Abnormal in vitro CXCR2 modulation and defective cationic ion transporter expression on polymorphonuclear neutrophils responsible for hyporesponsiveness to IL-8 stimulation in patients with active systemic lupus erythematosus

S.-C. Hsieh¹, T.-H. Wu², C.-Y. Tsai³, K.-J. Li⁴, M.-C. Lu⁵, C.-H. Wu¹ and C.-L. Yu¹

Objective. To elucidate the molecular basis of hyporesponsiveness of polymorphonuclear neutrophils (PMN) to interleukin-8 (IL-8) stimulation in patients with active SLE.

Methods. PMN obtained from active SLE and well-matched healthy individuals were studied. The expression of two IL-8 receptors, CXCR1 and CXCR2, in PMN were detected by flow cytometry and reverse transcriptase-polymerase chain reaction. The binding affinity of PMN with IL-8 was calculated by Scatchard plotting. Soluble CXCR2 level in IL-8-stimulated PMN culture supernatant was measured by sandwich enzyme-linked immunosorbent assay. The resting and IL-8-stimulated membrane potential (MP) changes, and membrane expression of cationic ion transporters including Na⁺-K⁺-ATPase, renal epithelial Na⁺ channel (ENaC) and renal outer medullary epithelial K⁺ channel 1 (ROMK1) on PMN were detected by flow cytometry.

Results. Compared with normal PMN, decreased CXCR2 gene expression, but normal IL-8-binding affinity of SLE-PMN, was found. For exploring the molecular basis of the defect, the modulation of CXCR2 in SLE-PMN was intensively investigated. We found that increased cytosolic CXCR2 expression in SLE-PMN was due to defective surface translocation, increased spontaneous internalization and/or increased spontaneous synthesis. The IL-8-induced CXCR2 down-regulation in SLE-PMN was also impaired due to decreased proteolytic cleavage of IL-8–IL-8 receptor complexes from the cell surface whereas IL-8-induced internalization of the complexes was normal. In addition, we originally found that increased resting but decreased IL-8-stimulated MP in SLE-PMN was relevant to defective expression of Na⁺-K⁺-ATPase, ENaC and ROMK1 on the cell surface.

Conclusion. The abnormal CXCR2 modulation and impaired cationic ion transporter expression cause SLE-PMN hyporesponsiveness to IL-8 stimulation in vitro.

Key words: Systemic lupus erythematosus, Polymorphonuclear neutrophil, Hyporesponsiveness, CXCR2 modulation, Membrane potential, Cationic ion transporters.

Introduction
Polymorphonuclear neutrophils (PMN) are the first-line defence cells against bacterial infections. PMN derived from patients with SLE exhibit a number of defects including phagocytosis [1], bactericidal activity [2], and spontaneous and bacterial lipopolysaccharide (LPS)-stimulated IL-8 production [3]. In our previous report, we found SLE-PMN were hyporesponsive to IL-8 stimulation [3]. However, the molecular basis of IL-8 hyporesponsiveness in SLE-PMN in vitro has not been explored in the literature. It is conceivable that IL-8, an important autocrinic chemokine, belongs to the glutamic acid-leucine-arginine ELR(+)–CXC chemokine family that specifically chemotaxacts and activates neutrophilic granulocytes [4, 5]. The cellular responses of PMN to IL-8 stimulation are initiated by the binding with specific cell surface receptors coupling to pertussis toxin-sensitive heterotrimeric G proteins [6]. Two IL-8 receptor subtypes have been identified on human PMN, receptor A or CXCR1 and receptor B or CXCR2 [7, 8]. Both receptors share 77% amino acid homology but differ in their binding properties [8–10]. CXCR1 is specific for IL-8 with high binding affinity, whereas CXCR2 binds with IL-8 and other ELR(+)–CXC chemokine family members including growth-related oncogene (GRO)-α, GRO-β, GRO-γ, epithelial cell-derived neutrophil attractant-78 (ENA-78) and neutrophil activating protein-2 (NAP-2) with equally high affinity [11, 12]. Both CXCR1 and CXCR2 undergo phosphorylation and desensitization upon agonist stimulation [13–15]. Normal PMN not only produces a large amount of IL-8 but promptly responds to IL-8 stimulation via these two receptors [16]. In contrast, SLE-PMN not only produced less IL-8 but hyporesponded to IL-8 stimulation in our previous study [3]. We hypothesize that IL-8 hyporesponsiveness of SLE-PMN may derive from defective expression and/or impaired functions of IL-8 receptors on the cell surface. On the other hand, cationic ion transporters on the cell surface control the transport of Na⁺, K⁺ and Ca²⁺ across the plasma membrane and membrane potential (MP) change that initiate the first step of cell activation [17, 18]. Whether defective expression and functions of these cationic ion transporters exist in SLE-PMN has not been reported in the literature. In the present study, we found that defective expression and abnormal modulation of CXCR2, and deranged MP changes from defective cationic ion transporter expression are responsible for IL-8 hyporesponsiveness of SLE-PMN.

