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Intracerebroventricular administration of lupus serum induces microglia activation and leukocyte adhesion in the cerebromicrovasculature of mice

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ABSTRACT

Background: Central nervous system (CNS) involvement is commonly seen in the patients with system lupus erythematosus (SLE). Mechanisms underlying CNS damage in SLE remain largely unknown. Accumulating evidence suggest that activation of microglia in CNS plays an important role in the inflammatory responses in neurological diseases. The aim of this study is to examine the involvement of microglia in the CNS inflammatory responses induced by circulating serum of SLE patients.

Methods: We performed intracerebroventricular (ICV) injection of serums collected from SLE patients or healthy controls to mice, and examined phenotypic changes of microglia, the levels of cytokines, chemokine and adhesion molecules in the brain. Intravital microscopy was used to observe leukocyte rolling and adhesion in the cerebromicrovasculature. We further examined whether minocycline can block inflammatory responses induced by SLE serum. In vitro experiments were conducted to examine whether IgGs from the sera of SLE patients or healthy control can activate the primary cultured microglia.

Results: We found that ICV injection of SLE serum increases morphological activation of microglia in the cortex and hippocampus. Inflammatory mediators including pro-inflammatory cytokines (IL-1, IL-6 and TNF-α), chemokine (CCL2 and CCL5) and adhesion molecules (P-selectin and ICAM-1) were significantly elevated in the brains of SLE-serum-treated mice. Using intravital microscopy, we demonstrated that SLE serum promotes leukocyte rolling and adhesion. Furthermore, suppression of microglia activation by systemically using minocycline could decrease the levels of inflammatory molecular, and prevent leukocyte rolling and adhesion. The in vitro experiments revealed that IgG from SLE sera could be engulfed by microglia and stimulated the microglia to secret pro-inflammatory cytokines.

Conclusion: Our data suggest that the activation of microglia, which promotes leukocyte adhesion to the brain microvasculature, is an important pathological mechanism of CNS involvement in SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by multisystemic involvement presenting with numerous clinical manifestations (Kaul et al., 2016; Rahman and Isenberg, 2008). Neuropsychiatric systemic lupus erythematosus (NPSLE) involves neurologic manifestations seen in the central, peripheral, and autonomic nervous systems as well as psychiatric disorders in patients with SLE in which other causes have been excluded (Monahan et al., 2017; Pikman et al., 2017; Unterman et al., 2011; Zirkzee et al., 2014). Extensive research into the pathogenesis of NPSLE has been conducted, with the identification of many potential contributing factors, including autoantibodies (such as anti-NMDA receptors, anti-GABA receptors, anti-ribosomal P and anti-U1 RNP et. al) (Gonzalez and Massardo, 2018; Karaaslan et al., 2017; Pisetsky, 2016; Sato et al., 2010; Tsuchiya et al., 2014; Viana et al., 2017), cytokines and chemokines in the serum of SLE patients (Ho et al., 2016; Hu et al., 2015; Jeltsch-David and Muller, 2014; Okamoto et al., 2010). Because of the protection of the blood-brain barrier (BBB), the CNS is traditionally regarded as an immunologically privileged site (Muldoon et al., 2013). The immune microenviroment in CNS is quite different from peripheral tissues. The immunological mechanisms of how the serum of SLE induces inflammatory responses in CNS a valuable research issue.

Microglia is a kind of innate immune cell in the CNS, which functions as first-line responders under inflammatory conditions. Once activated, microglia release cytokines pro-inflammatory molecules and reactive oxygen radicals, which can modulate astrocytes and neurons in a paracrine manner, and also affect microglia in an autocrine fashion by

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either positive or negative feedback loops (Kabba et al., 2018; Li and Barres, 2018). Activation of microglia has been implicated in the inflammatory responses in neurological and psychological diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis and stress (Clayton et al., 2017; Gentile et al., 2015; Slusarczyk et al., 2015; Subramaniam and Federoff, 2017; Wachholz et al., 2016). Animal studies on MRL-lpr mice, a model of NPSLE, also reported an increase of microglia activation with age (Ballok et al., 2006). Targeting microglia activation has been proposed as an effective strategy to treat neuroinfammatory diseases (Subramaniam and Federoff, 2017). Recently, we observed that stimulation of microglia cultures with serum collected from SLE patients could induce the activation of microglia, indicated by obvious morphological changes, increased expression of MHC II and CD86 protein, and overproduction of proinflammatory cytokines (Wang et al., 2017).

Based on our findings and previous knowledge, we hypothesize that inflammatory factors in serum of SLE patients may trigger an activation of microglia that results in imbalance of immunity hemostasis in CNS and the recruitment of immune cells. To test this hypothesis, we performed intracerebroventricular (ICV) injection of serums collected from SLE patients or healthy controls into mice. We examined phenotypic changes of microglia in the cortex and hippocampus, and measured the levels of cytokines, chemokine and adhesion molecules. To test whether ICV injection of SLE serum can promote the recruitment of blood circulating leukocytes, we used intravital microscopy to observe leukocyte rolling and adhesion in the cerebromicrovasculature. We further examined whether minocycline, an established microglial inhibitor (Bhandare et al., 2017; Bi et al., 2016; Stokes et al., 2017), can block inflammatory responses induced by SLE serum. We will provide evidence suggesting that an SLE-serum-induced activation of microglia is involved in the neuroinflammation in SLE and minocycline may be able to attenuate or dampen this potentially detrimental response.

2. Materials and methods

2.1. Animals

Experiments were performed using 8–12 weeks-old C57BL/6 male mice (Vital River Laboratory, Beijing, China). All animals were maintained in standard animal cages under conventional laboratory conditions (12 h/12 h light/dark cycle, 22 °C) with ad libitum access to food and water. The animals were maintained and treated in compliance with the policies and procedures detailed in the "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health. The animal experimental protocols of the "Guide" and the treatment procedures were reviewed and approved by the Animal Care and Use Committee of China Medical University (No. KT2018060). All surgeries were performed under anesthesia, and all efforts were made to minimize animal suffering.

2.2. Serum collection and processing

A total of 21 hospital patients, who fulfilled the American College of Rheumatology (ACR) classification criteria for SLE (Hochberg, 1997) from September 2017 to December 2017, and 17 healthy age- and gender-matched subjects were enrolled in this study. Patients with comorbidities of cancers or infections were excluded. Disease activity was scored using SLE Disease Activity Index (SLEDAI) scoring system (Bombardier et al., 1992). Only patients with moderate to severe disease activity were included in the study (defined as SLEDAI > 4). Clinical characteristics of the SLE patients are shown in Table 1. Sera from the SLE patients and healthy controls were prepared by centrifugation at 3000 rpm for 10 min in a clinical centrifuge and then stored at −80 °C prior to use. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University. The samples from the SLE patients or controls were pooled respectively for the application. For in vitro experiments, IgG was isolated from the pooled patient or normal serum using a protein A-resin (genScript, Piscataway, NY) and concentrated using Amicon Ultra Centrifugal Filter Units (Millipore, Billerica, MA).

Table 1
Clinical characteristics of the SLE patients (n = 21).

Age(years)	28.5 ± 10.3
Sex (male/female)	1/20
Disease duration (month)	4.8 ± 3.3
SLEDAI	12.6 ± 5.4
NPSLE (no.)	4
Fatigue (no.)	21
ESR (mm/h)	39.8 ± 18.4
CRP (mg/L)	9.1 ± 6.2
Anti-dsDNA + (no.)	12
Anti-P + (no.)	8
Anti-nuclear + (no.)	21
C3 (g/L)	0.5 ± 0.2
C4 (g/L)	0.07 ± 0.05

The healthy control group consisted of 1 men and 16 women with a mean ± SD, age of 29.9 ± 9.3 years. SLEDAI: systemic lupus erythematosus (SLE) disease activity index; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; C3 and C4: complements 3 and 4.

