

# Elevated levels of soluble fractalkine and increased expression of CX3CR1 in neuropsychiatric systemic lupus erythematosus

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**Abstract.** The aim of the present study was to determine the levels of soluble fractalkine (sFKN) and expression of CX3CR1 in neuropsychiatric systemic lupus erythematosus (NPSLE). Disease activity of SLE was assessed using the SLE Disease Activity Index (SLEDAI). The mRNA expression levels of CX3CR1 and FKN were quantified using reverse transcription-quantitative polymerase chain reaction. Levels of sFKN in the serum and cerebrospinal fluid (CSF) were measured using enzyme-linked immunosorbent assays. The mRNA expression levels of CX3CR1 in peripheral blood mononuclear cells from patients with NPSLE, non-NPSLE and Behcet's disease were significantly higher than that of rheumatoid arthritis and healthy persons. Levels of sFKN in the serum and CSF of cells with diffuse NPSLE (DNPSLE) were significantly higher than those of focal NPSLE (FNPSLE) cells. Serum levels of sFKN were higher in patients with NPSLE or non-NPSLE than healthy persons. sFKN in CSF were significantly higher in DNPSLE than non-NPSLE cells, but there were no significant difference between FNPSLE and control. Treatment reduced sFKN in serum and CSF in patients with NPSLE. There was significant correlation between sFKN in the serum of patients with SLE and the SLEDAI. sFKN levels were correlated with IgG in CSF from patients with NPSLE. The mRNA expression levels of CX3CR1 in the brain tissue of lupus mice were significantly higher than normal mice; however, the mRNA expression of FKN was lower than normal mice. These results suggest that sFKN and CX3CR1 may be involved in vasculitis and SLE, particularly in DNPSLE, which may occur by damaging the blood-brain barrier or recruiting expression microglial cells of CX3CR1. Additionally, sFKN appears to be

a serological marker in patients with SLE, and may be useful for the diagnosis and treatment of NPSLE.

## Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease in which the body's immune system mistakenly attacks healthy tissue (1). When SLE affects the central or peripheral nervous systems, it is called neuropsychiatric systemic lupus erythematosus (NPSLE) (2). The American College of Rheumatology (ACR) defines 19 neuropsychiatric syndromes in SLE in 1999, and NPSLE was divided into three clinical types: Diffuse (DNPSLE), focal (FNPSLE) and peripheral neurological SLE (2). Manifestations of DNPSLE include cognitive dysfunction, acute confusion states, psychosis, mood and anxiety disorders (2). Manifestations of FNPSLE include seizures, cerebrovascular disease, headache, demyelinating syndrome, aseptic meningitis, chorea and myelopathy (2). Autoantibody and inflammatory cells are important pathogenic factors of NPSLE. Infiltration and activation of inflammatory cell often require the involvement of chemotactic factors (3). Fractalkine (FKN) is the only known member of the CX3C family at present which is a recently discovered chemotactic factor, the chemokine receptor of which is CX3CR1 (4). FKN is primarily expressed and secreted by activated vascular endothelial cells, epithelial cells, synovial cells, and is highly expressed in "membrane-anchored" and "free" neurons. Studies of FKN regarding SLE are more concentrated in lupus nephritis (LN), while the role of FKN in NPSLE is less understood (5,6). Thus, the aim of the present study was to assess the mRNA expression of CX3CR1 in peripheral blood mononuclear cells (PBMCs), and levels of sFKN in the serum and cerebrospinal fluid (CSF) to investigate the involvement of sFKN in NPSLE. In addition, the mRNA expression levels of FKN and CX3CR1 in the brain tissue of lupus mice were evaluated to determine their effect on NPSLE.

