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Original Article

Circulating CCL20: A potential biomarker for active vitiligo together with the number of Th1/17 cells

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ABSTRACT

Background: Vitiligo is an autoimmune disease with varying pathological features. Activation of the CCL20-CCR6 axis plays an important role in chronic inflammatory diseases. However, whether CCL20-CCR6 and Th1/17 cells are indicative of active vitiligo is unclear.
Objective: To investigate the potential role of CCL20 and the involvement of Th1/17 and Tc1/17 cells in the mechanism in vitiligo.
Methods: One hundred patients with vitiligo, and 20 healthy controls were included. The serum and blister fluid IL-17, IFN-γ, CCL20, and CXCL10 were studied using enzyme-linked immunosorbent assays. The numbers of Th1/17 cells and Tc1/17 cells in circulation were quantified using flow cytometry. *CCR6* mRNA in peripheral blood mononuclear cells (PBMCs) was analyzed by real-time polymerase chain reaction and the protein level was confirmed by western blotting. CCR6 and CCL20 expression in lesions was analyzed by immunohistochemistry.
Results: The serum CCL20 level was significantly elevated in patients with vitiligo. The level of serum CCL20 was higher in active than in the stable stage, which correlated positively with the Vitiligo European Task Force spreading score and the Vitiligo Area Scoring Index score. Patients with active vitiligo had elevated numbers of circulating Th1/17 cells and Tc1/17 cells, and upregulated expression of *CCR6* in PBMCs and lesions. After effective treatment, the level of CCL20 in sera and blister fluid was significantly decreased, as were the numbers of circulating Th1/17 cells and Tc1/17 cells.
Conclusion: CCL20 might be a vital biomarker of active vitiligo, and circulating Th1/17 and Tc1/17 cells are involved in the pathogenesis of vitiligo.

1. Introduction

Vitiligo is an acquired benign depigmentation disorder that caused by autoimmune destruction of melanocytes [1]. Direct attack on melanocytes is primarily caused by cytotoxic CD8+ T cells, while the interplay of T helper cells is complicated and remains controversial [2,3]. Increased circulating Th1 and Th17 cells in vitiligo suggest the loss of self-tolerance to melanocytes [4]. The earlier theory of the immune response in vitiligo inclined toward a Th1 response [5], accompanied with increased levels of interferon gamma (IFN-γ) [6]. The influence of a complex Th17 cell-related cytokine environment in local depigmentation functions in autoimmune vitiligo [7]. In humans, a proportion of Th1/17 cells is proposed to be highly pathogenic in several autoimmune disorders that are also positive for IFN-γ, C-C Motif chemokine receptor 6 (CCR6) and C-X-C motif chemokine receptor 3 (CXCR3) [8]. In contrast to early reports of Th17 cells as a pure IL-17-secreting lineage, bifunctional Th1/17 cells producing both IFN-γ and IL-17 have been identified in rheumatoid arthritis synovial fluid [9]. Tc1/17 cells, characterized by IFN-γ-/IL-17–producing CD8+ T cells, has been reported infiltrating in the syphilitic lesions [10]. In a single-cell analysis of IFN-γ and IL-17A producing T cells in Sjogren's syndrome (SJS) mice, the number of Tc1/17 cells in the SJS mice was two-fold higher than those of B6 mice, indicating Tc1/17 cells might be associated with autoimmune mechanism in SJS [11]. Further studies are required

Abbreviations: ANOVA, analysis of variance; CCR, CC chemokine receptor; CXCL, CXC chemokine ligand; CXCR, CXC chemokine receptor; ELISA, enzyme-linked immunosorbent assay; FACS, flow cytometry analysis; IFN-γ, interferon-gamma; PBMCs, peripheral blood mononuclear cells; HCs, Healthy controls; qRT-PCR, quantitative real-time PCR; TNF-α, tumor necrosis factor-α; VASI, Vitiligo Area Severity Index; VETF, Vitiligo European Task Force; ROC, Receiver operating characteristic; Tregs, regulatory T cells; IBD, inflammatory bowel disease; SJS, Sjogren's syndrome; Th cells, helper T cells; Tc cells, cytotoxic T cells.

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to investigate the mechanism of Th1/17 cells and Tc1/17 cells in autoimmune vitiligo.

CCR6 was identified as a risk locus at 6q27 in a large-scale genome-wide associated study for vitiligo [12]. CCR6 is required for epidermal trafficking of γδCT cells in an IL-23-induced model of psoriasiform dermatitis, with abundant expression of C-C motif chemokine ligand 20 (CCL20) in psoriatic skin [13]. CCL20 interacts with its receptor CCR6 to recruit IL-17A-producing cells inside the skin [14]. CCL20 is produced in epithelial cell types [15], and this positive feedback loop of IL-17/CCL20 might perpetuate the inflammatory responses. The CCL20-CCR6 axis is activated in patients with chronic inflammatory conditions, such as psoriasis, arthritic conditions, and inflammatory bowel disease (IBD) [16–18]. However, the circulating level of CCL20 in vitiligo is unknown.

Identifying the underlying mechanism of vitiligo could identify potential molecular targets for therapeutic intervention. Therefore, the aim of the present study was to determine whether serum CCL20 levels correlate with disease activity and severity, and whether Th1/17 cells participate in the mechanism of vitiligo. We quantified the levels of CCL20, IL-17, IFN-γ, and C-X-C motif chemokine ligand 10 (CXCL10) in circulation and in blister fluid, and investigated the mRNA and protein levels of CCR6 in PBMCs, the numbers of Th1/17 cells in circulation, and the levels of CCL20 and CCR6 in vitiligous lesions in a large cohort of patients with vitiligo. We hypothesized that CCL20 is a vital biomarker for vitiligo and that Th1/17 and Tc1/17 cells are involved in vitiligo.

