Citrullination of Epithelial Neutrophil–Activating Peptide 78/CXCL5 Results in Conversion From a Non–Monocyte-Recruiting Chemokine to a Monocyte-Recruiting Chemokine

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Objective. To examine whether the citrullinated chemokines epithelial neutrophil–activating peptide 78 (ENA-78)/CXCL5, macrophage inflammatory protein 1α/CCL3, and monocyte chemotactic protein 1/CCL2 are detected in the biologic fluid of patients with rheumatoid arthritis (RA), and if so, to determine the biologic activities of these chemokines.

Methods. Recombinant human chemokines were citrullinated by peptidylarginine deiminase. Enzyme-linked immunosorbent assays were performed to measure the concentrations of citrullinated chemokines in sera from patients with rheumatoid arthritis (RA) and normal individuals and in synovial fluid from patients with RA, patients with osteoarthritis (OA), and patients with other inflammatory rheumatic diseases. The correlation between the citrullinated chemokine levels and clinical data was analyzed. Monocyte and neutrophil chemotaxis assays were performed, and native (non-citrullinated) or citrullinated ENA-78/CXCL5 was injected into mouse knees to evaluate the biologic activities of these chemokines.

Results. The concentration of citrullinated ENA-78/CXCL5 was significantly higher in RA sera and SF than in normal sera and in SF from patients with other rheumatic diseases including OA. In RA SF, a strong correlation between the amount of citrullinated ENA-78/CXCL5 and the C-reactive protein level or the erythrocyte sedimentation rate was observed. Citrullinated ENA-78/CXCL5 induced monocyte chemotaxis via CXCR1 and CXCR2, while noncitrullinated ENA-78/CXCL5 did not. In a mouse model of inflammatory arthritis, citrullinated ENA-78/CXCL5 induced more severe inflammation and recruited more monocytes than did noncitrullinated ENA-78/CXCL5.

Conclusion. Citrullinated ENA-78/CXCL5 is highly correlated with RA disease activity and, unlike noncitrullinated ENA-78/CXCL5, recruits monocytes. These results indicate that citrullinated ENA-78/CXCL5 may exert previously unrecognized inflammatory properties in RA by recruiting monocytes to inflamed joint tissue.

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by the infiltration of inflammatory cells into synovial tissue and synovial fluid (SF) (1). Anti–cyclic citrullinated peptide (anti-CCP) antibodies are present in the sera of nearly 70% of patients with RA and have become key diagnostic markers for this disease (2). Citrulline residues in target antigens are essential in the formation of anti–citrullinated peptide/protein antibody epitopes (3,4). These epitopes result from citrullination, a posttransla-

tional modification of arginine. Peptidylarginine deiminase (PAD) enzymes are calcium-dependent enzymes that catalyze citrullination of target proteins by converting arginine to citrulline (5). Five PAD family members, PAD type 1 (PAD1), PAD2, PAD3, PAD4, and PAD6, have been identified in many types of tissue, including synovial tissue, and in human leukocytes (6).

Chemokines play an important role as monocyte and polymorphonuclear neutrophil (PMN) recruiters in the setting of RA synovitis and tissue destruction (7,8). Chemokines are classified into 4 subfamilies based on the number and spacing of their first cysteine residues in the primary amino acid sequence (9). These chemokine families are designated as CXC, CC, C, and CX$_3$C. The CXC chemokines are further divided into 2 subgroups based on whether the Glu-Leu-Arg (ELR) motif precedes the first cysteine residue. Epithelial neutrophil–activating peptide 78 (ENA-78)/CXCL5, a CXC chemokine, is an 8.3-kd protein with 78 amino acids containing 4 cysteine residues positioned identically to those of interleukin-8 (IL-8/CXCL8) (10). We previously observed that the concentration of ENA-78/CXCL5, which is associated with neutrophil recruitment, is significantly higher in RA SF compared with osteoarthritis (OA) SF or SF from patients with other forms of arthritis (11). In addition, we demonstrated that neutralization of ENA-78/CXCL5 ameliorated the severity of adjuvant-induced arthritis in a rat model (12). These findings support the notion that ENA-78/CXCL5 is important in the pathogenesis of RA.

Although the presence of citrullinated proteins such as fibrinogen, α-enolase, and vimentin in RA has been reported (4,13–15), citrullinated chemokines have not yet been detected in RA. We chose to examine chemokines, because they are biologically relevant joint proteins. Several chemokines are highly expressed in the joints of patients with RA, and PAD2 and PAD4 are mainly present in synovial tissue. The expression levels of these PADs are correlated with inflammation, thickness of the synovial lining layer, and vascularity (16). These observations support the hypothesis that citrullinated chemokines may be present in rheumatic joints.

We compared the presence of citrullinated ENA-78/CXCL5, citrullinated macrophage inflammatory protein 1α (MIP-1α)/CCL3, and citrullinated monocyte chemotactic protein 1 (MCP-1)/CCL2 in the biologic fluid of patients with RA with that in the biologic fluid of patients with other arthritic diseases, using a newly developed enzyme-linked immunosorbent assay (ELISA) system, and examined the biologic activity of these chemokines in vitro and that of ENA-78/CXCL5 in vivo.

PATIENTS AND METHODS

Patients and normal control subjects. Serum samples were obtained from 11 patients with RA and 15 normal control subjects, and SF samples were obtained from 20 patients with RA, 15 patients with OA, and 13 patients with other inflammatory rheumatic diseases, including gout (n = 4), pseudogout (n = 2), psoriatic arthritis (n = 1), spondyloarthritis (n = 3), Behçet's disease (n = 1), Lyme disease (n = 1), and unclassified arthritis (n = 1). Rheumatoid factor (RF) was immunodepleted from RA sera and SF using goat anti-human IgM (μ-chain specific) agarose (Sigma-Aldrich) prior to measuring citrullinated chemokines by ELISA. All samples were obtained after approval by the Institutional Review Board and provision of informed consent by the subjects.

