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Citrullination of CXCL8 by peptidylarginine deiminase alters receptor usage, prevents proteolysis, and dampens tissue inflammation

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Biological functions of proteins are influenced by posttranslational modifications such as on/off switching by phosphorylation and modulation by glycosylation. Proteolytic processing regulates cytokine and chemokine activities. In this study, we report that natural posttranslational citrullination or deimination alters the biological activities of the neutrophil chemoattractant and angiogenic cytokine CXCL8/interleukin-8 (IL-8). Citrullination of arginine in position 5 was discovered on 14% of natural leukocyte-derived CXCL8(1–77), generating CXCL8(1–77)Cit$_5$. Peptidylarginine deiminase (PAD) is known to citrullinate structural proteins, and it may initiate autoimmune diseases. PAD efficiently and site-specifically citrullinated CXCL5, CXCL8, CCL17, CCL26, but not IL-1β. In comparison with CXCL8(1–77), CXCL8(1–77)Cit$_5$ had reduced affinity for glycosaminoglycans and induced less CXCR2-dependent calcium signaling and extracellular signal–regulated kinase 1/2 phosphorylation. In contrast to CXCL8(1–77), CXCL8(1–77)Cit$_5$ was resistant to thrombin- or plasmin–dependent potentiation into CXCL8(6–77). Upon intraperitoneal injection, CXCL8(6–77) was a more potent inducer of neutrophil extravasation compared with CXCL8(1–77). Despite its retained chemotactic activity in vitro, CXCL8(1–77)Cit$_5$ was unable to attract neutrophils to the peritoneum. Finally, in the rabbit cornea angiogenesis assay, the equally potent CXCL8(1–77) and CXCL8(1–77)Cit$_5$ were less efficient angiogenic molecules than CXCL8(6–77). This study shows that PAD citrullinates the chemokine CXCL8, and thus may dampen neutrophil extravasation during acute or chronic inflammation.

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Abbreviations: CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; CXCR, CXC chemokine receptor; ERK, extracellular signal-regulated kinase; GAG, glycosaminoglycan; HEK, human embryonic kidney; MBP, myelin basic protein; MMP, matrix metalloproteinase; MS, multiple sclerosis; PAD, peptidylarginine deiminase; PTH, phenyl thiohydantoin; RA, rheumatoid arthritis; RP-HPLC, reversed phase HPLC.

Chemokines are a family of small secreted proteins that activate and attract leukocytes during inflammation, but also play an important role in normal leukocyte trafficking, including lymphocyte homing. Chemokines exhibit affinity for seven transmembrane-spanning G protein–coupled signaling receptors and extracellular matrix or cell-bound glycosaminoglycans (GAGs). These chemotactic cytokines contain conserved cysteine residues in their amino (NH$_2$)-terminal structure, a characteristic used for classification into CXC, CC, CX$_3$C, and C chemokines (1, 2). CXCL8 (IL-8), which contains the tripeptide Glu-Leu-Arg (ELR) in front of the first Cys residue, is an inflammatory CXC chemokine with potent neutrophil chemotactic and angiogenic properties (3–6). CXCL8 promotes in vivo activation and recruitment of neutrophil granulocytes through the chemokine receptors 1 and 2 (CXCR1 and CXCR2) (7, 8).

Chemokine activity is controlled at different levels, including regulation of chemokine and chemokine receptor expression, the presence of "silent" or "decoy" chemokine receptors, binding to GAG, and posttranslational modification (9–13). Leukocytes have been

reported to produce a mixture of proteolytically modified forms of CXCL8, which are derived from secreted intact CXCL8, i.e., CXCL8(1–77) (6, 14). Limited N-terminal truncation by proteases such as thrombin, plasmin, and matrix metalloproteinases (MMPs) potentiates the in vitro CXCL8 activities (15–18). However, cleavage in or beyond the ELR motif abrogates CXCL8 activity (19). In vivo, no significant difference in neutrophil accumulation or plasma protein exudation was observed between CXCL8(1–77) and CXCL8(6–77) upon intradermal injection in rabbits or intra–air pouch administration in mice (17, 20). This apparent contradiction between in vitro and in vivo migration experiments may be explained by the rapid processing of CXCL8(1–77) in vivo, as indicated by studies with $Mmp8^{-/-}$ mice (17).

In an attempt to better understand such discrepancies, we studied alternatively modified chemokines and discovered a novel natural posttranslational modification of CXCL8, i.e., the conversion of one specific Arg into Cit. The enzyme responsible for this modification is peptidylarginine deiminase (PAD), for which a genetic association with rheumatoid arthritis (RA) has been uncovered (21). In addition, autoantibodies to citrullinated proteins are more specific markers for RA than IgM rheumatoid factor and are detected in most of the patients (22). Diagnostically, such autoantibodies were also shown to precede disease onset, implying the possibility of an early ontogenic immunological response toward citrullinated protein epitopes (23).

In this study, natural citrullinated CXCL8 was identified, and the effects of such citrullination were evidenced on the in vitro and in vivo activities of CXCL8. In contrast to N-terminal processing of CXCL8, the biological consequences of citrullination were more pronounced in neutrophil extravasation than in angiogenesis. These findings demonstrate a novel and important additional regulation of chemokine activity in inflammation. Citrullination affects the migration of neutrophils, the most abundant leukocyte type in human blood. Moreover, this study indicates that PAD directly modulates immune reactions through the modification of chemokines.

RESULTS
Identification of naturally citrullinated isoforms of CXCL8
Purified PBMCs from pooled buffy coats were induced at 5×10^6 cells/ml with 10 µg/ml polyriboinosinic:polyribocytidylic acid and 20 ng/ml IFN-γ in RPMI-1640 containing 2% FBS for chemokine production (24). Natural CXCL8 was purified from the conditioned medium by adsorption to controlled pore glass, heparin affinity, Mono S cation exchange, and C8 reversed phase HPLC (RP-HPLC), as previously described (6, 25, 26). In the column fractions, 11 different CXCL8 isoforms were detected by specific ELISA and identified by both mass spectrometry and amino acid sequencing using Edman degradation (Table I and Table S1, available at http://www.jem.org/cgi/content/full/jem.20080305/DC1). Although part of the PBMC-derived CXCL8 was truncated by five or more amino acids, many fractions contained intact CXCL8, i.e., CXCL8(1–77) and a CXCL8 isoform with two extra

N-terminal residues, CXCL8(-2–77), which is probably the result of an alternative cleavage of the signal peptide. Because of their instability in the Edman chemistry, the phenyl thiohydantoin (PTH)-Cys residues at positions 12 and 14 of CXCL8(1–77) (indicated as X) were not detected after Edman degradation and subsequent RP-HPLC. Remarkably, in some fractions, the PTH-Arg at position 5 (PTH-Arg_5) in the sequence of natural CXCL8(1–77) was also not identified (indicated as B), whereas the PTH-Arg_{11} present in the ELR motif was always detected (sequence AVLPBSAKEL-RXQXIK). In contrast, recombinant CXCL8(1–77) clearly showed the consistent presence of this PTH-Arg_5, whereas the PTH-Cys remained undetectable upon sequence analysis. Instead of a PTH-Arg_5 peak, in natural CXCL8(1–77) an unidentified compound eluted from the RP-HPLC column of the protein sequencer in between PTH-Thr and PTH-Gly (unpublished data). On classical, noncapillary protein sequencers with an HPLC column with a diameter of 2.1 mm instead of 0.8 mm, the unidentified residue could not be detected because it coeluted with the only partially separated PTH-Thr and PTH-Gly peaks. The high background signals of PTH-Thr and PTH-Gly and the weak signal for PTH-Arg on these noncapillary protein sequencers also explain why this modification was not reported before. In addition, the experimentally determined average M_r of 8,917.1–8,919 (Table S1) of natural CXCL8(1–77) with the observed modification of Arg on position 5 did not significantly differ from the theoretical M_r (8,918.44) of CXCL8(1–77). Based on these mass spectrometry and Edman degradation data, the difference between natural and recombinant CXCL8 could not be explained by a mutation of Arg to one of the other 19 classical amino acids. Therefore, amino acids generated by posttranslational modification of Arg were considered.

