IMMUNOLOGY ORIGINAL ARTICLE

The role of CCR1 and therapeutic effects of anti-CCL3 antibody in herpes simplex virus-induced Behçet's disease mouse model

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Summary

Behçet's disease (BD) is a chronic systemic inflammatory disease with unclear etiopathogenesis. Although gene variants of CC chemokine receptor type 1 (CCR1) have been reported, the protein expression of CCR1 in patients with BD remains unclear. The objective of this study was to analyze the frequencies of $CCR1^+$ cells in a herpes simplex virus-induced mouse model of BD. The frequencies of $CCR1^+$ cells on the surface and in the cytoplasm of peripheral blood mononuclear cells and lymph nodes were analyzed by flow cytometry. The $CCR1^+$ cells were significantly down-regulated in BD mice compared with the normal control and symptom-free control mice. Colchicine and pentoxifylline treatment improved the symptoms of BD and increased the frequencies of $CCR1^+$ cells in BD mice. Treatment with chemokine CC motif ligand 3 (CCL3), a ligand of CCR1, caused BD symptoms to deteriorate in 10 of 16 BD mice (62·5%) via down-regulation of $CCR1^+$ cells. Anti-CCL3 antibody treatment ameliorated BD symptoms in 10 of 20 mice (50%) and significantly decreased the disease severity score compared with CCL3-treated BD mice ($P = 0·01$) via up-regulation of $CCR1^+$ cell frequencies. In patients with BD, plasma levels of CCL3 in an active state were significantly higher than in healthy control individuals ($P = 0·02$). These results show that the up-regulation of $CCR1^+$ cells was related to the control of systemic inflammation of BD in mouse models.

Keywords: Behçet's disease; CCL3; CCR1; herpes simplex virus; inflammation; mouse model.

Introduction

Behçet's disease (BD) is a chronic systemic inflammatory disease with unclear etiopathogenesis. Gene variants of CC chemokine receptor type 1 (CCR1) in patients with BD have been identified by genome-wide association studies.[1,2] However, the CCR1 protein expression in patients with BD or in mouse models remains unclear.

CC chemokine receptor type 1 is expressed in cells of the immune system, including lymphocytes, monocytes, neutrophils and dendritic cells.[3,4] It is involved in host defense, hematopoiesis, T helper type 1/2 (Th1/2) cell balance, and chemotaxis.[5,6] The CCR1 expression is correlated with various inflammatory diseases, including rheumatoid arthritis,[7] asthma[8] and lupus nephritis.[9] CCR1 knockout ($-/-$) mice show reduced pulmonary inflammation,[10] and prolonged allograft survival.[11] CCR1 polymorphism might be a general risk factor for autoimmune or inflammatory disease. CCR1 antagonists have been used in clinical trials to treat multiple sclerosis, psoriasis and rheumatoid arthritis.[12]

Herpes simplex virus (HSV) has been reported as an etiological factor in BD.[13–15] A mouse model of HSV-induced inflammatory BD was developed and used in various studies.[16–19] The objective of the present study was to determine the expression of CCR1 in mice with inflammatory BD symptoms and to investigate whether CCR1 was regulated by ligands, agonists or antagonists. Flow

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Abbreviation: BD, Behçet's disease; BDN, herpes simplex virus-inoculated, but asymptomatic healthy mice; CCL3, chemokine CC motif ligand 3; CCR1, CC chemokine receptor type 1; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-10, interleukin-10

cytometry was used to examine the frequencies of CCR1^{+} cells on the surface and in the cytoplasm of lymph node (LN) cells and peripheral blood mononuclear cells (PBMC). Our results demonstrated the correlation between CCR1 surface expression on PBMC and the severity of BD symptoms in mice.

Materials and methods

Animal experiments

Male ICR mice (4 weeks old) were infected with HSV type 1 (1×10^6 plaque-forming units/ml, F strain) grown in Vero cells as described previously.[16] The virus was inoculated twice into mice at a frequency of 10 days. After the second HSV inoculation, mice were observed from week 4 to week 32. Animals were handled in accordance with guidelines provided by the Institutional Animal Care and Use Committee of Ajou University School of Medicine (AMC-2016-0010).

Symptoms of BD in mice

Among all the mice infected with HSV, 15% developed BD-like symptoms including skin ulcerations, skin pustules, genital ulcerations, erythema, oral ulceration, ear inflammation, arthritis, genital ulcerations, red eye (left, right), reduced vision (left, right), weight loss and facial swelling. Oral, genital and skin ulcers, and eye symptoms were classified as major symptoms. Other symptoms were identified as minor symptoms. Mice displaying more than one major and one minor symptom were diagnosed with BD. Each symptom score was assigned a single point. The sum of all symptom scores was used to determine the severity of BD. Disappearance of symptoms or ≥20% decrease in lesion size was classified as symptom improvement. The disease severity score of BD was calculated according to the BD activity index outlined in the BD Activity Form (www.behcet.ws/pdf/BehcetsDiseaseActivityForm.pdf). HSV-inoculated, but asymptomatic, healthy mice (BDN mice) served as the control group, as described previously.

Flow cytometry

Peripheral blood mononuclear cells and LN cells were isolated from mice. Erythrocytes were eliminated with ammonium–chloride–potassium solution and washed with phosphate-buffered saline (PBS) followed by surface staining of 1×10^6 cells with mouse CCR1 allophycocyanin-conjugated antibody (cat# FAB5986A; R&D Systems, Minneapolis, MN) for 30 min at 4° in the dark. For intracytoplasmic staining, cells were fixed in intracytoplasmic fixation buffer for 20 min at room temperature. After washing twice with a permeabilization buffer, 1×10^6 cells were stained with an anti-mouse CCR1 antibody,

followed by analysis of these cells with a flow cytometer (FACS Canto II; Becton Dickinson, San Jose, CA).

Western blot analysis

Fresh PBMC were washed twice with cold PBS and lyzed with lysis buffer (50 mM Tris–Cl, pH 7·4, 0·15 M NaCl, 1 mM EDTA, 1% Triton X-100, 10 µg/ml each of aprotinin and leupeptin, and 1 mM phenylmethylsulphonyl fluoride). The protein (30 µg) lysate was electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel. The proteins were transferred onto nitrocellulose membranes (Protran; GE Healthcare Life Sciences, Chicago, IL, USA) and subjected to immunoblot analysis using antibodies. The immunoblots were developed with the enhanced chemiluminescence system (GE Healthcare Life Sciences). Antibody against CCR1 (MAB5986) was purchased from R&D Systems and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (sc-32233) was from Santa Cruz Biotechnology, Inc. (Dallas, TX).

Administration of cytokines, ligand and antagonist

Normal and BD mice were treated in vivo with PBS, interleukin-10 (IL-10) (50 pg/mouse/day) (cat# CYT-497; Prospecbio, Ness-Ziona, Israel), granulocyte–macrophage colony-stimulating factor (GM-CSF) (200 pg/mouse/day) (cat# CYT-222; Prospecbio), chemokine CC motif ligand 3 (CCL3) (100 pg/mouse/day) (cat# CHM-320; Prospecbio), or anti-CCL3 antibody (20 ng/mouse/day) (cat# AF-450-NA; R&D Systems) for five consecutive days by intraperitoneal administration. Normal mice were 3·5 weeks old. PBMC and LN cells were isolated 3 hr after the last medication for analysis of CCR1^{+} cells by flow cytometry. BX471, a CCR1 antagonist (cat# SML0020; Sigma-Aldrich, St Louis, MO), was intraperitoneally administered to normal and BD mice at 0·5 mg/mouse three times (every other day). BD symptoms were observed before and after treatment. Images were obtained to document the treatment-related changes.