Chemokine DNA cloning. Full-length ENA-78/CXCL5 complementary DNA (cDNA) (NCBI accession no. NM_002994) was obtained from Open Biosystems. We generated cDNA libraries for MCP-1/CCL2 and MIP-1α/CCL3 from primary synovial fibroblasts derived from patients with RA. The cDNA fragments encoding mature proteins were amplified by polymerase chain reaction (PCR) using primers with an incorporated *Nco* I restriction site in the forward primers and with 6 codons encoding histidines followed by a stop codon and the *Eco* RI restriction site in the reverse primers (Primer Express software; Applied Biosystems). The primers used are as follows (forward and reverse, respectively): for ENA-78/CXCL5, 5′-TAATCCATGGGAGCTGGTCCT-GCCGCTGCTGT-3′ and 5′-TAAGAATTCTCAGTGATG-GTGATGGTGATGGTTTTCCTTGTTTCCACCGT-3′; for MIP-1α/CCL3, 5′-TAATCCATGGGAGCTGACACGCCG-ACCGCCTG-3′ and 5′-TAAGAATTCTCAGTGATGGTG-ATGGTGATGGGCACTCAGCTCCAGGTCGC-3′; for MCP-1/CCL2, 5′-TAATCCATGGGACAGCCAGATGCAA-TCAATGCC-3′ and 5′-TAAGAATTCTCAGTGATGGTG-ATGGTGATGAGTCTTCGGAGTTTGGGTTTG-3′. *Nco* I/*Eco* RI–flanked PCR fragments containing a C-terminus 6×His tag were initially cloned into a pQE-TriSystem Vector (Qiagen). All sequences were verified using BigDye Terminator sequencing (Life Technologies). For optimization of mammalian expression, inserts containing a C-terminus 6×His tag were further cloned into the mammalian expression vector pcDEF (17).

Transfection of HEK 293T cells and purification of recombinant human chemokines. All mammalian expression vectors were transfected into HEK 293T cells using polyethylenimine (Polysciences) to collect 6×His-tagged chemokines from cellular lysates prepared with 1% Triton X-100. Chemokines (6×His tagged) from HEK 293T cell lysates were purified with ProBond Nickel beads (Life Technologies), rinsed extensively with 10 mM imidazole, and eluted gradually with 50–200 mM imidazole. The quality and quantity of expressed recombinant proteins were assessed with appropriate DuoSet ELISA kits (R&D Systems) specific for these proteins and with colloidal Coomassie staining after sodium dodecyl sulfate–polyacrylamide gel electrophoresis resolution.

In vitro citrullination of chemokines. After the concentration of purified chemokines was measured using DuoSet ELISA kits, 100 microliters of purified recombinant human chemokine (~100 ng/ml) was incubated with 0.5 units of rabbit

skeletal muscle PAD (Sigma-Aldrich) in 40 mM Tris HCl, pH 7.6, 10 mM CaCl$_2$, and 2.5 mM dithiothreitol for 2 hours at 37°C. Deimination was stopped by chelating the calcium with 25 mM EDTA. These citrullinated chemokines were subsequently used as standards for ELISA of citrullinated chemokines. Alternatively, 5 μM recombinant human ENA-78 (rhENA-78)/CXCL5, recombinant human MIP-1α/CCL3, or recombinant human MCP-1/CCL2 (OriGene) was incubated with rabbit skeletal PAD (250 nM) at enzyme:substrate molar ratios of 1:20 in 40 mM Tris HCl with 2 mM CaCl$_2$ (pH 7.4) for 1.5 hours at 37°C. Deimination was stopped with 0.1% trifluoroacetic acid (TFA). These citrullinated chemokines were used in both in vitro chemotaxis assays and in vivo experiments. Citrullination of rhENA-78/CXCL5 was confirmed by Western blotting (18). For detection of citrullinated protein, citrulline residues were chemically modified to form a ureido group adduct before addition of an anti–modified citrulline antibody (Millipore) according to the manufacturer's protocol, which enables detection of citrulline residues independent of neighboring amino acid sequences (19). The diagnostic +1Da mass shift occurring upon citrullination was identified by liquid chromatography tandem mass spectrometry (MS Bioworks) (20).

Sandwich ELISA for citrullinated chemokines. An ELISA was designed to determine the concentrations of citrullinated chemokines in biologic fluid. Ninety-six–well plates (Thermo Fisher Scientific) were coated overnight at room temperature with mouse anti-human ENA-78/CXCL5, mouse anti-human MCP-1/CCL2, or goat anti-human MIP-1α/CCL3 (R&D Systems). Between each step, the plates were washed with wash buffer (0.05% Tween 20 in phosphate buffered saline [PBS]). The plates were blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature and incubated with serum, SF, or standards for 2 hours at room temperature. Citrullinated recombinant human chemokines and native (noncitrullinated) recombinant human chemokines purified from the transfected HEK 293T cells were used as standards and negative controls, respectively, for the ELISA. The samples were crosslinked onto the plates with 1% glutaraldehyde in PBS for 30 minutes at room temperature.

The plates were then incubated with 0.2M Tris HCl (pH 7.8) for 30 minutes at room temperature to block the crosslinking. The plates were then incubated overnight at 37°C in a citrulline-modification solution consisting of 2 parts solution A (0.025% [weight/volume] FeCl$_3$, 4.6M H$_2$SO$_4$, and 3.0M H$_3$PO$_4$), 1 part solution B (1% diacetylmonoxime, 0.5% antipyrine, and 1M acetic acid), and 1 part H$_2$O (18). The plates were incubated for 2 hours at room temperature with rabbit anti–modified citrulline (Millipore), diluted 1:2,500 in PBS containing 1% BSA. The plates were incubated for 2 hours at room temperature with horseradish peroxidase (HRP)–conjugated swine anti-rabbit IgG (Dako), diluted 1:1,000 in PBS containing 1% BSA. Biotin-Tyramide Reagent (PerkinElmer), diluted 1:1,000 in 0.05M Tris (base pH 8.5), was added, followed by HRP-conjugated streptavidin. The plates were developed using tetramethylbenzidine, development was stopped with 2N H$_2$SO$_4$, and the plates were read on a microplate reader at 450 nm. Serum and SF chemokine concentrations were also measured using DuoSet ELISA kits.

In vitro monocyte and polymorphonuclear neutrophil (PMN) chemotaxis assays. Monocytes and PMNs were isolated from normal human peripheral blood. Monocyte and PMN chemotaxis assays were performed using 48-well modified Boyden chambers (Neuro Probe) as described previously (21,22). Noncitrullinated chemokines (OriGene), citrullinated chemokines, and reaction buffer consisting of 40 mM Tris HCl, 2 mM CaCl$_2$, 250 nM rabbit skeletal PAD, and 0.1% TFA were diluted by PBS with calcium and magnesium in the same manner and tested for chemotaxis. The composition of the reaction buffer that contained PAD enzymes was the same as the composition of the stock solution of citrullinated chemokines. PBS with calcium and magnesium and fMLP (100 nM) (Sigma-Aldrich) were used as negative and positive stimuli, respectively. For the inhibitor studies, monocytes (2.5 $\times$ 10^6/ml) were pretreated with 500 ng/ml pertussis toxin (Sigma-Aldrich), which is a G protein–coupled receptor antagonist, 10 μg/ml anti-CXCR1, anti-CXCR2, or isotype control IgG2a (R&D Systems) in PBS with calcium and magnesium for 1 hour to examine whether G protein–coupled receptors are used by citrullinated ENA-78/CXCL5 to induce monocyte chemotaxis.