2. Materials & methods

2.1. Patients

The study was conducted with a cohort of 100 patients with vitiligo at our department between June 2016 and June 2017. Written informed consent was obtained from all participants. The study was approved by the Ethics Committee of Huashan Hospital, Fudan University.

Vitiligo was diagnosed according to the vitiligo guidelines [19]. All participants included were adult patients with non-segmental vitiligo (18 years or older) with no medication or only topical application within the preceding 6 months. All patients were selected on the criteria of not being accompanied by diabetes mellitus, thyroiditis, anemia pernicious, psoriasis, atopic dermatitis, or inflammatory skin diseases. Pregnant participants, those who had contraindications for systemic steroid therapy, or those on treatment were excluded from the study. Twenty healthy controls (HCs) were enrolled. Controls were selected from individuals visiting Health Examination Department of Huashan Hospital for a pre-scheduled general medical examination, and eligibility requirements were age 18 years or older, no history of vitiligo or other autoimmune diseases, absence of evidence of any active or chronic disease following a detailed medical and surgical history; patients were excluded if they had respiratory, renal, hepatic, gastrointestinal, hematological, lymphatic, neurological, cardiovascular or psychiatric diseases. Patients were classified into two clinical types: those with active vitiligo and those with stable vitiligo, as diagnosed by two dermatologists using wood lamps independently. Confetti-like macules and trichrome vitiligo were regarded as the clinical signs of active vitiligo. The extent of skin involvement was evaluated using the spreading part of the (VETF) score [20]. Active vitiligo was defined as a VETF score of +1 to +5, or displayed new lesions or spreading within 6 months. Stable vitiligo was defined as a VETF score of −5 to −1, and patients' self-reporting of no spreading nor new lesions within 6 months [20].

Sixty patients had active vitiligo, while 40 had stable vitiligo. Among the 60 patients with active vitiligo, 20 patients had a vitiligo area scoring index (VASI) <5 and 40 patients had a VASI > 5. The mean age of patients with active vitiligo (VASI > 5), active vitiligo (VASI < 5), stable vitiligo, and the HCs group were 35 ± 14, 31 ± 11, 31 ± 12, and 36 ± 10 years old, respectively. The duration of vitiligo varied from 1 month to 30 years. Patients in the active stage were treated with intramuscular injections of 1 m L of diprospan (containing 2 mg betamethasone sodium phosphate and 5 mg betamethasone dipropionate) once per month until the progression arrested, for a maximum of six injections. Topical tacrolimus ointment 0.1% was applied twice per day on all lesions. Samples of peripheral blood of patients with active vitiligo were collected at the first visit to our department at 1 month after the last injection of diprospan; 80% of the patients reported no spreading or new lesions and the VETF spreading score was from −5 to 0, among which 40% of the patients displayed various degrees of repigmentation. Samples from patients with stable vitiligo were collected only at the first visit.

2.2. ELISA detection of serum cytokines and chemokines

The levels of serum CCL20, IL-17, IFN-γ, and CXCL10 were measured using enzyme-linked immunosorbent assay (ELISA) kits (RD, USA), according to the manufacturer's instructions. The minimum detection limit was 2 pg/mL and the maximum detection limit was 2500 pg/mL.

2.3. Real-time PCR detection of CCR6 and expression

Total RNA was extracted from freshly isolated PBMCs using the TRIzol reagent (Invitrogen, US). First-strand cDNA was synthesized from DNAse-treated total RNA using an oligo-dT18 primer and RevertAid^{TM} M-MLV reverse transcriptase. Transcripts were quantified using real-time quantitative PCR on a 7500 Real-Time PCR System (Applied Biosystems, USA) with SYBR Premix Ex Taq II (Takara Biotechnology) according to the manufacturer's instructions. The real-time PCR included an initial denaturation at 95 °C for 10 min; followed by 40 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min; and one cycle of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. The relative abundance of the transcripts was normalized and calculated against that of the beta-actin gene (*β-actin*) as an endogenous reference using the $2^{-\triangle\triangle Ct}$ method. *CCR6* and *β-actin* primers were purchased from Sangon Biotech (Shanghai, China). The primers used for cDNA amplification were 5′-AGTCATGCATCTCTCAGATGGAGGAGGGAC-3′ (Forward), 5′-TCAGACTAGTAGAGGCCGTGTGAGTTAAAGAC-3′ (Reverse) for *CCR6*; 5′-TCACCCACACTGTGCCCATCTACGA-3′ (Forward), 5′-AGGGGAACCGCTCATTGCCAATGG-3′ (Reverse) for *β-actin*.

2.4. Western blotting analysis

Western blotting analyses were used to examine CCR6 expression in PBMCs from all the vitiligo cases and HCs. The protein concentration was determined using a BCA kit. Protein samples (20 μg) were subjected to electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gels, and then transferred to polyvinylidene fluoride (PVDF) membranes using a semidry electro transferring unit (Bio-Rad, USA). PVDF membranes were blocked with 3% albumin bovine V for 2 h and then incubated with diluted primary rabbit polyclonal antibodies against CCR6 (1:100, Abcam, USA) overnight at 4 °C. The membranes were washed with PBST (phosphate-buffered saline with 0.1% Tween-20) and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h. After extensive washing, immunoreactive proteins were detected using an enhanced chemiluminescent autoradiography reagent (Tiangen Biotech, China) according to the manufacturer's instructions. GAPDH was used as the internal control (1:1000, Abcam). The signal intensity was quantitatively analyzed with Quantity-One analysis software.