Drug treatments

Mice were treated orally with PBS, colchicine (2 µg/mouse/day), or pentoxifylline (400 µg/mouse/day) for five consecutive days. Changes in symptoms were monitored. At 3 hr after the last medication, the mice were killed by intramuscular injection into the hind leg with a ketamine/xylazine cocktail (15 mg/kg ketamine and 10 mg/kg xylazine). PBMC and LN cells were isolated for analysis of CCR1^{+} cells.

Patients with BD

The patient population consisted of 12 patients with BD, who presented for the first time or were monitored at the

Department of Dermatology, Ajou University Hospital. Sampling from patients with BD was random when the patients first arrived. Clinical characteristics and therapeutic histories of these patients are shown in Tables 1 and 2. According to the International Study Group for BD criteria, the presence of any two of the following symptoms, in addition to recurrent oral ulcerations, is considered as BD diagnosis: recurrent genital ulceration, cutaneous erythema nodosum, large-vessel vasculitis, arthritis, uveitis, and/or a positive pathergy test. The disease severity score was calculated from Behçet's disease current activity form 2006 (http://www.behcetdiseasesociety.org/behcetwsData/Uploads/files/BehcetsDiseaseActivityForm.pdf). Patients with active BD ($n = 12$, 11 men, one woman, 45.0 ± 10.2 years) were enrolled. Gender differences among BD patients with mucocutaneous manifestations were inconsistent among different nationalities.[20] Informed consent was obtained from patients before the study. The healthy control group ($n = 12$, 48.8 ± 9.2 years) comprised one man and 11 women. Serum was provided by the Biobank of Ajou University Hospital, a member of the Korea Biobank Network. This study was approved by the ethics committee of Ajou University Medical Center Institutional Review Board (AJIRB-GN3-07-098) and all experiments were conducted according to the Institutional Review Board permitted protocol.

CCL3 ELISA

Peripheral blood was collected in EDTA-coated test tubes and centrifuged at 1000 *g* for 5 min at 20°. The supernatant was stored at −70° until used. CCL3 levels were measured in plasma using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Cat# BMS2029INST; Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. For ELISA analysis, each sample was applied in duplicate wells and three times in different ELISA plates.

Statistical analysis

The data and statistical analysis complied with the recommendations of experimental design and analysis in pharmacology.

Results

Frequencies of CCR1$^+$ cells in BD mice

Flow cytometric analysis was performed to analyze the surface and cytoplasm CCR1$^+$ cells stained with anti-CCR1 antibody for PBMC and LN cells isolated from normal ($n = 10$), BDN ($n = 10$), and BD ($n = 10$) mice. Frequencies of CCR1$^+$ cells on the surface of PBMC were significantly down-regulated in BD mice compared with those in normal mice ($7.06 \pm 1.8\%$ versus $11.11 \pm 2.5\%$, $P = 0.0005$) or BDN mice ($7.06 \pm 1.8\%$ versus $10.58 \pm 1.92\%$, $P = 0.0006$). Frequencies of CCR1$^+$ cells on the surface of LN cells were also down-regulated in BD mice compared with normal mice ($10.6 \pm 3.2\%$ versus $12.99 \pm 1.5\%$, $P = 0.05$) or BDN mice ($10.6 \pm 3.2\%$ versus $11.12 \pm 2.1\%$, $P = 0.67$) (Fig. 1a).

Table 1. Clinical characteristics of patients with Behçet's disease

Patient no.	Age	Sex	OU	GU	Skin	OL	Arth	Neur	Vas	Path	HLA
Before treatment											
1	46	M	+	+	+	+	+				+
2	43	M	+		+				+		−
3	20	M	+	+	+	+					−
4	36	M	+	+	+	+		+			+
5	60	M	+	+	+	+				−	−
6	44	M	+	+		+					−
7	52	M	+		+					+	
8	45	F	+	+	+	+				−	−
9	52	M	+		+				+	+	+
10	47	M	+	+	+	+				−	+
11	54	M	+		+				+	+	−
12	41	M	+	+		+			+		−
After treatment											
2	43	M	+		+						−
3	20	M	+		+	+					−
6	44	M	+		+						−
7	52	M	+							+	
8	45	F	+							−	−

Arth, Arthritis; GU, genital ulcers; HLA, HLA-B51; Neur, neurological involvement; OL, ocular lesion; OU, oral ulcers; Path, pathergy test; Skin, skin lesion; Vas, vasculitis.

Table 2. Therapeutic history of patients with Behçet's disease

Patient	Colchicine	Azathioprine	Salazopyrine	Pentoxifylline	Minocycline	Aspirin	Vit.D	Methotrexate	Prednisolone	Methylprednisolone
1	+	+		+						
2	+			+	+				+	
3	+	+								
4	+		+	+		+	+			
5	+									
6	+			+	+		+	+	+	
7	+			+			+			
8	+		+	+			+			
9	+			+			+			
10	+			+						+
11	+			+						
12	+									

Figure 1. Frequencies of CCR1^{+} cells in normal, herpes simplex virus-inoculated, but asymptomatic healthy (BDN) and Behçet's disease (BD) mice. Peripheral blood mononuclear cells (PBMC) and lymph node cells were isolated from normal, BDN and BD mice. Frequencies of surface CCR1^{+} cells (a) and cytoplasm CCR1^{+} cells (b) of whole leukocytes were analyzed by FACS. Representative flow cytometric histograms of surface CCR1^{+} PBMC and lymph node cells are shown (c). (d) Protein expression of CCR1 by Western blot, (e) Expression ratio of CCR1 to GAPDH.

The frequencies of cytoplasm staining in CCR1^{+} PBMC were significantly lower in BD mice than in normal mice ($32.08 \pm 16.3\%$ versus $73.6 \pm 3\%$, $P < 0.0001$) or BDN mice ($32.08 \pm 16.3\%$ versus $58.4 \pm 11.05\%$, $P = 0.0005$). The frequencies of CCR1^{+} LN cells were also significantly lower in BD mice than in normal mice

(58·04 ± 22·39% versus 82·14 ± 8·15%, $P = 0·005$). There were no differences in frequencies of CCR1$^+$ cells between BD and BDN mice (58·04 ± 22·39% versus 59·10 ± 27·6, $P = 0·9$) (Fig. 1b). Representative histograms of surface staining results are presented in Fig. 1(c). Figure 1(d) shows the Western blot analysis for CCR1 expression in whole PBMC lysates in normal, BDN and BD mice ($n = 3$ in each group). The expression ratio of CCR1 compared with GAPDH was not different between BDN (1·42 ± 0·34) and BD (1·48 ± 1·15) mice (Fig. 1e, $n = 7$). This means the entire amount of CCR1 in PBMC was not correlated with the symptoms.

Interleukin-10 and GM-CSF affect frequencies of CCR1$^+$ cells in normal mice

Interleukin-10 synergistically enhanced GM-CSF-induced CCR1.[21] GM-CSF is known as a CCR1-enhancing cytokine.[22] Therefore, IL-10 and GM-CSF were administered to normal and BD mice. To determine the functional doses in mice *in vivo*, IL-10 and GM-CSF were injected into 3·5-week-old normal mice intraperitoneally at the following doses: IL-10 (25, 50 and 100 pg) and GM-CSF (50, 100 and 200 pg). At 3 hr after the last treatment, PBMC were isolated from mice for flow cytometry to measure the frequencies of CCR1$^+$ cells on the surface and cytoplasm.