2.3. Surgical procedures and injections

Canulae (#62001; RWD Life Science, Shenzhen, China) were stereotaxically implanted into lateral ventricle (coordinates with respect to bregma: −0.7 mm anteroposterior [AP], +1.5 mm mediolateral [ML], −2 mm dorsoventral [DV], according to the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin, 2001) in anesthetized mice (1.5% isoflurane). Animals were allowed to recover for 1 week. Animals were then injected with either pooled sera from SLE patients or healthy controls or vehicle [artificial cerebrospinal fluid (ACSF), an ionic composition in mmol/L: NaCL 132, KCL 2.95, $CaCL_2$ 1.71, $MgCL_2$ 0.64, $NaHCO_3$ 24.6, dextrose 3.71 and urea 6.7, pH 7.4]. Injection (1 µl at the rate of 0.4 µl/min) was conducted by cannulae PE tubing (# 62302, RWD Life Science) connected to a 10 µl Harvard Apparatus syringe pump system (Pump 11 Elite). The tubing was left in place for another 5 min at the end of injection, and the cannulae capped to prevent reflux of the injected solution.

2.4. Minocycline administration

Minocycline hydrochloride (#M9511; Sigma, St. Louis,MO, USA) was dissolved fresh in 0.9% NaCl and administered intragastrically (i.g.) at a dosage of 90 mg/kg body weight at 2 h prior to the ICV injection. The dose was selected on the basis of previous studies showing the beneficial effects of this dosage in animal models of cerebral brain ischemia, multiple sclerosis, and Parkinson's disease (Popovic et al., 2002; Wu et al., 2002; Yrjanheikki et al., 1998).

2.5. Intravital microscopy in mouse brain

Intravital microscopy of the mouse cerebromicrovasculature was performed as previously described (dos Santos et al., 2005). For this, a cranial window (2 mm in diameter) was opened using a high-speed drill (#78001, RWD Life Science) after implanted the canulae in lateral ventricle. A piece of cover glass (0.13 mm thick, Citotest, Haimen, China) was lowered on top of the dura, and dental cement was applied to the perimeter of the craniotomy to fix it. Animals were allowed to recover for 1 week. On the day of experiment, the mice were anesthetized by 1.5% isoflurane and the tail vein was cannulated for administration of fluorescent dyes. Blood flow in the cerebromicrovasculature was observed through the implanted glass window. Throughout the

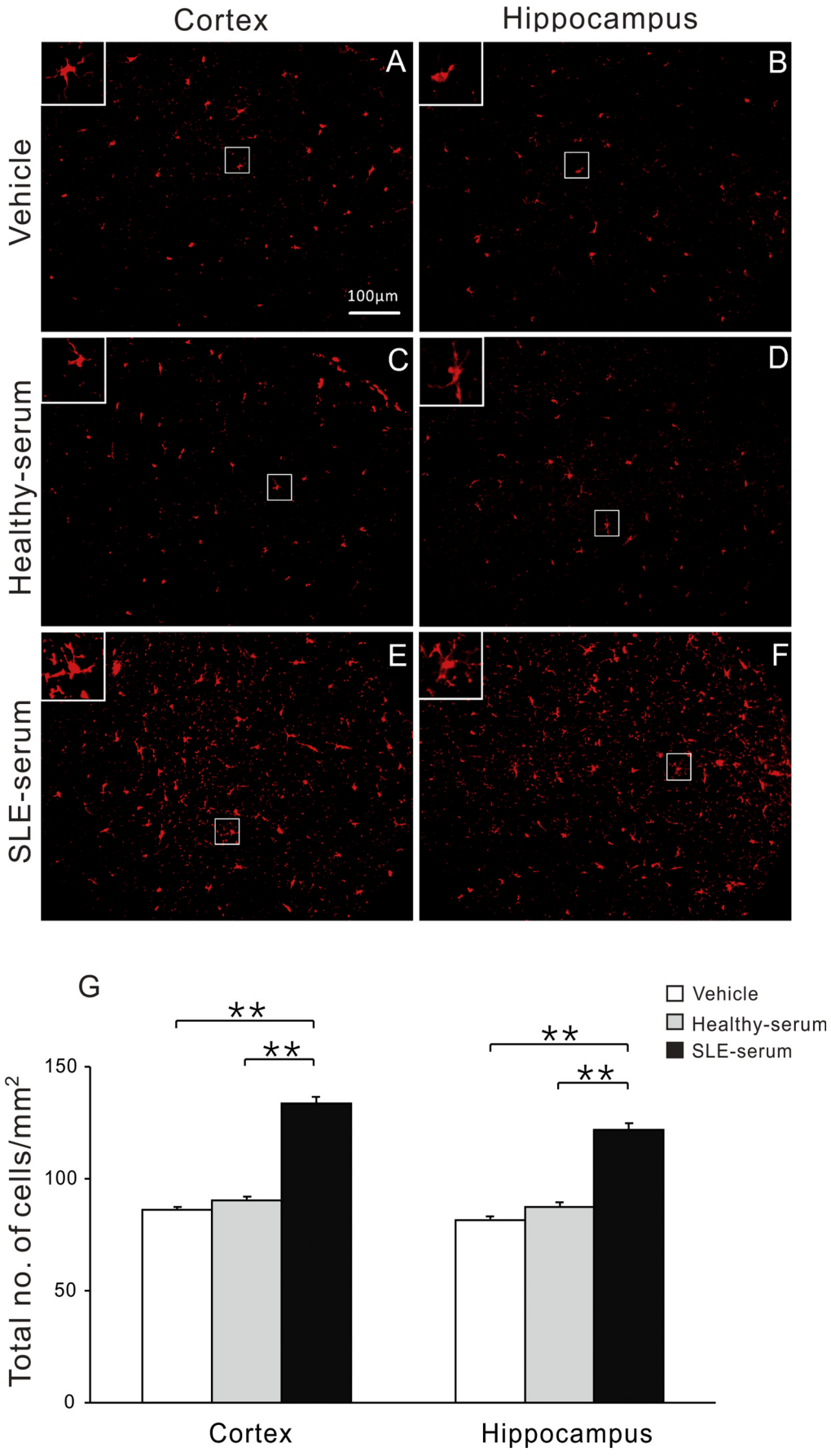

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Fig. 1. Microglial activation following SLE-serum injection. (A) – (F), microscopic images of the Iba-1 immunolabelled cortical and hippocampal sections depicting morphological transformation of microglia cells i.e. from resting state in vehicle control and healthy-serum injection to activated phenotype in SLE-serum injection. (G) count of Iba-1 immunolabelled cell in the cortical and hippocampal fields. SLE-serum caused a significant elevation in Iba-1 positive cell population. Values represent mean ± SEM. $^{**}p < .01$ (one way ANOVA followed by Tukey's post hoc test).

experiment, the mouse was maintained at 37.8 °C with a heating pad and the exposed brain was continuously superfused with ACSF at 37.8 °C. Leukocytes were fluorescently labeled by i.v. administration of rhodamine 6G (0.5 mg/kg body weight) and observed using a microscope (NE900, X20 objective lens, Novel, Zhejiang, China) outfitted with a fluorescent light source (epi-illumination at 510–560 nm, using a 590-nm emission filter). A silicon-intensified camera (MS55, Mshot, Guangzhou, China) mounted on the microscope projected the image onto a monitor. Rolling leukocytes were defined as white cells moving at a velocity less than that of erythrocytes cells. Leukocytes were considered adherent to the venular endothelium if they remained stationary for 30 s or longer.

2.6. Brain histology

After intravital microscopy observation, mice were sacrificed and extensively perfused with cold PBS. Brains were dissected into right and left hemispheres. The left hemisphere of the brain was fixed in 4% paraformaldehyde for 24 h at 4 °C. The right brain hemisphere was snap-frozen for RNA extraction. At least 24 h before sectioning, the fixed brains were transferred to a 20% sucrose (*w/v*) solution for cryoprotection. Coronal sections of 10 μm were cut on a freezing microtome, mounted on gelatin-coated slides.

2.7. Immunofluorescence

For immunofluorescence staining, sections were incubated with one of the following antibodies: Iba-1 (1:100,ab178847,Abcam), P-selectin (1:80,SC-8419, Santa Cruz) or ICAM-1 (1:250, ab119871, Abcam) followed by the secondary antibodies Alexa Fluor 594 (1:800, SA00006-8, Proteintech) or Fluorescein (FITC)(1:80,SA00003-1,Proteintech). Sections were analyzed under a fluorescence microscope (BX53, Olympus). To eliminate the potential correlation between distance to injection site and inflammatory activity, we selected five sections from each mouse brain between 1.5 and 2 mm posterior to ICV injection location in 100 μm interval. Data of the five sections were averaged for a single data point to represent that individual mouse. All quantifications were done in a blinded manner.