Mouse model of inflammatory arthritis. Female C57BL/6 mice (8–10 weeks old) were purchased from the National Cancer Institute. The mice were divided into the following 3 treatment groups: PBS, noncitrullinated ENA-78/CXCL5 (Origene), and citrullinated ENA-78/CXCL5. The mice were anesthetized, and the knee circumference was determined by caliper measurements before intraarticular injection and calculated using the following formula: circumference = $\pi (a + b)/2$, where a is the laterolateral diameter and b is the anteroposterior diameter. The anesthetized mice received 20 μl/knee joint of PBS, noncitrullinated ENA-78/CXCL5 (16 ng), or citrullinated ENA-78/CXCL5 (16 ng). In all of the mice, circumference measurements were obtained in a blinded manner 24 hours after the intraarticular injection.

Hematoxylin and eosin (H&E) and immunofluorescence staining. Mouse knee joints embedded in OCT compound were frozen and cut (10 μm). H&E staining was performed as described previously (23). Immunofluorescence staining was performed on cryosections from mouse knee joints to determine monocyte/macrophages, using rat anti-mouse F4/80 antibodies (GeneTex) at 1 μg/ml as primary antibody and Alexa Fluor 555–conjugated goat anti-rat IgG (Life Technologies) at a dilution of 1:200 as secondary antibody. The method of immunofluorescence staining has been described previously (21). The number of F4/80-positive monocyte/macrophages was calculated as the average of the number of cells in 3 fields (400$\times$) that showed the most remarkable infiltrates in the joint space where the injections were administered.

Statistical analysis. Statistical differences between experimental groups were determined by Student's t-test or one-way analysis of variance followed by Tukey's multiple comparison test for post hoc analysis. The clinical correlation between chemokine levels and clinical data was assessed by Pearson's correlation coefficient. Statistical analysis was performed with assistance from personnel at the Center for Statistical Consultation and Research at the University of Michigan. Results are expressed as the mean $\pm$ SEM. P values less than 0.05 were considered significant.

RESULTS

Detection of citrullinated rhENA-78/CXCL5 and verification of in vitro citrullination of ENA-78/CXCL5. As shown in Figure 1A, the standard curve of citrullinated rhENA-78/CXCL5, as determined by ELISA, demonstrated high correlation between the concentration of citrullinated rhENA-78/CXCL5 and absorbance (Figure 1A). Noncitrullinated ENA-78/CXCL5 (1000 pg/ml) was not detected by this ELISA. We then performed Western blotting to confirm that rhENA-78/CXCL5 was citrullinated in vitro. Citrullinated ENA-78/CXCL5 was recognized by anti–modified citrulline antibody, while noncitrullinated ENA-78/CXCL5 was not (Figure 1B). The diagnostic +1Da mass shift occurring upon citrullination of ENA-78/CXCL5, which has 2 arginine residues, was identified by mass spectrometry (20). The mass spectrometry data indicated that both arginine residues were detected and citrullinated (Figures 1C and D). Citrullination of MCP-1/CCL2 and MIP-1α/CCL3, which have 4 and 3 arginine residues,

respectively, was also identified by mass spectrometry (data not shown).