2.5. Immune cell phenotyping and quantification by flow cytometry

PBMCs were incubated with the following monoclonal antibodies: anti-CD3-BUV661, anti CD45-APC-Alexa700, anti-CD4-PE, anti-CD8-PE-Cy7, anti-CCR6-BUV737, anti-CXCR3-BUV400, and 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI)-BUV510 (1:50, all from BD Biosciences, USA). Immune cell phenotyping was performed on whole blood using antibody panels. Selected immune cells among PBMCs were gated as follows: Tc1/17 (CD8+CXCR3+CCR6+ T cells), Th1/17 (CD4+CXCR3+CCR6+ T cells), while monocytes and granulocytes were gated based on forward and side scatter. Flow cytometry was performed using a FortessaX20 instrument (BD Biosciences, USA).

2.6. In situ analysis of CCR6 and CCL20 expression by immunohistochemistry

Lesional biopsies were obtained from the site of the adjacent vitiligous-normal skin from seven patients with extensive vitiligo (VASI > 50). Control biopsies were obtained from the excision of seven HCs who were undergoing cosmetic surgery. Paraffin-embedded specimens were cut into 5-μm sections and treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, followed by incubation with 1% bovine serum albumin to block nonspecific binding. Anti-CCR6 (1:100, Abcam) and anti-CCL20 (1:100, Abcam) antibodies were incubated with the sections overnight at 4 °C. After washing, the tissue sections were treated with biotinylated anti-rabbit secondary antibodies (Abcam), followed by further incubation with streptavidin-horseradish peroxidase complex (Abcam). The expression of CCL20 and the number of CCR6 positive cells were measured and recorded using the Q500IW image analysis system (Leica, Germany) and Image-Pro Plus 6.0 software (Media Cybernetics, USA).

2.7. Blister fluid cytokine and chemokine assay by ELISA

Suction blisters (1 cm in diameter) were induced on the skin using a negative pressure instrument. The suction chambers were applied to the skin with 40 mm Hg of negative pressure and a constant temperature of 40 °C; blisters formed within 60 min after initiation of the procedure. After blister formation, the blister fluid was aspirated using a 1 mL insulin syringe from the lesional site and donor site, separately, and was collected and frozen for future ELISA analysis of CCL20, IL-17, IFN-γ, and CXCL10.

2.8. Statistical analysis

Differences between variables were analyzed using analysis of variance (ANOVA), Student's *t*-test, χ^2 test, and linear regression as appropriate; a P value <0.05 was considered significant. Receiver operating characteristic (ROC) analysis was performed to distinguish patients with vitiligo from normal controls based on serum CCL20 concentrations. The data were analyzed using SPSS statistical software (Version 19, IBM, New York, NY, USA).

Table 1
Clinical characteristics of patients with vitiligo.

	Active vitiligo	Stable vitiligo	P
Gender (male/female)	60 (28/32)	40 (14/26)	0.303
Age (years), median ± SD	34 ± 13	31 ± 12	0.1786
Disease duration (years), mean ± SD	5.51 ± 6.46	7.62 ± 6.23	0.2057
VASI score, mean ± SD	14.51 ± 18.21	20.01 ± 19.47	0.6984
Family history of vitiligo (%)	5 (8.3%)	2 (5%)	0.699
Confetti sign (%)	7 (11.7%)	2 (5%)	0.3089
Trichrome vitiligo (%)	5 (8.3%)	0	0.0813

The differences of gender, family history of vitiligo, frequency of confetti sign and frequency of trichome vitiligo between two groups were analyzed by χ^2 test. The differences of age, disease duration and VASI score were analyzed by unpaired Student's *t*-test.

3. Results

3.1. Description of patients with vitiligo

The clinical characteristics of the patients are summarized in Table 1. Detailed information for the active vitiligo subgroups is shown in Table 2. Among the 60 patients with active vitiligo, 11.7% had confetti-like depigmentation and 8.3% had trichrome vitiligo. Of the 60 patients with active disease, progression was arrested in 48 (80%) patients after treatment (Supplementary Fig. 1A). Typical photographs of stable vitiligo patients were also shown (Supplementary Fig. 1B). Patients with stable vitiligo presented totally depigmentation.

3.2. Elevated serum levels of CCL20 and associated IL-17, IFN-γ, and CXCL10 in patients with vitiligo

The serum CCL20 level was significantly elevated in patients with active vitiligo and stable vitiligo, compared with that in the HCs (Fig. 1A, both P < 0.001). In addition, the concentration of serum CCL20 in the active stage was significantly higher than that in the stable stage (Fig. 1A, P < 0.001). Furthermore, the concentration of serum CCL20 was higher in patients with active vitiligo with VASI > 5 than in those with VASI < 5 (Fig. 1A, P < 0.001). These results indicated that the level of CCL20 might be associated with the occurrence, activity, and severity of vitiligo.

The serum levels of CXCL10, IL-17, and IFN-γ were also upregulated in patients with vitiligo (Fig. 1A). In addition, the serum level of CCL20 was positively associated with that of CXCL10 (Fig. 1B, P < 0.001). The area under the ROC was 0.72 (Fig. 1C, P < 0.001). A cutoff value of 37.5 ng/mL resulted in a sensitivity of 80% and a specificity of 72.5% in the ROC analysis (Fig. 1C).

Table 2
Characteristics of active vitiligo subgroups (VASI < 5 and VASI > 5).