2.8. qRT-PCR

Total RNA was isolated from the brain of the mice using a TRizol RNA Extraction kit (MM032, ProbeGene, Jiangshu, China). cDNA synthesis was performed with the first strand synthesis kit (MM061, ProbeGene, Jiangshu, China). qRT-PCR was performed in triplicate for IL-1β, IL-6, TNF-α, CCL2, CCL5, ICAM-1, P-selectin. The primer sequences were as follows: 1) GAPDH: (forward) GTCAAGGCCGAGAATGGGA, (reverse) GCAGAAGGGGCGGAGATG 2) IL-1β: (forward) GTGTCTTTCCCGTGGACCTTC, (reverse) TCATCTCGGAGCCTGTAGTGC; 3) IL-6: (forward) CCATCCAGTTGCCTTCTTGG, (reverse) GGTCTGTTGGGAGTGGTATCCTC; 4) TNF-α: (forward) TTCACTGGAGCCTCGAATGTC, (reverse) TCAGGGAAGAATCTGGAAAGGT; 5) CCL2: (forward) GTGCTGACCCCAAGAAGGAA, (reverse) GGTGGTTGTGGAAAAGGTAGTG; 6) CCL5: (forward) GCTTCCCTGTCATTGCTTGC, (reverse) GATTTCTTGGGTTTGCTGTGC; 7) ICAM-1: (forward) TCGTGATGGCAGCCTCTTAT, (reverse) CTTGTCCCTTGAGTTTTATGGC; 8) P-selectin: (forward) GATGCCCTGCCCTCACGA, (reverse) GCGAGTTGGCTCCCCTGA.

PCR was performed using LineGene9600 PCR System (Bioer, Hangzhou, China). Amplification started with an initial denaturation step at 95 °C for 10 min, followed by gene-specific cycles of denaturation at 95 °C for 15 s, specific annealing for each gene for 30 s, and extension at 60 °C for 30 s. GAPDH was used as reference gene. The relative expression of target gene was calculated as ΔCq = Cq gene – Cq reference, and the fold change of target gene expression was calculated by the $2^{-\Delta\Delta Cq}$ method.

2.9. Primary microglia cultures and detection the binding of IgGs by immunofluorescence

Primary microglia were derived from postnatal C57BL/6 mice (P1-P3), using the "low concentration trypsin digestion" method. Briefly, cerebral cortices were devoid of meninges and blood vessels, dissected in Hank's salt (HBSS) and trypsinized with 0.125% trypsin-EDTA and 1μg/ml DNase I for 30 min at 37 °C. The mixed glial culture was incubated in DMEM/F-12 containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin (PS) and seeded at 7.5×10^6–10×10^6 cells per 75 cm^2 flask. After 8–13 days, microglia were separated from the mixed primary culture by trypsinized with 0.125% trypsin-EDTA for 20 min at 37 °C. The media were collected and centrifuged at 1000 rpm for 6 min to obtain a pellet of microglia. Thereafter, cells were plated at 1×10^5 cells/well in 24-well plates precoated with poly-L-lysine (30 μg/ml). Purity of microglia (above 95%) was confirmed by immunofluorescence staining with anti-iba1 antibody.

Microglia were seeded in 24-well culture plates until 90% confluence. The cells were incubated with 100 μg/ml of IgG from SLE patients or from healthy control in a 5% CO_2 incubator at 37 °C overnight. Thereafter, cells were fixed with 4% (*v*/v) paraformaldehyde in 1 × PBS at room temperature for 30 min and washed with 1 × PBS for 3 times, then permeabilized with 0.1% (v/v) Triton X-100 in 1 × PBS at room temperature for 20 min and washed with 1 × PBS for 3 times. They were then blocked with 1 × PBS containing 5% BSA at room temperature for 1 h. Primary antibody against ionized calcium binding adaptor molecule (Iba-1) (1:100 dilution in 1 × PBS, #10904–1-AP, Proteintech, Chicago, USA) was incubated at room temperature for 2 h. Subsequently, cells were incubated with goat anti-rabbit IgG (H + L)-FITC (1: 200 dilution in 1%BSA, #SA00003–2, Proteintech, Chicago, USA) and goat anti-rabbit IgG(H + L)-594 (1: 300 dilution in 1%BSA, #SA00006–4, Proteintech, Chicago, USA) at room temperature for 1 h in dark. After washing with 1 × PBS for 3 times, cell nuclei were stained with DAPI at room temperature for additional 5 min in dark. Washing steps following DAPI staining were three times with 1 × PBS containing 0.05% Tween-20. Cover slips were finally mounted with fluorescence decay resistance sealing reagent.

2.10. Cytokine assay to evaluate the microglia response to IgG stimulation

Microglia cells were seeded into 96-well plates at a density of 2×10^4 cells/well. After 24 h of incubation, 100 μg/ml of IgG from SLE patients or from healthy control were added to the plates. Twenty four hours later, supernatants of microglia cultured under different conditions were collected and centrifuged at 10,000 ×*g* for 10 min at 4 °C to remove cell debris. The supernatants were stored in aliquots at −80 °C prior to use. IL-1β, IL-6, and TNF-α were measured in the culture supernatants by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA). All treatments were completed eight times, and the data were expressed as the mean pg/ml ± SEM.

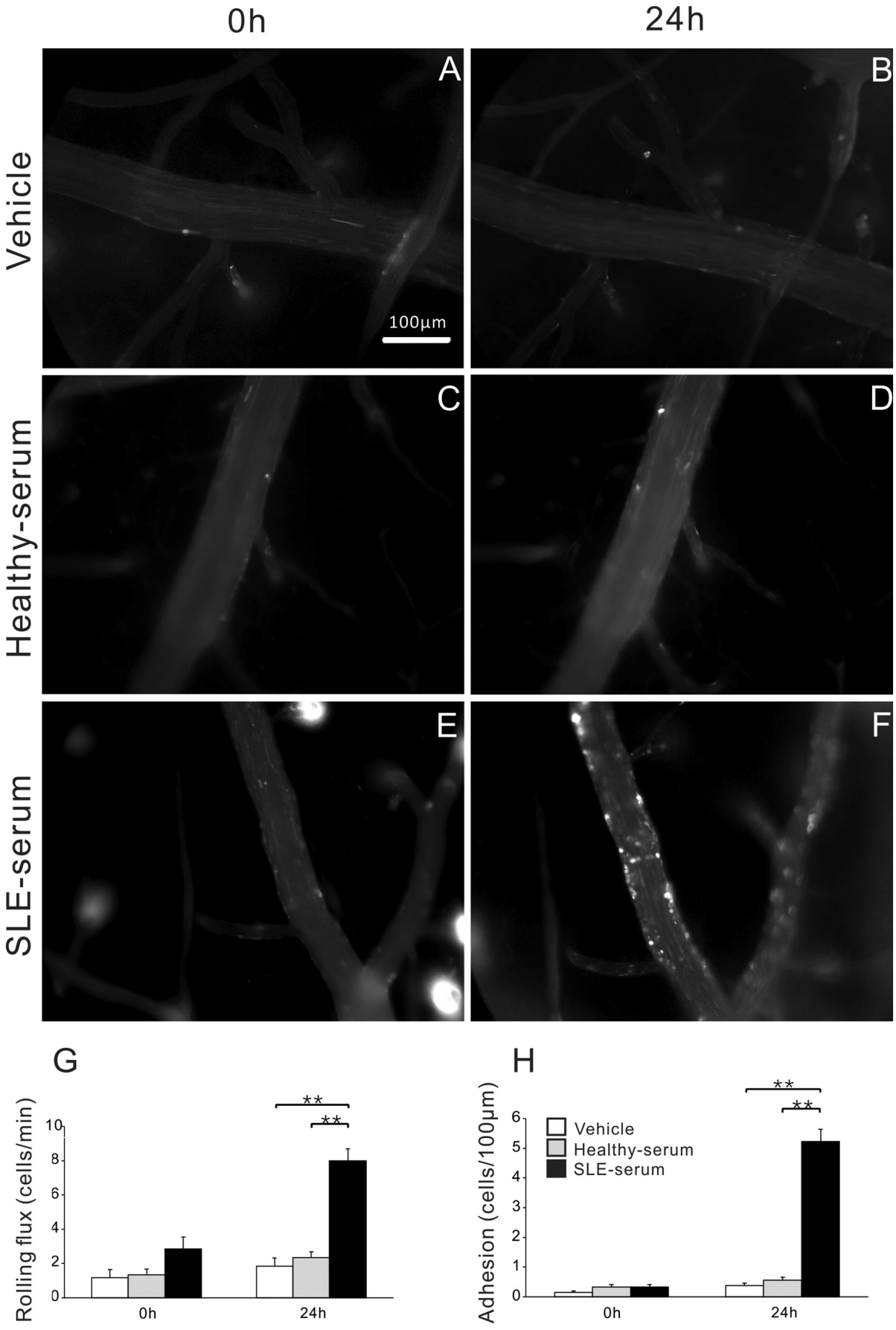

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Fig. 2. Leukocyte rolling and adhesion in the cerebromicrovessels induced by ICV injection of SLE-serum. (A) – (F), Inntravival microscopic images of leukocytes labeled with Rhodamine 6G before and 24 h after vehicle, SLE- and healthy-serum injection. (G) Quantitative analysis of leukocyte rolling and (H) adhesion. Values represent mean ± SEM. ** p < .01 (one way ANOVA followed by Tukey's post hoc test).