Table I. Identification of natural CXCL8 by Edman degradation and mass spectrometry

CXCL8 isoform	NH$_2$–terminal sequence[a]	Percentage of total amount of CXCL8[b]
	−2 1 5 10	
CXCL8(-2–77)	EGAVLPRSAKELRXQX	7.8
CXCL8(-2–77)Cit$_5$	EGAVLPBSAKELRXQX	0.7
CXCL8(1–77)	AVLPRSAKELRXQX	25.9
CXCL8(1–77)Cit$_5$	AVLPBSAKELRXQX	4.1
CXCL8(2–77)	VLPRSAKELRXQX	5.5
CXCL8(2–77)Cit$_5$	VLPBSAKELRXQX	0.7
CXCL8(3–77)	LPRSAKELRXQX	1.1
CXCL8(6–77)	SAKELRXQX	44.1
CXCL8(8–77)	KELRXQX	9.0
CXCL8(9–77)	ELRXQX	1.2

[a]X stands for an unidentified residue (on Cys positions) and B stands for citrulline. Numbering corresponds to the position of the amino acids in CXCL8(1-77).
[b]The relative amount of the different CXCL8 isoforms was obtained from the yields for the different amino acids during Edman degradation on >20 RP-HPLC fractions from a large batch of leukocytes from pooled blood donations (Table S1, available at http://www.jem.org/cgi/content/full/jem.20080305/DC1).

The only known side chain alteration on Arg that results in a minimal change in M_r (one mass unit) and has been reported to occur in some structural proteins is the modification of Arg into Cit (Fig. 1 A). Therefore, L-citrulline was loaded on the filter of the protein sequencer, and after one Edman degradation cycle the PTH-derivative was detected by RP-HPLC. PTH-Cit eluted at exactly the same position as the unidentified amino acid on position 5 in natural CXCL8(1–77), i.e., in between PTH-Thr and PTH-Gly (unpublished data). This confirms that the fifth amino acid in part of the natural PBMC-derived CXCL8(1–77) is posttranslationally modified from Arg into Cit. Because the experimentally determined M_r of natural CXCL8(1–77) did not significantly differ from the theoretical M_r and PTH-Arg_{11} was routinely detectable, it is expected that other Arg residues present in the CXCL8 structure are not or less efficiently converted to Cit. Simultaneous replacement of all Arg residues by Cit was excluded based on the mass spectrometry data on >20 fractions containing CXCL8 immunoreactivity from 6 different C8 RP-HPLC columns (Table S1). The relative amount of CXCL8(1–77) with Cit_5 instead of Arg_5 per RP-HPLC

fraction varied between 0 and 100%, as indicated by Edman degradation on all individual fractions. Moreover, citrullination of this Arg was also detected in the less abundant isoforms CXCL8(-2–77) and CXCL8(2–77). Arg_{11} of natural CXCL8(1–77) was not modified in any of these RP-HPLC fractions. Overall, 14% of natural CXCL8(1–77) and 5.5% of total natural CXCL8 from PBMC was citrullinated on position 5. The percentage of Cit_5 was calculated from the yield of the fifth cycle of the Edman chemistry. A detailed analysis indicated that CXCL8(1–77) and CXCL8(1–77)Cit_5 were only partially separated on heparin affinity, cation exchange, and C8 RP-HPLC. Compared with CXCL8(1–77), CXCL8(1–77)Cit_5 eluted at slightly lower NaCl concentrations from the heparin affinity and cation exchange column and at faintly higher acetonitrile concentrations from the C8 RP-HPLC column (Table S1). Because Cit and Arg differ in one mass unit only, the experimentally determined mass could not be used as a criterion to predict the percentage of citrullination. This also indicates that in body fluids, citrullination of CXCL8 cannot be detected at present by the proteomic approach using ion trap mass spectrometry, and that natural proteins need to be purified to homogeneity before identification of this modification by Edman degradation.
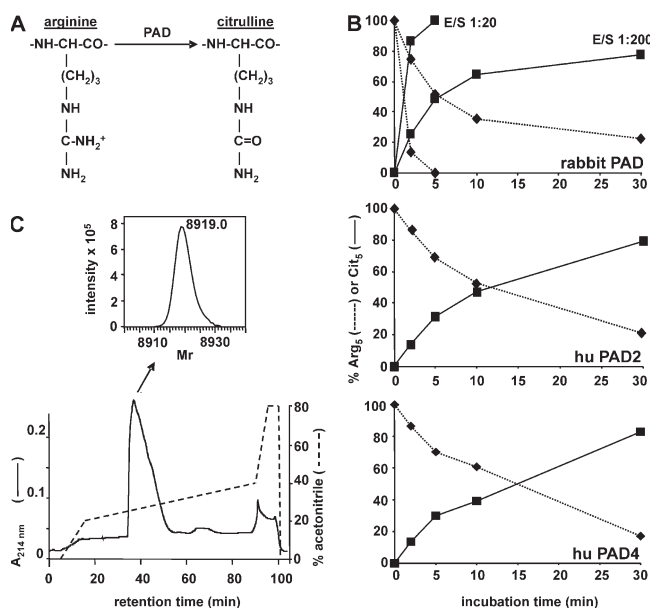
Citrullination of CXCL8 by PAD

The PAD enzymes catalyze the posttranslational hydrolysis of the guanidino group of Arg in proteins, resulting in a Cit in the primary structure of these proteins (Fig. 1 A). The purity of the commercial rabbit PAD preparation was verified by SDS-PAGE. Two bands were visible after proteins were blotted on PVDF membranes and stained with Coomassie blue, one corresponding to BSA and the other to PAD, as indicated by Edman degradation (unpublished data). Recombinant CXCL8(1–77) was incubated with PAD at a 1:20 or 1:200 enzyme/substrate (E/S) molar ratio. Amino acid sequencing revealed that rabbit PAD converted the Arg on position 5 (Arg_5) into Cit within 30 min at a 1:200 E/S molar ratio (Fig. 1 B). As indicated by both Edman degradation and mass spectrometry analysis, only this N-terminally located Arg_5 and no other Arg residues were citrullinated. At a 1:20 E/S molar ratio, the conversion of Arg_5 occurred more rapidly, and only Cit_5 and Arg_{11}, but neither Arg_5 nor Cit_{11} were detectable after 5 min (Fig. 1 B and not depicted). Although 1 mM dithiothreitol is reported to be added in most incubations of proteins with PAD (27–30), dithiothreitol was not essential for the conversion of Arg_5 in CXCL8(1–77) into a Cit, indicating that this Arg is highly accessible to the enzyme without partial disruption of the secondary structure of the protein (unpublished data). Recently, recombinant human PAD2 and PAD4 became commercially available. At a 1:200 E/S molar ratio and without addition of reducing reagents CXCL8(1–77) was found to be citrullinated by both human PAD2 and PAD4, with a half life for Arg_5 of 12 and 15 min, respectively (Fig. 1 B). Thus, both human enzymes converted CXCL8(1–77) into CXCL8(1–77)Cit_5 with an