In mice treated with 50 pg of IL-10 ($n = 13$), the frequencies of surface CCR1$^+$ cells were significantly up-regulated in PBMC compared with PBS-treated control mice ($n = 5$) (13·14 ± 0·9% versus 11·0 ± 0·3%, $P = 0·009$). Treatment with 25 pg of IL-10 ($n = 9$) did not affect the frequencies of CCR1$^+$ cells compared with PBS-treated control mice ($n = 5$) (11·74 ± 0·96% versus 11·0 ± 0·3%, $P = 0·47$). Treatment with 100 pg of IL-10 ($n = 6$) up-regulated the frequencies of CCR1$^+$ cells compared with PBS-treated control mice ($n = 5$), although the difference was not statistically significant (13·65 ± 2·05% versus 11·0 ± 0·3%, $P = 0·18$). The frequencies of cytoplasm CCR1$^+$ PBMC were slightly increased in mice treated with 50 pg of IL-10 and 100 pg of IL-10 compared with those in PBS-treated control mice (62·575 ± 9·89% versus 56·05 ± 23·12%, $P = 0·6$; 58·8 ± 23·33% versus 56·05 ± 23·12%, $P = 0·9$), although the differences were not statistically significant (Fig. 2a).

In normal mice exposed to 200 pg of GM-CSF ($n = 5$), the frequencies of surface CCR1$^+$ PBMC were up-regulated compared with PBS-treated control mice (15·65 ± 1·4% versus 11·0 ± 0·42%, $P = 0·05$). In mice treated with 100 pg of GM-CSF ($n = 6$), the frequencies of CCR1$^+$ cells were higher compared with the levels in PBS-treated control mice ($n = 5$) (13·75 ± 1·34% versus 11·0 ± 0·3%, $P = 0·11$). However, the difference was not statistically significant. Mice treated with 50 pg of GM-

CSF ($n = 6$) showed frequencies of CCR1$^+$ cells similar to those in PBS-treated control mice ($n = 5$). In the cytoplasm of PBMC, frequencies of CCR1$^+$ cells in mice treated with GM-CSF at 50, 100 and 200 pg (62·25 ± 3·18%; 63·95 ± 1·9%; 64·7 ± 1·5%, respectively) were not significantly ($P > 0·05$) different compared with those in the PBS control group (56·05 ± 23·12%) (Fig. 2b).

Interleukin-10 and GM-CSF up-regulate frequencies of CCR1$^+$ cells in BD mice

According to functional doses of IL-10 and GM-CSF in normal mice, 50 pg/mouse/day of IL-10 and 200 pg/mouse/day of GM-CSF were administered to BD mice to up-regulate CCR1$^+$ cells. After five consecutive days of treatment to BD mice, PBMC and LN cells were subjected to surface and cytoplasm staining for FACS analysis. In the surface of PBMC, the frequencies of CCR1$^+$ cells in BD mice treated with IL-10 ($n = 7$) and GM-CSF ($n = 7$) showed significant up-regulation compared with those in PBS-treated BD mice ($n = 10$) (IL-10 versus PBS: 13·61 ± 2·5% versus 7·06 ± 1·8%, $P < 0·0001$; GM-CSF versus PBS: 9·0 ± 1·3% versus 7·06 ± 1·8%, $P = 0·03$). The surface CCR1$^+$ LN cells were significantly up-regulated compared with PBS-treated BD mice (IL-10 versus PBS: 17·6 ± 2·7% versus 10·6 ± 3·23%, $P = 0·0002$; GM-CSF versus PBS: 15·4 ± 1·6% versus 10·6 ± 3·23%, $P = 0·003$) (Fig. 2c). In the cytoplasm, CCR1$^+$ PBMC also increased after treatment with IL-10 (54·67 ± 5·00% versus 32·04 ± 16·3%, $P = 0·02$) and GM-CSF (82·1 ± 6·4% versus 32·08 ± 16·3%, $P = 0·001$) compared with PBS-treated BD mice. Cytoplasm CCR1$^+$ cells in LN were increased only by GM-CSF (77·85 ± 5·9% versus 58·04%, $P = 0·04$). Frequencies of cytoplasm CCR1$^+$ LN cells were not changed after IL-10 treatment (Fig. 2d). IL-10 and GM-CSF up-regulated the frequencies of CCR1$^+$ cells in both normal and BD mice.

CCL3 down-regulates the expression of CCR1$^+$ cells in normal mice

CCR1 has multiple ligands. CCL3 is one of its ligands for CCR1 down-regulation function.[23] To determine whether CCR1 was regulated by CCL3, CCL3 was intraperitoneally injected into normal mice. Frequencies of CCR1$^+$ PBMC and LN cells were decreased after CCL3 treatment. In surface staining of PBMC, treatment with 100 pg of CCL3 ($n = 6$) significantly decreased the frequencies of CCR1$^+$ cells compared with PBS control ($n = 5$) (4·62 ± 1·3% versus 12·92 ± 1·89%, $P = 0·0002$). Treatment with 50 pg CCL3 decreased frequencies of CCR1$^+$ cells, although the decrease was not significant (9·05 ± 2·7% versus 12·92 ± 1·89%, $P = 0·06$). In surface staining of LN cells, treatment with CCL3 at 100 pg ($n = 6$) significantly down-regulated the frequencies of CCR1$^+$ cells
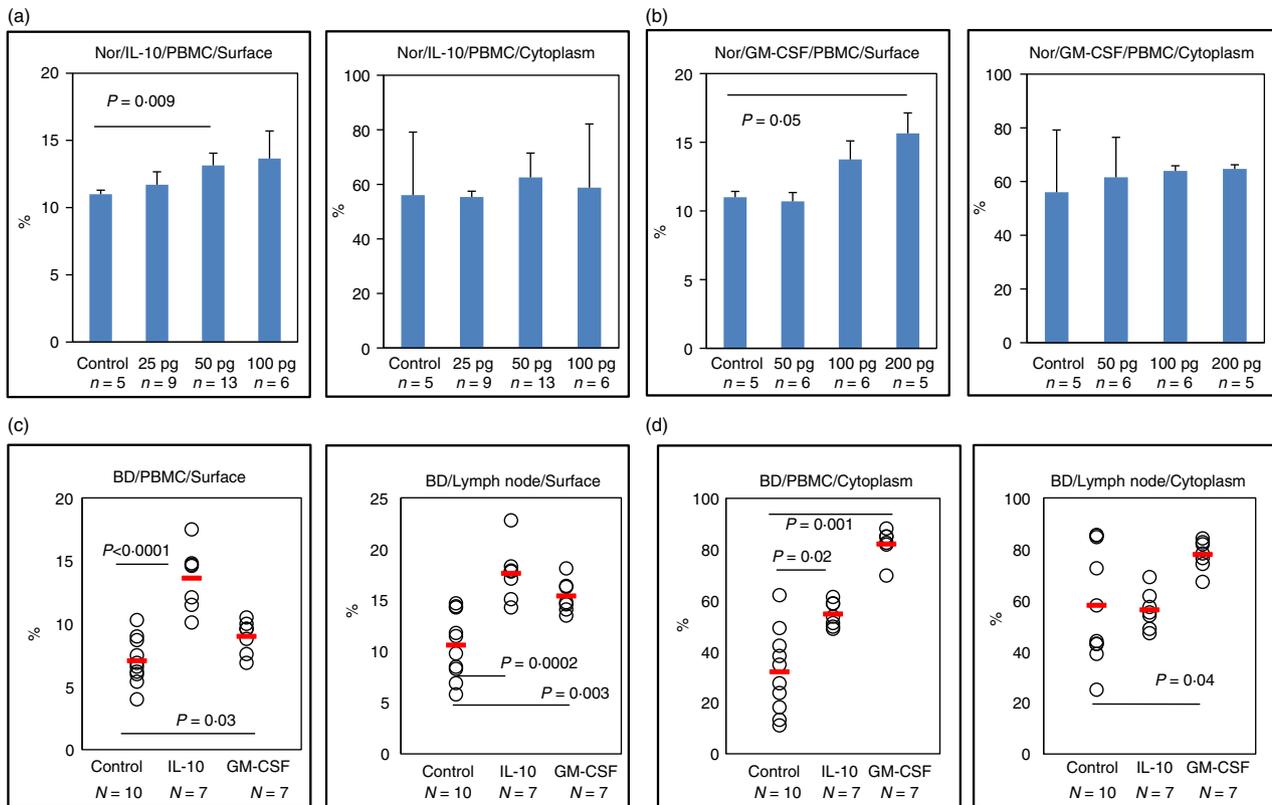
Figure 2. Frequencies of CCR1^{+} cells in cytokine-treated normal and Behçet's disease (BD) mice. Interleukin-10 (IL-10) (25, 50 and 100 pg) and granulocyte–macrophage colony-stimulating factor (GM-CSF) (50, 100 and 200 pg) were intraperitoneally injected into normal mice for five consecutive days and peripheral blood mononuclear cells (PBMC) were isolated. Frequencies of CCR1^{+} cells were analyzed by FACS for expression on the surface and in the cytoplasm of mice after treatment with IL-10 (a) or GM-CSF (b). IL-10 (50 pg) and GM-CSF (200 pg) were intraperitoneally injected into BD mice for five consecutive days. PBMC and lymph node cells were isolated. Frequencies of surface CCR1^{+} (c) and cytoplasm CCR1^{+} (d) PBMC and lymph node cells derived from IL-10- and GM-CSF-treated BD mice were analyzed by FACS.