Materials and methods
Reagents and antibodies
Mouse monoclonal antibodies against human CXCR1 and CXCR2 (R & D Systems, Inc. Minneapolis, MN, USA), human Na⁺-K⁺-ATPase (Santa Cruz Biotechnology, Inc. Santa Cruz,
CA, USA), human epithelial sodium channel (ENaC) (Alpha Diagnostic Intl. Inc., San Antonio, TX, USA) and rat renal outer medullary potassium channel 1 (ROMK1) (Alomone Labs Ltd, Jerusalem, Israel) were purchased from the respective manufacturers. Fluorescein isothiocyanate (FITC)-labelled goat anti-mouse immunoglobulins G (IgGs) and non-specific mouse IgG (for IgG isotype control) were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Human recombinant IL-8, GRO-α and TNF-α were obtained from R & D Systems.

Patients and controls

Patients fulfilling the 1982 revised ACR criteria for the classification of SLE were recruited. The age- and sex-matched healthy volunteers were the controls. These SLE patients were disease-active (SLEDAI score > 8 plus anti-dsDNA antibody titre > 50 IU/ml, normal < 12 IU/ml) judged by SLEDAI scoring system [19]. All of the SLE patients received prednisolone (25.3 ± 10.4 mg/day), hydroxychloroquine (200–400 mg/day) and/or azathioprine (50–100 mg/day). This study was approved by the Institutional Review Board and Medical Ethics Committee of National Taiwan University Hospital, Taipei, Taiwan. Informed consent was obtained from each participant.

Preparation of PMN from peripheral blood

Heparinized venous blood obtained from normal and SLE patients was mixed with one-quarter volume of 2% dextran solution (molecular weight 467 000 Da) and was incubated at 37°C for 20 min. Leucocyte-enriched supernatant was collected and diluted with the same volume of Hanks’ balanced salt solution. After Ficoll-Hypaque (specific gravity 1.077) density gradient centrifugation at 1000 r.p.m (150 g) for 30 min, PMN were obtained from the bottom. For further purification, the PMN suspension was positively selected by using monoclonal anti-Gr1 serum (FBS) in RPMI-1640 (10% FBS-RPMI).

Preparation of LPS-stimulated PMN supernatants

PMN (1 × 10⁶/ml) were stimulated with LPS (100 ng/ml, E. coli serotype 026:B6, Sigma-Aldrich Immunochromistry, St Louis, MO, USA) at 37°C in 5% CO₂–95% air for 24 h. The cell-free culture supernatants were collected and stored at −20°C until used.

Measurement of IL-8 concentration in PMN culture supernatants by enzyme-linked immunosorbent assay (ELISA)

Commercially available IL-8 ELISA kit (Quantikine, R & D Systems) was used for measuring the IL-8 levels in PMN culture supernatants. The detailed procedures are described in the manufacturer’s instruction booklet. The minimal detectable concentration of IL-8 was 18.1 pg/ml.