Citrullinated chemokine concentrations in RA sera and normal control sera. To ascertain whether citrullinated chemokines were detectable in biologic fluids, we assayed serum samples obtained from patients with RA and normal control subjects, using a citrullinated chemokine sandwich ELISA. In normal control subjects and patients with RA, the mean ± SEM levels of citrullinated ENA-78/CXCL5 were 1.2 ± 0.7 pg/ml and 286 ± 68 pg/ml, respectively (Figure 2A). The levels of citrullinated MIP-1α/CCL3 in normal and RA sera were 1.2 ± 1.2 pg/ml and 624 ± 96 pg/ml, respectively (Figure 2B), and the levels of citrullinated MCP-1/CCL2 in normal and RA sera were 0.9 ± 0.5 pg/ml and 143 ± 18 pg/ml, respectively (Figure 2C). The levels of citrullinated ENA-78/CXCL5, MIP-1α/CCL3, and MCP-1/CCL2 in patients with RA were all significantly higher than the levels in normal control subjects ($P < 0.05$). The mean ± SEM concentrations of ENA-78/CXCL5
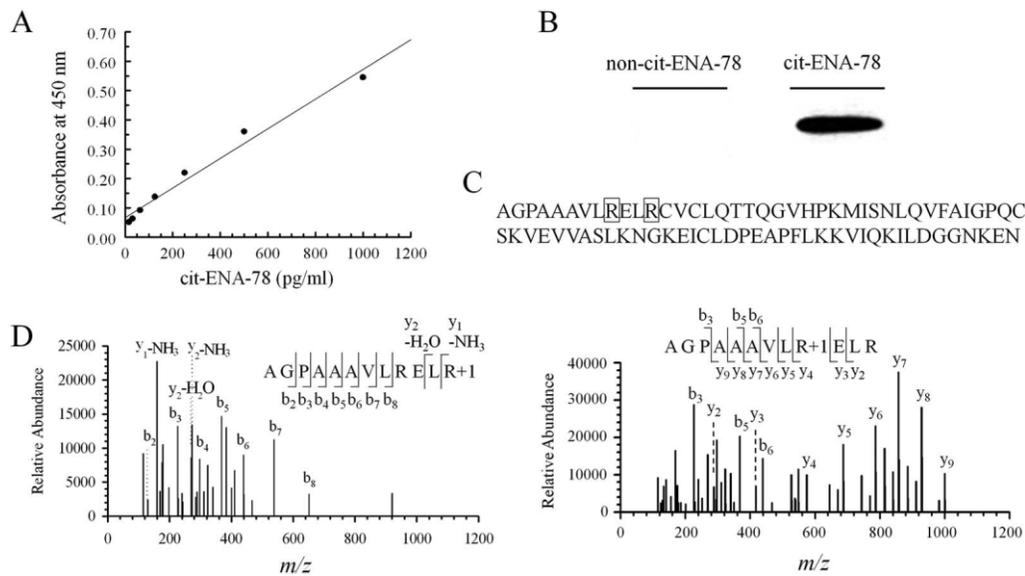
Figure 1. Detection of citrullinated recombinant human epithelial neutrophil–activating peptide 78 (ENA-78)/CXCL5 and verification of successful in vitro citrullination of ENA-78/CXCL5. **A,** Standard curve of citrullinated recombinant human ENA-78/CXCL5 as determined by enzyme-linked immunosorbent assay. Each point was determined using duplicate assays. **B,** Immunoblots showing that citrullinated recombinant human ENA-78/CXCL5 was recognized by anti–modified citrulline antibody (right) while noncitrullinated ENA-78/CXCL5 was not (left). The blot used to detect citrullinated protein was chemically modified prior to immunostaining. **C,** Amino acid sequence of ENA-78/CXCL5 (mature form 37–114). The boxed areas show the citrullinated arginines that were detected. **D,** Annotated tandem mass spectrometry (MS/MS) fragmentation spectra for the citrullinated ENA-78/CXCL5 peptides, showing the citrullinated arginine residues. Left, MS/MS spectrum confirming the presence of C-terminal citrullination at position 12 (R48) from the y_1 and y_2 ions present in the spectrum. Right, The presence of ions y_4 to y_9 in the spectrum confirms the presence of the "middle" R+1 (citrullination) at position 9 (R45). The MS/MS fragmentation data were annotated using Expert System (Max-Planck Institute of Biochemistry). The precursor ion was observed with a mass error of <1 parts per million, and the error for the fragment ions was ≤0.02 daltons.
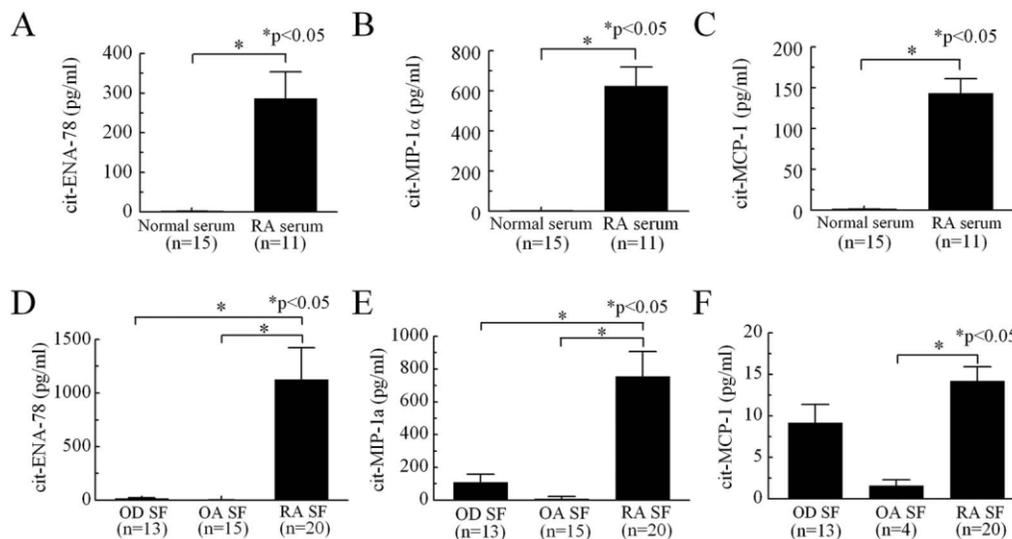
Figure 2. Expression of citrullinated chemokines in rheumatoid arthritis (RA) sera and synovial fluid (SF). Citrullinated chemokines were measured using an enzyme-linked immunosorbent assay (ELISA) in which chemokines were captured on an ELISA plate, followed by detection with anti–modified citrulline after chemical modification of citrulline residues. Rheumatoid factor was depleted from RA SF and RA sera prior to the ELISA. **A–C,** Concentrations of citrullinated epithelial neutrophil–activating peptide 78 (ENA-78)/CXCL5 (**A**), macrophage inflammatory protein 1α (MIP-1α)/CCL3 (**B**), and monocyte chemotactic protein 1 (MCP-1)/CCL2 (**C**) in normal sera and sera from patients with RA. **D–F,** Concentrations of citrullinated ENA-78/CXCL5, citrullinated MIP-1α/CCL3, and citrullinated MCP-1/CCL2 in SF from patients with other inflammatory rheumatic diseases (OD), patients with osteoarthritis (OA), and patients with RA. Bars show the mean ± SEM.

were 3,165 ± 825 pg/ml in normal sera and 3,942 ± 1,139 pg/ml in RA sera, the levels of MIP-1α/CCL3 were 230 ± 176 pg/ml and 727 ± 420 pg/ml, respectively, and the levels of MCP-1/CCL2 were 43 ± 22 pg/ml and 289 ± 271 pg/ml, respectively; none of the differences between groups were statistically significant (results not shown).

Citrullinated chemokine concentrations in SF from patients with RA, patients with OA, and patients with other rheumatic diseases. The concentrations of citrullinated chemokines in SF samples obtained from patients with RA, patients with OA, and patients with other inflammatory rheumatic diseases were measured by sandwich ELISA. The mean ± SEM concentration of citrullinated ENA-78/CXCL5 was significantly higher in SF from patients with RA (1,126 ± 297 pg/ml) than in SF from patients with OA (2.3 ± 1.0 pg/ml) and patients with other inflammatory rheumatic diseases (15 ± 9 pg/ml) ($P < 0.05$) (Figure 2D). The mean ± SEM concentration of citrullinated MIP-1α/CCL3 was also significantly higher in the SF of patients with RA (755 ± 152 pg/ml) compared with that in the SF of patients with other inflammatory rheumatic diseases (109 ± 49 pg/ml) and patients with OA (9 ± 15 pg/ml) ($P < 0.05$) (Figure 2E). Citrullinated MCP-1/CCL2 concentrations were

significantly higher in RA SF (14 ± 2 pg/ml) compared with OA SF (1.6 ± 0.7 pg/ml) ($P < 0.05$) (Figure 2F).

We also used ELISA kits to measure chemokine concentrations. The mean ± SEM concentrations of ENA-78/CXCL5 were 163 ± 107 pg/ml in RA SF, 0 ± 0 pg/ml in the SF of patients with other diseases, and 19 ± 13 pg/ml in OA SF. The concentrations of MIP-1α/CCL3 were 460 ± 171 pg/ml in RA SF, 31 ± 10 pg/ml in SF from patients with other rheumatic diseases, and 75 ± 52 pg/ml in OA SF. The concentrations of MCP-1/CCL2 were 1,085 ± 398 pg/ml in RA SF, 288 ± 42 pg/ml in SF from patients with other rheumatic diseases, and 390 ± 80 pg/ml in OA SF. There were no significant differences in SF chemokine concentrations between these groups.