	Active vitiligo (VASI > 5)	Active vitiligo (VASI < 5)	P
Gender (male/female)	40 (20/20)	20 (8/12)	0.5854
Age (years), median ± SD	35 ± 14	31 ± 11	0.1908
Disease duration (years), mean ± SD	6.57 ± 7.02	3.38 ± 4.60	0.0709
VASI score, mean ± SD	26.69 ± 21.35	1.45 ± 0.81	<0.0001
Family history of vitiligo (%)	4 (10%)	1 (5%)	0.6455
Confetti sign (%)	5 (12.5%)	2 (10%)	>0.99
Trichrome vitiligo (%)	4 (10%)	1 (5%)	0.6563

The differences of gender, family history of vitiligo, frequency of confetti sign and frequency of trichome vitiligo between two groups were analyzed by χ^2 test. The differences of age, disease duration and VASI score were analyzed by unpaired Student's *t*-test.

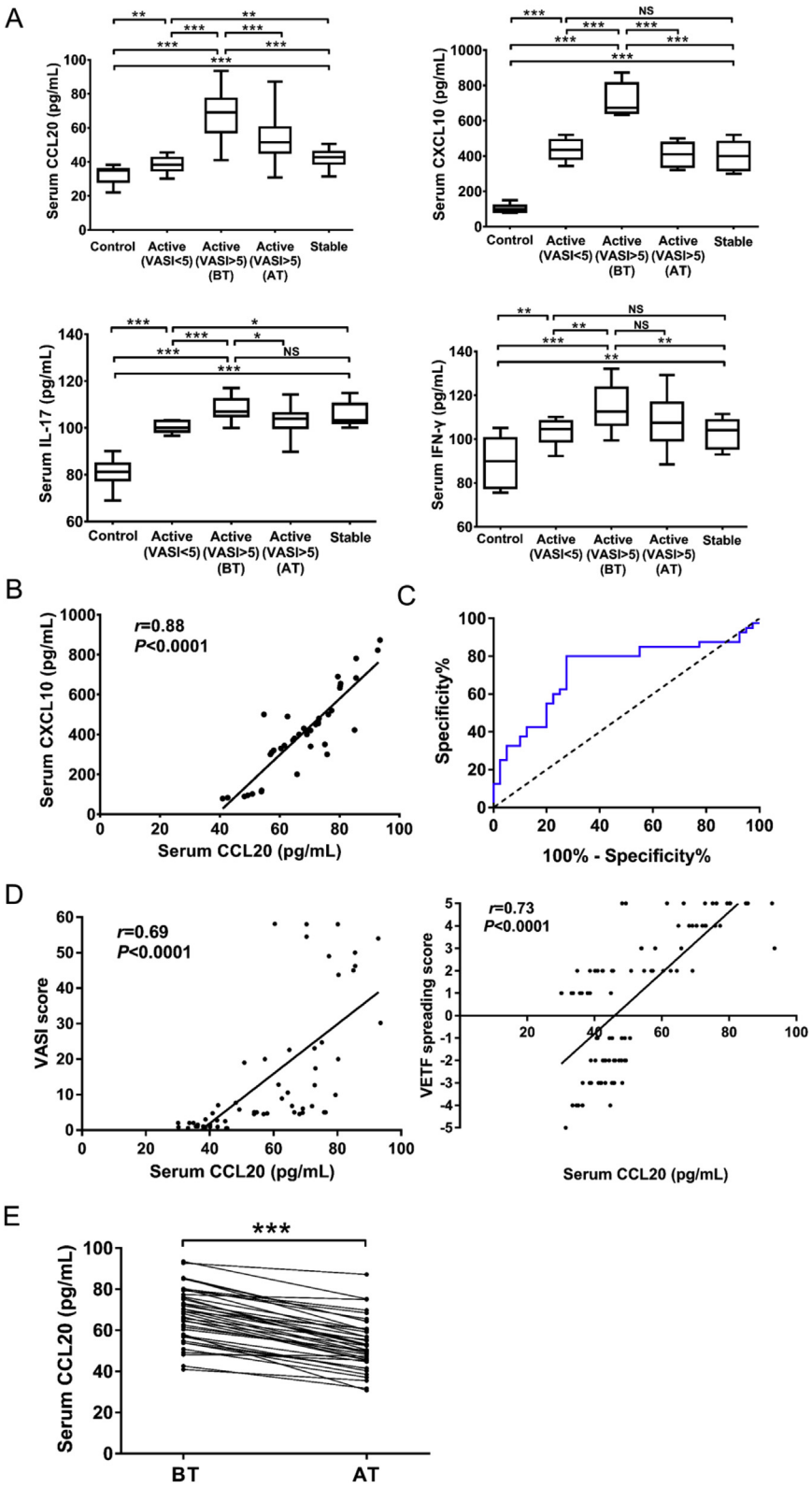

Fig. 1. Serum cytokines and chemokines associated with vitiligo. (A) The values of serum CCL20, IL-17, CXCL10, and IFN-γ were significantly elevated in patients with active vitiligo compared with their levels in patients with stable vitiligo. Serum levels of CCL20, IL-17, and CXCL10 were significantly higher in patients with active vitiligo before treatment than in those after treatment. (B) Serum CCL20 showed a positive link with serum CXCL10. (C) A ROC curve analyzing the specificity and sensitivity of CCL20 in vitiligo. (D) Serum CCL20 correlated positively with the VASI and VETF scores. (E) The serum CCL20 level decreased significantly after successful treatment, which occurred in parallel with the stabilization of the disease. AVONA was applied to compare the serum levels of CCL20, IL-17, CXCL10 and IFN-γ among different groups (A). Linear regression was applied to analyze the association between CCL20 and CXCL10, VASI score and VATF spreading score (B, D). ROC curve was used to analyze specificity and sensitivity of CCL20 in vitiligo (C). Paired Student's *t*-test was applied to compare the difference of CCL20 before and after treatment (E). *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant; VASI, vitiligo area scoring index; BT, before treatment; AT, after treatment; VETF, Vitiligo European Task Force; ROC, receiver operating characteristic.