Figure 1. Modification of CXCL8 by PAD and production of pure citrullinated CXCL8(1–77)Cit₅. (A) Arginine residues that are incorporated in protein sequences may be converted into citrulline by PAD. (B) Recombinant CXCL8 (5 μM) was incubated with rabbit PAD, human PAD2, or human PAD4 at an enzyme/substrate (E/S) molar ratio of 1:20 or 1:200 for different time periods. The presence of Arg (◆) or Cit (■) at position 5 was determined by Edman degradation. The percentage of Arg₅ or Cit₅ in the sequence was calculated from the amount of PTH-Arg and PTH-Cit that was detected by RP-HPLC after Edman degradation for five cycles. (C) Alternatively, recombinant CXCL8 was incubated for 90 min with PAD at an E/S molar ratio of 1:20, purified by C8 RP-HPLC, and eluted in an acetonitrile gradient, and UV absorption was detected at 214 nm. Part of the column effluent (0.67%) was connected online to an ion trap mass spectrometer, and the averaged spectra for the chromatographic peaks were deconvoluted to obtain the M_r of the proteins (top graph).

efficiency comparable to rabbit PAD, also without modification of Arg_{11} in the CXCL8 sequence. To investigate whether the citrullination was cytokine specific, recombinant IL-1β was incubated with PAD at a 1:20 E/S ratio, desalted, and subjected to Edman degradation. The Arg at position 4 in the protein sequence of mature biologically active IL-1β has been reported to be involved in receptor interactions (31). However, no citrullination was observed on either of the first two Arg of IL-1β (positions 4 and 11 in the protein sequence of mature IL-1β; unpublished data). Moreover, on ion trap mass spectrometry an average M_r for PAD-treated IL-1β of 17,375 was detected, which is comparable to its theoretical average M_r of 17,377. Although PAD was not able to citrullinate IL-1β, incubation of other chemokines with an Arg present in their N-terminal region (CXCL5, CCL17, and CCL26) was done with rabbit PAD, human PAD2, or PAD4. This resulted at the best tested E/S ratios and incubation times in 90–100% citrullination of the first N-terminally located Arg, as indicated by Edman degradation (Table II). However, CXCL5 and CCL26 were citrullinated more slowly compared with CXCL8. Human PAD4, but not human PAD2, citrullinated the first N-terminal Arg in CCL17 with an efficiency comparable to that in CXCL8. Moreover, the second Arg in CCL17 was also partially citrullinated by human PAD4, as well as by PAD2. In analogy with CXCL8, no conversion of the second and only other Arg was detected in CXCL5, even with 10-fold higher enzyme concentrations. In conclusion, citrullination does not appear to be a general phenomenon for all cytokines, and in addition occurs preferentially on specific positions such as Arg_5 in the CXCL8 sequence, which is in accordance with the citrullination of natural CXCL8 (vide supra). To exclude that the PAD activity in the leukocyte cultures originated from the FBS in the culture medium, FBS was tested after standard inactivation for 30 min at 56°C in a recently developed PAD activity assay (32).

PAD activity was not detected in either of three commercial FBS sources, confirming that the leukocytes were the actual source of the enzymatic activity (unpublished data).

To obtain sufficient amounts of pure citrullinated chemokine for bioassays, recombinant CXCL8(1–77) was incubated with rabbit PAD at a 1:20 E/S molar ratio for 90 min and purified by C8 RP-HPLC. Modified CXCL8 eluted in one major peak from the column with a M_r of 8,919.0, corresponding to the theoretical M_r of 8,919.4 (Fig. 1 C). The presence of Cit_5 and Arg_{11} was confirmed by Edman degradation. No CXCL8 that contained a remaining Arg_5 was detected in this preparation (unpublished data).

Effect of citrullination on the N–terminal processing of CXCL8 by proteases

CXCL8(1–77) produced by PBMCs is known to be N-terminally truncated into CXCL8(6–77) by thrombin and plasmin (15, 16). Moreover, this truncation has a significant impact on the receptor-dependent signaling and in vitro chemotactic activity of this chemokine. Because the major cleavage site in CXCL8 for the serine proteases thrombin and plasmin is located between Arg_5 and Ser_6, the effect of posttranslational modification of Arg_5 to Cit on the sensitivity of CXCL8 to both of these proteases was investigated. As expected, recombinant intact CXCL8(1–77) was almost completely converted into CXCL8(6–77) by thrombin within 5 min at an E/S ratio of 1:100 as detected by Edman degradation, ion trap mass spectrometry, and SDS-PAGE analysis (Fig. 2 A and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20080305/DC1). Also, plasmin was able to cleave CXCL8 (E/S ratio of 1:100), but plasmin was less proficient than thrombin because only 60% of CXCL8(1–77) was processed within 0.5 h (Fig. 2 B and Fig. S2). In contrast to CXCL8(1–77), CXCL8(1–77)Cit_5 was resistant to thrombin, as shown by Edman degradation, and it was also cleaved at a

Table II. PAD incubation of CXC and CC chemokines

Chemokine	NH₂-terminal sequence[a]	Enzyme	E/S[b]	Incubation time[c]	Percentage first Cit[d]	Percentage second Cit[d]	M_r shift[e]
CXCL5	AGPAAAVL**R**EL**R**CVC-	rabbit PAD	1/20	90	89.8	0	+ 1.0
		hu PAD2	1/200	120	14.0	0	+ 0.4
		hu PAD4	1/200	120	35.1	0	+ 0.6
CXCL8	AVLP**R**SAKEL**R**CQC-	rabbit PAD	1/20	90	100	0	+ 1.1
		hu PAD2	1/200	120	88.0	0	ND
		hu PAD4	1/200	120	94.5	0	+ 1.0
CCL17	A**R**GTNVG**R**ECC-	hu PAD2	1/200	120	24.8	22.6	+ 0.8
		hu PAD4	1/200	120	92.1	54.3	+ 2.4
CCL26	T**R**GSDISKTCC-	hu PAD	1/200	120	17.1	ND	+ 0.2
		hu PAD4	1/200	120	17.8	ND	+ 0.2
		hu PAD4	1/40	120	100	ND	+ 1.3