compared with PBS control ($n = 5$) ($9.42 \pm 1.32\%$ versus $13.15 \pm 1.7\%$, $P = 0.009$). Treatment with CCL3 50 pg ($n = 6$) resulted in similar frequencies of CCR1^{+} cells compared with PBS-treated control mice (Fig. 3a).

The frequencies of cytoplasm CCR1^{+} PBMC after treatment with CCL3 at 50 and 100 pg were significantly decreased to ($50.95 \pm 4.3\%$, $P < 0.0001$) and ($33.9 \pm 4.4\%$, $P < 0.0001$) compared with PBS control ($74.05 \pm 3.4\%$). Cytoplasm CCR1^{+} LN cells were significantly down-regulated in mice treated with 50 pg of CCL3 ($25.65 \pm 1.34\%$, $P < 0.0001$) or 100 pg of CCL3 ($39.48 \pm 2.4\%$, $P < 0.0001$) compared with PBS-treated control mice ($73.75 \pm 1.4\%$) (Fig. 3b). A histogram of surface and cytoplasm CCR1^{+} PBMC after treatment with CCL3 is presented in Fig. 3(c).

CCL3 down-regulates the expression of CCR1^{+} cells and deteriorates symptoms in BD mice

To down-regulate CCR1^{+} cells in BD mice, CCL3 100 pg/ day was administered to BD mice ($n = 11$) for five

consecutive days. Frequencies of surface CCR1^{+} PBMC were significantly lower compared with PBS-treated BD mice ($n = 10$) ($4.59 \pm 1.36\%$ versus $7.06 \pm 1.8\%$, $P = 0.003$). The frequencies of surface CCR1^{+} LN cells were not different after CCL3 treatment (Fig. 4a). Cytoplasm CCR1^{+} LN cells were down-regulated significantly after CCL3 treatment compared with PBS control ($38.38 \pm 7.74\%$ versus $58.04 \pm 22.39\%$, $P = 0.01$). However, CCR1^{+} expression was not changed in PBMC cytoplasm (Fig. 4b). Treatment with CCL3 deteriorated BD symptoms in 10 (62.5%) of 16 BD mice and the disease severity score increased from 2.73 ± 0.47 to 3.2 ± 1.00 after 5 days of treatment. Four BD mice showed new skin ulcer and arthritis, and the remaining BD mice showed increased ulcer size.

BX471, a CCR1 antagonist, does not down-regulate CCR1^{+} cells in BD mice

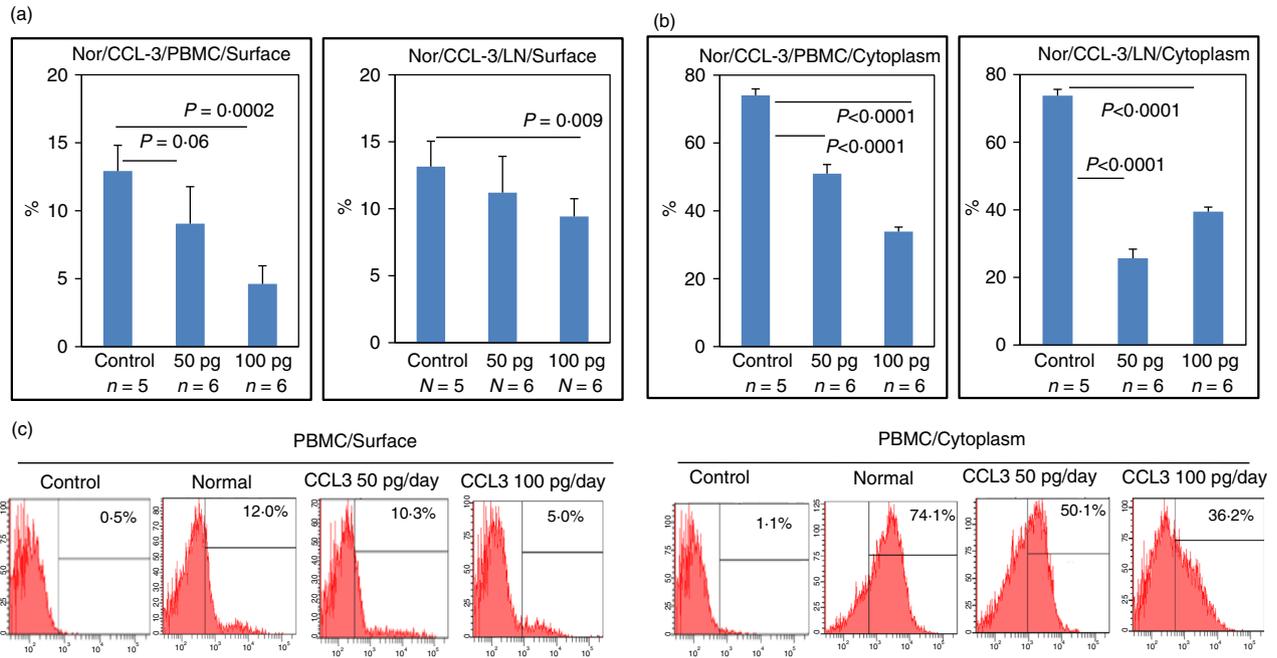BX471 was developed as a CCR1 antagonist to treat several inflammatory diseases, including rheumatoid

Figure 3. Frequencies of CCR1$^+$ cells in CCL3-treated normal mice. CCL3 (50 pg and 100 pg) was intraperitoneally injected into normal mice for five consecutive days. Peripheral blood mononuclear cells (PBMC) and lymph node cells were then isolated. Frequencies of CCR1$^+$ cells were analyzed by FACS on the surface (a) and in the cytoplasm (b) of PBMC and lymph node cells. Representative flow cytometric histograms of CCR1$^+$ cells on the surface (a) and in the cytoplasm (b) of PBMC are shown in (c).

arthritis.[12] BX471 was administered to BD mice to down-regulate CCR1. BX471-treated BD mice ($n = 3$) showed 8·23 ± 6·17% of surface CCR1$^+$ PBMC, 11·53 ± 3·71% of surface CCR1$^+$ LN cells, 54·53 ± 14·59% of cytoplasm PBMC, and 67·17 ± 11·49% of cytoplasm LN cells. In BD mice, BX471 did not play a role in the down-regulation of CCR1$^+$ cells (Fig. 4a,b). In normal mice, BX471-treated mice showed higher frequencies of surface CCR1$^+$ PBMC than in PBS-treated normal mice (17·22 ± 8·66% versus 12·15 ± 7·92%, $P = 0·3$) and LN cells (15·66 ± 1·14% versus 13·15 ± 1·73%, $P = 0·05$). Inhibition of CCR1 expression by BX471 was only found in the cytoplasm of PBMC (BX471 versus PBS: 65·94 ± 5·12% versus 74·05 ± 3·47%, $P = 0·02$) (Fig. 4c,d).