Positive correlation between citrullinated ENA-78/CXCL5 concentrations and the C-reactive protein (CRP) level and erythrocyte sedimentation rate (ESR) in RA SF. We analyzed the relationship between the citrullinated chemokine concentrations in SF and clinical data in patients with RA. As shown in Table 1, the clinical characteristics of these patients were as follows: mean ± SEM age 52.7 ± 3.4 years, disease duration 145.7 ± 39.2 months, ESR 38.1 ± 4.3 mm/hour, CRP level 40.5 ± 9.9 mg/liter, Disease Activity Score in 28

Table 1. Clinical characteristics of the patients with rheumatoid arthritis whose synovial fluid was studied*

Male/female, % (n = 20)	20/80
Age, years (n = 20)	52.7 ± 3.4
Disease duration, months (n = 20)	145.7 ± 39.2
Tender joint count in 28 joints (n = 13)	5.7 ± 2.1
Swollen joint count in 28 joints (n = 13)	4.5 ± 1.9
Bone erosion, yes/no, % (n = 20)	40/60
ESR, mm/hour (n = 15)	38.1 ± 4.3
CRP, mg/liter (n = 14)	40.5 ± 9.9
DAS28 (n = 12)	5.1 ± 0.4
Rheumatoid factor, units/ml (n = 10)	74.8 ± 43.2
Anti-CCP, AU/ml (n = 10)	2,296.1 ± 1,559.1

* Except where indicated otherwise, values are the mean ± SEM. ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = Disease Activity Score in 28 joints; anti-CCP = anti–cyclic citrullinated peptide.

joints (24) 5.1 ± 0.4, tender joint count 5.7 ± 2.1, swollen joint count 4.5 ± 1.9, RF level 74.8 ± 43.2 units/ml, and anti-CCP antibody level 2,296.1 ± 1,559.1 AU/ml. As shown in Figure 3, citrullinated ENA-78/ CXCL5 concentrations were significantly correlated with CRP levels (r = 0.69, $P < 0.05$) and the ESR (r = 0.77, $P < 0.05$), and citrullinated MCP-1/CCL2 concentrations were significantly correlated with the ESR (r = 0.64, $P < 0.05$). There was no significant correlation between the concentration of citrullinated MIP-1α/CCL3 in RA SF with the other clinical parameters examined.

Citrullinated ENA-78/CXCL5–induced monocyte migratory activity via CXCR1 and CXCR2. Under normal conditions, ENA-78/CXCL5 induces PMN migration, but it does not induce monocyte migration (10,25,26). We performed monocyte and PMN chemotaxis assays using a modified Boyden chamber, and the results showed that the fold increase in monocyte migration was significantly higher in response to citrullinated ENA-78/ CXCL5 at 0.1 nM, 1 nM, and 10 nM (mean ± SEM 1.6 ± 0.1, 1.9 ± 0.2, and 1.9 ± 0.2, respectively) than in response to noncitrullinated ENA-78/CXCL5 and PBS (Figure 4A). We also tested various concentrations of reaction buffer in citrullinated ENA-78/CXCL5 stock solution for chemotaxis of monocytes. The reaction buffer including PAD enzymes did not induce monocyte migration at the concentrations tested. In contrast to citrullinated ENA-78/CXCL5, citrullinated MCP-1/ CCL2 recruited significantly fewer monocytes at 10 nM and 100 nM than did noncitrullinated MCP-1/CCL2 (data not shown), and citrullinated MIP-1α/CCL3 tended to have the same level of monocyte-recruiting activity as noncitrullinated MIP-1α/CCL3 at 0.01–100 nM (data not shown).

PMN chemotaxis assays showed that noncitrulli-

nated ENA-78/CXCL5 had significantly higher PMN chemotactic activity at 0.1 nM (1.5 ± 0.2) and 1 nM (1.6 ± 0.2) compared with PBS ($P < 0.05$), while citrullinated ENA-78/CXCL5 had significantly higher activity at 10 nM (1.4 ± 0.1) compared with PBS ($P < 0.05$) (Figure 4B). Thus, the fold increase in PMN
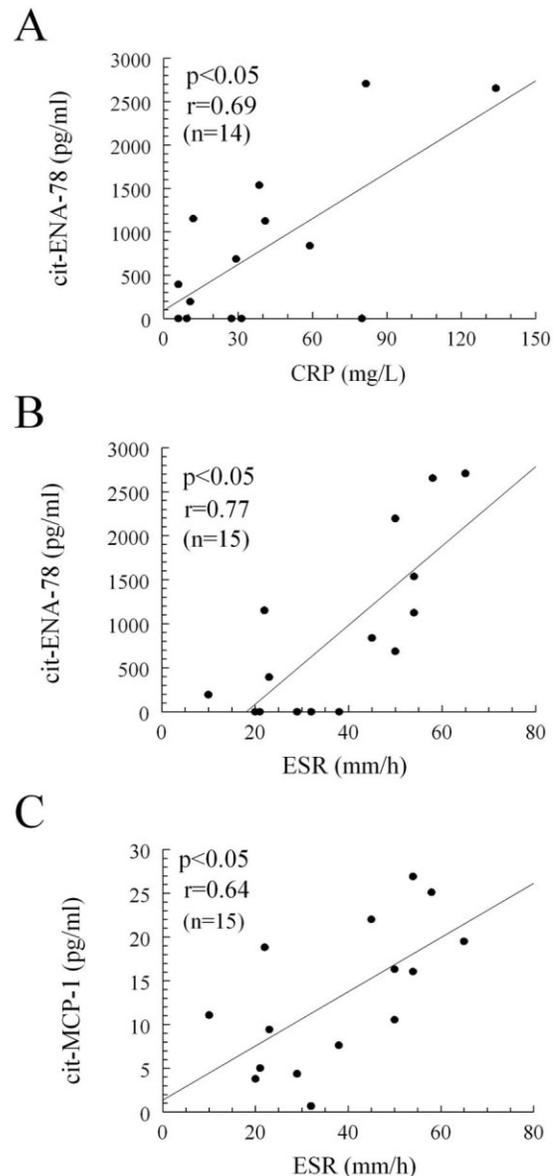
Figure 3. Correlation of clinical data with citrullinated chemokine levels in RA SF. Positive correlations between citrullinated ENA-78/ CXCL5 and the C-reactive protein (CRP) level (**A**), citrullinated ENA-78/CXCL5 and the erythrocyte sedimentation rate (ESR) (**B**), and citrullinated MCP-1/CCL2 and the ESR (**C**) were observed. See Figure 2 for other definitions.
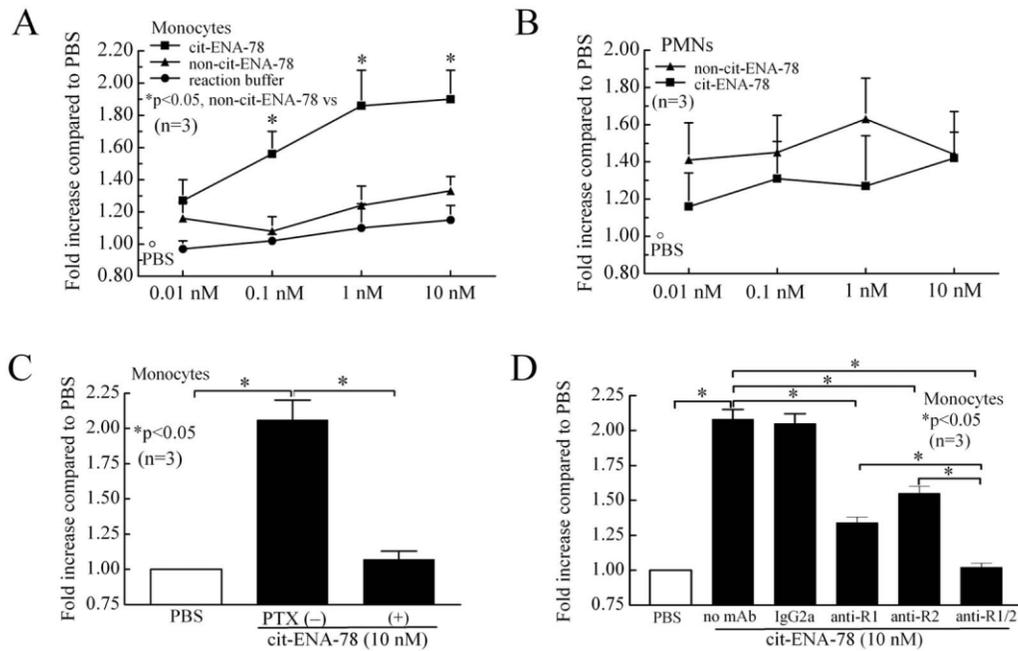
Figure 4. Monocyte recruitment by citrullinated epithelial neutrophil–activating peptide 78 (ENA-78)/CXCL5. **A,** Fold increase in monocyte migration in response to citrullinated ENA-78/CXCL5 and noncitrullinated ENA-78/CXCL5, as determined by monocyte chemotaxis assay. At 0.1 nM, 1 nM, and 10 nM, the response to citrullinated ENA-78/CXCL5 was significantly higher than the response to noncitrullinated ENA-78/CXCL5 and phosphate buffered saline (PBS). As controls, various concentrations of reaction buffer including peptidylarginine deiminase enzymes were tested for chemotaxis of monocytes. There was no significant difference between the response to reaction buffer and the response to PBS. The mean ± SEM fold increase in monocyte migration in response to fMLP (100 nM) was 4.4 ± 0.5. **B,** Fold increase in polymorphonuclear neutrophil (PMN) migration in response to citrullinated ENA-78/CXCL5 and noncitrullinated ENA-78/CXCL5, as determined by chemotaxis assay. At either 0.1 nM or 1 nM, the response to noncitrullinated ENA-78/CXCL5 was significantly higher than the response to PBS ($P < 0.05$). The response to citrullinated ENA-78/CXCL5 was lower than the response to noncitrullinated ENA-78/CXCL5, but the difference was not significant. The mean ± SEM fold increase in PMN migration in response to fMLP (100 nM) was 7.5 ± 2.5. **C,** Effect of pertussis toxin (PTX) on the increased monocyte migration in response to citrullinated ENA-78/CXCL5. **D,** Effect of anti-CXCR1 (anti-R1) and anti-CXCR2 (anti-R2) on the increased monocyte migration in response to citrullinated ENA-78/CXCL5. All assays were performed in quadruplicate, with 3 high-power fields (400×) counted in each replicate well. The fold increase was determined by dividing the number of cells migrated by the number of cells migrated to the negative control PBS. Values are the mean ± SEM.