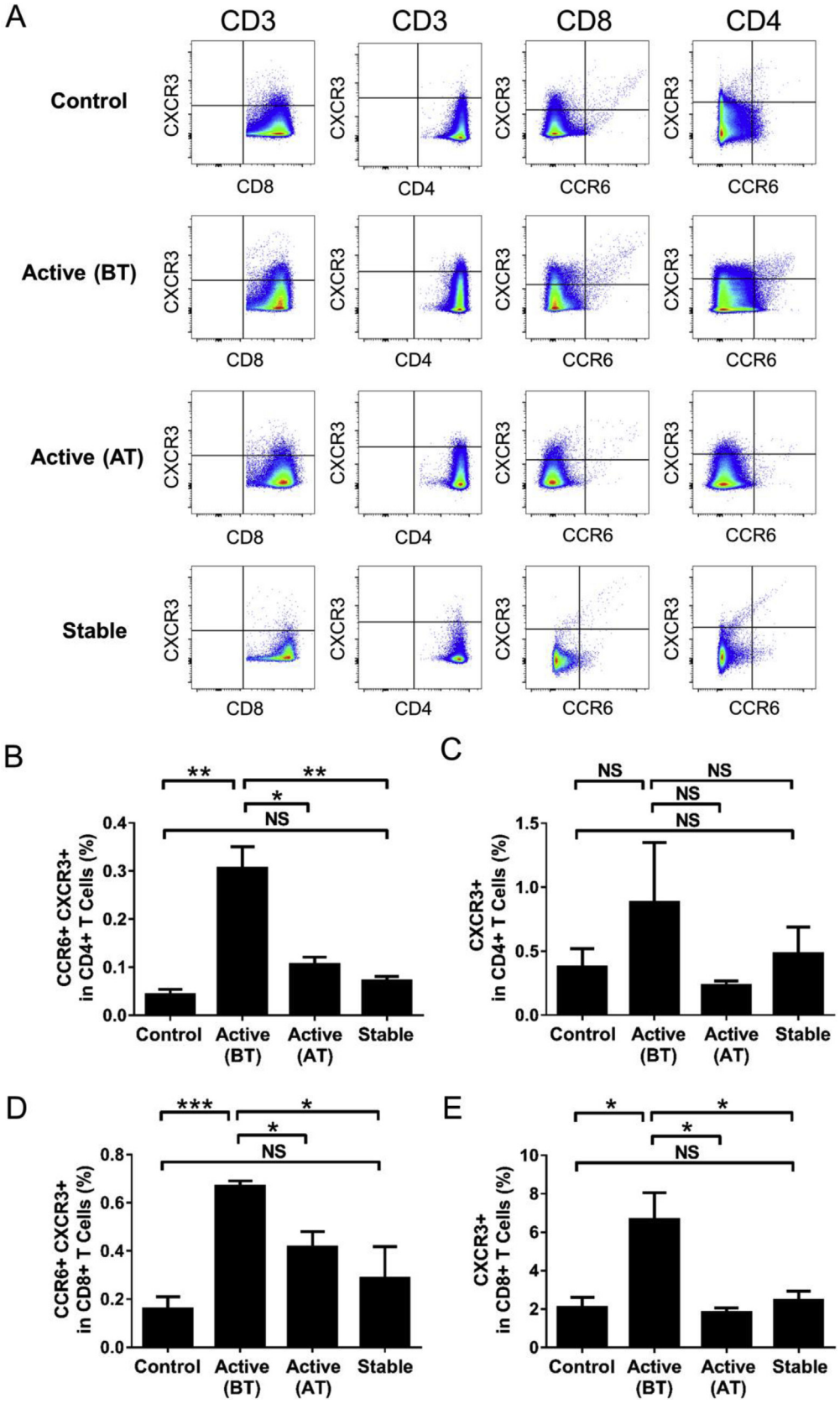

Fig. 2. Proportions of Th1/17 cells and Tc1/17 cells among PBMCs. Plots gated on T lymphocytes first by DAPI, CD3, and CD45. CD4+ or CD8 + T cells were gated, followed by CCR6+ or CXCR3 + . (A) Representative flow cytometry data is shown for the proportions of CD8+CXCR3+ T cells and CD4+CXCR3+ T cells in HCs, in patients with active vitiligo before treatment, in patients with active vitiligo after treatment, and in patients with stable vitiligo. (B) Representative flow cytometry data of Th1/17 cells (CD4+CCR6+CXCR3 + T cells) are shown in the last column of the left panel. Tc1/17 (CD8+CCR6+CXCR3+ T cells) are shown in the third column of the left panel. The proportions of Th1/17 cells were significantly higher in the patients with active vitiligo than in the patients with stable vitiligo or HCs. (C) The proportions of CD8+CXCR3+ T cells were significantly higher in patients with active vitiligo than in patients with stable vitiligo or HCs. (D) The proportions of Tc1/17 cells were elevated in patients with active vitiligo compared with those in

