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ORIGINAL ARTICLE

mRNA Expression of Chemokine Receptors on Peripheral Blood Mononuclear Cells and Correlation with Clinical Features in Systemic Lupus Erythematosus Patients[△]

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Objective To investigate the expressions of chemokine receptors and interleukin (IL) receptors on the peripheral blood mononuclear cells (PBMCs) from systemic lupus erythematosus (SLE) patients and their correlations with clinical features as well as SLE disease activity index (SLEDAI).

Methods The mRNA expressions of chemokine receptors and IL receptors on PBMCs of 93 SLE patients and 30 healthy controls were detected by reverse transcription-polymerase chain reaction, including CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CXCR3, CXCR5, CX3CR1, XCR1, IL-4R, and IL-10R. The clinical features of SLE patients were recorded. The correlations of chemokine receptors and IL receptors mRNA expressions with clinical features as well as SLEDAI were assayed using linear regression analysis.

Results The level of CCR5 mRNA in SLE patients (including active and inactive SLE) was significantly higher than that in healthy controls ($P < 0.05$), and there was no significant difference between active and inactive patients in this respect ($P > 0.05$). CX3CR1 mRNA expression significantly increased from healthy control to inactive SLE to active SLE in sequence. The others (except for CCR8, CXCR3, and IL-10R) in active SLE patients were significantly higher than those in both inactive SLE patients and healthy controls (all $P < 0.05$). There were positive correlations between SLEDAI and CCR2 ($r = 0.424$, $t = 4.313$, $P < 0.001$), CCR3 ($r = 0.518$, $t = 5.410$, $P < 0.001$), CCR4 ($r = 0.376$, $t = 3.851$, $P < 0.001$), CCR6 ($r = 0.457$, $t = 4.513$,

$P<0.001$), CXCR5 ($r=0.455$, $t=4.629$, $P<0.001$), CX3CR1 ($r=0.445$, $t=4.523$, $P<0.001$), as well as XCR1 ($r=0.540$, $t=5.445$, $P<0.001$). And CCR5 mRNA expression level was positively correlated with IL-4R mRNA ($r=0.313$, $t=2.353$, $P<0.05$). The patients with myositis and cutaneous vasculitis simultaneously showed lower levels of CCR5 and CX3CR1, and CCR5 expression was negatively correlated with the scores of SLEDAI in SLE cases accompanied by photosensitivity ($r=0.426$, $t=-2.155$, $P<0.05$).

Conclusion Increased expressions of CCR5 and CX3CR1 on PBMCs may be indicators in clinical survey for SLE.

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SYSTEMIC lupus erythematosus (SLE) is a chronic, inflammatory autoimmune disease characterized by antinuclear autoantibodies, immune complex formation, and systemic vasculitis.¹ The involved organs and systems in SLE include the skin, joints, lungs, peripheral blood cells, kidneys, and the central and peripheral nervous systems. The disease course of SLE is characterized by unpredictable flare and remission. Thus, biomarkers need to be developed for more accurate diagnosis and better assessment of the disease activity and organ involvement.

The efficient operation of the immune system is critically dependent on a complex series of cellular interactions and migrations to specific locations. Chemokines are small chemotactic cytokines characterized by critically positioned cysteine residues, which recruit distinct leukocyte subsets to the sites of inflammation and specific microenvironments within secondary lymphoid tissues.^{2,3} Chemokine receptor expression is exquisitely regulated depending on the stage of activation and differentiation of T cells, and coordinates tissue localization and encounters with antigen presenting cells.⁴⁻⁶ We propose that this receptor interaction also promotes the internalization, processing, and presentation of the autoantigens, thus enhancing their immunogenic capabilities. Over 40 chemokines have been identified as major traffic directors of leukocytes engaged in innate and adaptive immune responses.⁷ The subsets of leukocytes responding to particular chemokines are determined based on their interaction with selected G protein-coupled receptors.⁸