Drug treatment regulates frequencies of CCR1$^+$ cells in normal mice

Colchicine and pentoxifylline are the most frequently prescribed medications for BD.[24] They are also used in BD mice.[25] To determine the correlation between CCR1 expression and these two agents, colchicine ($n = 7$) and pentoxifylline ($n = 7$) were administered to 4-week-old normal mice. The surface expression of CCR1 in PBMC was significantly up-regulated compared with PBS-treated normal mice ($n = 10$) (colchicine versus PBS: 16·02 ± 2·2% versus 11·11 ± 2·39%, $P = 0·0006$; pentoxifylline versus PBS: 17·02 ± 3·8% versus 11·11 ± 2·2%,

$P = 0·001$). Colchicine significantly up-regulated the expression of CCR1 on the surface of LN cells, compared with PBS-treated normal mice (16·02 ± 2·2% versus 12·99 ± 1·56%, $P = 0·005$). Pentoxifylline also up-regulated the expression of CCR1 compared with PBS-treated normal mice, although the up-regulation was not statistically significant (14·8 ± 3·6% versus 12·99 ± 1·56%, $P = 0·18$) (Fig. 5a). Colchicine and pentoxifylline significantly down-regulated the expression of cytoplasm CCR1$^+$ PBMC (59·37 ± 11·5%, $P = 0·002$; 59·7 ± 18·8%, $P = 0·03$, respectively) compared with PBS control (73·65 ± 3·0%). Cytoplasm CCR1 expression in LN cells was not altered under colchicine or pentoxifylline treatment (Fig. 5b).

Drug treatment regulates frequencies of CCR1$^+$ cells in BD mice

Frequencies of CCR1$^+$ cells were determined after BD mice were treated with medication. Treatment with colchicine ($n = 7$) or pentoxifylline ($n = 7$) significantly up-regulated CCR1 surface expression in PBMC of BD mice compared with PBS-treated BD mice ($n = 10$) (15·92 ± 4·5% versus 7·06 ± 1·8%, $P < 0·0001$; 19·083 ± 3·4% versus 7·06 ± 1·8%, $P < 0·0001$, respectively). The frequencies of surface CCR1$^+$ LN cells were also increased significantly by colchicine or pentoxifylline compared with the PBS-treated BD mice (17·68 ± 7·9%
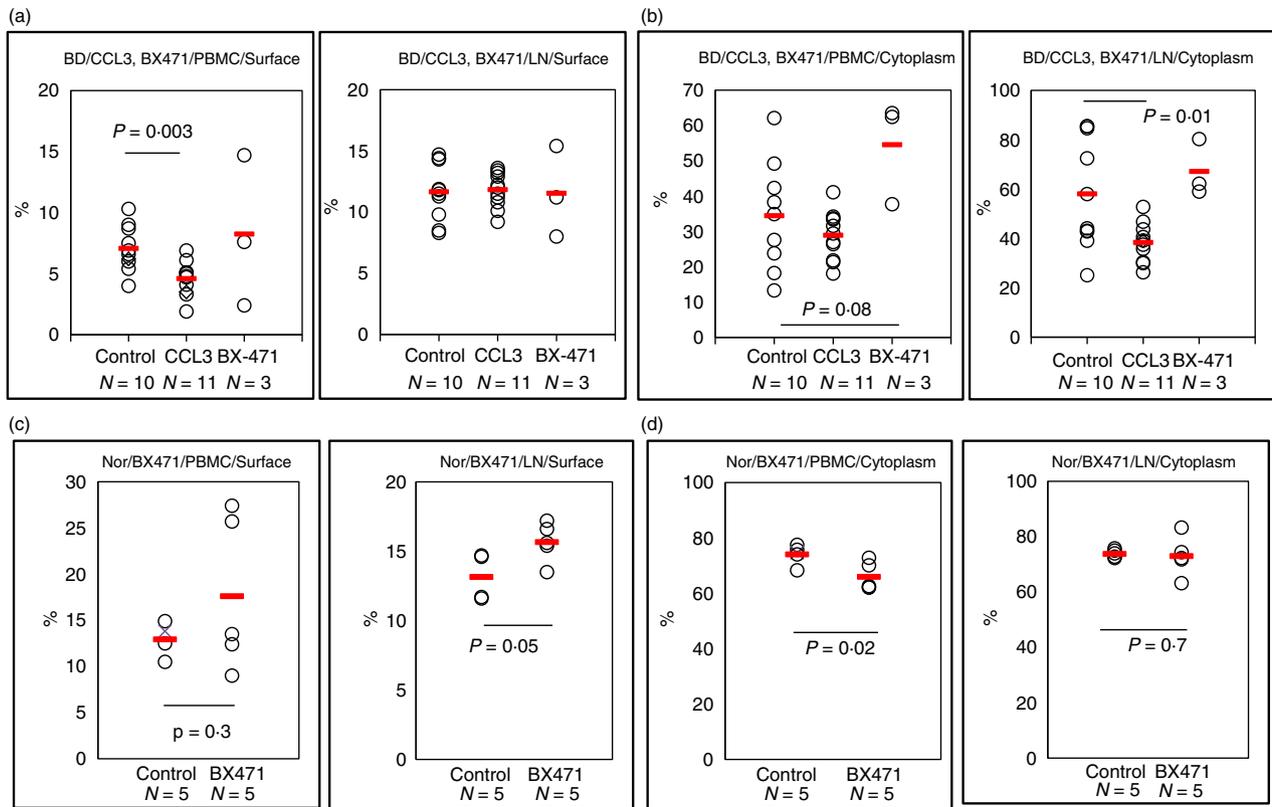
Figure 4. Frequencies of CCR1^{+} cells in CCL3- and BX471-treated Behçet's disease (BD) mice. CCL3 and BX471 were used to treat BD mice and frequencies of CCR1^{+} cells were analyzed by FACS on the surface (a) and in the cytoplasm (b) of peripheral blood mononuclear cells (PBMC) and lymph node cells. Frequencies of CCR1^{+} cells in normal mice after treatment with BX471 are shown in (c) and (d).

versus 10·6 ± 3·23%, $P = 0.02$; 21·36 ± 4% versus 10·6 ± 6·9%, $P < 0.0001$, respectively) (Fig. 5c). In the cytoplasm of PBMC, treatment with colchicine and pentoxifylline increased the frequencies of CCR1^{+} cells. However, only pentoxifylline treatment resulted in a significant increase (77·4 ± 6·97% versus 32·08 ± 16·39%, $P < 0.0001$), colchicine or pentoxifylline did not affect the frequency of cytoplasm CCR1^{+} LN cells compared with PBS-treated BD mice (Fig. 5d). The histogram of CCR1^{+} expression in the surface and cytoplasm of PBMC after treatment with colchicine and pentoxifylline is presented in Fig. 5(e). Results show that the frequencies of surface CCR1^{+} expression rather than cytoplasm expression strongly correlated with the regulation of BD symptoms.