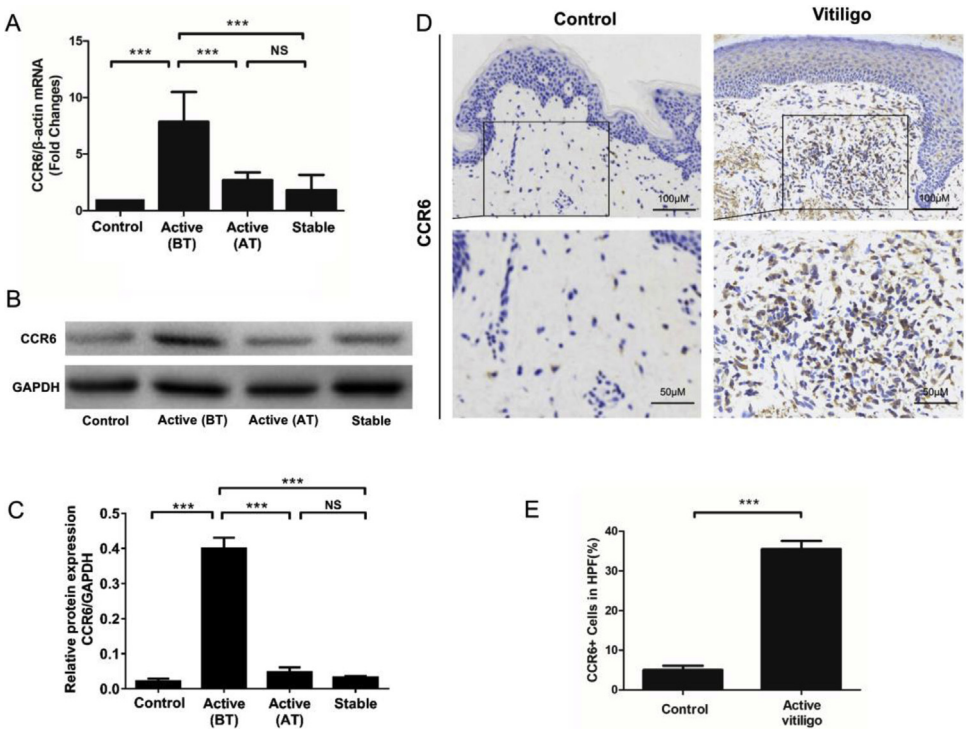

Fig. 3. *CCR6* mRNA and protein in PBMCs and in vitiligo lesions. (A) Real-time PCR demonstrated that the relative expression of *CCR6* mRNA in the PBMCs of patients with active vitiligo was higher than that in HCs. (B–C) Western blotting demonstrated that the gray scale level of CCR6 was significantly greater in the active vitiligo group than the group of HCs and significantly decreased after treatment. (D) Representative immunostaining images of CCR6 expressions in skin biopsies from HCs and patients with active vitiligo are shown. (E) Quantitative analysis demonstrated that CCR6+ cells increased significantly in lesions of active vitiligo group in comparison with HC group. AVONA test was applied to compare the difference between 4 groups (A and C). Unpaired Student's *t*-test was applied to compare the difference between HCs and vitiligo patients (E). $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant; BT, before treatment; AT, after treatment; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction.

3.3. CCL20 correlates positively with VASI and VETF scores

The serum level of CCL20 correlated positively with the VASI score (Fig. 1D, $P < 0.001$). Interestingly, the serum level of CCL20 correlated positively with the VETF score (Fig. 1D, $P < 0.001$).

3.4. Decreased level of serum CCL20 after treatment in patients with active vitiligo

The serum CCL20 concentration in patients with active vitiligo decreased significantly after therapy (Fig. 1A, $P < 0.001$), which occurred in parallel with the disease's stabilization (Fig. 1E, $P < 0.001$). In addition, serum IL-17 level in patients with active vitiligo decreased significantly after therapy (Fig. 1A, $P < 0.001$). The serum levels of CXCL10 also decreased significantly after effective treatment (Fig. 1A, $P < 0.001$). However, there was no statistical difference in IFN-γ levels before and after treatment (Fig. 1A, $P > 0.05$).

3.5. High proportions of Th1/17 cells and Tc1/17 cells in circulation in patients with vitiligo

Fluorescence Minus One (FMO) Controls for flow cytometry analysis were shown Supplementary Fig. 2. in We quantified Th1/17 and Tc1/17 cells using flow cytometry in HCs, patients with active vitiligo before treatment, patients with active vitiligo after treatment and patients with stable vitiligo (Fig. 2A). Patients with active vitiligo had higher proportions of circulating Th1/17 cells than HCs and patients with stable vitiligo (Fig. 2B, both $P < 0.001$). There were no statistical differences in the numbers of CD4+CXCR3+ T cells between the healthy control group, the active vitiligo group (before treatment), the active vitiligo group (after treatment), and the stable vitiligo group (Fig. 2C, $P > 0.05$, respectively).

Higher proportions of circulating Tc1/17 cells were observed in the active vitiligo group than in the healthy control and stable vitiligo groups (Fig. 2D, $P < 0.001$, $P < 0.05$, respectively), and higher proportions of circulating CD8+CXCR3+ T cells were observed in the active vitiligo group compared with that in the healthy control group (Fig. 2E, $P < 0.05$). Moreover, the proportions of CD8+CXCR3+ T cells were higher in the active vitiligo group than in the stable vitiligo group (Fig. 2E, $P < 0.05$).

3.6. Th1/17 and Tc1/17 cell numbers were downregulated after effective treatment

Most patients experienced complete resolution of the lesions or marked improvement. The proportion of circulating CD8+CXCR3+ T cells was downregulated after effective treatment (Fig. 2E, $P < 0.05$). Treatment reduced the proportions of circulating Th1/17 cells (Fig. 2B, $P < 0.05$) and Tc1/17 cells (Fig. 2D, $P < 0.05$) in patients with active vitiligo.

Real-time PCR demonstrated that the relative expression of *CCR6* mRNA in the PBMCs of patients with active vitiligo after treatment was lower than that before treatment (Fig. 3A, $P < 0.05$).

the HCs. (E) There was no significant difference in the proportions of CD4+CXCR3+ T cells between the HCs, the patients with active vitiligo, and the patients with stable vitiligo. AVONA was applied (B–E). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant; BT, before treatment; AT, after treatment; PBMCs, peripheral blood mononuclear cells; DAPI, 2-(4-amidinophenyl)-1H-indole-6-carboxamidine.