[a]NH₂-terminal Arg residues are indicated in bold, cysteine motifs are underlined.
[b]Molar Enzyme/Substrate ratio.
[c]Incubation time in minutes.
[d]Percentages of conversion of NH₂-terminal Arg residues to Cit were determined by Edman degradation.
[e]Shift compared to the theoretical average M_r of the uncitrullinated chemokine as determined by ion trap mass spectrometry.

much lower rate with plasmin. Indeed, a peptide smaller than CXCL8(1–77) appeared on the gel upon incubation of CXCL8(1–77)Cit$_5$ with plasmin for 1 h or more (Fig. 2 B). However, plasmin did not cleave CXCL8(1–77)Cit$_5$ (theoretical M_r 8,919.5) into CXCL8(6–77) (theoretical M_r 8,381.8), but rather into CXCL8(9–77) (theoretical M_r

Figure 2. Sensitivity of citrullinated CXCL8 to thrombin and plasmin cleavage. Recombinant CXCL8(1–77) and CXCL8(1–77)Cit$_5$ were incubated with thrombin (A) or plasmin (B) for different time periods at an enzyme/substrate molar ratio of 1:100. SDS-PAGE was performed under reducing conditions on Tris/tricine gels and proteins were visualized by silver staining (25). The bovine trypsin inhibitor (M_r 6,200) is visible as the M_r marker (arrows). Edman degradation and ion trap mass spectrometry analysis were used to calculate the percentage of conversion of CXCL8(1–77) or CXCL8(1–77)Cit$_5$ (black) into CXCL8(6–77) (white) or CXCL8(9–77) (dashed).

8,095.5; Fig. S2) and upon prolonged incubation also into CXCL8(9–72) as indicated by mass spectrometry (unpublished data). It can be concluded that Arg$_5$ citrullination in intact CXCL8(1–77) protects this chemokine from being processed rapidly into the more active inflammatory form, i.e., CXCL8(6–77).

Effect of citrullination on CXCR1, CXCR2, and GAG binding properties of CXCL8

Cells transfected with the two high-affinity CXCL8 receptors, CXCR1 and CXCR2, were used to compare the binding efficiency of CXCL8 isoforms. CXCL8(6–77) competed more efficiently than CXCL8(1–77) for binding of iodinated CXCL8(6–77) to CXCR1 (Fig. 3 A). In addition, CXCL8(1–77)Cit$_5$ was a more potent competitor for CXCL8 binding to CXCR1 compared with CXCL8(1–77). In contrast, on cells transfected with CXCR2, citrullination of Arg$_5$ did not affect the binding efficiency (Fig. 3 B). Removal of the five N-terminal amino acids resulted in increased CXCL8 binding to CXCR2. This indicates that the conversion of the positively charged Arg into a neutral Cit on the fifth position in CXCL8(1–77) enhances the binding efficiency of this chemokine on CXCR1, but not on CXCR2.

In addition to binding to seven-transmembrane spanning G protein-coupled receptors, CXCL8 interaction with GAG is important for in vivo biological activity (9, 33). Because chemokine binding to GAG primarily depends on positively charged amino acids, and because citrullination induces a reduction of the chemokine charge, the binding of CXCL8(1–77), CXCL8(1–77)Cit$_5$, and CXCL8(6–77) to GAG was compared on EpranEx plates treated with heparin (Fig. 3 C) or heparan sulfate (Fig. 3 D). Although the first Arg in the CXCL8 sequence is missing in CXCL8(6–77), CXCL8(1–77) and CXCL8(6–77) had a comparable affinity for heparin and heparan sulfate. In contrast to truncation, citrullination of the first Arg provoked reduced binding properties on heparin and heparan sulfate. Approximately threefold higher concentrations of CXCL8(1–77)Cit$_5$ compared with CXCL8(1–77) and CXCL8(6–77) were required to obtain an equal effect in the GAG binding assays.

Effect of citrullination on receptor signaling and in vitro chemotactic properties of CXCL8

In the calcium signaling assay on neutrophils, CXCL8(1–77) and CXCL8(1–77)Cit$_5$ provoked a comparable increase of the $[Ca^{2+}]_i$. As expected, CXCL8(6–77) was fivefold more potent (Fig. 4 A). In addition, CXCL8(6–77) was 5- to 10-fold more potent in comparison with intact, unmodified, and citrullinated CXCL8 at desensitizing CXCL8(1–77) (Fig. 4 B). The signaling capacity of CXCL8(1–77)Cit$_5$ was also evaluated on receptor-transfected cells. Despite its higher affinity for CXCR1, CXCL8(1–77)Cit$_5$ provoked a comparable calcium signal and was equally potent to desensitize CXCR1 compared with CXCL8(1–77) (Fig. 4, C and D). However, on CXCR2-transfected cells, CXCL8(1–77)Cit$_5$ was about twofold less potent in comparison with unmodified
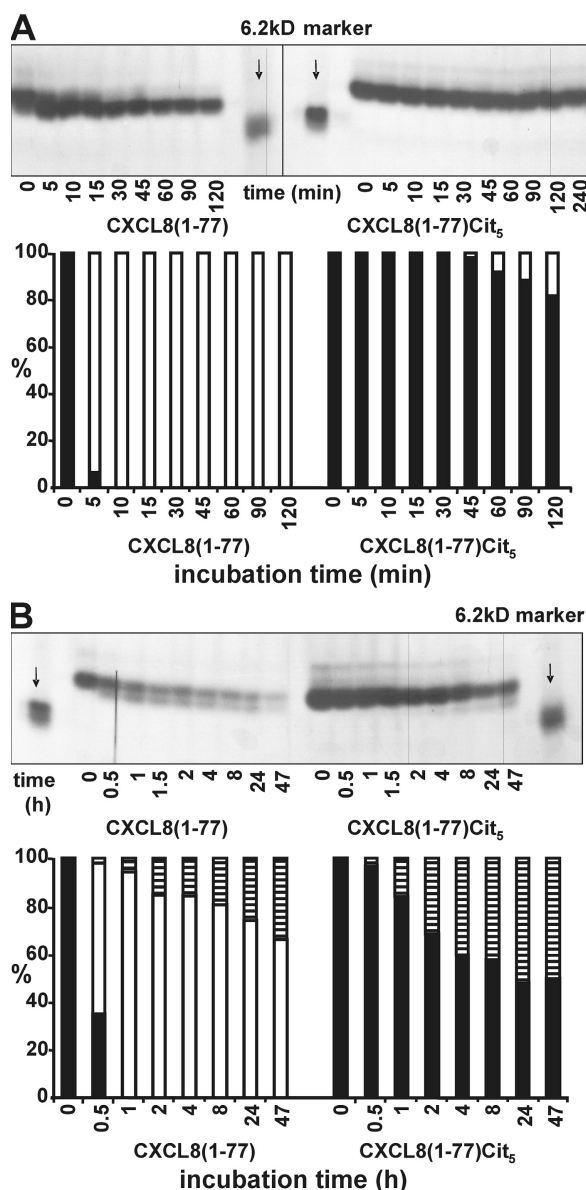
CXCL8(1–77) in provoking a calcium release and in desensitizing CXCR2 (Fig. 4, E and F).