## Materials and methods

**Patients.** Subjects admitted to Renji Hospital (Shanghai, China) were retrospectively analyzed from November 2005 to October 2008, including 152 patients and 51 healthy persons (HPs). The patient population consisted of non-NPSLEs

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(female, 29; male, 5; age, 29±7 years), RA (female, 26; male, 4; age, 30±5 years), BD (female, 38; male, 10; age, 32±7 years). Healthy control population consisted of 43 females and 8 males (age, 31±6 years). In addition, brain tissues from 8 lupus mice were analyzed, obtained from the Model Animal Research Center, Nanjing University (Nanjing, China). Patients with SLE conformed to the standard of classification revised by ACR in 1997 (1), while NPSLE conformed to the class definition proposed by the ACR in 1999 (2). Neuropsychiatric symptoms include: i) Abnormal electroencephalogram, which are predominantly with slow wave and diffuse activity; ii) abnormal CSF, which mainly manifested as increased pressure, total protein, white blood cell count and/or total IgG; and iii) abnormal computed tomography or magnetic resonance imaging. Infection, drugs and metabolic factors were excluded; such as infection/drugs/metabolic factors including intracranial tuberculosis, bacteria and streptococcus, the nerve symptoms caused by drugs such as corticosteroids, low sodium and low chlorine encephalopathy. Furthermore, rheumatoid arthritis (RA) was identified according to the diagnostic criteria of ACR in 1987 (7) and Behcet's disease (BD) according to the diagnostic criteria of the International Study Group for Behcet's disease in 1990 (8). This study was approved by the ethics committee at Dongying People's Hospital and was conducted in accordance with the provisions of the Declaration of Helsinki, Good Clinical Practice guidelines, and local laws and regulations.

*mRNA expression levels of CX3CR1 in PBMCs.* Investigated subjects include 40 cases with NPSLE, 34 cases with SLE but no neuropsychiatric symptoms (non-NPSLE), 30 cases with RA, 48 cases with BD and 51 HPs. Average age of the NPSLE group was 33±14 years old, including 16 cases with DNPSLE and 24 cases with FNPSLE. Leucocytes from the peripheral blood of above investigated subjects were collected and dissolved in TRIzol to extract total RNA. Integrity, purity and concentration were tested (9), then total RNA was reverse transcribed to cDNA. mRNA expression of CX3CR1 was quantified using reverse transcription-quantitative polymerase chain reaction, using a SYBR *Premix Ex Taq* kit (Takara Bio, Inc, Otsu, Japan). Primers were designed as following: Reference gene RPL13A forward, 5'-CCTGGAGGAGAA GAGGAAAGAGA-3' and reverse 5'-TTGAGGACCTCT GTGTATTTGTCAA-3'; CX3CR1 forward, 5'-AGCAGGCAT GGAAGTGTCT-3' and reverse 5'-GTTGTTTTGTGTGCA TTGGG-3'. Primers were stored at -20°C after dissolution and dilution. The operation was performed according to the instructions of a DRR041A Takara Kit (Takara Bio, Inc.). A 5- $\mu$ l reaction system included 2.5  $\mu$ l 2X SYBR Premix, 0.1  $\mu$ l Forward Primer (10  $\mu$ M), 0.1  $\mu$ l Reverse Primer (10  $\mu$ M), 0.1  $\mu$ l ROX Reference Dye, 1.2  $\mu$ l deionized water and 1.0  $\mu$ l cDNA. Reaction plates were centrifuged at 2,000 rpm for 1 min at 4°C. PCR was performed according to the instruction of ABI 7900 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc., Foster City, CA, USA). Reaction conditions are set as follows: Pre denaturation (95°C for 10 sec, Repts: 1), PCR (95°C for 5 sec, 60°C for 30 sec, Repts: 40) and a dissociation Stage with the same parameter. Sample volume was 5  $\mu$ l.  $2^{-\Delta\Delta Ct}$  value was considered to be proportional to the mRNA expression of the corresponding gene in the specimen.