Measurement of phagocytic activity of PMN

Fluoresbrite carboxylate microspheres (0.75 μm, Polysciences Inc., Washington, PA, USA) were opsonized with fresh normal human serum by incubation at 37°C for 60 min. One hundred microlitres of freshly prepared PMN (2 × 10⁶/ml) were incubated with 10 μl of opsonized fluorescence beads (2 × 10⁹ beads/ml) at 37°C for 60 min in the presence of medium or different PMN activators; LPS (100 ng/ml), GRO-α (50 ng/ml), TNF-α (25 ng/ml) or IL-8 (25 ng/ml). IL-8 at concentrations of 10 and 25 ng/ml are optimum and not cytotoxic to lymphocytes or PMN demonstrated in our previous study [3]. The endotoxin level in PMN activators except LPS is < 1.0 EU/μg cytokine as determined by the limulus amoebocyte coagulation (LAL) method, according to the manufacturer’s sheet. The non-phagocytosed beads were removed from PMN by three washes at 2000 r.p.m (300 g) for 10 min. The percentage (%) and mean fluorescence intensity denoted by mean channel number (MFI#) of bead-engulfing PMN were measured by FACSsort flow cytometry (Becton Dickinson) with 488 nm excitation using Lysis II Software analysis [20].

Detection of CXCR1 and CXCR2 expression in PMN

Flow cytometric determination for protein expression. Freshly prepared PMN (1 × 10⁶/ml) were incubated with 5 μl of monoclonal antibody (50 μg/ml) against CXCR1 or CXCR2 in an ice-bath for 30 min. The isotype-matched mouse non-specific IgG was the control. After three washes with PBS, pH 7.2, the cells were stained with FITC-labelled goat anti-mouse IgGs in ice-bath for another 30 min. After several washes, both percentage and MFI# of the positive cells were measured by FACSsort flow cytometer (Becton Dickinson) with 488 nm excitation.

Reverse transcriptase-polymerase chain reaction (RT-PCR) for mRNA expression

Total cellular RNA extraction and cDNA synthesis. The total cellular RNA was extracted from normal and SLE PMN (1 × 10⁶ cells/ml) using Ultrascript RNA isolation kit (Biotek Lab, Houston, TX, USA). cDNA was synthesized by priming 1 μg/ml of total RNA at 42°C for 1 h in a final volume of 20 μl containing 1 μg of oligo-dT primer (Pharmacia Fine Chemicals, Piscataway, NJ, USA), 200 nmol of each dNTP (Pharmacia) and MMLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD, USA) at 200 U/μg RNA.

Amplification of cDNA by PCR. An aliquot of cDNA was amplified by PCR using oligonucleotide pair primers specific for human CCR1 (as positive control), CXCR1 and CXCR2. Human G3PDH mRNA expression was the universal internal control. Two different sets of pair primers for CCR1 and CXCR2 [denoted as set (a) and set (b) subsequently] were purchased for comparison. A HYBAID OmnisGene DNA Thermal Cycler (Tedderington, UK) was run 26 cycles for denaturation at 94°C for 1 min and annealing/extension at 65°C for 2 min in the case of G3PDH. Thirty-five cycles of denaturation at 95°C for 1 min and annealing/extension at 60°C for 2 min were carried out for CCR1, CXCR1 and CXCR2.

Human CCR1:

Set (a): 5'-ACA CCA CAG AGG ACT ARG AC-3' (sense)
5'-AGG AAG GGG AGC CAT TTA AC-3' (antisense)

Set (b): 5'-CGA CTA CAA GGT GAA GGA TGA CT-3' (sense)
5'-GGC TTT CGT GAG GAA AGT GAA C-3' (antisense)

Human CXCR1:

5'-AGG GCC CAC ACC AAC CTG CTG-3' (sense)
5'-AGT GCC TGC CTC AAT GTC TCC A-3' (antisense)

Human CXCR2:

Set (a): 5'-CAA TGT TAG CCC AGC CTG CTA TGA G-3' (sense)
5'-GAA TCT CGG TGC CAT CCA GAG-3' (anti-sense)
Set (b): 5'-CAG TTA CAG CTC TAC CCT GCC-3' (sense)
5'-CCA GGA AGA AGC GAV CCC-3' (anti-sense)

Human G3PDH:
5'-ACC ACA CAT GCC ATC AC-3' (sense)
5'-TCC ACC GG TTG CTG TA-3' (anti-sense)

The cDNA fragments amplified by these sets of primers were 335 bp (set a) or 300 bp (set b) of CCR1, 364 bp of CXCR1, 979 bp (set a) or 451 bp (set b) of CXCR2 and 452 bp of G3PDH. The PCR products were electrophoresed in 1.8% agarose gel with phiX174 digested by HaeIII enzyme as DNA calibration marker.