Further investigation on CXCR2 signaling was performed by measuring the amount of phosphorylated extracellular signal–regulated kinase 1/2 (ERK1/2). Truncated CXCL8(6–77), intact CXCL8(1–77), and CXCL8(1–77)Cit$_5$ provoked significant phosphorylation of ERK1/2 at a concentration of 10 nM (Fig. 5). ERK1/2 phosphorylation induced by CXCL8(1–77)Cit$_5$ was significantly lower than CXCL8(6–77)–induced ERK1/2 phosphorylation after 5, 10, and 20 min of stimulation, whereas CXCL8(1–77)-induced stimulation only differed from CXCL8(6–77) after 5 min. A significantly more pronounced ERK1/2 phosphorylation with CXCL8(1–77) compared with citrullinated CXCL8(1–77)Cit$_5$ was also observed after stimulating the cells for 20 min (Fig. 5). Thus, both the calcium and ERK1/2 signaling assays on CXCR2-transfected cells indicate that CXCL8(1–77)Cit$_5$ is weaker than authentic CXCL8(1–77). It must be concluded that citrullination of CXCL8 results in a CXCR2-specific reduction of its in vitro signaling potency.

Authentic CXCL8(1–77), CXCL8(1–77)Cit$_5$, and truncated CXCL8(6–77) were compared for their ability to attract neutrophils in the Boyden chamber assay. Although the chemotactic response to CXCL8(1–77) was moderately higher than to CXCL8(1–77)Cit$_5$, no statistically significant difference in activity was detected (Fig. 6). In contrast, CXCL8(6–77) provoked a significantly higher chemotactic response in comparison with CXCL8(1–77)Cit$_5$ and CXCL8(1–77). Truncated

CXCL8(6–77) reached its maximal chemotactic activity at a concentration of 1 nM, which was 10-fold lower than the optimal concentration of CXCL8(1–77) and CXCL8(1–77)Cit$_5$.

Collectively, reduced in vitro CXCR2 signaling and GAG binding by citrullination of CXCL8 and protection of CXCL8(1–77)Cit$_5$ from proteolytic cleavage into the most active CXCL8(6–77) would allow us to speculate that in vivo neutrophils are less activated to migrate in response to CXCL8(1–77)Cit$_5$ than to CXCL8(1–77). In addition, CXCL8 and other CXC chemokines with an ELR motif were reported to have angiogenic properties (5, 34). Therefore, we compared these CXCL8 forms using in vivo assays for angiogenesis and leukocyte infiltration.

Effect of citrullination on the angiogenic properties of CXCL8 after local application in vivo

Because the angiogenic activity of the different CXCL8 forms was never compared in vivo, Hydron pellets containing different amounts of CXCL8(1–77), CXCL8(1–77)Cit$_5$, or CXCL8(6–77) were implanted into corneal micropockets in rabbits. Maximal neovascularization occurred between days 5 and 7 after implantation. At 3 pmol, CXCL8(1–77) and CXCL8(1–77)Cit$_5$ induced significant angiogenesis in comparison with control pellets, whereas CXCL8(6–77) already provoked angiogenesis at 0.3 pmol (Fig. 7 A). At 1 pmol, CXCL8(6–77) induced maximal angiogenic activity with a significantly higher score compared with CXCL8(1–77) ($P < 0.05$) and CXCL8(1–77)Cit$_5$ ($P < 0.01$). No variation in
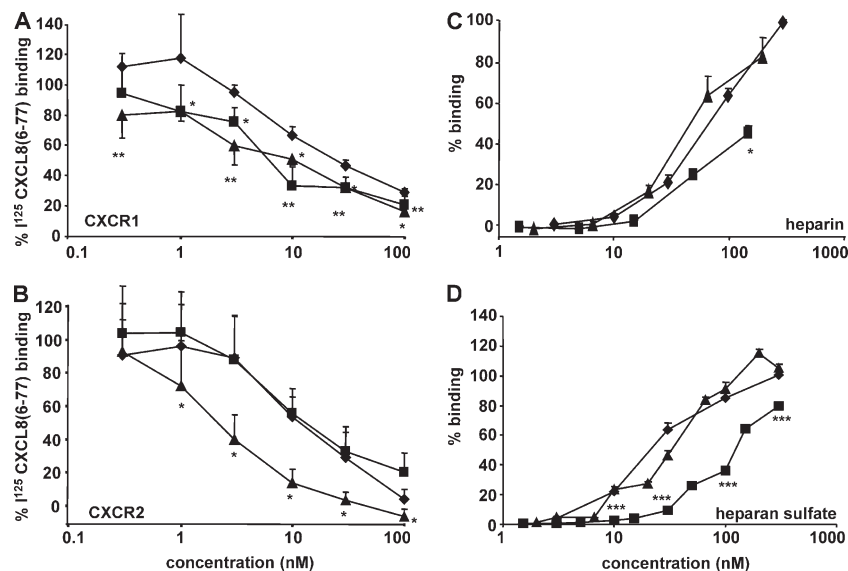

Figure 3. Effect of citrullination on the receptor and GAG binding properties of CXCL8. Increasing concentrations of unlabeled chemokine were added together with ^{125}I-CXCL8 to HEK293 cells transfected with CXCR1 (A) or CXCR2 (B). Results represent the mean percentage ($\pm$ the SEM) of remaining specific ^{125}I-CXCL8 binding ($n = 4$). Alternatively, GAG binding was evaluated by immobilizing low molecular weight heparin (C) or heparan sulfate (D) on EpranEx plates. CXCL8 isoforms bound to GAG were detected with biotinylated anti-CXCL8 antibodies and peroxidase-conjugated streptavidin. The optical densities obtained without addition of CXCL8 and with addition of 300 nM CXCL8(1–77) were set to 0 and 100%, respectively. Results represent the mean percentage of specific chemokine binding (six or more independent experiments) $\pm$ the SEM to heparin or heparan sulfate for human CXCL8(1–77) ($\blacklozenge$), CXCL8(6–77) ($\blacktriangle$), or CXCL8(1–77)Cit$_5$ ($\blacksquare$). Statistical differences between CXCL8(1–77) and CXCL8(1–77)Cit$_5$ or CXCL8(6–77) were detected using the Mann-Whitney U test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

neovascularization was observed between CXCL8(1–77) and CXCL8(1–77)Cit$_5$ despite the latter being weaker in CXCR2 signaling and GAG binding (Fig. 3, Fig. 4, and Fig. 7 A). However, the enhanced CXCR1 binding of CXCL8(1–77) Cit$_5$ may counteract this imbalance.