In response to the accumulation of immune complexes at the kidney in SLE, monocytes, T lymphocytes, and neutrophils infiltrate the kidney and mediate tissue injury and renal dysfunction. Chemotactic factors induced by immune complexes are responsible for recruiting inflammatory cells to the kidney. Considerable attention has focused on the role of the chemokine network in regulating renal leukocyte recruitment in autoimmune glomerular diseases. Intervention studies directed at chemokines or chemokine receptors in animal models of lupus nephritis have provided definitive

proof that specific chemokines are involved in the pathogenesis of renal inflammation. These chemokines and chemokine receptors are expressed in human kidney during lupus nephritis, and correlate with the markers of renal injury and inflammation. CCR5⁺ cells were detected in the interstitial tissue in specimens from lupus nephritis patients, meanwhile no CCR5⁺ cells were documented in normal controls. A strong positive correlation was found between tubulointerstitial immunoexpression of regulated upon activation, normal T cell expressed and secreted protein (RANTES) and the number of interstitial CCR5⁺ cells as well as between immunoexpression of RANTES and the immunoexpression of transfer growth factor beta1, alpha-smooth muscle actin, renal cortical volume, and serum creatinine in patients with lupus nephritis.⁹ Lupus erythematosus profundus skin lesions were characterized by lobular panniculitis, dominated by cytotoxic CXCR3⁺ lymphocytes.¹⁰

These studies showed that chemokines and chemokine receptors play an important role in the pathogenesis and tissue damage of SLE. In the present study, we extended the analysis of chemokine receptor expression profiles on the peripheral blood mononuclear cells (PBMCs) of SLE patients. We also analyzed the correlation between expression level of chemokine receptors and the scores of SLE disease activity index (SLEDAI) or clinical features.

PATIENTS AND METHODS

Patients

After the approval of Medical Ethics Committee of Chinese Academy of Medical Sciences, 93 patients (84 females and 9 males) who met at least four of the revised American Rheumatism Association (ARA) criteria¹¹ were enrolled into the study from the cohort of Chinese SLE patients in Hospital for Skin Diseases. The average age was 31.73 ± 1.88 years (range, 12-59). SLE disease activity was assessed by SLEDAI.¹² Inactive disease was defined as SLEDAI score ≤ 7 (40 cases), and active disease as SLEDAI score >7 (53 cases). Thirty healthy volunteers (4 males and 26 females), whose average age was 30.63 ± 1.05 years

(range, 18-48), were also enrolled. The clinical manifestations and the results of laboratory tests including complete blood count, routine urine test, erythrocyte sedimentation rate, lupus erythematosus cell test, serum level of complements (C3, C4, and CH50), circulating immune complex, antinuclear antibody (ANA), anti-ENA mutipeptide, anti-dsDNA, etc, were recorded for analyzing the clinical features. All the enrolled volunteers signed informed consent forms approved by the Ethics Committee.

Sampling peripheral blood

Blood was collected into sterile tubes containing 1.5% EDTA. PBMCs were isolated with lymphocyte-separation medium by the density gradient separation.

Protocol for reverse transcription-polymerase chain reaction (RT-PCR)

mRNA was extracted from PBMCs with TRIzol agent (GIBICO/BRL, Paisley, Scotland, UK). After precipitation with ethanol, the first-strand cDNA was reversely transcribed using SuperScript™ First-strand Synthesis System (GIBICO/BRL). Amplification of cDNA was performed as described in protocol (Tag polymerase, Promega, USA). cDNAs of chemokine receptors and interleukin (IL) receptors were amplified by PCR on GeneAmp PCR System

2700 (Applied Biosystems, CA, USA). The sequences of sense and antisense primers used are listed in Table 1.^{13,14} The PCR conditions included preheating at 94°C for 5 minutes followed by 38 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C-66°C for 30 seconds, elongation at 72°C for 1 minute, and the final elongation at 72°C for 7 minutes. PCR products were electrophoresed on 2% agarose gel and visualized with ethidium bromide. The densities of target gene mRNA bands were semi-quantified with β -actin mRNA by Quantity One 1-D (BioRad Corporation, Sundbyberg, Sweden).

Statistical analysis

All data were expressed as means $\pm$ SEM. The differences in mRNA expressions of chemokine receptors and IL receptors among active SLE group, inactive SLE group, and healthy control group were analyzed by *t*-test. The correlations of clinical data including SLEDAI with chemokine receptors and IL receptors mRNA expression levels were investigated using linear regression analysis (Pearson's correlation). To determine the statistical significance of each comparison, random permutation was performed to define the *P*-value thresholds. Differences were considered significant if *P* values were lower than 0.05. Analysis was performed with SPSS 16.0.