Detection of spontaneous cytosolic expression and IL-8-induced internalization of CXCR2 in PMN

The expression of CXCR2 in the cytosol of human PMN was determined according to the report of Avrameas et al. [21]. Briefly, the freshly prepared PMN (1 x 10^6/ml) were incubated with medium (spontaneous cytosolic receptor expression) or IL-8 (25 ng/ml, IL-8-induced receptor internalization) at 37°C for 60 min in 5% CO₂–95% air. The cells were then washed three times with PBS, pH 7.2, followed by fixation with 2% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The fixed and permeabilized cells were trypsinized with 0.125% trypsin at 37°C for 10 min to remove the surface proteins for preventing the interference from surface-expressed CXCR2. The cells were then washed with anti-CXCR2 and 200 x diluted FITC-labelled goat anti-mouse IgGs in an ice-bath for 30 min. The percentage and MFI of cytosolic CXCR2 in PMN were determined by FACSort flow cytometry at 488 nm excitation. The spontaneous cytosolic CXCR2 expression in PMN is denoted by percentage expression in PMN incubation with medium. The IL-8-induced receptor internalization (%) was calculated by subtracting the value (%) of incubation with medium from incubation with IL-8.

Determination of binding affinity of IL-8 receptors on PMN with recombinant IL-8

We followed the method reported by Katancik et al. [22] with the modification that radiolabeled IL-8 was replaced by ELISA method. Briefly, 50 µl of freshly prepared normal or SLE-PMN (2 x 10^6/ml) were incubated with human recombinant IL-8 (Quantikine) at different concentrations of 100, 500, 1000, 20,000, 40,000, 50,000 or 100,000 pg/ml at 4°C in continuous rotation for 2 h. The mixture was then spun at 3000 r.p.m. (450 g) for 10 min. After three washes to get rid of non-cell-bound IL-8, PMNs were lysed with 200 µl of lysis buffer containing 50 mM borate, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 25 mM phenylmethyl-sulphonylfluoride, pH 8.0. All of the supernatants were collected after centrifugation for IL-8 quantification. The IL-8 content in the supernatants (free IL-8) and cell lysate (bound) was measured by commercially available IL-8 ELISA kit. A Scatchard plot was drawn and analysed by the ‘Pristmon’ statistical program provided by GraphPad Software (http://www.curvefit.com/scatchard-plots.htm).

Measurement of CXCR2 down-regulation on PMN surface after binding with IL-8

Freshly prepared PMN (1 x 10^6/ml) were incubated with medium or human IL-8 (25 ng/ml) at 37°C for 60 min. After three washes, the cells were then stained with monoclonal anti-human CXCR2 antibody as mentioned in the previous paragraph. Down-regulation of CXCR2 on PMN surface was evaluated by difference of percentage expression between medium and IL-8 incubation.

Determination of soluble CXCR2 (sCXCR2) levels in PMN culture supernatants after incubation with IL-8 by ELISA

To determine the role of proteolytic cleavage of IL-8–IL-8 receptor complex in IL-8-induced CXCR2 down-regulation on PMN, the amount of soluble CXCR2 release from PMN surface membrane to the culture supernatants was measured by sandwich ELISA after incubation of PMN with IL-8 for 1 h. Briefly, a mouse monoclonal antibody (E2, Santa Cruz Biotechnology, Inc.) against amino acid sequence of 1–19 at the N-terminus of human IL-8RB was pre-coated on microwells at pH 9.0 (0.5 µg/well) followed by blocking with 0.5% BSA solution at 4°C for 2 h for blocking the non-specific binding. The PMN culture supernatant was added to the wells and incubated at 4°C overnight. The microwells were washed several times with PBS, pH 7.2. Rabbit polyclonal antibodies against amino acid sequence of 18–39 at N-terminus of human CXCR2 (Abcam, Inc., Cambridge, MA, USA) was then added to the microwells and further incubated at 37°C for 4 h. After sufficient washes, horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgGs were added. The amount of sCXCR2 in the culture supernatants was detected by ELISA readers at A_{450} nm after colour development.