Effect of CXCL8 citrullination on neutrophil extravasation

To study the effect of CXCL8 citrullination on leukocyte emigration from the blood circulation into tissues, CXCL8(1–77), CXCL8(6–77), and CXCL8(1–77)Cit$_5$ were injected i.p. in mice. Mice were killed after 2 h (Fig. 7, B and C) or 4 h (not depicted) and leukocyte accumulation in the peritoneal cavity was evaluated. None of the three CXCL8 isoforms induced an increase in the number of peritoneal lymphocytes or macrophages (unpublished data). In this model, maximal accumulation of neutrophils in the peritoneum was obtained at 2 h after injection. CXCL8(6–77) was threefold more potent than CXCL8(1–77) to attract neutro-

Figure 5. Phosphorylation of ERK1/2 by CXCL8 forms. The amount of phosphorylated ERK1/2 (pg phospho ERK1/2 per mg total protein) was measured by a specific ELISA after stimulation of serum-starved CXCR2-transfected HEK293 cells for 5, 10, or 20 min with medium (co), 10 nM of CXCL8(1–77), CXCL8(6–77), or CXCL8(1–77)Cit$_5$. Results represent the percentage ERK1/2 phosphorylation (± the SEM) compared with medium-treated control cells (three to four independent experiments). Statistical analysis was performed using the Mann-Whitney U test on paired values (*, P < 0.05 for comparison with CXCL8[1–77]).

phils at 2 h after injection, confirming the observed in vitro difference in chemotactic potency between these two CXCL8 forms (Fig. 7, B and C). In contrast to authentic CXCL8(1–77), which is active at 30 pmol, up to 100 pmol of CXCL8(1–77)Cit$_5$ was still unable to induce an increase of the total number or the percentage of peritoneal neutrophils. Thus, despite the lack of an effect of citrullination on the in vitro chemotactic activity of CXCL8, citrullination of CXCL8 significantly reduced neutrophil extravasation to the peritoneal cavity. These findings are in agreement with reduced GAG binding, CXCR2 signaling, and resistance to N-terminal proteolytic potentiation. The inhibitory effect of citrullination
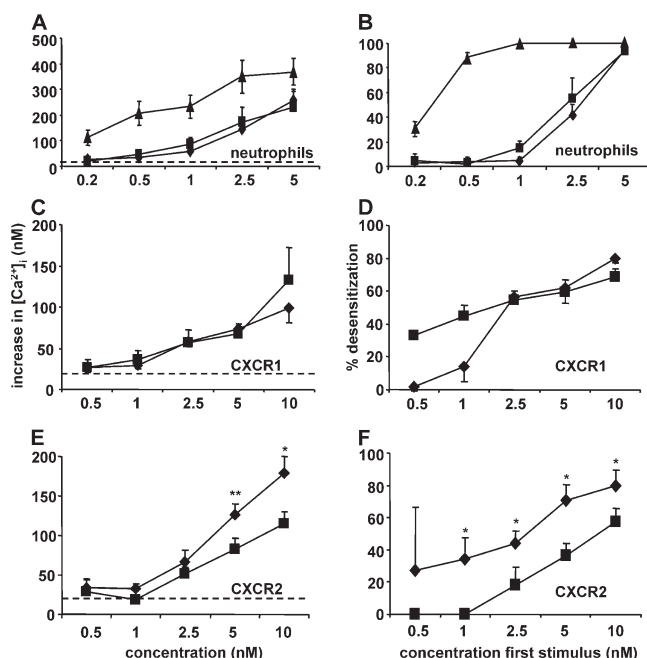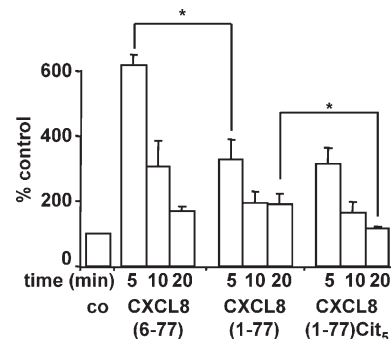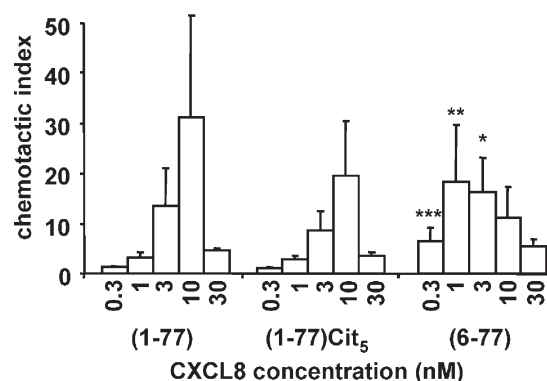
Figure 4. Calcium signaling capacity and receptor desensitization of citrullinated CXCL8 in neutrophils and HEK293 cells transfected with CXCR1 or CXCR2. The increase in intracellular calcium concentration ([Ca^{2+}]$_i$) in neutrophils and HEK293 cells transfected with CXCR1 or CXCR2 was measured using the ratiometric dye Fura-2. The cells were stimulated with CXCL8(1–77) (◆), CXCL8(6–77) (▲), or CXCL8(1–77)Cit$_5$ (■). Values represent the mean (± the SEM) increase of [Ca^{2+}]$_i$ (nM) with a detection limit at 20 nM (dotted line; neutrophils, $n = 5$ [A]; HEK-CXCR1, $n = 3$ [C]; HEK-CXCR2, $n = 5$ [E]). Desensitization experiments were performed by rechallenging the cells with 5 nM of CXCL8(1–77) 100 s after the first stimulus. Results (mean ± the SEM) represent the percentage of inhibition of the second agonist by the first stimulus in comparison with buffer as the first stimulus (neutrophils, $n = 5$ [B]; HEK-CXCR1, $n = 3$ [D]; HEK-CXCR2, $n = 5$ [F]). Significant differences were calculated using the Mann-Whitney U test on paired values (*, P < 0.05; **, P < 0.01 for comparison between CXCL8[1–77] and CXCL8[1–77]Cit$_5$).