Table 1. Primer sequences for PCR reactions

Genes	Primer sets	Sequence	Size (bp)	Annealing temperature (°C)
β -actin	Sense	5'-CAACTCCATCATGAAGTGTA-3'	180	60
	Antisense	5'-CCACACGGAGTACGCGCTG-3'		
CCR2	Sense	5'-CTCGCTGGTGTTCATCTTTG-3'	583	57
	Antisense	5'-AGGCGTAGATCACTGGGTTG-3'		
CCR3	Sense	5'-TGGCGGTGTTTTTCATTTTC-3'	315	59
	Antisense	5'-CCGGCTCTGCTGTGGAT-3'		
CCR4	Sense	5'-TACTTGGTGGGCTTTTACAG-3'	382	55
	Antisense	5'-TCATCTTCACCGCCTTGTC-3'		
CCR5	Sense	5'-CTTCTGGGCTCCCTACAACA-3'	311	57
	Antisense	5'-CAGATATTTCTGCTCCCCA-3'		
CCR6	Sense	5'-GAGCCCATCAGGAAGCTGCTG-3'	316	58
	Antisense	5'-GGCAGCAGTGCAGGAAAGCCAGGAC-3'		
CCR8	Sense	5'-CATCCTGGTCTTGTGGTCT-3'	540	56
	Antisense	5'-GGCTTGGTCTTGTGTGGTT-3'		
CXCR3	Sense	5'-TCCTTGAGGTGAGTGACCACAAA-3'	584	59
	Antisense	5'-CTCGTCGTGGTGGGCCGACAG-3'		
CXCR5	Sense	5'-GGTCTTCATCTTGCCCTTTG-3'	340	59
	Antisense	5'-ATGCGTTTCTGCTTGGTTCT-3'		
CX3CR1	Sense	5'-GTAGTGTTCGCCCTACCAACA-3'	502	59
	Antisense	5'-ACAGCGTCTGGATGATTCTGAA-3'		
XCR1	Sense	5'-TCCAGATGGAGTCCTCAGGCAACC-3'	531	66
	Antisense	5'-CACAGCCCCAAGAAAGCACCTT-3'		
IL-4R	Sense	5'-CCCCACCACTGGCTATC-3'	162	56
	Antisense	5'-GCCCCAAACCCACATTTTC-3'		
IL-10R	Sense	5'-GTACCACAGCAATGGCTACC-3'	672	60
	Antisense	5'-TGAGGTCCAAGTTCTTCAGC-3'		

PCR: polymerase chain reaction; IL-4R: interleukin-4 receptor; IL-10R: interleukin-10 receptor.

To assess the possible role of chemokine receptors in the organ-specific disease involvement, we identified those patients who showed evidence of active disease as determined by the organ specific features of Physician's Global Assessment, SLEDAI, or laboratory testing. The patients were accordingly divided into positive and negative groups, and Student's unpaired *t*-tests were used to compare the difference in chemokine receptor levels between the two groups. Some cases without sufficient clinical data were excluded from the analyses.

RESULTS

mRNA expression levels of chemokine and IL receptors on PBMCs from SLE patients

The mean expression levels of CCR2, CCR3, CCR4, CCR6, CX3CR1, CXCR5, XCR1, and IL-4R mRNAs in active SLE group were significantly higher than those in the other two groups (all $P < 0.05$). The mean level of CCR5 mRNA in SLE patients (including active and inactive) was significantly higher than that in healthy control ($P < 0.05$), and there was no difference between active and inactive patients ($P > 0.05$). CX3CR1 mRNA in inactive group was also significantly higher than that in controls ($P < 0.05$). The levels of CXCR3 and IL-10R mRNAs in active SLE group were significantly higher than those in inactive SLE group ($P < 0.05$), while there was no statistical difference in the mean expression levels of CCR8, CXCR3, and IL-10R between active SLE group and healthy control group (all $P > 0.05$) (Fig. 1, Table 2).

Correlation between SLEDAI and mRNA expression levels of chemokine receptors and IL receptors

There were positive correlations between SLEDAI and mRNA expression levels of some chemokine receptors, including CCR2 ($r = 0.424$, $t = 4.313$, $P < 0.001$), CCR3 ($r =$

0.518 , $t = 5.410$, $P < 0.001$), CCR4 ($r = 0.376$, $t = 3.851$, $P < 0.001$), CCR6 ($r = 0.457$, $t = 4.513$, $P < 0.001$), CXCR5 ($r = 0.455$, $t = 4.629$, $P < 0.001$), CX3CR1 ($r = 0.445$, $t = 4.523$, $P < 0.001$), XCR1 ($r = 0.540$, $t = 5.445$, $P < 0.001$), except for CCR5 ($r = 0.062$, $t = -0.589$, $P > 0.05$). No significant correlation was found between SLEDAI and IL-4R ($r = 0.085$, $t = -0.611$, $P > 0.05$) or IL-10R mRNA expression levels ($r = 0.066$, $t = 0.473$, $P > 0.05$).