Western blotting confirmed the real-time PCR results (Fig. 3C, $P < 0.05$, respectively).

3.7. Elevated expression of CCR6 in PBMCs and lesions in patients with vitiligo

Real-time PCR demonstrated that the relative expression of *CCR6* mRNA in PBMCs from patients with active vitiligo was higher than that in PBMCs from HCs (Fig. 3A, $P < 0.001$). In addition, the relative expression of *CCR6* mRNA in PBMCs in the active stage was significantly higher than that in the stable stage (Fig. 3A, $P < 0.001$). Western blotting (Fig. 3B) confirmed the real-time PCR results. The protein expression of CCR6 in PBMCs from patients with active vitiligo was higher than that in PBMCs from HCs (Fig. 3C, 0.40 ± 0.07 vs. 0.02 ± 0.02, $P < 0.001$). The protein expression of CCR6 in PBMCs in the active stage was significantly higher than that in the stable stage (Fig. 3C, 0.40 ± 0.07 vs. 0.03 ± 0.01, $P < 0.01$).

As shown in Fig. 3D, most epidermal cells stained negative for CCR6, despite sporadic positive staining. The numbers of CCR-stained cells were significantly enhanced in vitiligo lesions compared with those in HCs and were mainly lymphocytes. In the vitiligo group, CCR6 expression was significantly increased compared with the weak CCR6 staining in the HCs (Fig. 3E, $5.0 \pm 2.2\%$ vs. $35.5 \pm 4.1\%$, $P < 0.001$).

3.8. Abundant expression of CCL20 in vitiligo skin and blister fluid

CCL20 expression increased in vitiligous lesions compared with that in lesions from the HCs (Fig. 4A–B, $20.4 \pm 16.1\%$ vs. $43.0 \pm 12.1\%$, $P < 0.001$). These findings suggested that the CCR6/CCL20 axis might be involved in recruiting T lymphocytes into vitiligous areas.

The level of CCL20 was higher in blister fluid from vitiligous site than in blister fluid from normal sites (Fig. 4C, $P < 0.001$). Similar results were observed for CXCL10, IL-17, and IFN-γ (Fig. 4D–F, $P < 0.001$, respectively).

4. Discussion

CCL20 is involved in pathological immune processes and is the only identified chemokine ligand of CCR6. CCL20 is expressed by Th17 cells, regulatory T cells (Tregs), and other T cells [21]. We evaluated the levels of CCL20 and associated cytokines in serum and blister fluid, and found increased levels of CCL20 in samples from patients with vitiligo. Moreover, patients in the active stage had higher levels of CCL20 than those with stable disease, and serum levels of CCL20 correlated positively with the VETF spreading score. These results suggested that CCL20 correlates with disease activity and has a pathogenic role in vitiligo. The serum CCL20 level also correlated with VASI in patients with active vitiligo (VASI > 5), suggesting the CCL20 could be used as a disease and severity biomarker. It was reported that the levels of CCL20 in PBMCs were correlated with disease severity of other autoimmune diseases such as IBD [22], which was in agreement with our findings. Furthermore, we found that the serum CCL20 expression correlated with VASI in active vitiligo and decreased significantly after effective treatment, suggesting the potential utility of serum CCL20 as an indicator of therapeutic action in active vitiligo.