Anti-CCL3 antibody up-regulates the expression of CCR1^{+} cells and improves symptoms in BD mice

To determine whether CCR1 was regulated by CCL3 inhibition, anti-CCL3 antibody was intraperitoneally injected into normal mice. Frequencies of CCR1^{+} PBMC and LN cells were increased after anti-CCL3 antibody treatment. In surface staining of PBMC, treatment with anti-CCL3

antibody at 1–200 ng significantly increased frequencies of CCR1^{+} cells compared with PBS-treated normal mice (1 ng, 12·84 ± 1·98%, $P = 0.009$; 2 ng, 13·37 ± 2·28%, $P = 0.002$; 20 ng, 15·28 ± 2·57%, $P < 0.0001$; 50 ng, 16·92 ± 1·32%, $P < 0.0001$; 200 ng, 15·6 ± 2·12%, $P = 0.0005$; versus PBS, 8·3 ± 2·04%). In surface staining of LN cells, treatment with anti-CCL3 antibody significantly up-regulated frequencies of CCR1^{+} LN cells compared with PBS-treated normal mice (1 ng, 15·16 ± 2·83%, $P < 0.0001$; 2 ng, 17·62 ± 4·24%, $P < 0.0001$; 20 ng, 19·4 ± 1·59%, $P < 0.0001$; 50 ng, 19·82 ± 1·76%, $P < 0.0001$; 200 ng, 16·75 ± 5·58%, $P = 0.0008$; versus PBS, 8·72 ± 1·21%) (Fig. 6a). In the cytoplasm, frequencies of CCR1^{+} PBMC after treatment with anti-CCL3 antibody at 1–200 ng were not altered significantly in PBMC cytoplasm compared with PBS-treated normal mice (1 ng, 64·36 ± 11·82%; 2 ng, 55·75 ± 3·33%; 20 ng, 53·94 ± 5·04%; 50 ng, 64·65 ± 8·83%; 200 ng, 40·05 ± 2·33%; versus PBS, 52·45 ± 13·42%) (Fig. 6b). Cytoplasm CCR1^{+} LN cells were significantly up-regulated in mice treated with 1 ng of anti-CCL3 antibody (42·12 ± 8·93%, $P = 0.02$) or 200 ng of anti-CCL3 antibody (59·0 ± 7·77%, $P = 0.0002$) compared with PBS-treated normal mice
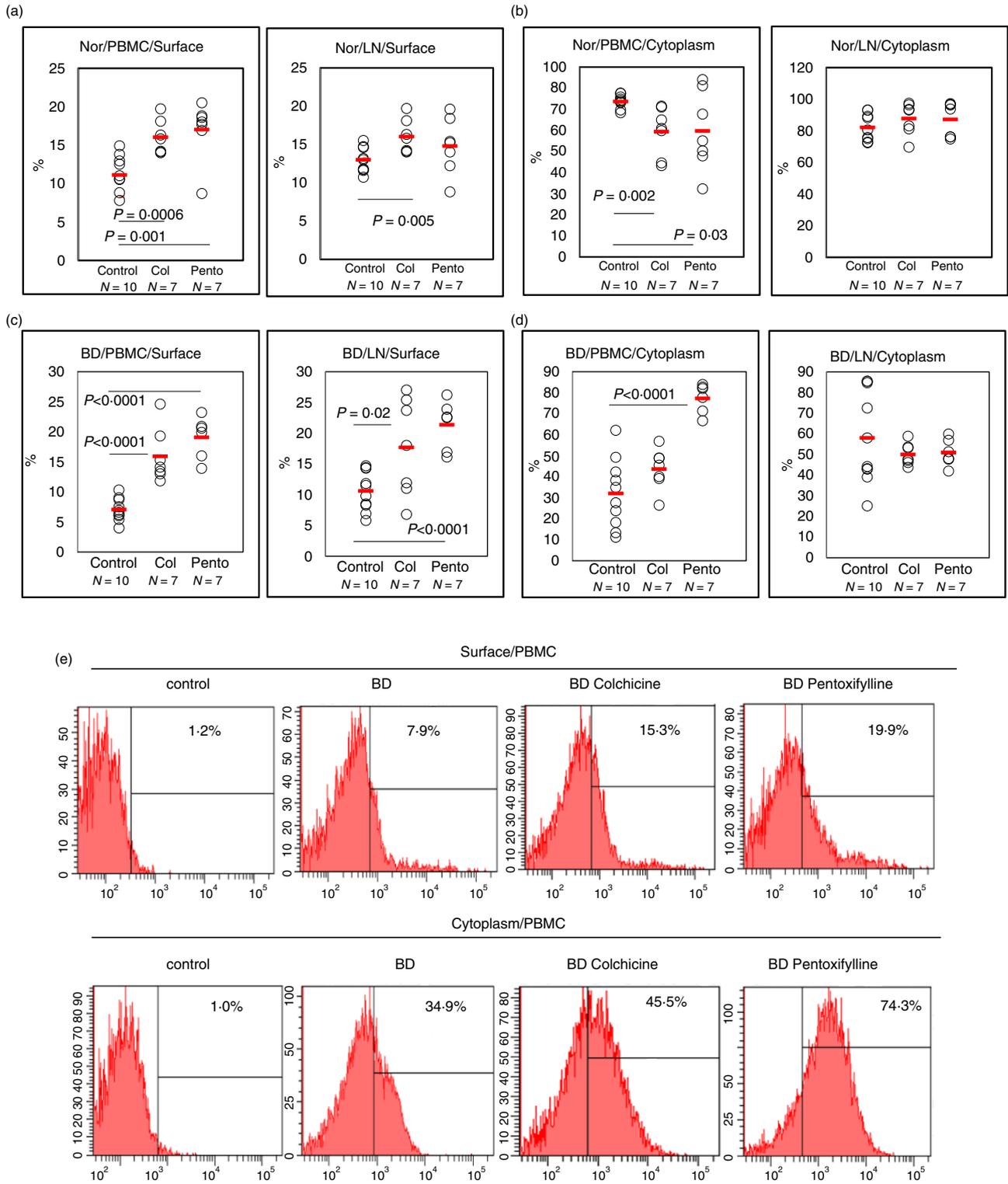
Figure 5. Frequencies of CCR1^{+} cells in colchicine (Col) and pentoxifylline (Pento) -treated normal and Behçet's disease (BD) mice. Colchicine (2 µg/mouse/day) and pentoxifylline (400 µg/mouse/day) were used to treat mice. Frequencies of surface and cytoplasm CCR1^{+} peripheral blood mononuclear cells (PBMC) and lymph node cells were analyzed in normal mice (a, b) and BD mice (c, d) by FACS. (e) Representative flow cytometric histogram of surface and cytoplasm CCR1^{+} PBMC following colchicine or pentoxifylline treatment of BD mice.
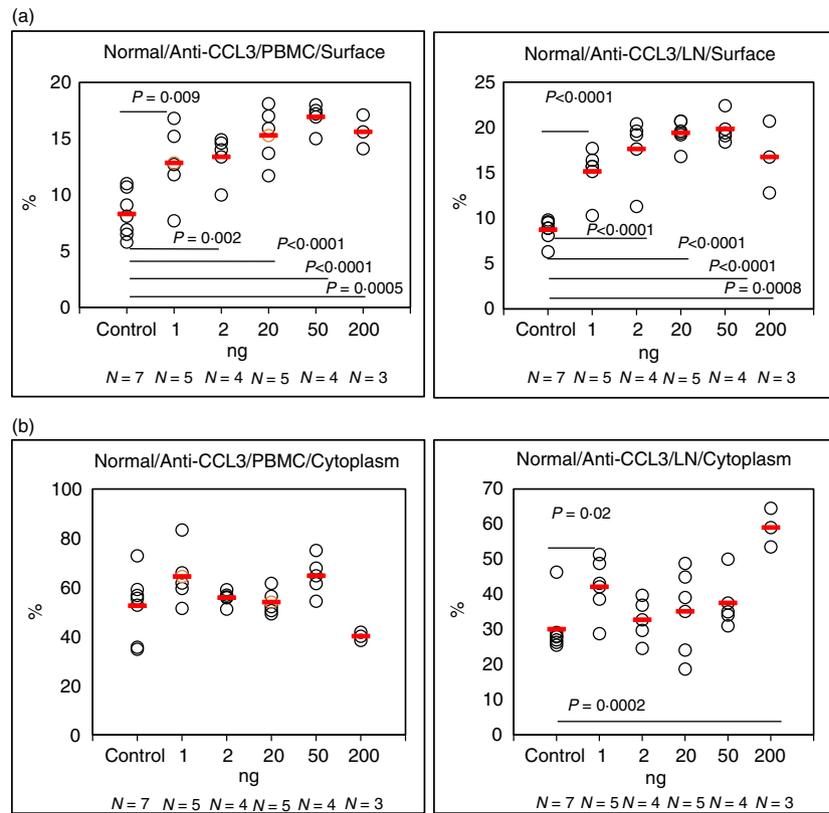
Figure 6. Frequencies of CCR1^{+} cells in anti-CCL3 antibody-treated normal mice. Anti-CCL3 antibody (1, 2, 20, 50 and 200 ng) was intraperitoneally injected into normal mice for five consecutive days. Peripheral blood mononuclear cells (PBMC) and lymph node cells were isolated. Frequencies of surface CCR1^{+} (a) and cytoplasm CCR1^{+} (b) PBMC and lymph node cells were analyzed by FACS.