Measurement of resting and IL-8-stimulated MP on normal and SLE-PMN

The method reported by Shapiro et al. [23] was employed for measuring the resting and IL-8-activated MP changes of normal and SLE-PMN. Briefly, 3,3'-dihexyl-oxocarboxycinamide iodide (DiOC6(3), Eastman Kodak, Rochester, NY, USA) was added to freshly prepared PMN suspension (1 x 10^6/ml) in the presence of medium (resting) or recombinant IL-8 (25 ng/ml) and the mixture was incubated at room temperature for 30 min. The MP changes were detected by FACSort flow cytometry with 488 nm excitation.

Measurement of cationic ion transporter expression on PMN by flow cytometry

The expression of cationic ion transporter molecules including Na⁺-K⁺-ATPase, ENaC and ROMK1 on PMN was stained with respective antibody and measured by FACSort flow cytometry with 488 nm excitation as mentioned above.

Statistical analysis

Results represent mean±s.d. throughout the study. Continuous variables were analysed by the non-parametric Mann-Whitney rank-sum test using a commercially available software package: Stata/SE 8.0 for Windows. A P-value ≤0.05 was considered statistically significant.

Results

Decreased IL-8 production and phagocytosis of SLE-PMN after stimulation

The functional defects of SLE-PMN are demonstrated by decreased IL-8 production after LPS (100 ng/ml) stimulation (Fig. 1A) and impaired phagocytosis either spontaneously or stimulated with IL-8 (25 ng/ml), TNF-α (25 ng/ml) and GRO-α (50 ng/ml) (a representative case is demonstrated in Fig. 1B). We chose IL-8 at a concentration of 25 ng/ml in the present study because a dose-response experiment in our previous study revealed that this dose was optimum for PMN activation [3].
These results indicate a non-specific hyporesponsiveness of SLE-PMN in vitro to different PMN activators.

**Decreased surface but increased cytosolic expression of CXCR2 molecule in unstimulated SLE-PMN**

We measured both protein and mRNA (Fig. 2B) expression of CXCR1 and CXCR2 on normal and SLE-PMN. As shown in Fig. 2A and C, the expression of CXCR2, but not CXCR1, was decreased on SLE-PMN compared with normal PMN. Two representative cases using different pair primers, set (a) and set (b), were conducted to compare the mRNA expression of CXCR1 and CXCR2 in normal and SLE-PMN. The results revealed that the CXCR2/G3PDH mRNA expression ratio was 0.48 in SLE vs 0.74 in normal using set (a) pair primers, and 0.94 in SLE vs 1.44 in normal using set (b) pair primer. In contrast, the mRNA expression ratio of chemokine receptors/G3PDH was not different in CXCR1 [1.26 in SLE vs 1.28 in normal using set (b)] or CCR1 [0.85 in SLE vs 0.83 in normal using set (a)] pair primer and 1.4 in SLE vs 1.4 in normal using set (b)]. The inconsistency in the ratio of CXCR2/G3PDH mRNA expression in (a) and (b) was reasonable since the percentage expression of CXCR2 in either normal or SLE-PMN was highly variable as shown in Fig. 2C. We believe that defective CXCR2 expression on SLE-PMN is a major contributing factor for PMN hyporesponsiveness to IL-8.