*Levels of sFKN in the serum and CSF.* Serum samples were collected from 30 NPSLE patients, 28 female and 2 male, including 13 cases with DNPSLE and 17 cases with FNPSLE (average age, 34±13 years). Serum samples were collected from 53 non-NPSLE cases, 49 female and 4 male (average age, 33±11 years). Serum samples were additionally collected from 39 HPs, including 37 females and 2 males (average age, 34±8 years). CSF samples were collected only from 8 cases with DNPSLE and 15 cases with active stage FNPSLE. In addition, serum samples from 17 cases and CSF of 8 cases with NPSLE were collected after treatment with 200-1,000 mg methylprednisolone per day for 3-5 days (Pfizer Manufacturing Belgium NV, Puurs, Belgium) and immunosuppressive drugs including cyclophosphamide 0.4-0.6 g per week and hydroxychloroquine sulfate 0.1-0.2 g bid (Baxter Oncology GmbH, Halle, Germany), for 1-2 weeks. Dosage and course of treatment were determined according to the severity of the illness and the patient's weight. For ethical reasons, CSF samples were not collected from HPs. As the inspection results of SLE patients did not conform to the diagnostic criteria of NPSLE, CSF from these SLE patients was considered to be non-NPSLE. Age and gender of all above groups had no statistically significant difference. The active disease stage of each patient was determined according to the SLE Disease Activity Index (SLEDAI), with an SLEDAI >10 indicating the active stage (10). The concentration of total IgG in the CSF of cases with NPSLE was obtained from patient medical records. Levels of sFKN in serum and CSF were evaluated using an enzyme-linked immunosorbent assay (DY365) and substrate kit (DY999; R&D Systems, Inc., Minneapolis, MN, USA).

*mRNA expression of FKN and CX3CR1 in the brain tissue of lupus mice.* Investigated samples consisted of brain tissues from 8 lupus mice, including 3 NZB, 1 NZW, 2 NZB/NZWF1 and 2 BXSB mice (6 females and 2 males). Six matched normal mice were also included (B57; Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China). The rats were anesthetized with 0.4-0.6 ml 0.25% pentobarbital Sodium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and sacrificed by cutting the abdominal cavity and thoracic cavity, exposing the heart and then blood letting. Brain tissues of mouse were obtained through operation and stored in a refrigerator. Brain tissues were ground in liquid nitrogen to dissociate cells. After removing impurity, total RNA was extracted to determine the mRNA expression levels of FKN and CX3CR1. Primers were designed as follows:  $\beta$ -actin, forward 5'-ATGCTCCCCGGGCTGTAT-3' and reverse 5'-CATAGGAGTCCTTCTGACCCATTC-3'; mouse CX3CR1, forward 5'-TGTTATTTGGGCGACATTG-3' and reverse 5'-CGAGGACCAACAGATT-3'; and mouse FKN, forward 5'-TGCGACAAGATGACCTCAC-3' and reverse 5'-TCTGTGCTGTGTCGTCTCC-3'. PCR protocols were the same as those used for detecting CX3CR1.

*Statistical analysis.* SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA) was used to analyze data. Data were manipulated using normal distribution and homogeneity of variance tests, then with one-way analysis of variance or non-parameter statistics. All data are expressed as the mean  $\pm$  standard error. Data from before or after treatment

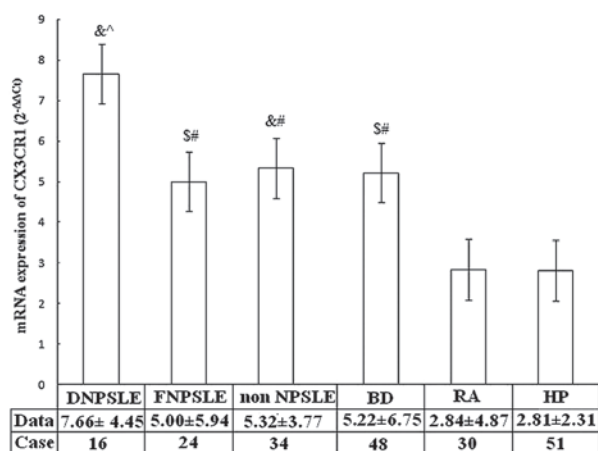


Figure 1. mRNA expression of CX3CR1 in PBL. &^P<0.01 vs. HP; \$P<0.05 vs. HP; ^P<0.01 vs. RA; #P<0.05 vs. RA. DNPSLE, diffuse neuropsychiatric systemic lupus erythematosus; FNPSLE, focal neuropsychiatric systemic lupus erythematosus; BD, Behcet's disease; RA, rheumatoid arthritis; HP, healthy person.

were compared using *t*-test. Correlation was performed using Pearson correlation analysis. P<0.05 were considered to indicate a statistically significant difference.

**Results**

*mRNA expression levels of CX3CR1 in PBMCs.* The mRNA expression levels of CX3CR1 in the PBMCs of NPSLE, non-NPSLE and BD patients were all significantly higher than the RA and HP PBMCs. However, no statistically significant differences were detected among the DNPSLE, FNPSLE, non-NPSLE and BD groups (Fig. 1).