(30·0 ± 7·27%). Treatment with 2, 20 and 50 ng of anti-CCL3 did not yield significant changes compared with PBS-treated normal mice (Fig. 6b).

To up-regulate CCR1^{+} cells in BD mice, anti-CCL3 antibody 20 ng/day was administered to BD mice (n = 16) for five consecutive days. The frequencies of CCR1^{+} expression on the surface of PBMC were significantly increased compared with those in PBS-treated BD mice (n = 10) (15·61 ± 2·92% versus 7·92 ± 2·61%, P < 0·0001). In LN cells, frequencies of surface CCR1^{+} expressions were significantly increased after anti-CCL3 antibody treatment (22·8 ± 2·7% versus 10·6 ± 3·1%, P = 0·0001) (Fig. 7a). In the cytoplasm of PBMC, the frequencies of CCR1 expression after treatment with anti-CCL3 antibody were up-regulated significantly compared with PBS control (51·42 ± 13·08% versus 32·11 ± 16·40%, P < 0·0001). However, the cytoplasm CCR1^{+} LN cells were down-regulated after treatment with anti-CCL3 antibody compared with PBS control (40·62 ± 7·81% versus 58·04 ± 22·43%, P = 0·008; Fig. 7b). Anti-CCL3 antibody treatment improved BD symptoms in 10 (50·0%) of 20 BD mice on day 5 after initiation with anti-CCL3 antibody treatment. The list of improved mice is provided in the Supplementary material (Table S1). The disease severity score in BD mice changed from 3·1 ± 0·3 to 2·45 ± 0·68 on day 5 with symptom improvement after anti-CCL3 antibody treatment

(P < 0·0001). Disease severity score was also significantly varied between CCL3-treated BD mice (3·2 ± 1·0) and anti-CCL3 antibody-treated BD mice (2·45 ± 0·68) on day five (P = 0·004) (Fig. 7c). Figure 7(d) show the changes in symptoms after treatment with CCL3 or anti-CCL3 antibody in BD mice.

Plasma levels of CCL3 in patients with BD

The levels of CCL3 in patients with active BD (76·88 ± 74·48 pg/ml) were higher than in patients with inactive BD (24·58 ± 7·54 pg/ml, P = 0·09) and healthy control individuals (22·82 ± 7·87 pg/ml, P = 0·02) (Fig. 8).

Discussion

This study quantitatively analyzed the expression of chemokine receptors in PBMC and LN cells of BD mice. Our results suggest that BD-like symptoms were correlated with frequencies of CCR1^{+} cells. Percentages of CCR1^{+} cells were significantly down-regulated in BD mice compared with normal or BDN mice. Cytokine IL-10- and GM-CSF-treated BD mice exhibited significant up-regulation of CCR1^{+} cells compared with PBS-treated BD mice. CCR1 ligand CCL3-treated BD mice showed significant down-regulation of CCR1^{+} cells compared with PBS-
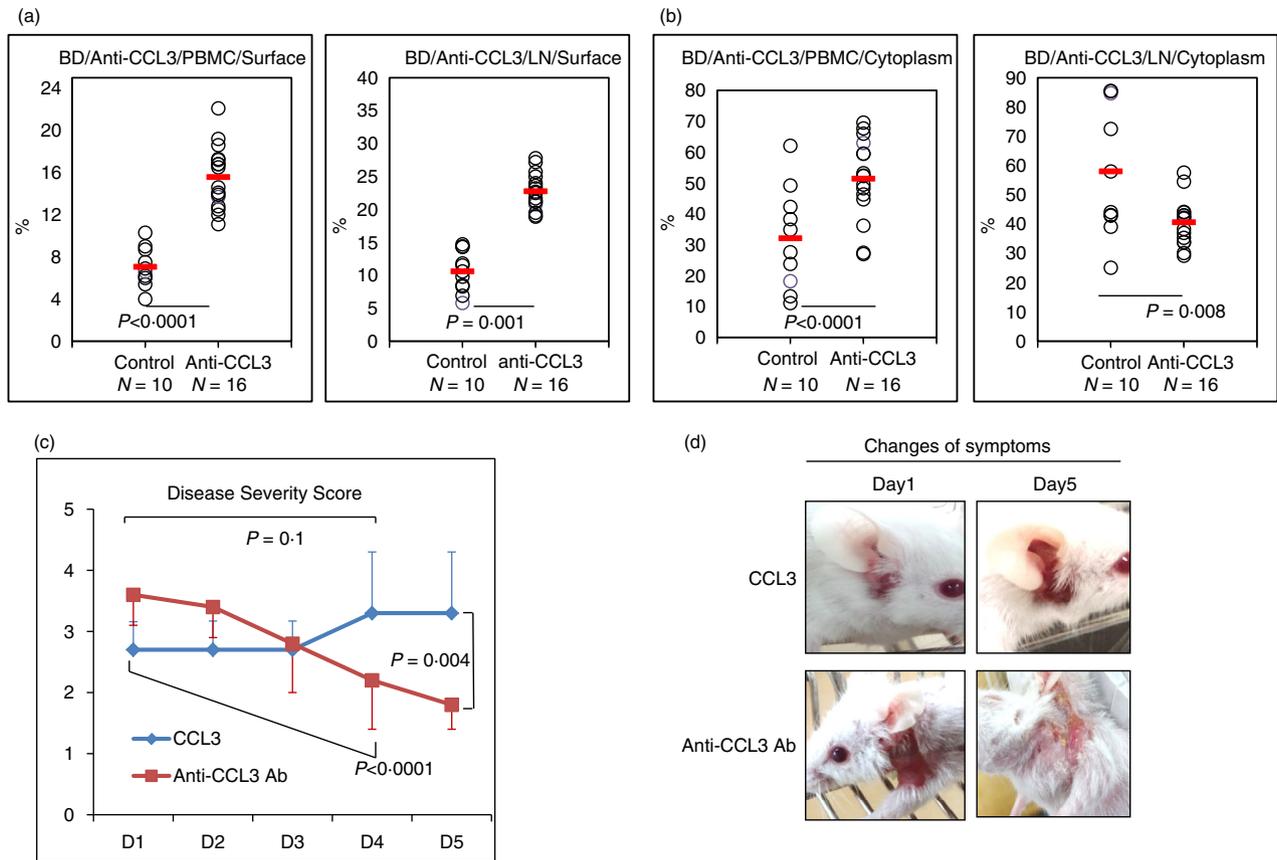
Figure 7. Frequencies of CCR1$^+$ cells in anti-CCL3 antibody-treated Behçet's disease (BD) mice. Anti-CCL3 antibody 20 ng was intraperitoneally injected into BD mice for five consecutive days. Peripheral blood mononuclear cells (PBMC) and lymph node cells were then isolated. Frequencies of surface CCR1$^+$ (a) and cytoplasm CCR1$^+$ (b) PBMC and lymph node cells were analyzed by FACS. (c) The changes in disease severity score after CCL3 or anti-CCL3 antibody treatment of BD mice. (d) Symptoms of BD mice after CCL3 or anti-CCL3 antibody treatment.
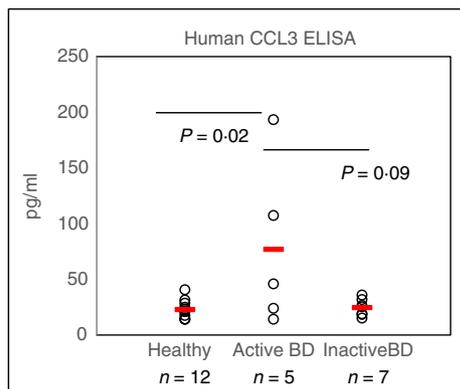