**Abnormal modulation of CXCR2 expression in SLE-PMN spontaneously and in IL-8 stimulation**

The binding of CXCR2 with its ligand, IL-8, would induce down-regulation and desensitization of the receptors on PMN [14]. Three mechanisms are involved in CXCR2 down-regulation on PMN after binding with its ligand: (i) Endocytosis or internalization of ligand–receptor complex [24]. (ii) The degree of recycling of the internalized receptors back to the surface [25, 26]. (iii) Release of the proteolytic enzyme-cleaved soluble receptors into the exterior [27]. Figure 3A and B suggest that the spontaneous cytosolic CXCR2 expression (incubation with medium) in SLE-PMN (34.43 ± 13.17%) was higher than normal PMN (21.52 ± 7.57%). However, the IL-8-induced cytosolic CXCR2 expression (internalization of the receptors) calculated after densitometric determination is demonstrated at the bottom. (C) Comparison of percentage CXCR2 protein expression on normal vs SLE-PMN by flow cytometry.
However, decreased down-regulation capacity of SLE-PMN (38.21 ± 5.4% decrease) was observed after reacting with IL-8 (25 ng/ml) for 60 min compared with normal PMN (73.39 ± 7.18% decrease) (Fig. 5A and B). For elucidating the molecular basis of decreased CXCR2 down-regulation in SLE-PMN, we then measured the amount of soluble CXCR2 in the PMN culture supernatants with and without incubating with IL-8 for 90 min by sandwich ELISA. It is conceivable that sCXCR2 represents the proteolytic enzyme-cleaved products from surface-expressed CXCR2. As demonstrated in Fig. 5C, the spontaneous release of sCXCR2 from normal PMN (5.30 pg/2 x 10^6 cells/ml) and SLE-PMN (4.67 pg/2 x 10^6 cells/ml) was minimal. However, a significant decrease in sCXCR2 release was found from IL-8-stimulated SLE-PMN culture supernatant (17.45 ± 9.71 pg/ml) compared with normal PMN (27.39 ± 15.17 pg/ml). It is not surprising that IL-8-induced CXCR2 release is decreased in SLE-PMN when surface CXCR2 expression is reduced (Fig. 2A and C). These results indicate that reduced IL-8–IL-8 receptor complex degradation rate on SLE-PMN may retard the regeneration of IL-8 receptors by the cells. The differences of CXCR2 modulation between normal and SLE-PMN are summarized in Table 1.

Comparison of surface MP changes before and after IL-8 stimulation and spontaneous cationic ion transporter expression on normal and SLE-PMN

Changes in the MP of cells are the first step in cell activation [23]. The transport of cationic ions including Na^+, K^+ and Ca^{2+} across the cell surface is crucial for MP changes and subsequent cell activation [17, 18]. For correlating IL-8 hyporesponsiveness, MP changes and cationic ion transporter expression on SLE-PMN, we measured the MP of SLE-PMN in resting and activating states. We found elevated resting but decreased IL-8-stimulated MPs on SLE-PMN (Fig. 6A). In addition, decreased spontaneous expression of the three cationic ion transporters, Na^+-K^+-ATPase (Fig. 6B), ENaC (Fig. 6C), and ROMK1 (Fig. 6D) was found, which is the underlying cause for defective IL-8-stimulated MP changes. Putting these results together, we conclude that the abnormal CXCR2 modulation and defective cationic ion transporter expression cause SLE-PMN hyporesponsiveness to IL-8 stimulation.

Discussion

A number of functional defects in SLE-PMN had been reported including spontaneous and IL-8-induced phagocytosis, and LPS-stimulated IL-8 production [1–3]. These defects predispose SLE patients susceptible to bacterial infections [1, 28]. IL-8 is a potent autocrine chemokine specific for PMN chemoattraction and activation [4]. Hyporesponsiveness of SLE-PMN to IL-8 stimulation is one of the serious defects for impairing the amplification loop of IL-8–IL-8 receptor-mediated inflammatory reactions [3]. In our preliminary study, we failed to find a difference in the expression of Toll-like receptors 2, 4, 7 and 9 between normal and SLE-PMN (data not shown). Accordingly, defect in IL-8-induced inflammatory reactions seems specific for SLE-PMN. In the present study, we try to explore the molecular basis of IL-8 hyporesponsiveness in SLE-PMN. Several original findings...
Defective CXCR2 and ion transporters on SLE-PMN