*Levels of sFKN in the serum and CSF.* Levels of sFKN in serum samples from patients with DNPSLE were significantly higher than those from FNPSLE patients. In addition, the DNPSLE and FNPSLE groups had significantly higher sFKN levels than non-NPSLE and HP groups, whereas non-NPSLE was only significantly higher than HP (Fig. 2). sFKN in CSF were significantly higher in DNPSLE compared with FNPSLE and non-NPSLE (P<0.01), but there were no significant differences between FNPSLE and non-NPSLE (Fig. 3). Moreover, IgG in CSF samples from DNPSLE (31.3±28.8 mg/dl) were higher than FNPSLE (7.1±6.6 mg/dl) (data not shown).

*Correlation between sFKN and patient treatment, SLEDAI and IgG.* Comparisons of levels of sFKN in the serum and CSF before and after treatment with methylprednisolone and immunosuppressive drugs are shown in Figs. 4 and 5. Before treatment, sFKN in serum and CSF were 5,232±3,039 and 2,632±2,272 pg/ml, respectively. However, after treatment the data became 2,414±2,507 and 705±404 pg/ml, respectively. sFKN in the serum of patients with SLE were positively correlated with SLEDAI (*r*=0.671, P<0.01; Fig. 6). In addition, sFKN in CSF from patients with NPSLE were positively correlated with IgG (*r*=0.945, P<0.01; Fig. 7).

*mRNA expression of FKFN and CX3CR1 in the brain tissue of lupus mice.* The mRNA expression levels of CX3CR1 in

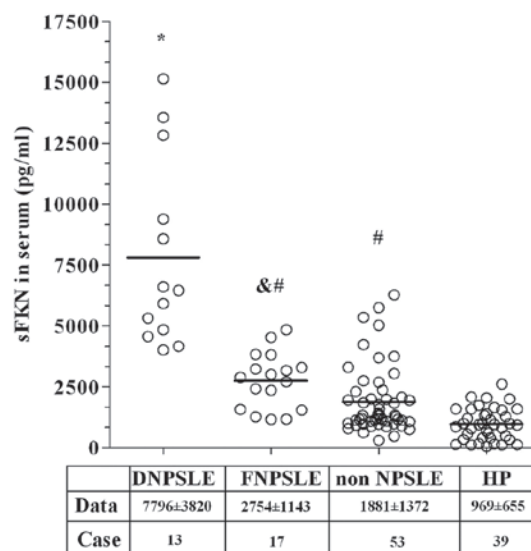


Figure 2. Levels of sFKN in serum. \*P<0.01 vs. FNPSLE, non-NPSLE or HP; &#P<0.05 vs. non-NPSLE; #P<0.01 vs. HP. sFKN, soluble fractalkine; DNPSLE, diffuse neuropsychiatric systemic lupus erythematosus; FNPSLE, focal neuropsychiatric systemic lupus erythematosus; non-NPSLE, non-neuropsychiatric systemic lupus erythematosus; HP, healthy person.

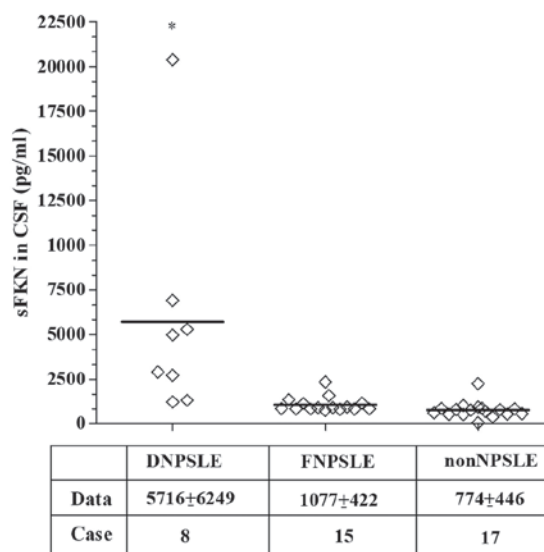


Figure 3. Levels of sFKN in CSF. \*P<0.01 vs. FNPSLE and non-NPSLE. sFKN, soluble fractalkine; CSF, cerebrospinal fluid; DNPSLE, diffuse neuropsychiatric systemic lupus erythematosus; FNPSLE, focal neuropsychiatric systemic lupus erythematosus; non-NPSLE, non-neuropsychiatric systemic lupus erythematosus.

the brain tissue of lupus mice were significantly higher than normal mice, while the mRNA expression of FKFN was lower than normal mice. By contrast, the former showed a significant difference (P=0.028) and the latter did not (P=0.543; Fig. 8).