Figure 8. Plasma levels of CCL3 in patients with active and inactive Behçet's disease (BD).

treated BD mice. Ten of 16 CCL3-treated BD mice showed deteriorated BD symptoms. Anti-CCL3 antibody treatment improved BD symptoms, decreased the disease severity score, and up-regulated CCR1$^+$ cells significantly compared with PBS-treated BD mice. Treatment of BD mice with colchicine and pentoxifylline significantly up-

regulated CCR1$^+$ cells compared with PBS treatment. Frequencies of CCR1$^+$ cells were strongly associated with symptoms in BD mice.

Chemokine receptors are essential moderators of leukocyte trafficking in inflammatory diseases. CCR1 may be a strong therapeutic target in a variety of diseases.[26] CCR1 has been shown to play a role in recruiting monocytes and Th1 cells according to active inflammation.[27] CCR1 and its ligands are associated with the development of experimental arthritis. In rheumatoid arthritis, CCR1 is an efficient therapeutic target because it mediates the regulation of tumor necrosis factor-α and IL-10.[28] However, in patients with BD, the frequencies of CCR1$^+$ cells are decreased in patients with active BD than in normal controls, even though the difference was not statistically significant.[29] Similarly, this study also shows that the frequencies of CCR1$^+$ cells in BD mice were down-regulated compared with the control mice. Down-regulated CCR1 expression has been reported after infection with *Leishmania infantum*[30] or coronavirus.[31] Correlation between HSV infection and CCR1 expression does not show consistent results.[32,33]

Chemokine CCL3 expression has been reported in arthritic inflammation.[34] CCL3 recruits inflammatory leukocytes by binding to CCR1.[35] It affects chronic joint inflammation.[36] Neutralizing antibodies to CCL3 alleviate inflammation.[37] Higher serum levels of CCL3 have been found in patients with active BD than in patients with inactive stages of BD.[38] Results of the present study demonstrate that treatment with CCL3 deteriorated symptoms in BD mice and down-regulated the frequencies of CCR1^{+} cells. Increased CCL3 expression exacerbates BD symptoms through down-regulation of CCR1.

Interleukin-10 is known as a CCR1 stimulator.[39] It is mainly expressed in monocytes, macrophages, Th2 cells, and B cells. IL-10 is one of the key anti-inflammatory cytokines expressed in the human immune response. It is a strong inhibitor of Th1 cytokines IL-2 and interferon-γ.[40,41] Anti-inflammatory cytokine IL-10 suppresses pro-inflammatory cytokines and protects the host against tissue damage.[42] In patients with BD, IL-10 polymorphisms have been identified.[43,44] Such gene variation might be correlated with insufficient expression of IL-10. Results of the present study show that IL-10 up-regulated the frequencies of CCR1^{+} cells in BD mice compared with PBS control. IL-10 and GM-CSF also synergistically increased the frequencies of CCR1^{+} cells in BD mice.

Granulocyte–macrophage colony-stimulating factor is a stimulator of CCR1 expression.[22] It enhances the maturation of dendritic cells and plays a role in recruiting circulating neutrophils, monocytes and lymphocytes for host defense.[45] Different types of inflammatory and immune cells such as dendritic cells, monocytes, neutrophils, microglia and macrophages are modulated by GM-CSF under autoimmune conditions.[46] GM-CSF may influence chronic inflammatory diseases such as arthritis,[47] experimental autoimmune encephalitis[48] and airway inflammation.[49] GM-CSF also enhances resistance to viral infection[50] and bacterial translocation.[51] In patients with BD, GM-CSF transcription was down-regulated more than in controls.[52] However, the protein expression or serum levels of GM-CSF in patients with BD have yet to be elucidated. According to a case report, intra-lesional injection with recombinant human GM-CSF induces wound healing in genital ulcers of BD.[53] GM-CSF also enhances CCR1 expression.[21,22] Our data demonstrated that treatment with GM-CSF significantly up-regulated CCR1^{+} expression in PBMC and LN cells.

BX471 was developed as a potent antagonist against CCR1.[54] BX471 treatment attenuated systemic inflammation of sepsis,[55] nephritis,[56] arthritis[57] and myocarditis.[58] In our study, BX471 was administered to BD mice to confirm the correlation of CCR1 expression with BD by inhibiting CCR1. BX471 treatment failed to inhibit the frequencies of surface or cytoplasm CCR1^{+} PBMC and LN cells in BD mice. In normal mice, BX471 inhibited the expression of CCR1 only in the cytoplasm of PBMC.

These results indicate that BX471 treatment failed to adequately inhibit CCR1^{+} cells *in vivo*, which partly explains why clinical trials of BX471 inhibition of CCR1 were discontinued.

In conclusion, our study demonstrated down-regulated frequencies of CCR1^{+} cells, which were closely correlated with BD symptoms and the expression of CCR1^{+} cells was controlled by cytokine IL-10, GM-CSF and ligand CCL3. Even though BD is a multi-factorial inflammatory disease associated with immune dysregulation, treatment with single chemokine or chemokine receptor alone contributes to immune regulation resulting in the control of systemic symptoms of BD.

Acknowledgements

Disclosure

The authors declare no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 List of symptoms in anti-CCL3 antibody-treated Behçet's disease mice.