-reactive protein (CRP), MPO-ANCA titer, and Birmingham Vasculitis Activity Score were performed using the Spearman coefficient test. All statistical analyses were performed with IBM SPSS Statistics for Windows, Version 19.0. (IBM Corp., Armonk, NY), and $P < 0.05$ was considered to be statistically significant.

RESULTS

CCR8 Expressions in Various Forms of Vasculitis
A total of 133 vasculitis patients with various forms of vasculitis were enrolled in this study. Among them, 60 patients were referred to our hospital without treatment; their baseline characteristics are summarized in Table 1. The number of patients with MPO was 30, whereas the number with PR3 was 4, because the major type of ANCA is MPO in a Japanese population. All MPO, PR3, and non-ANCA patients revealed an

Table 1. Population and types of vasculitis

	Total	MPO	PR3	ANCA-negative	Anti-GBM	Lupus	HSPN
Patients, n	60	30	4	9	4	3	10
Age, yr	70 ± 15	76 ± 8	72 ± 9	78 ± 7	74 ± 6	49 ± 13	48 ± 15
Male, %	48.3	46.7	75.0	66.7	25.0	0.0	50.0
Creatinine, mg/dl	3.1 ± 3.0	2.7 ± 2.0	3.2 ± 1.8	4.6 ± 2.0	9.3 ± 6.6	0.7 ± 0.2	1.0 ± 0.4
C-reactive protein, mg/dl	6.0 ± 6.5	8.0 ± 6.5	2.6 ± 4.2	2.1 ± 2.1	13.6 ± 6.1	0.9 ± 0.7	3.6 ± 6.8
BVAS		20.3 ± 6.6	18.3 ± 6.7	19.0 ± 8.9			

Data are presented as number, percentage, or mean ± SD. ANCA, antineutrophil cytoplasmic antibody; BVAS, Birmingham Vasculitis Activity Score; GBM, anti-glomerular basement membrane disease; HSPN, Henoch-Schonlein purpura; Lupus, lupus nephritis; MPO, myeloperoxidase; PR3, proteinase 3.

elevated Birmingham Vasculitis Activity Score (20.3 ± 6.6, 18.3 ± 6.7, and 19.0 ± 8.9, respectively). Corticosteroid was used as an initial treatment for ANCA-associated vasculitis, and i.v. cyclophosphamide was used in combination in some cases. One case of propylthiouracil-induced ANCA-associated vasculitis was treated with discontinuation of propylthiouracil without any immunosuppressive drugs.

All 4 GBM patients progressed to end-stage kidney failure, but none of them manifested pulmonary hemorrhage. Histological classification of 3 lupus patients included class III, IV and III+V. All lupus patients showed protein and blood in the urine; however, renal function was normal, with serum creatinine levels of 0.7 ± 0.2 mg/dl, possibly because they were referred to the hospital within several months of disease onset.

CCR8 gene expression in PBMCs was significantly upregulated in MPO- and ANCA-negative patients compared to healthy controls ($P < 0.001$ and $P = 0.001$, respectively) whereas no upregulation was seen in PR3, GBM, lupus, and Henoch-Schonlein purpura (HSPN) (Figure 1a). A receiver operating characteristic curve was calculated between MPO and healthy controls and revealed that CCR8 predicted the activity of MPO-ANCA−associated vasculitis with the area under the curve of 0.91 (95% confidence interval [95% CI] = 0.82–1.00), sensitivity of 87.6%, and specificity of 100% with the cut-off point of 2.1 (Figure 1b). CCR8 protein expression was assessed by flow cytometry; mean fluorescence intensity of CCR8 in lymphocytes increased significantly in MPO-ANCA−associated vasculitis patients compared to healthy controls ($P = 0.01$), which was equivalent to that observed with quantitative polymerase chain reaction (Figure 1c).

Immunohistological analysis was performed for detection of CCR8-positive cells in the kidney biopsy samples from MPO-ANCA−associated vasculitis, but the CCR8 stain was blunted (Figure 1d).