Figure 6. In vitro neutrophil chemotactic activity of citrullinated CXCL8. The chemotactic activity of CXCL8(1–77), CXCL8(1–77)Cit$_5$, and CXCL8(6–77) for neutrophils was measured using a Boyden microchamber (four to six independent experiments). The chemotactic index (± the SEM) was calculated by dividing the number of migrated cells toward test samples by the number of spontaneously migrated cells toward buffer. Statistical analysis was performed using the Mann-Whitney U test on paired values (*, P < 0.05; **, P < 0.01; ***, P < 0.001 for comparison with the corresponding concentration of CXCL8[1–77]).
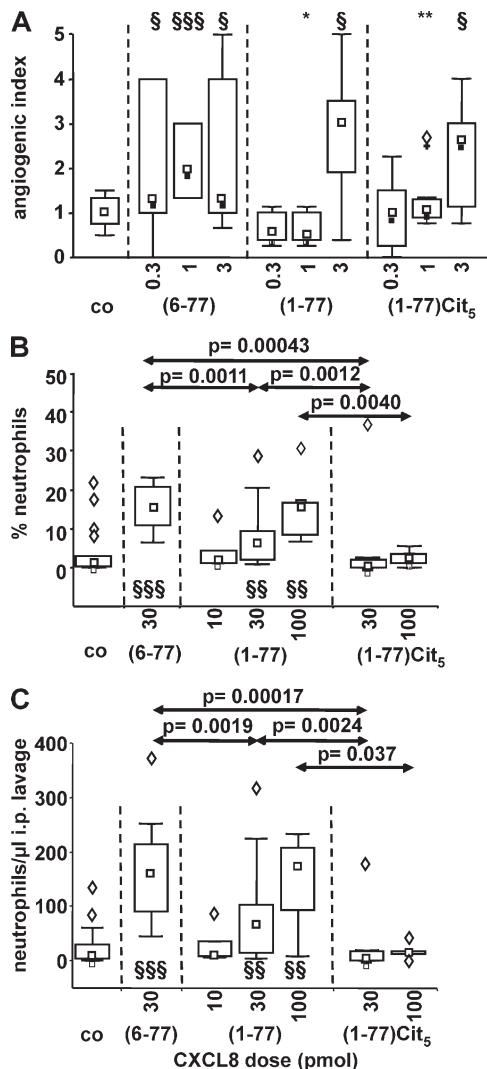
Figure 7. Effect of citrullination on CXCL8–induced angiogenesis and neutrophil extravasation in vivo. (A) Neovascularization induced by CXCL8(1–77), CXCL8(1–77)Cit$_5$, and CXCL8(6–77) was tested in a rabbit corneal micropocket. Angiogenesis was scored daily (score 0–4) from days 4 to 8. The angiogenic index was calculated by dividing the maximal neovascularization scores (occurring between days 5 and 7) by the spontaneous angiogenesis score obtained with PBS (co). (B and C) Induction of neutrophil infiltration was measured in NMRI mice by i.p. injection of 200 µl vehicle (0.9% NaCl = co), CXCL8(1–77), CXCL8(6–77), or CXCL8(1–77)Cit$_5$. After 2 h, mice were killed and the peritoneal cavity was washed. The total amount of leukocytes per microliter of peritoneal lavage solution was determined, and cytospins were stained with Hemacolor solutions to determine the percentage of neutrophils. The figures denote the median (squares), the 25–75% range (boxes), the nonoutlier range (whiskers), and the extreme values (◇) acquired from 5–9 replicates (eyes; A) or 12–21 mice (B and C) per CXCL8 dose. Statistical analysis was performed using the Mann-Whitney U test (§, $P < 0.05$; §§, $P < 0.01$; §§§, $P < 0.001$ for comparison with vehicle alone; *, $P < 0.05$; **, $P < 0.01$ for comparison with CXCL8[6–77]).

of CXCL8 on neutrophil recruitment in vivo was even more profound than the enhancing effect of N-terminal truncation, yielding a difference of at least 10-fold between CXCL8(1–77) and CXCL8(1–77)Cit$_5$.

DISCUSSION

During the past decade, posttranslational modification of chemokines has been reported to affect their in vitro and in vivo activities (11). Primarily N-terminal processing alters the receptor affinity and specific biological activity of chemokines. This includes minimal modification of an N-terminal Gln to pyroglutamic acid in the three monocyte chemotactic proteins, CCL2/MCP-1, CCL8/MCP-2, and CCL7/MCP-3, for which this pyroglutamic acid is essential for full biological activity (26, 35). Proteolytic processing of the N terminus of chemokines results in enhanced or reduced activity depending on the chemokine, protease, and degree of processing involved. In addition to the numerous naturally occurring forms of N-terminally truncated chemokines, a limited number of C-terminally processed chemokines have been identified (CCL2, CXCL7, and CXCL10) (36–38). Some chemokines, e.g., CCL2 and CCL11, may also be glycosylated (39, 40). Despite the significant increase in M$_r$, glycosylation only moderately (twofold) influences the in vitro activities of natural human CCL2 (38). N-terminal truncation of CXCL8 by thrombin, plasmin, MMP-8, or MMP-9 by five to eight amino acids has been reported to significantly increase its in vitro receptor signaling and chemotactic activity (15–18). C-terminal truncation of CXCL8 resulted in reduced heparin-binding properties (33). In addition to N-terminal proteolytic processing, this study reveals a novel biologically relevant enzymatic modification of Arg into citrulline (Cit) at position five of natural leukocyte-derived CXCL8.

The presence or absence of a Cit in natural CXCL8 was indicated by a combination of mass spectrometry and capillary protein sequencing (Table I). Purification of CXCL8 immunoreactivity from PBMC-conditioned medium to homogeneity by heparin affinity, cation exchange, and RP-HPLC revealed that 14% of CXCL8(1–77) and 5.5% of total CXCL8 was posttranslationally modified on Arg$_5$. However, it can be speculated that the amount of citrullinated CXCL8 produced by fibroblasts or endothelial cells in the synovial cavity of RA patients is substantially higher because CXCL8 produced by these cell types is predominantly not CXCL8(6–77) lacking Arg$_5$, but intact CXCL8 (6, 14, 41–45). Because citrullination alters the M$_r$ of proteins by only one mass unit, this posttranslational modification could not be detected by mass spectrometry alone and had to be indicated by capillary Edman degradation on purified proteins. At present, the requirement for extensively purified proteins is a significant handicap in the analysis of natural CXCL8 in complex samples such as body fluids of patients in which CXCL8 is present in only nanogram/milliliter concentrations within mixtures of milligram/milliliter contaminating proteins. Because all miniaturized protein purification techniques to analyze small volumes of complex samples that have been

developed during the last decade depend on identification of proteins by mass spectrometry, the detection of citrullinated CXCL8 in patient samples is hampered. Indeed, N-terminally truncated chemokine forms that differ in >100 mass units have been identified in body fluids of cancer patients by mass spectrometry (46). Furthermore, a good correlation with the in vitro occurrence of these truncated chemokine forms was observed.

Incubation of CXCL8 with rabbit PAD, human PAD2, and human PAD4 rapidly converted recombinant CXCL8(1–77) into $CXCL8(1-77)Cit_5$, indicating that in all likelihood this enzyme is responsible for the observed posttranslational modification of natural CXCL8 (Fig. 1). Deimination of an Arg to Cit, which results in the loss of one positive charge and in only a single mass unit increase of the M_r, is a known natural posttranslational modification. Citrullination has previously been observed for proteins such as vimentin, keratin, filaggrin, α-enolase, myelin basic protein (MBP), the protease inhibitor anti-thrombin, and some nuclear proteins, e.g., histones, nucleophosmin, and p300, but was never reported before for cytokines or chemokines (28, 30, 47–53). To date, five human genes have been described that code for PAD, i.e., *PADI1, 2, 3, 4* and *6* (54–57). PAD is differentially expressed in various organs depending on the isotype (58). Most PAD protein is present in the cytoplasm, although during inflammation PAD enzymes have been detected extracellularly (41) and the extracellular calcium concentration is sufficiently high for PAD activity and citrullination of extracellular proteins such as fibrin (59). PAD1 is primarily expressed in the uterus, and both PAD1 and PAD3 are found in the epidermis, where they citrullinate structural proteins such as keratin, filaggrin, and trichohyalin (58). Both PAD1 and PAD3 are absent in PBMCs (41, 60). PAD2 is known to process vimentin and MBP, and is widely expressed and highly abundant in skeletal muscle, brain, spleens, and secretory glands. In contrast to PAD1, PAD3, and PAD6 proteins, both PAD2 and PAD4 have been detected in hematopoietic cells. PAD4 expression is mainly restricted to leukocytes and in contrast to other PADs, PAD4 has been detected in the nucleus. The most recently identified PAD, PAD6, is found in male and female germ cells and is a crucial enzyme in fertility. The enzyme is essential for normal development of an embryo beyond the two-cell stage (54, 61).