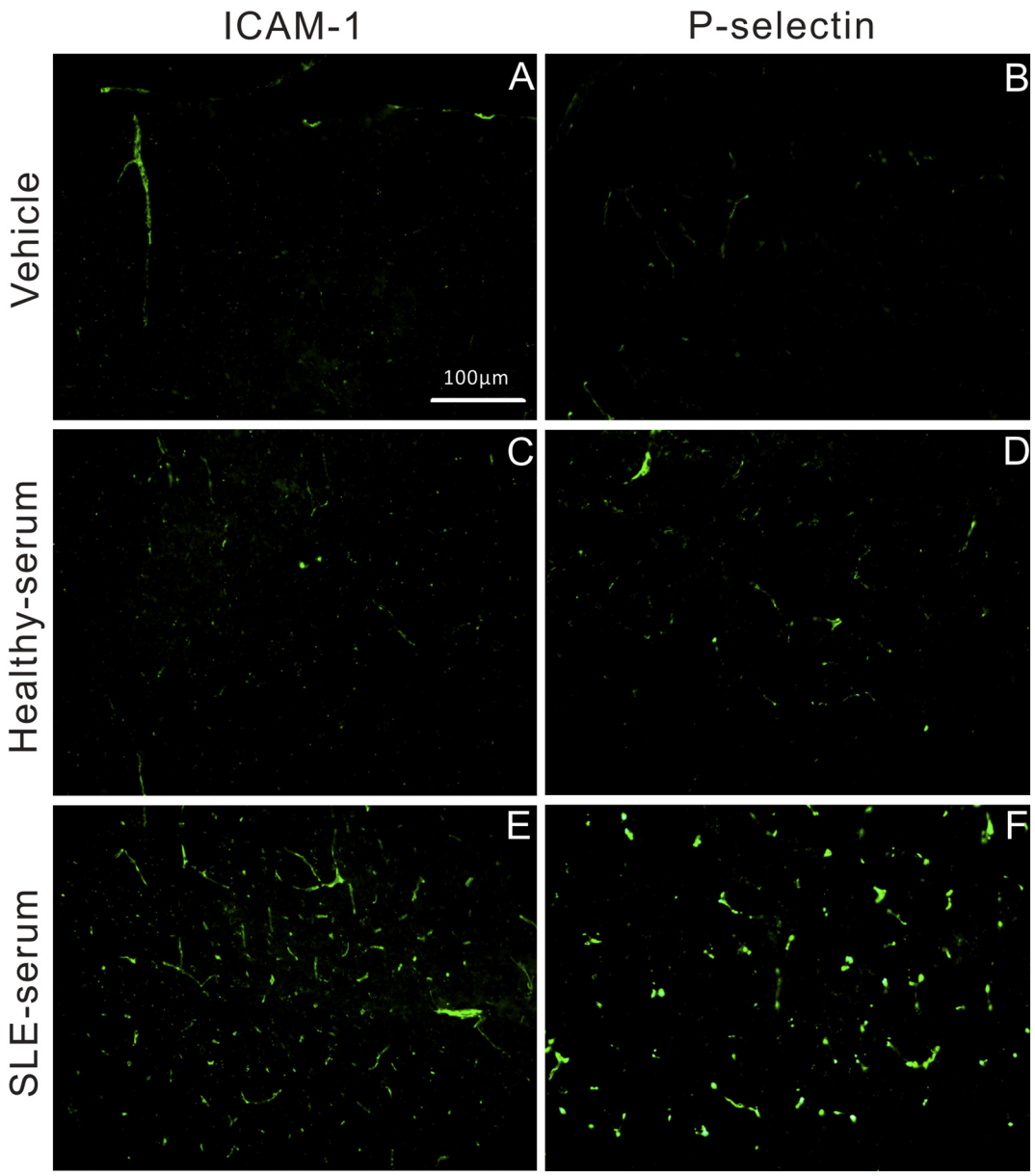

Fig. 3. Localization of the cellular adhesion molecule ICAM-1 and P-selectin in the brain cortex. In the mice treated with vehicle, ICAM-1 (A) and P-seletctin (B) only occasionally expressed in the region of small blood vessels. Healthy-serum treated mice show a low level of ICAM-1 (C) and P-seletctin (D) expression. A clear increase of ICAM-1 (E) and P-selectin (F) expression is observed in SLE-serum treated mice.

2.11. Statistical analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analysis. Each graph depicts the mean ± standard error of the mean. Differences in the immunohistochemical data and qRT-PCR results were detected by Student's *t*-test or one-way analysis of variance (ANOVA). Each ANOVA reporting significant effects was followed by Tukey's post hoc test of multiple comparison. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. ICV injection of SLE-serum induces microglia activation

Twenty-four mice, randomly divided into 3 groups, were administered by a single ICV injection of SLE sera or health sera or vehicle, respectively. The mice had no any obvious signs of sickness within 24 h after ICV injection. We gauged and compared the distribution of cells immunoreactive for the microglial (Iba-1) markers in the cortex and hippocampus of mice at 24 h after received ICV injection. The activated state of microglia was judged by phenotypic changes and increase in cell number. Iba-1 protein expressed on the cell surface is prevalently used to study the activated states (Imai et al., 1996; Patro et al., 2010)

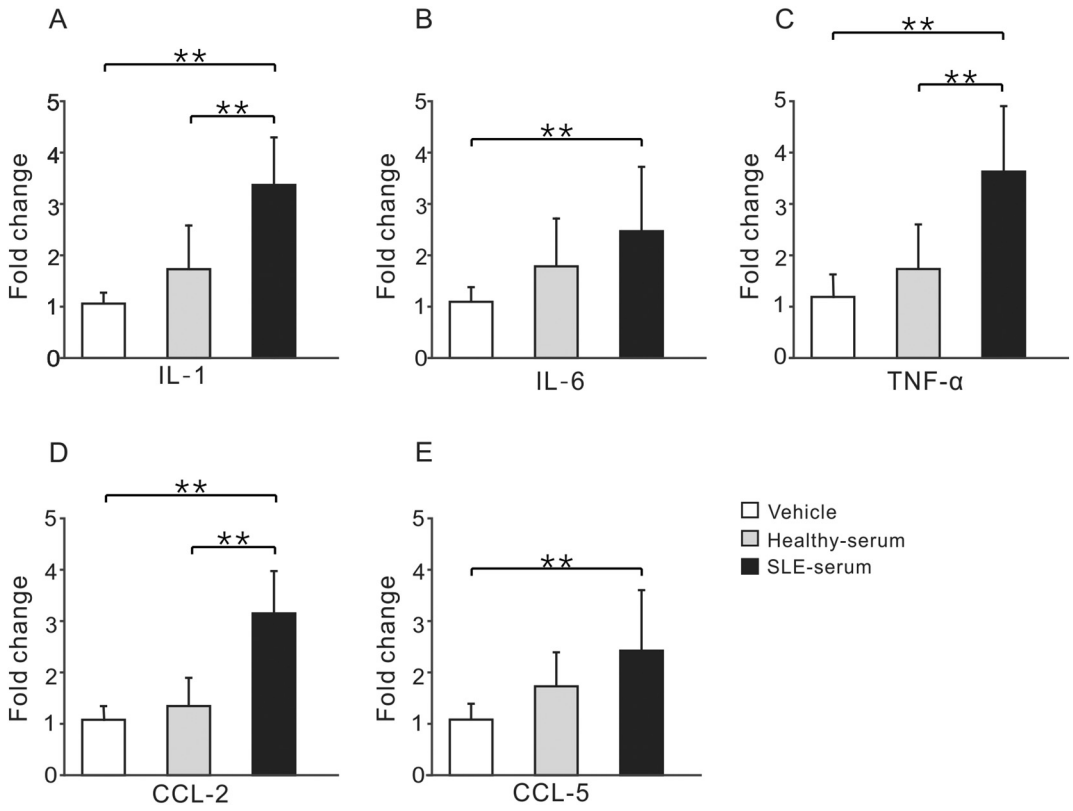

Fig. 4. mRNA quantification of cytokines (IL-1β, IL-6 and TNF-α) and chemokines (CCL2 and CCL5) in the brain tissues treated by vehicle, healthy- and SLE-serum. Experiments were performed on 10 mice in each group. Values represent mean ± SEM. **p < .01 (one way ANOVA followed by Tukey's post hoc test).

and morphological changes in the microglia (De Geyter et al., 2012; Ito et al., 2001).