CCR8 Expression Among MPO-ANCA− Associated Vasculitis Patients
Next, we examined CCR8 gene expression among patients with MPO-ANCA−associated vasculitis who were in the active, remission, and infectious phases (Figure 2a). Patients with infectious complications during active vasculitis were excluded. Upregulated CCR8 level in the active phase decreased significantly in remission ($P = 0.002$). A reduction was also shown in each patient before and after the treatment (Figure 2b). The CCR8 gene expression in MPO-ANCA−associated vasculitis patients at the time of infection, including bacterial, cytomegalovirus infection, pneumocystis pneumonia, aspergillosis, and cryptococcosis, did not increase, suggesting that this marker is not influenced by infection-associated inflammation ($P = 0.02$) (Table 2, Figure 2a). Receiver operating characteristic curves between the active and remission phases and between the active and infectious phases were calculated; areas under the curve were 0.91 (95% CI = 0.83–0.99) and 0.92 (95% CI = 0.82–1.00) with the cut-off point of 2.1 respectively, which suggests that CCR8 can accurately predict vasculitis activity (Figure 2c).

Serum CCL18 and CCL1 Levels
Serum CCL18 increased significantly in patients with MPO-ANCA−associated vasculitis compared to healthy controls ($P < 0.001$) (Figure 3). Patients with PR3, ANCA negativity, and GBM also showed elevated CCL18 levels, but no significant differences were found. Serum CCL1 did not show statistical differences among vasculitis patients with various types of vasculitis and healthy controls, but patients with GBM tended to show higher values. Then we calculated CCL18/CCL1 ratio for each patient. Even though there was no statistical difference demonstrated, the ratio was higher in patients with pauci-immune types of vasculitis (MPO, PR3, and ANCA-negative), which reflected a similar tendency observed in CCR8 levels in PBMCs.