**Discussion**

The present study investigated the effect and significance of sFKN and CX3CR1 in NPSLE using the following three parameters: i) mRNA expression of CX3CR1 in PBMCs from human subjects; ii) levels of sFKN in serum and CSF from human subjects; and iii) mRNA expression of FKFN

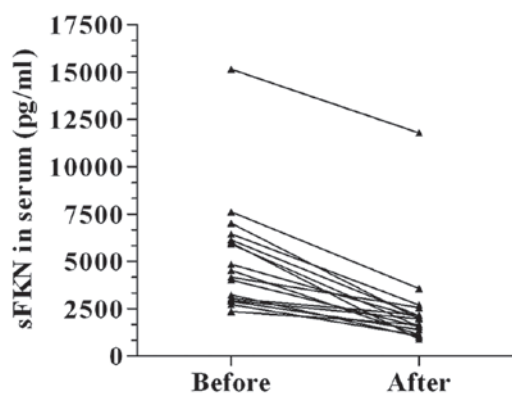


Figure 4. Correlation of sFKN in serum from 17 patients with neuropsychiatric systemic lupus erythematosus prior to and after treatment. sFKN, soluble fractalkine.

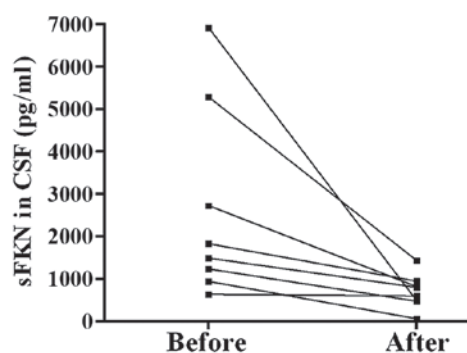


Figure 5. Correlation of sFKN in CSF from 8 patients with neuropsychiatric systemic lupus erythematosus before and after treatment. sFKN, soluble fractalkine; CSF, cerebrospinal fluid.

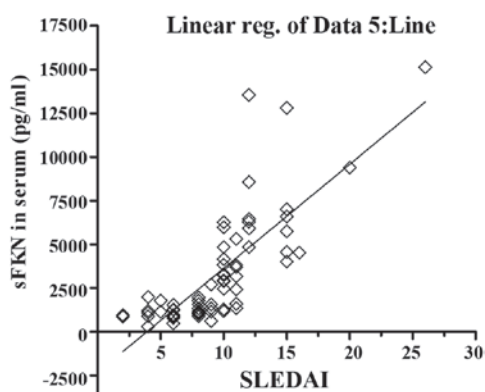


Figure 6. Correlation between sFKN in serum and SLEDAI from patients with SLE. sFKN, soluble fractalkine; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index.

and CX3CR1 in the brain tissue of lupus mice. We initially confirmed the significant difference between DNPSLE and FNPSLE patients in their serum levels of sFKN. Furthermore, the mRNA expression of FKFN and CX3CR1 in the brain tissue of lupus mice was initially reported in the present study.

The present study found that the mRNA expression of CX3CR1 in PBMCs from patients with SLE and BD was higher than HPs and patients with RA. The pathological basis of SLE and BD is vasculitis, while RA is synovitis (11). The

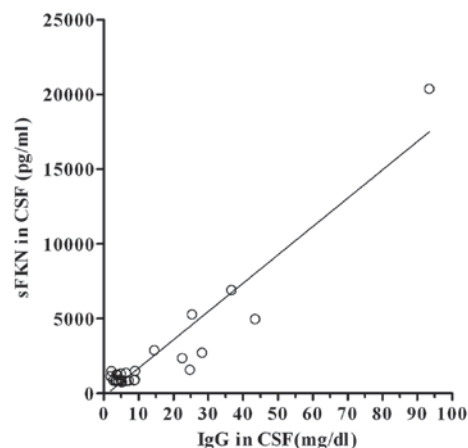


Figure 7. Correlation between sFKN and IgG in CSF from patients with neuropsychiatric systemic lupus erythematosus. sFKN, soluble fractalkine; CSF, cerebrospinal fluid.