changes on SLE-PMN. (v) Defective spontaneous expression of cationic ion transporters on SLE-PMN including Na\(^{+}\)-K\(^{+}\)-ATPase, sodium channel ENaC and potassium channel ROMK. We deduce that the underlying patho-physiological basis of these defects ex vivo is derived from pre-excitation of PMN in SLE in vivo. Molad et al. [29] reported that high basal expression and low stimulation induced enhancement of CD11b/CD18 on active SLE-PMN correlating inversely with SLE disease activity. But our preliminary results revealed decrease in both spontaneous and IL-8-stimulated CD11b expression concomitantly with reduced phagocytosis in active SLE-PMN different from those of Molad et al. [29] (data not shown). This may be due to the difference in disease activity and anti-SLE medications in the two studies. The pre-excited SLE-PMN is generated by the effects of different activating factors including pro-inflammatory cytokines/chemokines, immune complexes, reactive oxygen species and inflammatory mediators in active SLE serum. In our previous reports, we found that anti-dsDNA [30] and anti-SSB/La [31] auto-antibodies were responsible for the increased IL-8 production by SLE-PMN and activation-induced cell death in vitro. These results are quite consistent with the finding that increased serum IL-8 levels correlate with disease activity in SLE in vivo [32, 33]. The increased IL-8 in SLE serum would bind to IL-8 receptors on PMN and induces receptor internalization and proteolytic cleavage [14, 24, 34, 35]. It remains possible that defective SLE-PMN CXCR2 expression is affected by anti-SLE medications, six untreated active SLE patients were studied. We found decreased IL-8 production (3140.7 ± 876.6 vs 5537.8 ± 665.4 pg/ml), CXCR2 expression (32.7 ± 11.3 vs 65.3 ± 6.4%) and IL-8-stimulated phagocytosis (36.8 ± 7.13 vs 77.2 ± 10.0%) in these untreated patients compared with normal persons. Tse et al. [34] demonstrated that pro-inflammatory cytokines, TNF-α, IL-1β and reactive oxygen species induce the third signal for enhancing adaptive T-cell immune responses and hyperresponsiveness to further stimulation. In a similar way, pre-excitation of SLE-PMN in vivo by pro-inflammatory cytokines may lead PMN hyperresponsiveness to further stimulation. Our study clearly demonstrates that pre-excitation of SLE-PMN hyporesponsiveness to further stimulation. Different from CXCR1, CXCR2 can bind with IL-8 as well as other ELR(+)CXCR chemokines with high affinity. Accordingly, CXCR2 plays more pleiotropic roles in the inflammation than CXCR1 [11, 12]. Biologically, CXCR2 on PMN surface can be promptly down-regulated after binding with TNF-α and bacterial endotoxins [24, 33, 35–37] through clathrin-mediated endocytosis [24], actin filament-mediated intracellular trafficking [25–27] and a tyrosine kinase-dependent mechanism [37]. For further understanding the molecular basis of decreased CXCR2 expression on SLE-PMN, the modulation of CXCR2 in normal and SLE-PMN was compared. We noted that the cytosolic CXCR2 expression in SLE-PMN was greater than normal PMN in either the resting state or after IL-8 stimulation (Fig. 3A and B). However, the binding affinity of IL-8 receptor on PMN and PMN-SLE with IL-8 was not different (Fig. 4A and B). These results suggest that decreased CXCR2 expression on SLE-PMN, but not binding affinity, is a crucial factor responsible for IL-8 hyporesponsiveness in SLE-PMN. The mechanisms of decreased surface CXCR2 expression on SLE-PMN are still not conclusive in this study. Four possibilities are suggested: (i) Defective translocation of the cytosolic receptors to the surface. (ii) Increased internalization of surface-expressed receptors after binding with ligands. (iii) Increased spontaneous synthesis of CXCR2 by SLE-PMN per se. (iv) Decreased down-regulation of CXCR2 after binding with IL-8. However, study on the CXCR2 molecules trafficking in PMN after ligand binding is necessary to explore the real mechanism. Our results also indicate that decreased IL-8-induced CXCR2 down-regulation in SLE-PMN is the result of impaired proteolytic cleavage, rather than reduced internalization of the

\[\text{Table 1. Comparison of CXCR2 modulation in normal and SLE-PMN}\]

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<tr>
<th>Modulation of CXCR2 expression</th>
<th>Normal PMN</th>
<th>SLE-PMN</th>
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<tr>
<td>Surface expression</td>
<td>High</td>
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<td>Cytosolic expression</td>
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<td>Binding affinity with IL-8</td>
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<td>(B_\text{max})</td>
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<td>(K_\text{d})</td>
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<tr>
<td>IL-8-induced down-regulation</td>
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<tr>
<td>Internalization of IL-8–IL-8R complex</td>
<td>Same</td>
<td>Same</td>
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<tr>
<td>Proteolytic cleavage of complex</td>
<td>High</td>
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were derived: (i) Decreased expression of IL-8 receptor CXCR2, but not CXCR1, on SLE-PMN whereas the binding affinity of the receptors with IL-8 is not impaired. (ii) Abnormal modulation of CXCR2 in SLE-PMN including increased spontaneous cytosolic expression, decreased IL-8-stimulated down-regulation and defective proteolytic cleavage of IL-8–CXCR2 complex. (iii) Normal IL-8–induced internalization of CXCR2 by SLE-PMN. (iv) Increased resting but decreased IL-8-stimulated MP