Cases
Representative cases of MPO-ANCA−associated vasculitis are shown in Figure 4. In all cases, upregulated CCR8 gene expression in the active phase consistently decreased in remission. In cases 1 and 2, CCR8 stayed low when cases were complicated by bacterial pneumonia or cryptococcal meningitis during remission. Case
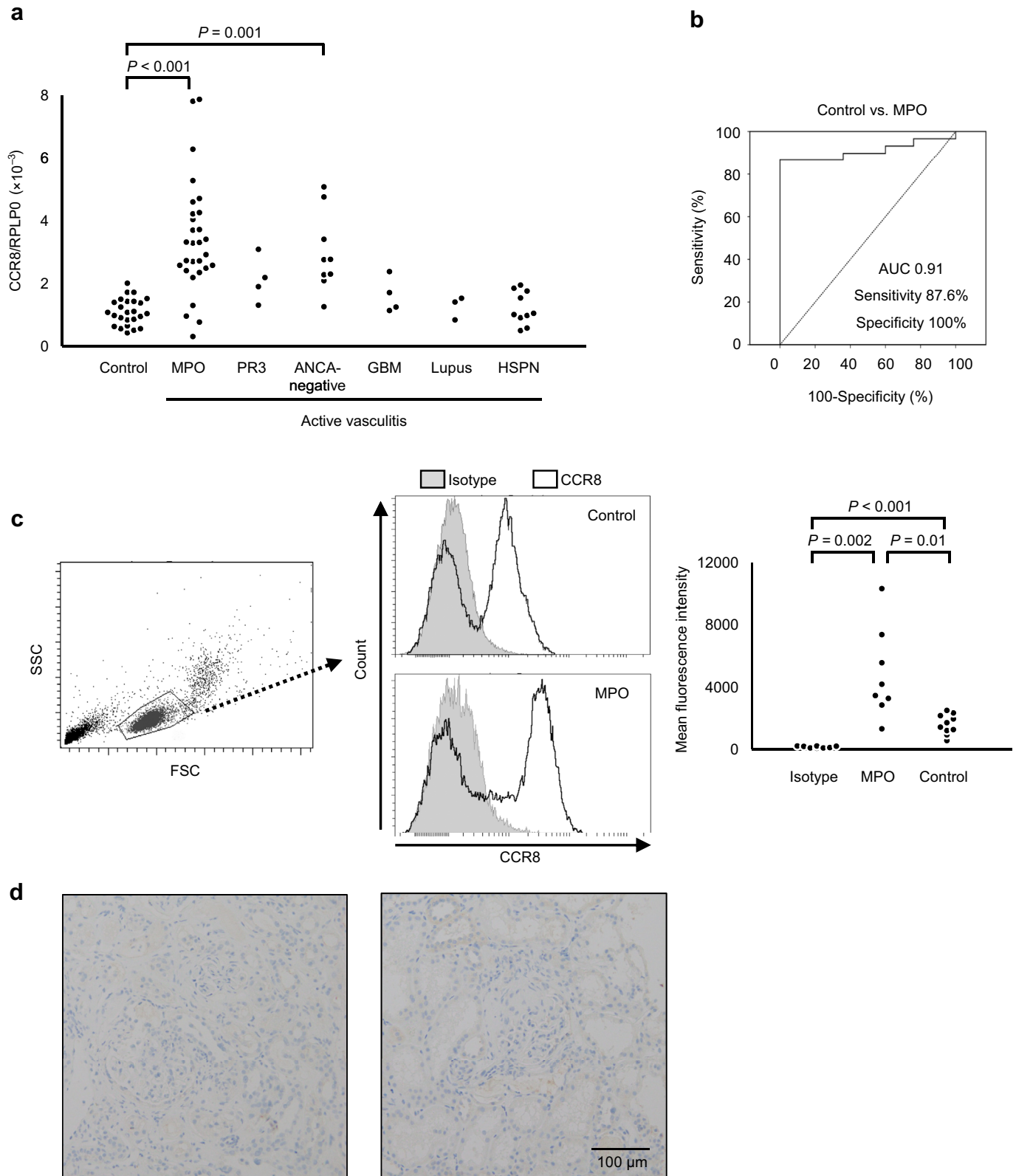
Figure 1. Chemokine receptor 8 (CCR8) expressions in various forms of vasculitis. (a) CCR8 gene expression in peripheral blood mononuclear cells among patients with various types of active vasculitis before initiating any treatment. (b) A receiver operating characteristic curve of CCR8 gene expression for distinguishing vasculitis activity between myeloperoxidase (MPO)–antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis patients and healthy controls. (c) CCR8 expression on lymphocytes subset in healthy controls and MPO-ANCA—associated vasculitis patients. (Left) Lymphocytes were identified by forward scatter (FSC) and side scatter (SSC). (Middle) Representative fluorescence histogram of lymphocytes stained with anti-CCR8 or isotype control. (Right) Mean fluorescence intensity of CCR8 on lymphocytes from healthy control and MPO patients and of isotype from MPO patients. (d) Immunohistological examination of CCR8 in 2 distinct MPO-ANCA—associated vasculitis kidneys. Original magnification ×20. AUC, area under the curve; GBM, anti—glomerular basement membrane disease; HSPN, Henoch-Schonlein purpura; Lupus, lupus nephritis; PR3, proteinase 3.
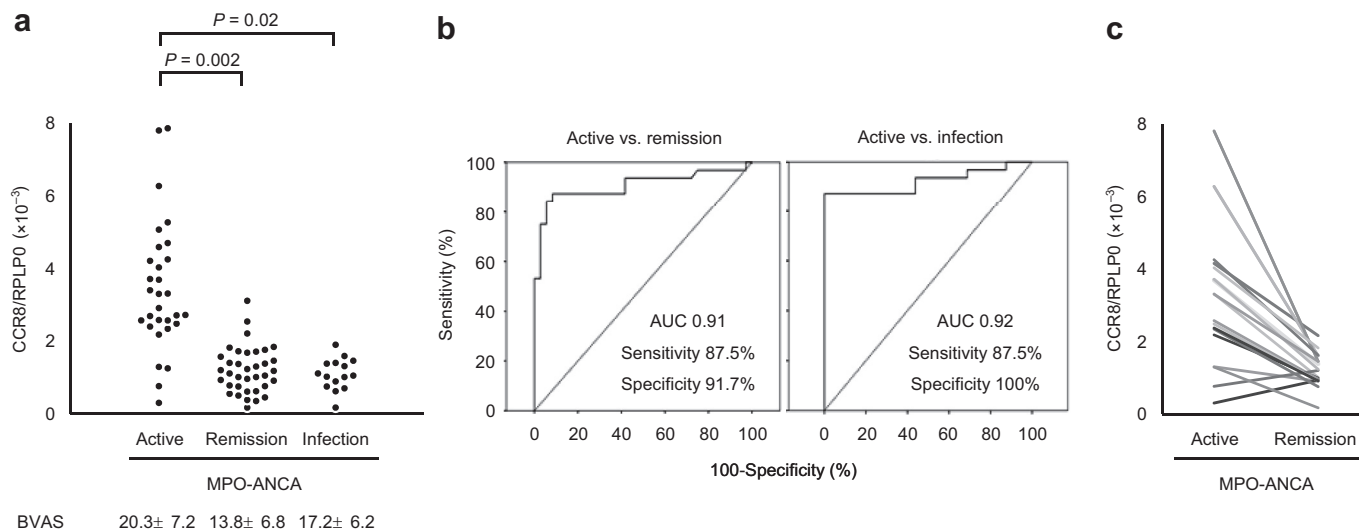
Figure 2. Chemokine receptor 8 (CCR8) expression in myeloperoxidase (MPO) antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis. (a) CCR8 gene expression in peripheral blood mononuclear cells among various phases of MPO-ANCA−associated vasculitis. (b) Receiver operating characteristic curve of CCR8 gene expression for distinguishing the active from remission phases (left) and the active from infectious phases (right). (c) Sequential changes in CCR8 gene expression in each MPO-ANCA−associated vasculitis patient. AUC, area under the curve; BVAS, Birmingham Vasculitis Activity Score.