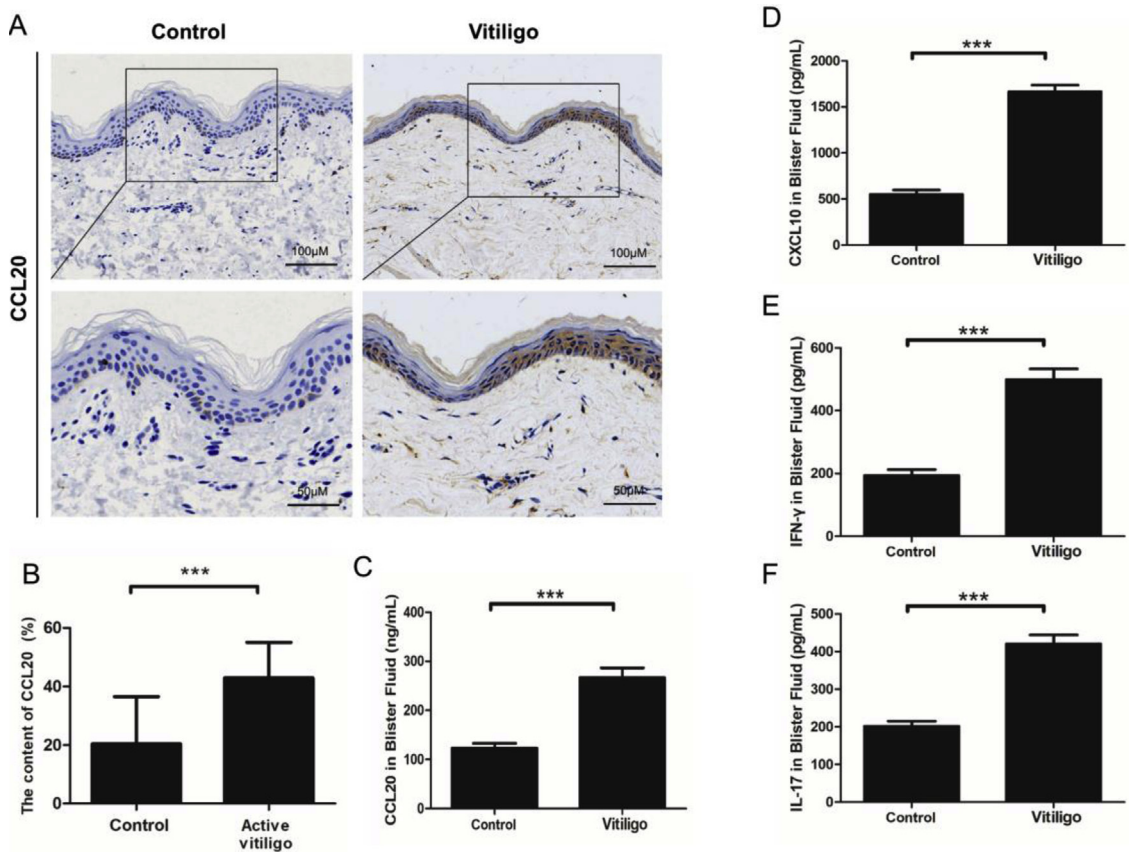

Fig. 4. Elevated expression of CCL20 in vitiligo lesions and in blister fluid. (A) Representative immunostaining images of CCL20 expression of skin biopsies from HCs and patients with active vitiligo are shown. Patients with vitiligo had high expression of CCL20. (B) Quantitative analysis of CCL20 demonstrated that the CCL20 area rate increased significantly in active vitiligo group in comparison with HC group. (C) The values of CCL20 in the blister fluid were significantly higher in the vitiligo groups compared with those in the donor site blister fluid. (D–F) The values of CXCL10, IFN-γ, and IL-17 were elevated in the vitiligo-derived blister fluid. Unpaired Student's *t*-test was applied (B–F). ***$P < 0.001$; HPF, high-power field.

CCL20 is inducible and is upregulated in response to cytokines IL-17 A, IL-23, and TNF-α [23]. In human keratinocytes, IL-17 A elicits *CCL20* gene expression and protein secretion via spleen tyrosine kinase (Syk)-dependent NF-kB mechanism [24]. Increased levels of IL-17 A and decreased expression of melanogenesis associated transcription factor suggested a role of these cytokines in dysregulation of melanocytic activity in lesions of active vitiligo [25]. An oligonucleotide microarray demonstrated a dramatic increase in proinflammatory cytokine and chemokine transcripts, including CCL20, which might be involved in the pathogenesis of vitiligo [26]. It was reported the serum levels of IL-17 are elevated in patients with active vitiligo [4,25,27]. Our study revealed higher IL-17 levels in the serum of patients with active and stable vitiligo. However, no significance difference was detected between active vitiligo and stable vitiligo, which suggested that IL-17 might not be suitable as an activity marker.

The mRNA and protein levels of CCR6 were upregulated in PBMCs from patients with vitiligo, especially in the active stage, accompanied by upregulated serum CCL20. CCR6, together with CXCR3, is a vital surface marker of Th1/17 cells in humans [28,29], allowing them to migrate to sites of both Th1- and Th17-mediated inflammation. Our previous study demonstrated that CXCL10, in parallel with its receptor, CXCR3, is a potential clinical biomarker for vitiligo [30]. In the present study, CCL20 was positively correlated with CXCL10 in patients with vitiligo, confirming the role of CCL20 as a biomarker in vitiligo. Higher frequencies of CXCR3+CD8 + T cells in PBMCs of patients with vitiligo were detected in the active stage than in the stable stage [30]. The present study further demonstrated that the frequencies of these cells decreased after treatment of active vitiligo.

Th1/17 cells are a subset of Th cells characterized by their ability to coproduce IL-17 and IFN-γ, together with co-expression of the Th17 and Th1 lineage-specifying transcription factors RORγt and T-bet [31]. In our study, increased proportions of circulating Th1/17 cells and Tc1/17 cells were detected in patients with active vitiligo. Notably, CD8 + Tc cells, especially Tc1/17 cells, were upregulated in the active stage, suggesting a possible sustaining role of Tc1/17 cells in destroying melanocytes. These prolonged Tc1/17 cells continuously attack any remaining or new melanocytes, and maintain a rather active immune process, thus resulting in persistent depigmentation. The mixed character of Th1/17 cells raises important questions regarding their differentiation, specificity, and functional stability. Recent studies have shown that Th1/17 cells can differentiate from Th17 cells when stimulated via their T cell receptor in the presence of IL-12, causing the cells to produce only IFN-γ, the so-called exTh17 cells [32–34]. CCR6+ CXCR3+ Th1/17 cells are highly polyfunctional, and have a unique specificity profile and surface phenotype associated with activation in mucosal tissue sites.

To date, treatments for active vitiligo have focused on inhibiting local or systemic immune responses. Based on the present findings, blocking T cell recruitment might represent an alternative therapeutic approach. CCR6 and its ligand, CCL20, are possible therapeutic targets. Although there is no relevant study in vitiligo, this theory has been tested in psoriasis. Adalimumab and methotrexate responses are differentiated by patterns of normalization of CCL20 mRNA expression and may explain the varied onset and degree of clinical responses to each treatment [35]. Decreased CCL20 concentrations in patients with rheumatoid arthritis could be reversed by aberrant IL-17 production, and a CCR6 genetic defect might contribute to activation of a downstream pathway [36].

In summary, we investigated peripheral Th1/17 cells and the CCR6-CCL20 axis in vitiligo, and identified increased proportions of Th1/17 and Tc1/17 cells, accompanied by increased levels of CCR6 and CCL20 in PBMCs. In addition, a profound skewing of CCR6 and CCL20 was observed in vitiligous lesions (Supplementary Fig. 3). We concluded that serum levels of CCL20 could be a novel biomarker to monitor disease activity and to guide treatment of active vitiligo.

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Conflict of interest

All authors declare that they have no competing interests.

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None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2018.12.005.

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