migration in response to citrullinated ENA-78/CXCL5 tended to be lower than that in response to noncitrullinated ENA-78/CXCL5. However, there was no significant difference in PMN chemotaxis between noncitrullinated and citrullinated ENA-78/CXCL5.

In monocyte chemotaxis assays using pertussis toxin (a G protein–coupled receptor antagonist), anti-CXCR1, and anti-CXCR2 antibodies, the fold increase in the response to citrullinated ENA-78/CXCL5 with pertussis toxin was significantly lower (mean ± SEM 1.07 ± 0.06) compared with that without pertussis toxin (2.06 ± 0.14; $P < 0.05$) (Figure 4C). We then examined whether monocyte migration is mediated via the G protein–coupled receptors CXCR1 and CXCR2. The fold increases in the response to citrullinated ENA-78/CXCL5 with anti-CXCR1 or anti-CXCR2 antibodies

were significantly lower (1.34 ± 0.04 and 1.55 ± 0.05, respectively) compared with that without the antibodies (2.05 ± 0.07), and the fold increase in the response to citrullinated ENA-78/CXCL5 with a combination of anti-CXCR1 and anti-CXCR2 antibodies was significantly lower (1.02 ± 0.03) compared with that without the antibodies, with anti-CXCR1, or with anti-CXCR2 ($P < 0.05$). IgG2a was used as an isotype-matched control monoclonal antibody that did not inhibit monocyte migration (Figure 4D). These results indicated that citrullinated ENA-78/CXCL5 induced monocyte migration via CXCR1 and CXCR2.