3 was an anuria patient on maintenance hemodialysis with a previous history of vasculitis-associated interstitial lung disease. He was admitted to the hospital 3 times with a cough, fever, and high CRP level. He was treated with steroids for relapsed vasculitis at the first admission, when his serum MPO-ANCA titer increased and his CCR8 was high. On the second and the third admissions, only antibiotics were used against infectious pneumonia when CCR8 was low. This case indicates that CCR8 is useful in diagnosing vasculitis activity even in patients without urinalysis. Case 4 was a patient with relapsed vasculitis after remission. At the time of relapse, his MPO-ANCA titer did not increase, whereas his CCR8 was high, implying that CCR8 is more sensitive or prompt than MPO-ANCA titer in predicting vasculitis activity.

Relationship Between CCR8 and Vascular Inflammation

The correlation between disease activity and CCR8 gene expression was examined among patients with

Table 2. Characteristics of infectious complications in myeloperoxidase−antineutrophil cytoplasmic antibody−associated vasculitis patients

Infection type	Complications	n
Bacterial	Bronchitis	1
	Pneumonia	7
	Enterocolitis	1
	Sepsis	2
Viral	Cytomegalovirus	1
Fungal	Cryptococcus	1
	Pneumocystis jirovecii	2

MPO-ANCA−associated vasculitis. MPO patients whose cases were complicated with infection were excluded from this analysis. On the Spearman correlation test, CCR8 was positively related to erythrocyte sedimentation rate ($r = 0.41$, $P < 0.001$) and CRP ($r = 0.22$, $P = 0.02$) (Figure 5). A positive but weak correlation was shown between CCR8 and MPO-ANCA titer ($r = 0.18$, $P = 0.13$) and Birmingham Vasculitis Activity Score ($r = 0.12$, $P = 0.23$), which was not statistically significant.