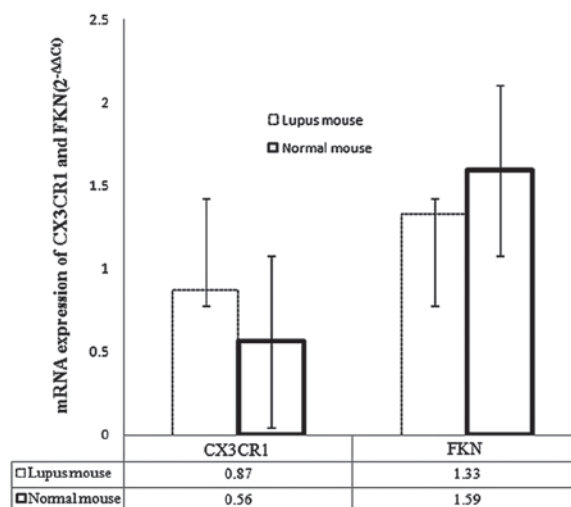


Figure 8. mRNA expression of CX3CR1 and sFKN in brain tissue of lupus mouse and normal mouse. sFKN, soluble fractalkine.

present study found that the mRNA expression of CX3CR1 in PBMCs from patients with SLE and BD was higher than HPs and patients with RA. This result indicated that the expression of CX3CR1, which is the receptor of FKFN, increased in vasculitis. Matsunawa *et al* found that the levels of sFKN in serum from patients with RA combined with rheumatoid vasculitis (RV) was higher than patients with RA only (12). In addition, Yajima *et al* similarly found that the serum levels of sFKN in patients with SLE were higher than patients with RA (13). Sato *et al* found levels of sFKN in serum from patients with SLE were higher than those from RA and primary Sjogren's syndrome, whose pathological feature is inflammation of the exocrine glands (14). Bjerkeli *et al* confirmed that the mRNA expression of CX3CR1 in PBMCs and levels of sFKN in the serum of patients with granulomatosis with polyangiitis (GPA) were both higher than HPs (15). Matsunawa *et al* discovered serum levels of sFKN in patients with microscopic polyangiitis (MPA) were also higher than HPs (16). Thus, FKFN and CX3CR1 may serve a crucial function in SLE, the pathological cause of which vasculitis. Vascular endothelial cells have been

shown to be activated by interleukin-1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , interferon (IFN)- $\gamma$  and lipopolysaccharide (17,18). Moreover, the nuclear factor- $\kappa$ B pathway was activated, expression of FKN increased on the surface of vascular endothelial cells, which is known as membrane-anchored FKN (17,18). If membrane-anchored FKN released from the cytomembrane, it was called soluble FKN (sFKN). Membrane-anchored FKN functions as an adhesion molecule and sFKN as a chemotactic factor (19). They captured natural killer cells, monocytes cell and T cells, which can express CX3CR1 on their surfaces (20). In order to drive them near endothelial cells of vessel wall and induce transmembrane transport. These inflammatory cells can release IFN- $\gamma$ , TNF- $\alpha$ , perforin and granzyme following activation, which can damage vascular endothelial cells and other tissue cells, causing vasculitis and organ damage (21).