Citrullinated ENA-78/CXCL5–enhanced induction of inflammatory arthritis and recruitment of monocyte/macrophages in vivo. To test the inflammatory activity of citrullinated ENA-78/CXCL5 in vivo, we
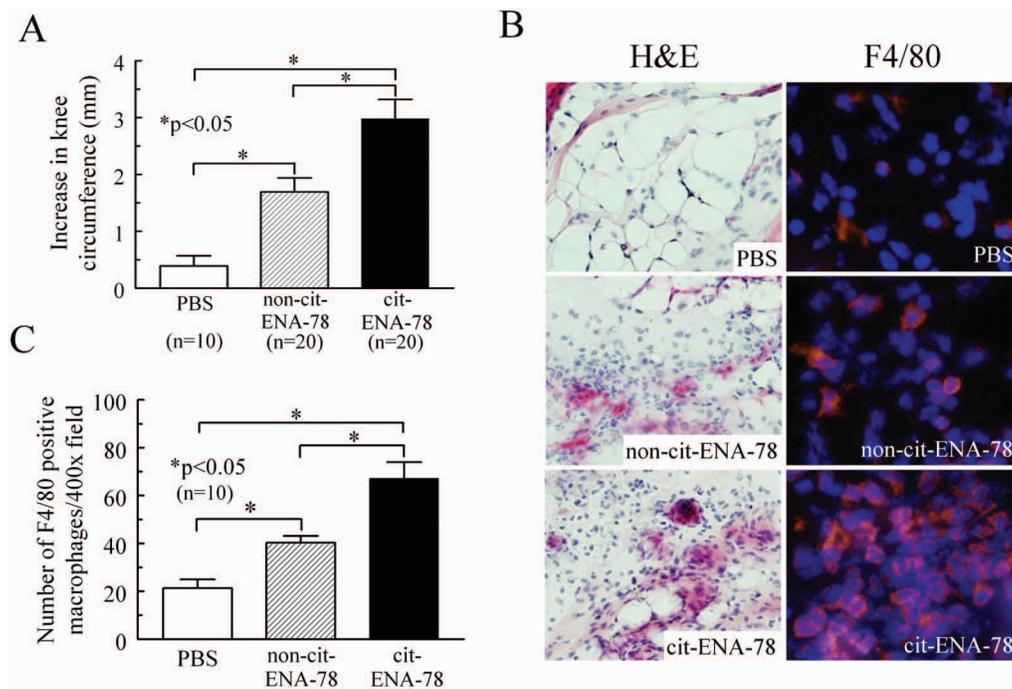
Figure 5. Effect of citrullinated ENA-78/CXCL5 in vivo. The knees of female C57BL/6 mice were injected intraarticularly with PBS, noncitrullinated ENA-78/CXCL5, or citrullinated ENA-78/CXCL5 to induce inflammation. **A,** Circumference of the mouse knees 24 hours after administration of the intraarticular injections. The increases in knee circumference were significantly greater in mice that received citrullinated ENA-78/CXCL5 compared with those that received PBS or noncitrullinated ENA-78/CXCL5 and were also significantly greater in mice that received noncitrullinated ENA-78/CXCL5 compared with those that received PBS. **B,** Left, Hematoxylin and eosin (H&E)–stained mouse knee joint sections, showing inflammatory cell infiltration in all groups and severe inflammation in mice treated with citrullinated ENA-78/CXCL5. Right, Immunofluorescence-stained mouse knee joint sections, showing a greater number of F4/80-positive monocyte/macrophages in mice treated with citrullinated ENA-78/CXCL5. Fluorescent red and blue staining show F4/80-positive monocyte/macrophages and cell nuclei, respectively. Original magnification $\times$ 400. **C,** Numbers of F4/80-positive monocyte/macrophages in mice treated with PBS, noncitrullinated ENA-78/CXCL5, and citrullinated ENA-78/CXCL5. Values are the mean $\pm$ SEM (n values are the number of joints). See Figure 4 for other definitions.

injected PBS, noncitrullinated ENA-78/CXCL5, or citrullinated ENA-78/CXCL5 into mouse knee joints. Joints injected with citrullinated ENA-78/CXCL5 had a significantly greater increase in circumference (mean $\pm$ SEM 3.0 $\pm$ 0.3 mm), and hence joint swelling, compared with knees injected with PBS (0.4 $\pm$ 0.2 mm; $P < 0.05$) or noncitrullinated ENA-78/CXCL5 (1.7 $\pm$ 0.3 mm; $P < 0.05$). Joints injected with noncitrullinated ENA-78/CXCL5 had significantly increased joint swelling compared with joints injected with PBS ($P < 0.05$) (Figure 5A). H&E staining showed inflammatory cell infiltration in each treatment group, and inflammation was especially severe in the group that received citrullinated ENA-78/CXCL5 (Figure 5B). Immunofluorescence staining revealed that the number of F4/80-positive monocyte/macrophages was significantly higher in mouse knee joints injected with citrullinated ENA-78/CXCL5 (mean $\pm$ SEM 67 $\pm$ 7 macrophages) compared with joints injected with noncitrullinated ENA-78/

CXCL5 (40 $\pm$ 3 macrophages) or PBS (21 $\pm$ 4 macrophages). In addition, the number of F4/80-positive monocyte/macrophages was significantly higher in the mice treated with noncitrullinated ENA-78/CXCL5 compared with those that received PBS ($P < 0.05$) (Figures 5B and C).

DISCUSSION

We observed the presence of citrullinated ENA-78/CXCL5, MIP-1α/CCL3, and MCP-1/CCL2 in RA sera and RA SF. There is evidence that some chemokines can be citrullinated in vitro (27–30); however, not all cytokines can be citrullinated. Proost et al showed that PAD efficiently and site-specifically citrullinated ENA-78/CXCL5 and IL-8/CXCL8 but not IL-1β (27). Struyf et al demonstrated that stromal cell–derived factor 1 (SDF-1)/CXCL12 can be citrullinated in vitro, and that SDF-1/CXCL12 and PAD were coexpressed in

the colon tissue of patients with Crohn's disease (30). However, those investigators did not directly demonstrate the presence of citrullinated SDF-1/CXCL12 in diseased colon tissue.

In the current study, we demonstrated that the concentrations of citrullinated ENA-78/CXCL5, MIP-1α/CCL3, and MCP-1/CCL2 were all significantly higher in RA sera than in normal control sera. Furthermore, citrullinated ENA-78/CXCL5 and MIP-1α/CCL3 concentrations were significantly higher in RA SF than in SF from patients with OA and patients with other rheumatic diseases, and the level of citrullinated MCP-1/CCL2 was significantly higher in RA SF than in OA SF. In particular, the concentration of citrullinated ENA-78/CXCL5 was high in RA SF but was barely detected in SF from patients with OA or other rheumatic diseases. Additionally, the PAD4 level measured by ELISA is significantly elevated in RA SF compared with SF from patients with OA and patients with ankylosing spondylitis (31). This fact supports the notion that chemokines, including ENA-78/CXCL5, are citrullinated more efficiently in RA joints than in the inflamed joints of patients with other diseases. In our study, the concentrations of ENA-78/CXCL5 and MIP-1α/CCL3 in SF as measured by standard ELISA kits (R&D Systems) were lower than the concentrations of citrullinated ENA-78/CXCL5 and MIP-1α/CCL3 in SF as measured by the new ELISA system developed by our group. We observed that after citrullination, purified chemokines were not detected by standard ELISAs (data not shown). It is possible that these ELISAs may not completely recognize citrullinated chemokines in RA SF.

We showed that among 3 citrullinated chemokines tested in RA SF, the concentrations of citrullinated ENA-78/CXCL5 correlated most closely with clinical data (the CRP level and the ESR). Chemokines are readily detectable in RA SF (11,32–36), and the concentrations of MIP-1α/CCL3 and IL-8/CXCL8 in SF correlate with CRP levels in serum (35,36). In our study, we observed significant positive correlations between citrullinated ENA-78/CXCL5 and not only the CRP level but also the ESR, whereas the ENA-78/CXCL5 concentration did not correlate with any clinical parameters examined (data not shown). These results suggest that compared with the other chemokines that we examined, citrullinated ENA-78/CXCL5 is more closely related to disease activity in RA.