DISCUSSION

Diagnosing vasculitis is difficult because of its non-distinguished and nonspecific physical signs, such as fever, cough, myalgia, and arthralgia. Meanwhile, treatment should be started immediately to prevent progressive organ damage due to inflamed vessels. Moreover, even after successful remission via initial treatment, the disease is likely to relapse. In this study, we focused on PBMC gene expressions to find a new biomarker for vasculitis. Because T cells and monocytes play a central role in the immune system, we think that these cells could be involved in the pathway of vasculitis activity and may promptly reflect inflammatory changes. As a result, CCR8 gene expression in PBMCs enhances disease activity of pauci-immune−type vasculitis (MPO, PR3, and ANCA-negative) but does not reflect that of antibody-mediated and immune complex−mediated vasculitis such as GBM, lupus, and Henoch-Schonlein purpura. Although the sample number was small, CCR8 could be a sensitive indicator of active ANCA-associated vasculitis. The remarkable point of this biomarker is that
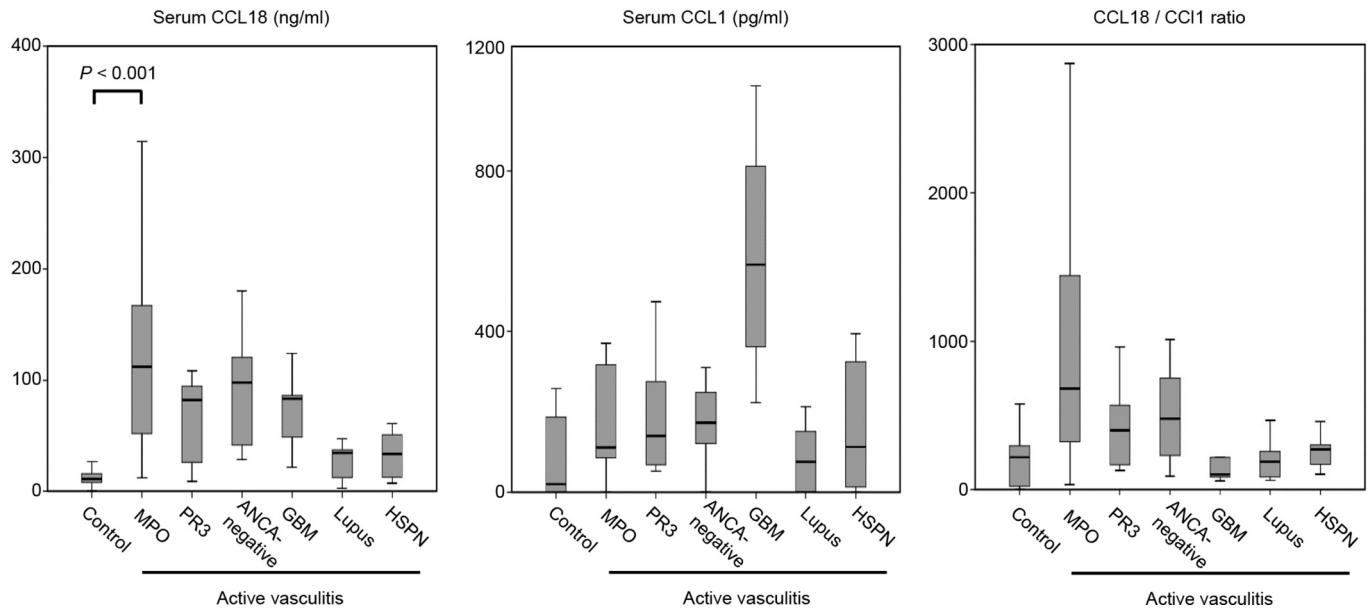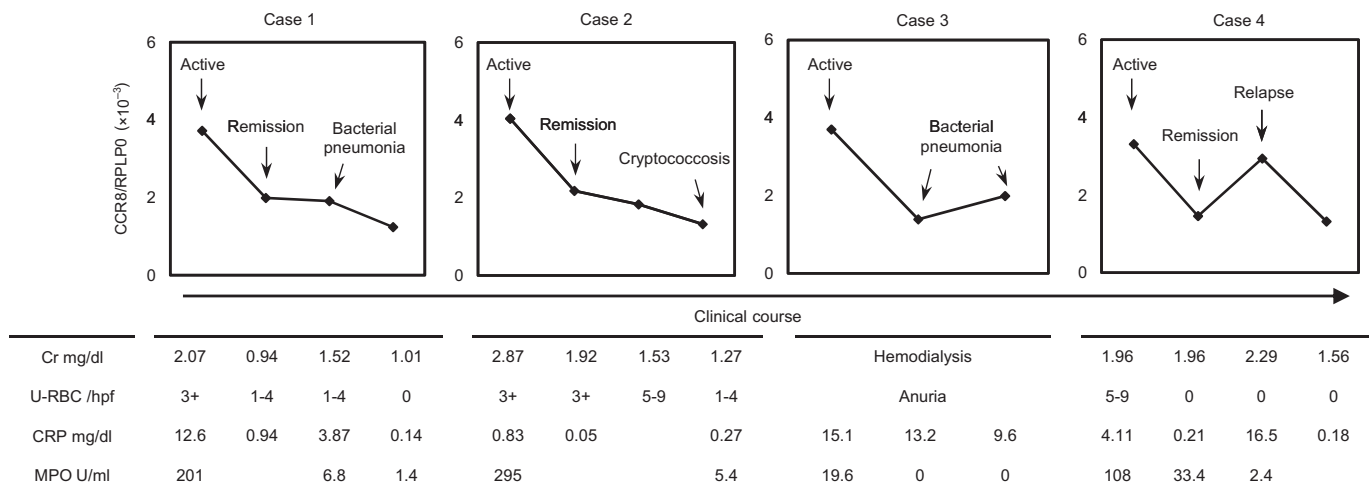
Figure 3. Serum CCL18 and CCL1 levels. Serum levels of chemokine ligand 18 and 1 were determined by enzyme-linked immunosorbent assay among various types of active vasculitis. ANCA, antineutrophil cytoplasmic antibody; CC, chemokine ligand; GBM, anti—glomerular basement membrane disease; HSPN, Henoch-Schonlein purpura; Lupus, lupus nephritis; MPO, myeloperoxidase; PR3, proteinase 3.