Correlation between mRNA expression levels of chemokine receptors and IL receptors

Between chemokine receptors and IL receptors, there was a positive correlation between CCR5 and IL-4R mRNA expression levels ($r = 0.313$, $t = 2.353$, $P < 0.05$).

Associations between chemokine receptor expression and clinical features

CCR2 mRNA expression significantly lowered in patients with myositis (95%CI 0.48-2.61, $P < 0.01$) or thrombocytopenia (95%CI 0.46-3.12, $P < 0.01$) than that in patients without these two features. The other similar links between the lower mRNA levels of chemokine receptors and clinical features were as follows: CCR3 associated with myositis (95%CI 1.85-4.91, $P < 0.001$); CCR4 with cutaneous vasculitis (95%CI 0.01-2.10, $P < 0.05$); CCR5 with myositis (95%CI 1.84-5.34, $P < 0.001$), cutaneous vasculitis (95%CI 0.88-5.72, $P < 0.01$), and photosensitivity (95%CI 1.26-5.69, $P < 0.05$); CXCR3 with renal disorder (95%CI 0.13-1.73, $P < 0.05$) and C3 decrease (95%CI 0.32-2.09, $P < 0.001$); CXCR5 with myositis (95%CI 1.67-4.42, $P < 0.001$), hemolytic anemia (95%CI 2.23-5.31, $P < 0.001$), leukopenia (95%CI 0.72- 4.69, $P < 0.01$), elevated serum IgA (95%CI 1.54-4.74, $P < 0.001$), and positive anti-Ro/SSA (Sjögren's syndrome antigen) antibody (95%CI 0.18-4.36, $P < 0.05$); CX3CR1 with myositis (95%CI 1.02- 3.89, $P < 0.01$), cutaneous vasculitis (95%CI 0.63-4.69, $P < 0.05$),

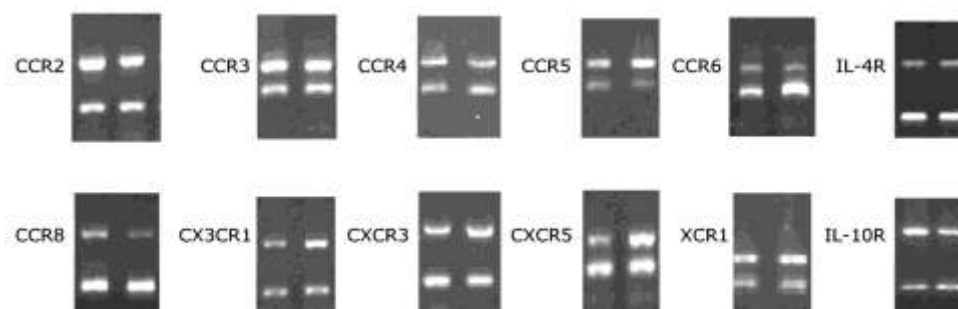


Figure 1. RT-PCR products for chemokine receptor and interleukin receptor mRNAs on PBMCs of SLE patients detected with ethidium bromide-stained agarose gel.

Upper row: chemokine receptor and interleukin receptor mRNA amplification products; lower row: β -actin amplification products.

RT-PCR: reverse transcription-PCR; PBMCs: peripheral blood mononuclear cells; SLE: systemic lupus erythematosus.

renal disorder (95%CI 0.13-1.73, $P<0.05$), and thrombocytopenia (95%CI 0.86-4.69, $P<0.01$); and XCR1 with positive anti-Ro/SSA antibody (95%CI 0.59-3.60, $P<0.01$) (Table 3).

Correlation between the mRNA level of chemokine receptor with clinical features and SLEDAI

We found that only the lowered level of CCR5 expression was negatively correlated with the scores of SLEDAI in the SLE cases with photosensitivity ($r=0.426$, $t=-2.155$, $P<0.05$).