Following injection of SLE sera, Iba-1+ cells in the cortex and hippocampus present activated morphology as compared to the vehicle controls. In vehicle control, resting microglia cells exhibited ramified wispy appearance with round lightly stained cell body (Fig. 1A and B). Microglia in the mice treated by healthy sera did not show an obvious morphological change as compared to the vehicle controls (Fig. 1C and D). In contrast, injection of SLE sera induced a phenotypic transformation from ramified to activated states bearing retracted, thick processes and large irregular cell body (Fig. 1E and F).

Cell quantification of Iba-1 immunopositive cells revealed that Iba-1+ cell count in the cortex and CA1 hippocampus of SLE-serum treated mice was significantly higher than those in the healthy-serum and vehicle treated mice (Fig. 1G, ANOVA followed by Tukey's post hoc test), and there was no significant difference between the healthy-serum and vehicle treated mice.

3.2. SLE-serum promotes leukocyte recruitment into the brain

We used intravital microscopy to directly visualize the leukocyte - endothelial cell interaction in the cerebromicrovessels before and 24 h after vehicle, SLE- or healthy-serum administration (Fig. 2A-F). Negligible numbers of both rolling leukocytes (Fig. 2G) and adherent leukocytes (Fig. 2H) were noted under control condition. After SLE-serum injection, the number of rolling and adherent cells increased 8-fold (Fig. 2G and H). Such a change was not observed in the mice treated by healthy-serum.

3.3. SLE-serum induced endothelial activation in the brain microvasculature

A key event in leukocyte recruitment is the ability of the endothelium to express cellular adhesion molecular to allow for leukocyte rolling. To test whether endothelium in the CNS was activated after ICV injection of SLE-serum, ICAM-1 and P-selectin expression in the brain was measured using immunofluorescence staining. As shown in Fig. 3, although very little ICAM-1 and P-selectin expression was noted in the brain microvasculature of vehicle- (Fig. 3A and B) and healthy-serum treated mice (Fig. 3C and D), an increase in ICAM-1 and P-selectin expression was observed in the SLE-serum treated mice (Fig. 3E and F). Clearly, SLE-serum induced endothelium activation in the brain.

3.4. SLE-serum injection up-regulated the production of cytokines and chemokines

We also examined the levels of cytokines and chemokines in the brain tissues using qRT-PCR analysis and found that the mRNA expression of IL-1β, IL-6, TNF-α, CCL2 and CCL5 was up-regulated by SLE-serum injection (Fig. 4).

3.5. Minoncyline inhibited SLE-serum induced microglia activation and leukocytes recruitment

Minocycline has been reported by many groups to be an effective inhibitor for microglial activation, which can rapidly cross the blood-brain barrier (Bhandare et al., 2017; Bi et al., 2016; Stokes et al., 2017). We used a subset of mice to examine whether minocycline can inhibit the microglia activation in response to ICV administration of SLE-serum and suppress the subsequent inflammatory responses. The first group of mice (n = 5) received an i.g. administration of minocycline (90 mg/kg, dissolved in saline) at 2 h before ICV injection of SLE-serum (minocycline + SLE-serum group). The second group (n = 5) received the same dose of saline and SLE-serum as the control group (Saline + SLE-serum group). The third group (n = 5) received only minocycline without the SLE-serum injection (minocycline group). We found that pretreatment of minocycline effectively inhibited the morphological changes and

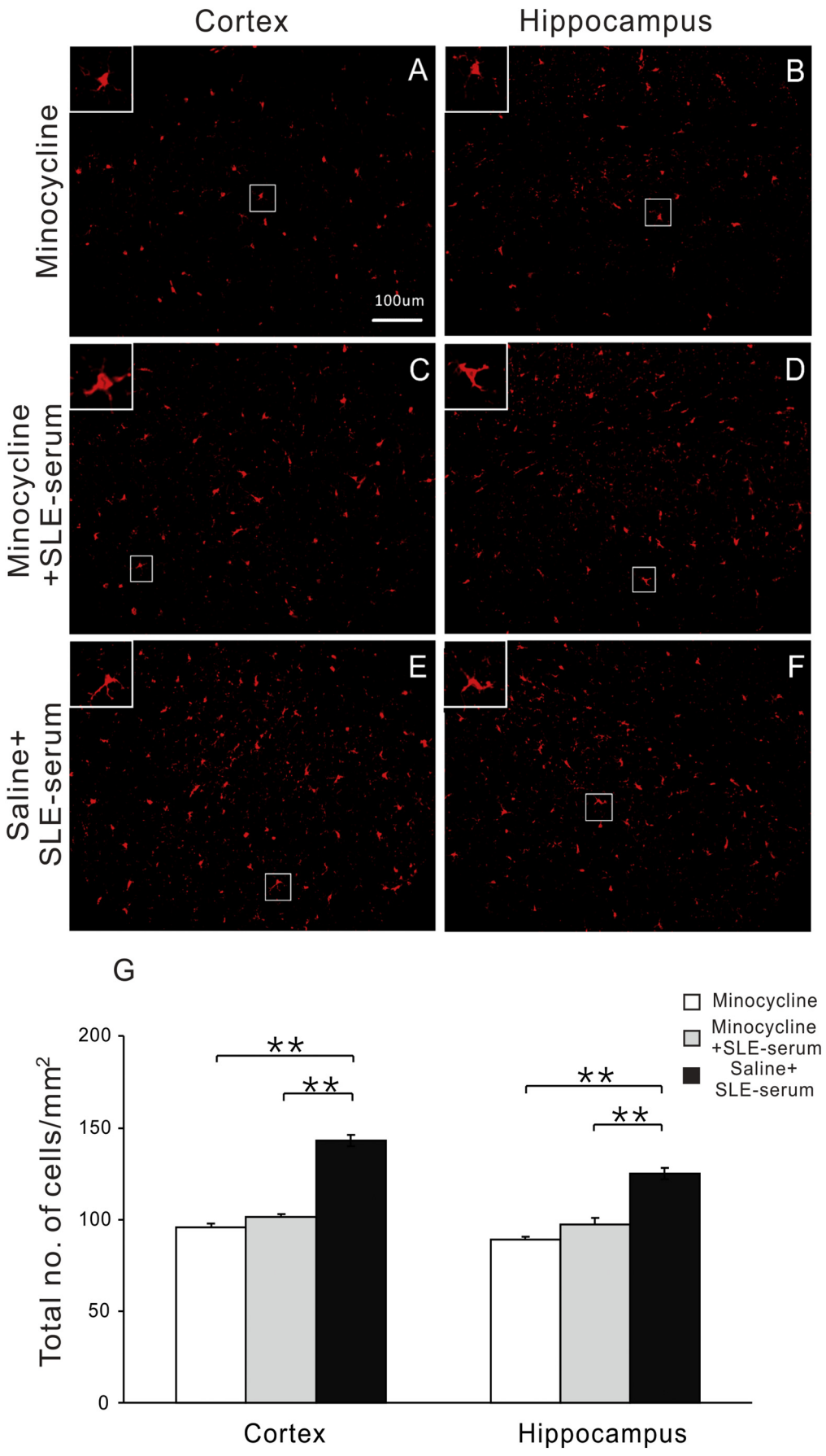

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Fig. 5. Minocycline inhibited the SLE-serum induced microglia activation. (A) – (B), application of minocycline alone had no obvious effect on the phenotype of microglia. (C) – (D), pretreatment with minocycline at 2 h prior to the SLE-serum injection effectively inhibited the morphological changes of microglia, comparing to treatment with SLE-serum alone (E) – (F). (G), count of Iba-1 immunolabelled cell in the cortical and hippocampal fields. Values represent mean ± SEM. **p < .01 (one way ANOVA followed by Tukey's post hoc test).

proliferation of microglia induced by ICV injection of SLE-serum (Fig. 5). Minocycline treatment dramatically reduced leukocyte rolling and adhesion (Fig. 6), and the expression of ICAM-1 and P-selectin (Fig. 7). The increases in the mRNA expression of IL-1β, IL-6, TNF-α, CCL2 and CCL5 caused by SLE-serum stimulation were also largely inhibited by pretreatment of minocycline (Fig. 8).