it is not affected by any type of infectious complications, including bacterial, fungal, or viral. Distinguishing disease activity and infection has been the most controversial issue in vasculitis treatment, because the major cause of death in ANCA-associated vasculitis is infection, of which nearly one-half of the patients die.[1] Therefore, preventing infectious complications and avoiding unnecessary immunosuppression with accurate diagnosis is considered as the highest priority. Consequently, this marker could have a great significance in vasculitis treatment. As shown in Figure 3, patients with relapsed ANCA-associated vasculitis demonstrated elevated CCR8

levels even on treatment with immunosuppressive drugs, suggesting that immunosuppressants do not undermine the CCR8 expression in active vasculitis.

Another strength of this marker is that it enables rapid diagnosis. A result can be acquired within 4 hours from collection of the blood sample, using the polymerase chain reaction method. In addition, as shown in case 4, CCR8 may reflect vasculitis activity more promptly than serum ANCA titer, indicating that this marker is suitable for a rapid diagnostic test. In most cases, vasculitis patients need to be treated as soon as possible to minimize acute organ damage.

Cr mg/dl	2.07	0.94	1.52	1.01	2.87	1.92	1.53	1.27	Hemodialysis				1.96	1.96	2.29	1.56
U-RBC /hpf	3+	1-4	1-4	0	3+	3+	5-9	1-4	Anuria				5-9	0	0	0
CRP mg/dl	12.6	0.94	3.87	0.14	0.83	0.05		0.27	15.1	13.2	9.6		4.11	0.21	16.5	0.18
MPO U/ml	201		6.8	1.4	295			5.4	19.6	0	0		108	33.4	2.4	