DISCUSSION

Many autoimmune diseases are mainly characterized by lymphocyte and monocyte infiltration in target tissues, leading to the damage of these tissues characterized by

accumulation and activation of specific leucocytes attracted by chemokines. The composition of infiltrating cells mainly depends on the chemokine receptors expressed on these cells and the kind of chemokine produced at the location, which is somewhat associated with the activity of the disease. The levels of serum chemokines were proposed to be convenient biomarkers for disease activity in lupus.¹⁵ In our research, the chemokine receptor expression profiles were found associated with the disease activity in SLE patients.

To compare the expression levels of these markers, we applied semi-quantitative RT-PCR since it is a more precise tool to quantify gene expression. First, this study discovered over-expressions of CCR2, CCR3, CCR4, CCR6, CX3CR1, CXCR5, XCR1, and IL-4R in active SLE patients compared with inactive patients and controls. The increased mRNA expressions of CCR2, CCR3, CCR4, CCR6, and

Table 2. Comparison of chemokine receptor and IL receptor mRNA expressions on PBMCs among active SLE, inactive SLE, and healthy control groups[§]

Group	<i>n</i>	CCR2	CCR3	CCR4	CCR5	CCR6	CCR8
Healthy control	30	0.263±0.035**	0.602±0.277**	0.192±0.041**	0.373±0.032**	0.229±0.041**	0.120±0.018
Inactive SLE	40	0.388±0.033**	0.585±0.036**	0.259±0.030**	0.591±0.054	0.306±0.034*	0.219±0.031
Active SLE	53	1.595±0.436	2.400±0.429	1.041±0.281	0.531±0.070	0.985±0.257	0.703±0.285
Group	<i>n</i>	CXCR3	CXCR5	CX3CR1	XCR1	IL-4R	IL-10R
Healthy control	30	0.207±0.043	0.431±0.031**	0.146±0.020**#	0.192±0.031**	0.375±0.022**	0.734±0.119
Inactive SLE	40	0.159±0.029*	0.562±0.050**	0.530±0.045**	0.308±0.040**	0.387±0.130**	0.691±0.204**
Active SLE	53	0.822±0.255	1.784±0.318	3.735±0.557	2.087±0.343	0.604±0.147	0.870±0.290

§: Plus-minus values are means±SEM.

* $P<0.05$, ** $P<0.01$ compared with active SLE group; # $P<0.01$ compared with inactive SLE group.

Table 3. Correlation of chemokine receptor expression with clinical parameters in SLE patients[§]

Chemokine receptors	Clinical features	Total case number	Clinical parameter positive group		Clinical parameter negative group		<i>t</i> value	<i>P</i> value	95%CI
			Number	mRNA level	Number	mRNA level			
CCR2	Myositis	80	6	1.88±0.14	74	3.44±0.52	2.90	0.005	0.48-2.61
	Thrombocytopenia	80	11	1.77±0.38	69	3.56±0.56	2.69	0.009	0.46-3.12
CCR3	Myositis	75	6	2.38±0.11	69	5.76±0.76	4.42	<0.001	1.85-4.91
CCR4	Cutaneous vasculitis	83	22	1.49±0.28	61	2.55±0.45	2.00	0.049	0.01-2.10
CCR5	Myositis	84	7	1.07±0.20	77	4.66±0.86	4.08	<0.001	1.84-5.34
	Cutaneous vasculitis	83	22	1.99±0.62	61	5.29±1.05	2.71	0.008	0.88-5.72
CXCR3	Photosensitivity	84	23	1.88±0.32	61	5.36±1.06	3.14	0.040	1.26-5.69
	Renal disorder	74	17	0.89±0.18	57	1.82±0.36	2.31	0.024	0.13-1.73
	C3 decrease	73	24	0.81±0.17	49	2.01±0.41	2.72	0.008	0.32-2.09
CXCR5	Myositis	77	5	2.04±0.12	72	5.08±0.68	4.40	<0.001	1.67-4.42
	Hemolytic anemia	77	6	1.41±0.35	71	5.18±0.67	4.91	<0.001	2.23-5.31
	Leukopenia	79	13	2.54±0.67	66	5.25±0.73	2.74	0.009	0.72-4.69
	IgA increase	76	7	2.08±0.37	69	5.21±0.71	3.93	<0.001	1.54-4.74
CX3CR1	Anti-Ro/SSA antibody	75	17	3.21±0.65	58	5.48±0.82	2.17	0.034	0.18-4.36
	Myositis	77	7	2.41±0.21	70	4.87±0.67	3.42	0.001	1.02-3.89
	Cutaneous vasculitis	76	19	2.67±0.61	57	5.34±0.81	2.62	0.011	0.63-4.69
	Renal disorder	77	19	3.63±0.94	58	4.86±0.77	2.31	0.024	0.13-1.73
	Thrombocytopenia	77	10	2.23±0.62	67	5.01±0.71	2.94	0.005	0.86-4.69
XCR1	Anti-Ro/SSA antibody	68	17	1.37±0.30	51	3.47±0.69	2.78	0.007	0.59-3.60