3.6. In vitro experiments indicate that IgG in SLE-serum induce microglia activation

Above results raise a question of what factors in the SLE-serum cause the effect of microglia activation. One reasonable deduction is that immune complexes may play a role in the microglia activation. To test this, we isolated IgG from the pooled patient sera, and examined its effects on primary cultured microglia. IgG from pooled normal sera was used as control. Histochemical staining revealed that both patient and normal IgGs could bind with the microglia, and were engulfed by the microglia (Fig. 9 A and B). However, the expression levels of pro-inflammatory cytokines were significantly higher in the microglia stimulated by SLE IgG than in those stimulated by normal IgG (Fig. 9C – E). This result suggests that the microglia were activated after they engulfed the IgG from the SLE sera, while the microglia engulfed normal IgG were not activated.

4. Discussion

In the present study, we aimed to examine whether the circulating serum of SLE patients can evoke microglia activation and inflammatory reaction in the CNS. We found that ICV administration of SLE-serum induced a morphological change of microglia from ramified to activated states, and up-regulated the expression of IL-1β, IL-6, TNF-α, CCL2 and CCL5 in the brain. Intravital microscopy also revealed an increase of leukocyte rolling and adhesion in the brain microvasculature, which may associate with an elevated expression of ICAM-1 and P-selectin on the blood vessels. We further found that pretreatment with minocycline to inhibit microglia activation effectively suppressed the up-regulation of cytokine and chemokine expression, and inhibited the leukocyte rolling and adhesion. The in vitro experiments revealed that the activation of microglia may be attributable to IgG in the SLE sera. Our data suggests that SLE-serum can induce a series of inflammatory responses in CNS, and microglia play a key role in this process.

As the major immune cell type resident in the brain, microglia continuously scans the extracellular brain environment and becomes activated in response to various inflammatory stimuli, which may further lead to abundant cytokine production or phagocytosis of cellular debris (Kabba et al., 2018; Li and Barres, 2018). Microglial activation takes place in various neurological disorders including CNS and peripheral infections, neurodegenerative diseases, traumatic brain injury, ischemic and hemorrhagic stroke (Doens and Fernandez, 2014; Dudvarski Stankovic et al., 2016; Hernandez-Ontiveros et al., 2013; Puntener et al., 2012). Microglia activation was also found in the brain of animal model of NPSLE (Ballok et al., 2006). Previously, we have observed that SLE-serum could activate the cultured microglia in vitro (Wang et al., 2017). The microglia activated by SLE-serum showed thick processes and large irregular soma, and released more proinflammatory cytokines IL-1β, IL-6, and TNF-α. However, due to the biological complexity of neurological homeostasis, it is not enough just to study glia in isolation from their extracellular environment. We need to understand how changes in the brain microenvironment trigger glial activation and inflammatory activity in brain tissues. Here, we directly injected the SLE-serum into the lateral ventricle of healthy mice to examine the effect of SLE-serum on microglia. Consistent with the results of in vitro observations, we found that SLE-serum could activate the microglia under in vivo condition. It should be mentioned that the activation of microglia was accessed by immunofluorescence stain of Iba-1, which is upregulated during microglia reactive responses (Ito et al., 1998). Iba-1 has been established as a microglial marker, and frequently used for immunohistochemical identification of microglia. However, Iba-1 is widely expressed by myeloid cell types (Prinz and Priller, 2014; Prinz et al., 2011), meaning that the immunohistochemical antibodies against Iba-1 cannot discriminate microglia from CNS-infiltrating macrophages. Microglia-specific markers have not been well defined until recently. Although expressions of some molecules (such as TMEM119) have been found to restrict to microglia in the CNS, these markers are not fully specific. For example, TMEM119 expression is absent in immature microglia (Bennett et al., 2016). Because in the intravital image experiments we just observed that leukocytes rolled and adhered to the vessel wall (Fig. 2), no obvious leukocyte infiltration was found, the number of infiltrating macrophages in our Iba-1 positive cells may not be large.

It has been well established that activated microglia can produce proinflammatory cytokines (Wang and Dore, 2007) and chemokines (Matsushita et al., 2000). Indeed, we observed a significant increase in the expression of IL-1β, IL-6, TNF-α, CCL2 and CCL5 in the SLE-serum-treated brain. These up-regulated cytokines and chemokines may contribute to leukocyte recruitments into CNS. The proinflammatory cytokines, such as TNF-α, have been proved to be able to stimulate endothelium to cause an increase in adhesion molecule expression (Li et al., 2015; Rajan et al., 2008). To migrate into sites of inflammation, leukocytes must first tether and roll along the vessel before they firmly adhere and emigrate out of the vasculature (Kerfoot and Kubes, 2002; McCafferty et al., 2000). Firm adhesion is mediated by the expression of adhesion molecules and their ligands on the surface of leukocytes. ICAM-1 and P-selectin are typical adhesion molecules that participate in leukocytes rolling and adhesion (Broide et al., 1998; Su et al., 2012). Indeed, our results showed that the expressions of ICAM-1 and P-selectin were elevated by SLE-serum treatment. Moreover, chemokines such as CCL2 and CCL5 produced by microglia have chemotactic activity on leukocyte. Firm adhesion between leukocytes and endothelium cells is triggered by the action of the chemoattractant molecules (dos Santos et al., 2005).

Using in vivo imaging, we demonstrated that SLE-serum can promote leukocyte rolling and adhesion in the brain microvasculature. In vivo imaging is a non-invasive method, which has been well used to observe the dynamic of cell function in CNS and has provided invaluable insights for the cellular mechanisms of disease onset and progression (Adams et al., 2007; Davalos et al., 2012; Ryu et al., 2015). An advantage of in vivo imaging over immunohistochemical staining is to continuously observe the pathological changes in the same subject. Taken our immunohistochemical and in vivo imaging results together, we propose that microglia activation plays a key role in SLE-serum induced inflammatory response, that is, SLE-serum induces an activation of microglia, and the activated microglia release amount of proinflammatory cytokines and chemokines, which then trigger leukocyte recruitment into the CNS.

The essential pathophysiological role of microglia in SLE-serum-induced inflammation suggests that inhibition of microglia activation may have therapeutic potential. Minocycline, a tetracycline derivative, has been reported to block LPS-induced microglial release of cytokines,