Citrullination has been reported to decrease the activities of certain chemokines in some instances (37). In vitro, ENA-78/CXCL5 (0.1–10 nM) induces PMN migration in a dose-responsive manner (10,25). However, citrullination of ENA-78/CXCL5 has been reported to reduce in vitro and in vivo PMN chemotaxis, intracellular calcium signaling, phosphorylation of extracellular signal–regulated kinase, and internalization of CXCR2 compared with noncitrullinated ENA-78/CXCL5 (38). We showed that citrullination of ENA-78/CXCL5 directly supports monocyte migration but not PMN migration. These results indicate the possibility that citrullination of ENA-78/CXCL5 does not increase the function of ENA-78/CXCL5 as a PMN recruiter but instead dampens it.

Surprisingly, we observed that citrullinated ENA-78/CXCL5 acquired a monocyte-recruiting function that noncitrullinated ENA-78/CXCL5 did not have. Normally, ENA-78/CXCL5 is not a potent chemotactic factor for monocytes but rather for PMNs and endothelial cells and also possesses angiogenic properties (10,25,39,40). Monocyte chemotaxis assays showed that citrullinated ENA-78/CXCL5 recruited monocytes in a dose-dependent manner, while noncitrullinated ENA-78/CXCL5 did not. These results suggest that citrullination of ENA-78/CXCL5 results in its conversion from a non–monocyte-recruiting chemokine to a monocyte-recruiting chemokine.

We then investigated whether citrullinated ENA-78/CXCL5 recruited monocytes through the G protein–coupled receptors CXCR2, which is the primary ENA-78/CXCL5 receptor, and CXCR1, which has 78% homology with CXCR2 at the amino acid level (41,42). Citrullinated ENA-78–induced monocyte migration was completely inhibited by pertussis toxin (a G protein–coupled receptor antagonist) or by a combination of anti-CXCR1 and anti-CXCR2 antibodies. These results suggest that citrullinated ENA-78/CXCL5 recruits monocytes via both CXCR1 and CXCR2. ENA-78/CXCL5 elicits PMN chemotaxis by interacting with the chemokine receptor CXCR2 present on the PMN cell surface (43,44). This receptor is expressed on many different cells including PMNs and monocytes (45). CXCR1 is also present on the surface of human monocytes (26,46). CXCR1 and CXCR2 are expressed on 23–90% and 22–93% of human monocytes, respectively (26). IL-8/CXCL8, neutrophil-activating peptide/CXCL7, growth-related oncogene α/CXCL1, and ENA-78/CXCL5 bind to CXCR2 with high affinity, whereas IL-8/CXCL8 exhibits high affinity for CXCR1 as well (47,48). Our results suggest that citrullination may increase the affinity of ENA-78/CXCL5 for CXCR1 (38).

We clearly show that at physiologically relevant concentrations, citrullinated ENA-78/CXCL5 recruits

monocytes. We also show data confirming that citrullinated ENA-78/CXCL5 preferentially binds to the CXCR1 receptor instead of the normal receptor for ENA-78/CXCL5, namely CXCR2. This indicates that modification of ENA-78/CXCL5 changes its receptor-binding affinity (and likely its conformation). This also explains, at least in part, the ability of citrullinated ENA-78/CXCL5 to recruit monocytes. Nonetheless, assuming that both citrullinated ENA-78/CXCL5 and noncitrullinated ENA-78/CXCL5 are present in SF and are binding to the same receptor, the modified or unmodified forms of ENA-78/CXCL5 that are present in greater quantities would be the forms that are more competitive for binding the available receptor. However, we now present evidence that citrullinated ENA-78/CXCL5 utilizes an alternative receptor (CXCR1), indicating that the concentration of citrullinated ENA-78/CXCL5 is likely more important when considering the monocyte-recruiting activity of citrullinated ENA-78/CXCL5. We believe that the conversion of ENA-78/CXCL5, a potent neutrophil recruitment factor to citrullinated ENA-78/CXCL5, an active monocyte recruitment factor, represents a change to a more chronic inflammatory response in RA-affected joints.

Last, we examined whether citrullination of ENA-78/CXCL5 results in increased inflammation and monocyte migration in vivo compared with noncitrullinated ENA-78/CXCL5. To test this hypothesis, we injected noncitrullinated or citrullinated ENA-78/CXCL5 into mouse knee joints and evaluated joint inflammation and monocyte migration. Citrullinated ENA-78/CXCL5 increased the knee circumference more than noncitrullinated ENA-78/CXCL5. Immunofluorescence staining showed that citrullinated ENA-78/CXCL5 induced more F4/80-positive monocyte/macrophage ingress into the synovial tissue of mouse knees compared with noncitrullinated ENA-78/CXCL5. These results suggest that citrullination of ENA-78/CXCL5 induces more severe inflammation in mouse knee joints through monocyte recruitment.

In the current study, although RA sera and RA SF were obtained from different groups of patients, the concentration of citrullinated ENA-78/CXCL5 in RA SF was higher than that in RA sera. These results suggest that citrullinated ENA-78/CXCL5 may be produced mainly in the joints and functions to recruit monocytes to the joints. Other citrullinated proteins (e.g, citrullinated vimentin) stimulate proinflammatoy cytokine production from fibroblast-like synoviocytes or peripheral blood mononuclear cells in RA (49,50). It is possible that citrullinated ENA-78/CXCL5 may also stimulate cells other than monocytes in mouse knee joints to release inflammatory mediators and aggravate the inflammatory response.

In addition, our study demonstrated that noncitrullinated ENA-78/CXCL5 recruited more monocyte/macrophages in vivo compared with PBS. In general, ENA-78/CXCL5 recruits neutrophils in vitro and in vivo. However, it is possible that other types of cells besides neutrophils respond to ENA-78/CXCL5 in vivo. It has been reported that macrophages, which are either inflammatory monocyte-derived cells or reemerging tissue-resident macrophages, increase in number during acute inflammatory responses (51). It is quite possible that the number of monocyte/macrophages increases in vivo during ENA-78/CXCL5–induced acute inflammation.

In conclusion, citrullinated ENA-78/CXCL5 was detected in RA SF and sera. The concentrations were significantly higher in SF from patients with RA than in SF from patients with OA or other inflammatory rheumatic diseases and correlated with the CRP level and the ESR. Citrullinated ENA-78/CXCL5 induced monocyte migration via CXCR1 and CXCR2 in vitro, while noncitrullinated ENA-78/CXCL5 did not. Mouse knees injected with citrullinated ENA-78/CXCL5 had more inflammation and monocyte/macrophages than did those injected with noncitrullinated ENA-78/CXCL5, suggesting that citrullination enhances the proinflammatory activity of ENA-78/CXCL5 and accelerates disease progression in inflammatory arthritis.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Yoshida had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study conception and design. Yoshida, Korchynskyi, Tak, Ruth, Campbell, Amin, Koch.
Acquisition of data. Yoshida, Korchynskyi, Isozaki, Baeten, Gerlag, Amin.
Analysis and interpretation of data. Yoshida, Korchynskyi, Ruth, Campbell, Koch.

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