The present study found levels of sFKN in serum from patients with SLE were higher than those in serum from HPs, which is consistent with previous reports (13,14). NPSLE was significantly higher than non-NPSLE, which was consistent with a previous report by Sato *et al* (14), but conflicted with the results of a report by Yajima *et al* (13). Levels of sFKN in serum and CSF from patients with DNPSLE were significantly higher than those with FNPSLE, particularly patients with clinical manifestations of acute confusion states, cognitive dysfunction and psychosis. A prior study found that sFKN levels in CSF from patients with HIV combined with cognitive dysfunction was higher than patients without cognitive dysfunction (22) and a similar results was observed in the present study except in patients with various types of NPSLE. However, the results of Yajima *et al* showed sFKN in CSF from patients with FNPSLE was not significantly higher than in patients with DNPSLE (13), which may be due to the small size of the study sample. Moreover, the present study showed that sFKN levels in CSF from patients with DNPSLE was significantly higher than that of non-NPSLE patients, which was different with results obtained by Sato *et al* (14). Thus, sFKN may serve a crucial function in the pathogenesis of neuropsychiatric symptoms in DNPSLE. In the present study, sFKN levels in serum from patients with SLE were positively correlated with SLEDAI. Furthermore, sFKN levels in CSF from patients with NPSLE were positively correlated with IgG. sFKN in serum and CSF decreased markedly after treatment. These results suggested levels sFKN in the serum and CSF were consistent with disease activity and can be used as an index to evaluate disease activity of SLE and guide its treatment.

Upon the first detect of FKN in the nerve tissues of mice, it was called neurotactin (23). Reports regarding the distribution of FKN and CX3CR1 in the central nervous system of human, mouse and rat differ (23-25). The majority of studies have shown that the expression of FKN is elevated in neurons under physiological conditions, while CX3CR1 is predominantly expressed in microglia, which are a type of mononuclear macrophage in the central nervous system (26,27). Under physiological conditions, FKN can inhibit the activation of microglia and the release of inflammatory factors to protect brain tissue (28,29). Under pathological conditions, active microglia can release excitatory neurotoxins such as glutamic acid to stimulate in part the release of FKN from neurons (30), which was called sFKN. sFKN induced microglia to migrate to inflammatory site, and expression of FasL in microglia and

Fas in brain cell increased simultaneously (31). The Fas/FasL pathway can result in brain cell apoptosis. By contrast, FKN is able to block the Fas/FasL pathway and inhibit apoptosis of microglia by interacting with CX3CR1 on microglia (32). Thus, microglia can continuously release toxic substances such as free radicals, nitric oxide, TNF- $\alpha$  and IL-1 $\beta$  which can aggravate the inflammatory reaction. In the present study, mRNA expression levels of CX3CR1 in brain tissue of lupus mice was significantly higher than in normal mice, suggesting that FKN and CX3CR1 were involved in pathogenesis of NPSLE through local action in the brain tissue. Harrison *et al* observed that the proliferation of microglia in the nucleus of facial nerve was associated with significantly increasing mRNA expression of CX3CR1 following myotomy of rat facial nerve axons (27). Furthermore, Jiang *et al* detected increased mRNA expression of CX3CR1 in the lumbar spinal cord from experimental autoimmune encephalomyelitis rats (33). These previous reports have shown that FKN-CX3CR1 were involved in injury of nervous system. As sFKN can function as a chemotactic factor, unlike membrane-anchored FKN, we hypothesized that FKN activity was regulated by modification after translation rather than transcription in the inflammation of nervous system. The mRNA expression levels of FKN in the present study were lower than normal mice, which was consistent with the results of Harrison *et al* (27), but there was no statistical significance in our study. This result was consistent with the finding that decreased mRNA expression of FKN can weaken protective effect on nerve tissue under pathological conditions.

In the present study, the results showed that FKN and CX3CR1 are crucially involved in the pathogenesis of vasculitis, including SLE and BD. Moreover, the present study indicates that FKN and CX3CR1 may participate in the pathogenesis of NPSLE, particularly DNPSLE. Future studies are required to evaluate the expression of FKN and CX3CR1 in various types of systemic vasculitis, such as Takayasu arteritis, polyarteritis nodosa, BD and anti-neutrophil cytoplasmic antibody associated vasculitis. It has been shown that levels of sFKN in serum from patients with GPA and MPA were increased (15,16). However, Ceyla *et al* found that sFKN levels in serum were not changed in active and inactive BD and neuro-BD (34). Therefore, further large-scale studies are required to assess the role of FKN/CX3CR1 in patients with systemic vasculitis. Numerous experiments have shown that antagonism of FKN or CX3CR1 can inhibit inflammation (5,35-38). It is thus possible that FKN-CX3CR1 may be a novel target in the treatment of SLE and systemic vasculitis.

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