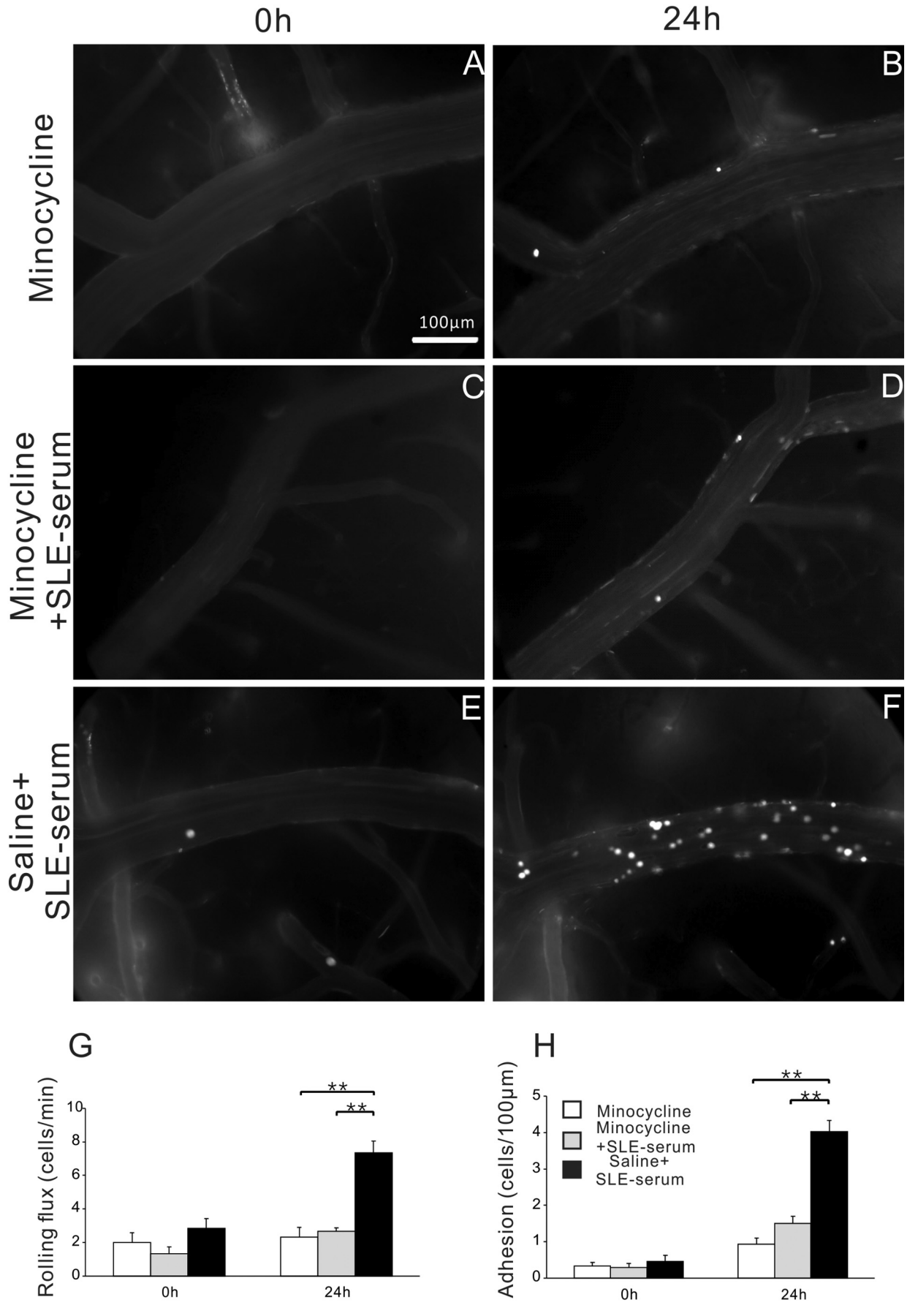

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Fig. 6. Minocycline reduced the SLE-serum induced leukocyte rolling and adhesion. (A) – (B), treatment with minocycline alone had no obvious effect on leukocyte rolling and adhesion in the cerebromicrovessels. Pretreatment with minocycline dramatically reduced leukocyte rolling (C) and adhesion (D), comparing to treatment with SLE-serum alone (E) – (F). (G) Quantitative analysis of leukocyte rolling and (H) adhesion. Experiments were performed on 5 mice in each group. Values represent mean ± SEM. **p < .01 (one way ANOVA followed by Tukey's post hoc test).

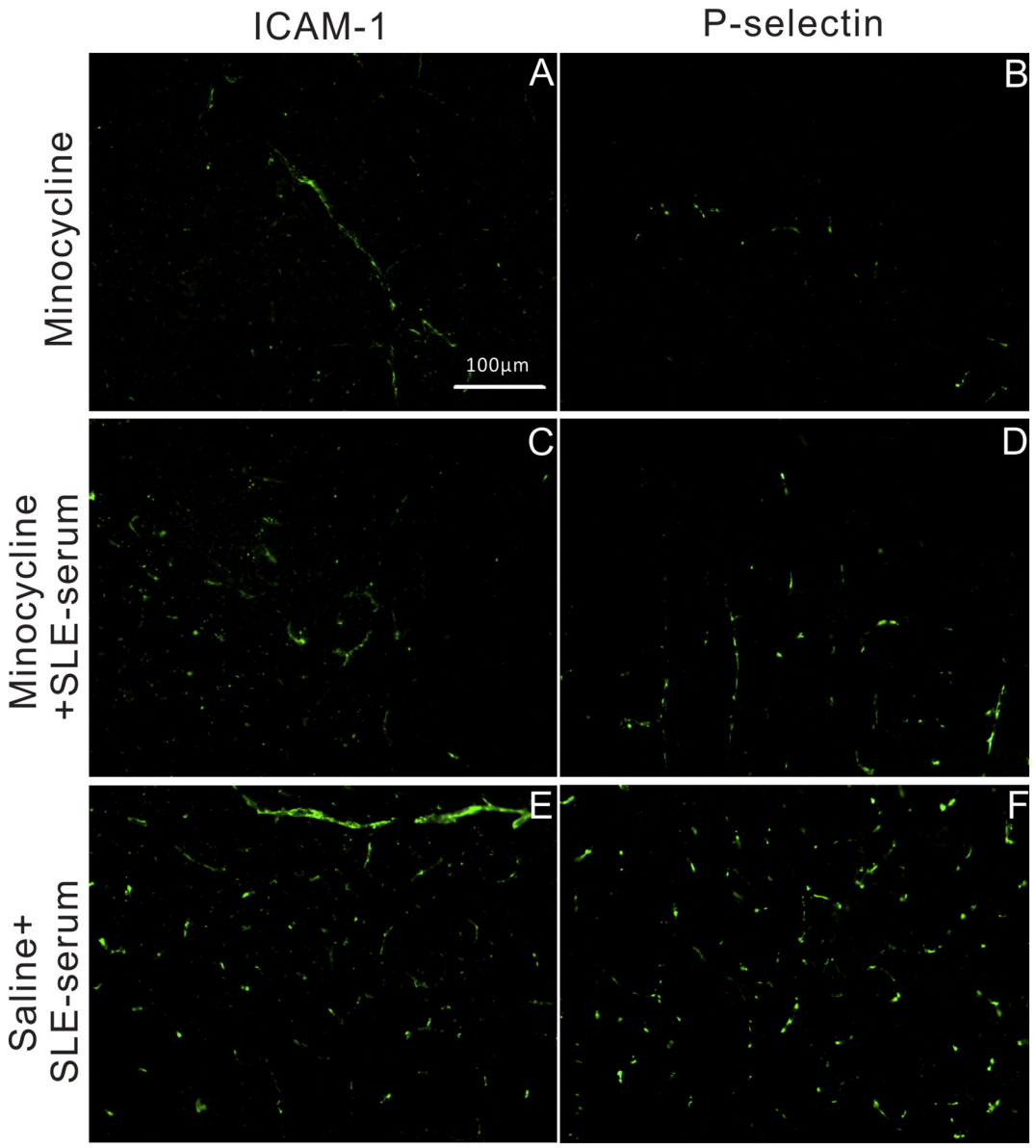

Fig. 7. Minocycline reduced the SLE-serum induced ICAM-1 and P-selectin expression. Treatment with minocycline alone had no effect on the expression of ICAM-1 (A) and P-seletctin (B). Pretreatment with minocycline dramatically reduced the expression of ICAM-1 (C) and P-seletctin (D), comparing to treatment with SLE-serum alone (E) – (F).

inducible NO synthase production, and oxidant production in vitro (Wang et al., 2005). Because minocycline has surprising ability to penetrate the blood-brain barrier (Zhu et al., 2002), studies on animal models have found some remarkable protective effects of minocycline against neurodegenerative diseases, including Huntington's disease (Kumar et al., 2013), Parkinson's disease (Thomas and Le, 2004), and Alzheimer's disease (Budni et al., 2016), and brain injuries caused by trauma (Haber et al., 2018) or ischemia/reperfusion (Jin et al., 2015). Our data provide further information on minocycline's inhibitory effects on microglia. Also, leukocyte rolling and adhesion induced by SLE-serum administration were attenuated by minocycline. However, it should be noted that minocycline is also an inducer of lupus in humans (Sarzi-Puttini et al., 2005). We used minocycline in this animal study just to prove that inhibition of microglia activation is a potential way to modulate or dampen the neuroinflammation associated with SLE. Finding other safe tetracyclines or antibiotics that are protective for microglia and cross the BBB is a clinically significant issue.