\[\text{Fig. 5. Comparison of CXCR2 modulation in normal and SLE-PMN after incubation with IL-8 (25 ng/ml) for 60 min. (A) IL-8-induced down-regulation of CXCR2 on SLE-PMN vs normal PMN detected by flow cytometry. (B) A representative case showing down-regulation of CXCR2 on PMN after incubation with IL-8 detected by flow cytometry. Negative control denotes IgG isotype control for representative case showing down-regulation of CXCR2 on PMN after incubation with IL-8. (C) Comparison of spontaneous and IL-8-induced sCXCR2 release from normal and SLE-PMN detected by sandwich ELISA method.}\]
ligand–receptor complexes (Fig. 5). It is possible that impaired proteolytic cleavage of IL-8–IL-8 receptor complexes on SLE-PMN surface retards CXCR2 regeneration on the cells. Although the recycling of the CXCR2 after binding with IL-8 was measured, the preliminary results were quite variable (data not shown) and not conclusive. A number of cytokines/chemokines including IL-8 and other important ELR(þ)-CXC chemokines (GROs, ENA-78 and NAP-2) are present in the inflammatory tissues of SLE. These inflammatory mediators can activate PMN through CXCR1 and CXCR2 to accelerate the inflammatory amplification loop in patients with SLE. We believe that CXCR2 on PMN is more important than CXCR1 in IL-8-mediated inflammatory responses. Khandaker et al. [37] reported that genistein attenuated the LPS-mediated down-regulation of CXCR1 and CXCR2. In our preliminary study, we noted that expression of CXCR2 on normal and SLE-PMN was variably affected by different protein tyrosine kinase inhibitors and anti-oxidants (data not shown). These results indicate that the regulatory mechanisms and ligand-induced down-regulation of CXCR2 in normal and SLE-PMN are different.

The changes of MP are the early events in the cell activation after ligand–receptor binding. The regulation of MP is a very complex mechanism of electrolyte transport across the cell membrane. Cationic ion transporters, membrane phospholipids, intracellular calcium [17, 38] and Na⁺/H⁺ exchangers [39] are all involved in the regulatory mechanism of MP. Among these, Na⁺-K⁺-ATPase[18], ENaC [40] and ROMK1 [41] play key roles in modulating transmembrane potential. Our data demonstrate that increased resting but decreased IL-8-induced MP of SLE-PMN in vitro is also due to pre-activation of the cells in vivo that results in reduced expression of these three cationic ion transport molecules on the cells. These results are consistent with Musch et al. [42] that cell activation inhibited epithelial Na⁺-K⁺-ATPase expression on SLE cell surface in vivo. Reduced cation-

ion-transporter expression on SLE-PMN may cause IL-8 hyporesponsiveness of these cells in vitro. In conclusion, abnormal modulation of CXCR2 and defective cationic ion transporter expression are important factors for IL-8 hyporesponsiveness in SLE-PMN responsible for susceptibility to bacterial infections.

FIG. 6. Comparison of MP changes and cationic ion transporter expression on normal and SLE-PMN detected by flow cytometry. (A) Comparison of resting and IL-8-stimulated MP changes on normal and SLE-PMN. (B) Comparison of Na⁺-K⁺-ATPase expression on resting normal and SLE-PMN. (C) Comparison of human renal epithelial Na⁺ channel (ENaC) expression on resting normal and SLE-PMN. (D) Comparison of rat renal outer medullary epithelial K⁺ channel 1 (ROMK1) expression on resting normal and SLE-PMN.

Rheumatology key messages

- SLE-PMN are hyporesponsive to IL-8 stimulation.
- Abnormal CXCR2 modulation and defective cationic ion transporter expression are key factors for SLE-PMN hyporesponsiveness to IL-8 stimulation.

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