§: Plus-minus values are means±SEM.

SSA: Sjögren's syndrome antigen.

CXCR5 on PBMCs in active SLE patients were positively correlated with disease activity. These data conform with the previously reported studies and suggest that Th2 cells play a predominant role in SLE.

Second, the mRNA levels of CXCR3 and IL-10R in active patients were significantly higher than those in inactive patients, but there were no significant difference between active patients and controls or between inactive patients and controls. The reason might be the limited size of samples in this study. The expressions of CXCR3 are possibly enriched in kidney-infiltrating cells, as reported in previous studies,^{16,17} but there were only 19 cases with renal disorder in our study.

Last, the level of CCR5 mRNA in both active and inactive SLE patients was significantly higher than that in controls. This result is slightly different from Al-Saleh's report in which patients in active SLE group had significantly elevated expression of CCR5 on the surface of CD4⁺ T lymphocytes compared with SLE patients in remission and healthy controls.¹⁸ In this study, CX3CR1 mRNA expression was the highest in active group, and it showed positive correlation with SLEDAI. CX3CR1 is the receptor of CX3CL1 (fractalkine), which is released by activated endothelial cells, activated platelets, injured subendothelial parenchymal cells, and dendritic cells.¹⁹⁻²¹ Up-regulated in inflammatory tissues, CX3CL1 not only serves as an adhesion molecule and chemokine, but also participates in an amplification circuit of polarized Th1 responses.²² The up-regulation of CX3CR1 in our study suggests its involvement in the Th1/Th2 imbalance of SLE, and in multi-organ injury except for vascular damage.

In order to observe whether there were abnormal IL expressions in the same SLE patients, we analyzed the correlation between IL-4R and IL-10R with chemokine receptors. There was positive correlation between the mRNA expression of CCR5 and that of IL-4R. We previously reported a positive correlation between IL-4R Ile50/Ile50 genotype and susceptibility to SLE ($P=0.022$).²³ It was showed that IL-4R gene dysfunction led to its abnormal over-expression. Although IL-10R expression in active patients was significantly higher than that in inactive patients, there was no significant difference between SLE patients and controls in this respect. This revealed that IL-4R was more related to the pathogenesis of SLE than IL-10R. Interestingly, in the present study, there was a positive correlation between CCR5 and IL-4R mRNA levels, suggesting some possible linkage between these two receptors. The up-regulated expression of CCR5 mRNA in PBMCs of SLE patients suggests its roles in the pathogenesis of SLE, which may be involved in newly activated

CD4⁺ T cells although there is no correlation between CCR5 level and SLEDAI. Further study is still needed.

Furthermore, the lowered expression levels of some chemokine receptors in SLE patients were found associated with some clinical features, suggesting that these receptors probably play some roles in preventing or interdicting the development of these symptoms or signs. Each individual clinical parameter is linked to one or more declining chemokine receptor, but their clinical significance is unclear and needs further investigation. Among these receptors, only CCR5 and CX3CR1 showed over-expressions in both active and inactive SLE patients, and down-expressions with both cutaneous vasculitis and myositis in SLE patients; the lowered level of CCR5 expression was negatively correlated with the scores of SLEDAI in cases accompanied by photosensitivity. We therefore propose the following scenario: injury induces the release of self-antigen, sending a tissue-specific signal to cells of innate and adaptive immune system to migrate to the site of injury and eliminate pathogens and cellular debris, inducing molecular marker expression on lymphocytes. The CCR5 HHE/HHG*2 genotype was associated with the maximal risk of developing SLE. These findings implicate a key role of CCR5 and CX3CR1 in the pathogenesis of SLE.²⁴ The levels of CCR5 and CX3CR1 mRNAs might act as indicators of cutaneous vasculitis and myositis in SLE.

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