There is still an important question to be addressed in the future, that is, what are the molecular links between increased BBB leakage, microglia activation and inflammation? Previous studies have revealed a number of factors associated with neurological disorders in the circulating blood of SLE patients, including autoantibodies (anti-phospholipid, anti-ribosomal P and anti-DNA antibodies) and cytokines (IL-2, IL-6, IL-8, IL-10, IFN-α, and TNF-α) (Abe et al., 2017; DeGiorgio et al., 2001; Efthimiou and Blanco, 2009; Ho et al., 2016; Segovia-Miranda et al., 2015). Some of these immunological mediators may

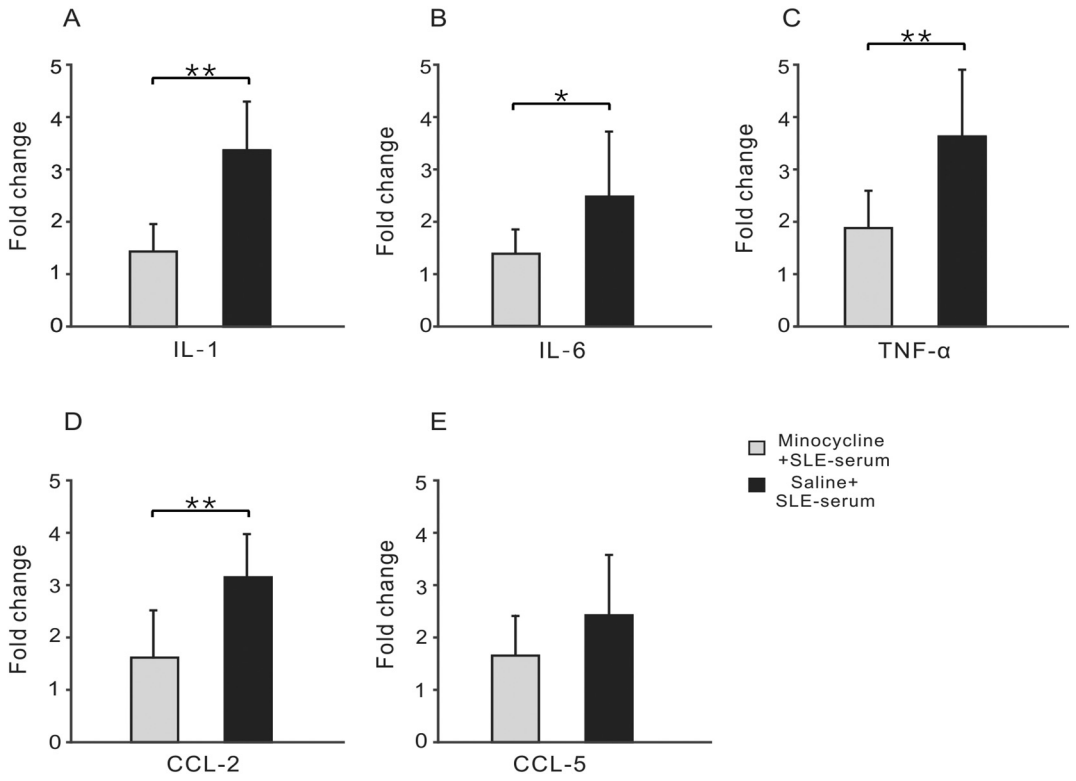

Fig. 8. Comparison of the mRNA expression of cytokines (IL-1β, IL-6 and TNF-α) and chemokines (CCL2 and CCL5) in the mice received minocycline pretreatment or not. Pretreatment of minocycline significantly inhibited the SLE-serum induced the increases in IL-1β, IL-6, TNF-α, CCL2 and CCL5. Values represent mean ± SEM. * $p < .05$, **p < .01, Student's *t*-test.

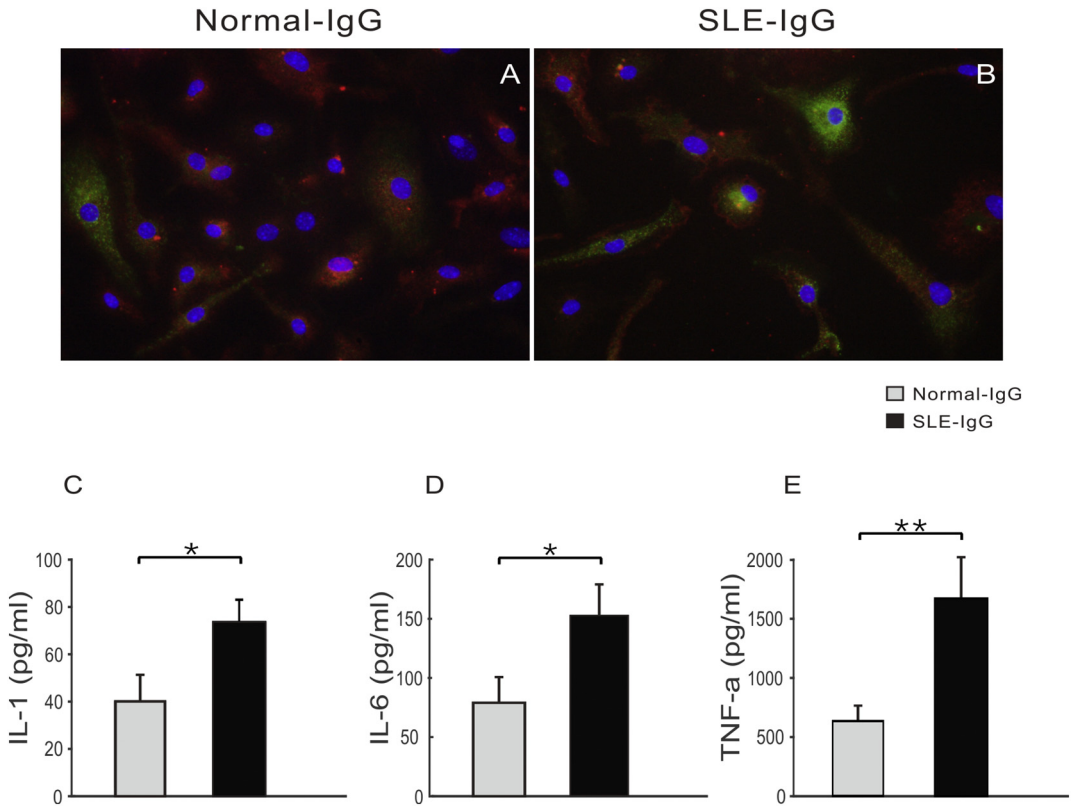

Fig. 9. The effects of IgG from the sera of healthy control and SLE patient on the primary cultured microglia. (A) and (B), microscopic images of the microglia incubated with the IgG from healthy control or SLE patient sera. Red: Iba-1, Green: IgG, Blue: DAPI. (C) – (E), concentrations of IL-1, IL-6 and TNF-αin the microglial culture supernatants. Values represent mean ± SEM. * p < .05, **p < .01, Student's *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contribute to the initial disruption of BBB by acting on vascular endothelial cells (Abbott et al., 2003; Jacob et al., 2010; Stock et al., 2013; Wen et al., 2015; Wen et al., 2013). Leakage of BBB can cause more serum proteins enter CNS, which then promote microglial activation, inflammatory mediator production and leukocyte recruitment in a positive feedback loop. The limitation of this study is lack of proof of specific factors for microglia activation in SLE-serum. The results of in vitro experiments showed that IgG of SLE patients could be engulfed by the microglia, and stimulated the microglia to produce pro-inflammatory cytokines. Limited by the volume of samples, we could not purify the specific antibodies and examine their effects on microglia one by one. In the future, identifying the key factors for microglia activation in SLE-serum not only deepens our understanding of the mechanisms of NPSLE but also reveals a novel target for therapeutic intervention.

5. Conclusion

Our study suggests that microglia in the brain can detect the pathological factors in the SLE-serum and release cytokines that activate the endothelium to recruit leukocytes. Although it is an important immune response to internal environment changes, if the inflammatory response is too robust, neuron injury can also occur and nervous functions may be damaged. Our data also demonstrate that minocycline can significantly reduce the inflammatory response, raising the possibility that this drug could be used to relieve NPSLE if and when necessary.

Conflicts of interest

All authors declare no conflict of interest.

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