Figure 4. Longitudinal change in chemokine receptor 8 (CCR8) gene expression in myeloperoxidase (MPO)—antineutrophil cytoplasmic antibody—associated vasculitis patients. Status at blood sampling is indicated with arrows, and laboratory data are presented below. Cr, creatinine; CRP, C-reactive protein; U-RBC, red blood cells in the urine.
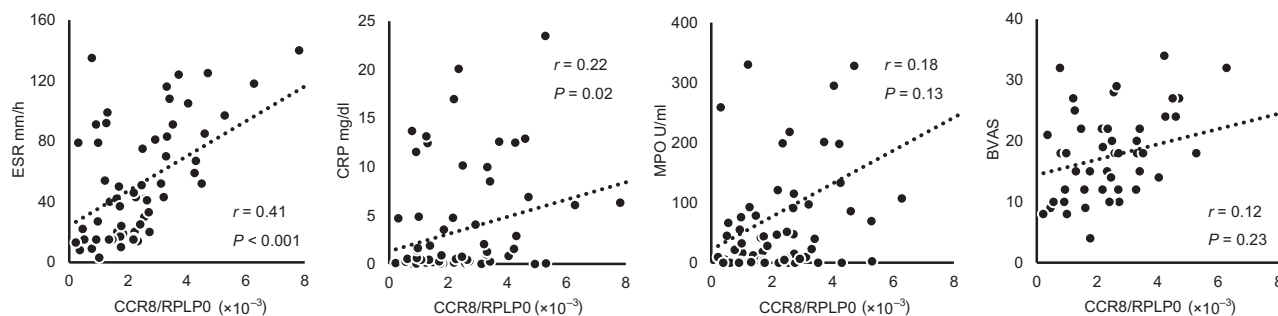
Figure 5. Correlation between chemokine receptor 8 (CCR8) and erythrocyte sedimentation rate, C-reactive protein, serum myeloperoxidase−antineutrophil cytoplasmic antibody titer, and Birmingham Vasculitis Activity Score (BVAS). Linear regression analysis and Spearman correlation coefficient between CCR8 gene expression and inflammatory markers in myeloperoxidase (MPO)−antineutrophil cytoplasmic antibody−associated vasculitis. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; RPLP0, ribosomal protein, large, P0.

Therefore, rapid diagnosis is beneficial and may lead to improved treatment outcome.

Interestingly, CCR8 is involved only in pauci-immune vasculitis. Meanwhile, in the kidney biopsy samples, every type of vasculitis, including GBM, lupus, and Henoch-Schonlein purpura, manifests crescent formation in glomeruli. Thus, we thought that the underlying mechanism of crescent formation could be different among various strains of vasculitis. We examined CCR8 immunohistochemistry in the kidney to confirm CCR8 involvement in glomerular injury. As a result, CCR8 staining was negative in ANCA-associated crescentic vasculitis, indicating that there is no apparent evidence that CCR8-positive mononuclear cells attack glomeruli directly.

In this study, patients with overlapping features of vasculitis were excluded. However, the patients with ANCA-positive lupus nephritis who showed low CCR8 levels were successfully treated with multi-target therapy, which is a regimen for lupus. Although further study is required, CCR8 could be a diagnostic tool to discriminate between pauci-immune and other types of vasculitis.

Previous reports have shown that CCR8 is involved in atopic dermatitis and bronchial asthma, both of which are associated with Th2 immune responses, suggesting that CCR8-mediated inflammation is not limited to vasculitis.[13,14] Like these allergic diseases, pauci-immune vasculitis may be characterized by delayed hypersensitivity. Takeuchi *et al*. showed evacuation of silica influenced by the development of ANCA-associated vasculitis after the Great East Japan earthquake.[15] Although the causes of vasculitis are largely unknown, CCR8-mediated allergic stimuli could be part of the pathogenesis in pauci-immune types of vasculitis.

CCL18 and CCL1 are both ligands of CCR8, and they are known to compete for CCR8 binding.[11] We speculate that the CCL18−CCR8 axis is essential for ANCA-associated vasculitis; then CCL1 may block the CCL18−CCR8 interaction as a competitive inhibitor, because the previous report revealed that CCL18 is associated with vasculitis activity. The CCL18/CCL1 ratio in serum showed a pattern analogous to that observed in CCR8 in PBMCs, suggesting that dominance of CCL18 over CCL1 could be involved in the development of pauci-immune types of vasculitis.

In conclusion, we found that CCR8 enhances the activity of MPO-ANCA−associated vasculitis. Using this marker, accurate and rapid diagnosis is possible by distinguishing between vasculitis and infection. We hope that this study can be contributory to the treatment of vasculitis and lead to better prognosis in vasculitis patients.

DISCLOSURE

All the authors declared no competing interests.

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