Modification of Arg into Cit has been linked to autoimmune diseases such as RA and multiple sclerosis (MS) (27, 62, 63). Citrullination of proteins is even suggested to initiate the generation of autoimmune reactions. In serum of RA patients, citrullinated fibrin appeared to be the major target of the anti–citrulline peptide antibodies. A synthetic cyclic citrullinated peptide (CCP) construct is used nowadays as a diagnostic tool to distinguish RA from other arthritic disorders and possesses a highly predictive value for future development of RA in healthy individuals and patients with undifferentiated arthritis (22, 23). In addition to citrullination of structural proteins, increased levels of citrullinated anti-thrombin were detected in plasma of RA patients, which

could have consequences for angiogenic and inflammatory processes (64). Suzuki et al. reported that in Japanese population cohorts, SNPs in the human *PADI4* gene were strongly associated with susceptibility to RA, although this association was not found in studies that were conducted in the UK and France (21, 65, 66). Deimination of Arg was also reported in association with MS (62, 67). PAD2-dependent citrullination of MBP was suggested to play an important role in MS patients (68). Citrullination of MBP is increased in MS patients (69) and exposes immunodominant epitopes (70); it renders MBP more susceptible to cleavage with cathepsin D (29), and may therefore initiate loss of myelin stability.

This study reports natural and site-specific citrullination of a cytokine, i.e., the chemokine CXCL8 by rabbit PAD, human PAD2, and human PAD4. Moreover, another CXCR2 ligand, CXCL5, was also site-specifically citrullinated by these enzymes, but less rapidly than CXCL8. Two CC chemokines, CCL17 and CCL26, were also citrullinated by human PAD2 and PAD4 with different effectiveness. Citrullination of CCL17 with human PAD2 or PAD4 was slower compared with that of CXCL8. In contrast to the other chemokines tested, the first and the second N-terminally located Arg of CCL17 were both modified. In addition, CCL17 appeared to be the only substrate on which PAD4 was more efficient compared with PAD2 in posttranslationally modifying the Arg residues. In contrast to most other previously identified PAD substrates, which were primarily abundant structural proteins, chemokines directly affect immune functions and ELR+ CXC chemokines such as CXCL8 promote angiogenesis. Therefore, PAD-activity may not only interfere with autoimmunity in the long term through the stimulation of the production of anti-citrulline antibodies, but PAD may also directly alter immunological processes. Because cytokines such as IL-1β and TNF-α are important players in autoimmune diseases such as RA, the possible interaction of PAD with IL-1β was also investigated (71–73). The failure of PAD to convert Arg to Cit in IL-1β (with N-terminal arginines at positions comparable to those of CXCL8) shows that citrullination is not only a site-specific but also a cytokine-specific phenomenon. The rapid in vitro citrullination of CXCL8 and other chemokines by PAD without addition of reducing agents and the isolation of natural citrullinated $CXCL8(1-77)Cit_5$ from PBMCs both underscore the importance of this enzyme in chemokine biology. Actually, most of the in vitro incubations with PAD on other reported substrates were performed in the presence of reducing agents. These conditions may render Arg residues, which are protected by the three-dimensional structure of the protein, accessible for enzymatic processing (27–30). In vitro, $CXCL8(1-77)Cit_5$ and uncitrullinated CXCL8(1–77) were equally potent in neutrophil chemotaxis assays. The significantly enhanced CXCR1 binding, and the reduced CXCR2-dependent signaling efficiency, as detected on receptor-transfected cells, could account for this unaltered in vitro chemotactic activity (Figs. 3–6). However, in contrast to CXCL8(1–77), $CXCL8(1-77)Cit_5$ was resistant to cleavage

by thrombin into CXCL8(6–77) (Fig. 2). Conversion of CXCL8(1–77) into CXCL8(6–77) by thrombin rapidly occurs in inflamed tissues, and this proteolytic N-terminal truncation results in enhanced in vitro activity of CXCL8 (15). Depending on the cells and enzymes that are present in a particular in vivo setting, the increased stability of CXCL8(1–77)Cit_5 may thus affect the availability and hence chemotactic activity of this chemokine. In addition to the loss of the proteolytic cleavage site in CXCL8, citrullination of the first Arg also reduced the GAG-binding properties of this chemokine (Fig. 3). The combination of the enhanced stability against proteolytic activation, the reduced binding to GAG, and the reduced CXCR2 signaling capacity may explain the failure of CXCL8(1–77)Cit_5 to induce extravasation of neutrophils in vivo upon i.p. injection (Fig. 7). Indeed, CXCL8(1–77)Cit_5, unlike CXCL8(1–77), was devoid of neutrophil chemotactic activity when up to 100 pmol was injected i.p. In contrast, truncated CXCL8(6–77) had increased in vivo chemotactic activity compared with intact CXCL8(1–77) confirming the previously reported in vitro observations (15). Thus, citrullination of a single Arg in CXCL8 appears to dampen local neutrophil-driven inflammation, despite the retained in vitro chemotactic activity. Upon in vivo administration, chemokine–GAG interaction on the endothelial layer is required to maintain the chemotactic gradient. Reduced interaction with GAG may destabilize the gradient, partially explaining the reduced activity of CXCL8(1–77)Cit_5 to recruit neutrophils into the peritoneal cavity. Alternatively, it is possible that CXCR2 signaling is more important than CXCR1 binding for the extravasation of neutrophils in vivo.

In addition to its function as a neutrophil chemotactic and activating protein, CXCL8 also has potent angiogenic activity. In accordance with the in vitro and in vivo chemotactic activity on neutrophils, CXCL8(6–77) was more potent compared with intact CXCL8(1–77) in inducing neovascularization in the rabbit cornea assay (Fig. 7 A). However, no difference in angiogenic activity could be detected between CXCL8(1–77) and CXCL8(1–77)Cit_5. These findings suggest that the angiogenic activity of CXCL8 is primarily less dependent on CXCR2- and GAG-mediated pathways. However, the reduced GAG-binding properties may be compensated for by enhanced CXCR1 binding capacity and/or altered integrin-dependent adhesion (74). Alternatively, the angiogenic activity of CXCL8 may occur through different signal transduction mechanisms compared with those essential for transendothelial migration.

In summary, this study describes natural posttranslational citrullination of a cytokine, i.e., IL-8 or CXCL8 but not IL-1β, by PAD, an enzyme that is associated with the initiation of autoimmune diseases. Despite the minor structural changes in CXCL8 and the limited effects of PAD on the in vitro signaling potency and chemotactic activity of CXCL8, citrullination of CXCL8 results in a drastically reduced in vivo chemotactic activity in a model for local inflammation, while keeping full angiogenic activity. Thus, PAD may not only generate epitopes on structural proteins that cause autoimmune diseases but may initially function as an enzyme that dampens inflammation by reducing the local influx of neutrophils.

MATERIALS AND METHODS

Reagents and cells. Recombinant human chemokines and cytokines were obtained from PeproTech. Human thrombin (2,532 NIH U/mg) and plasmin (3–6 U/mg), PAD purified from rabbit skeletal muscle (200 U/mg), and double-stranded RNA polyriboinosinic:polyribocytidylic acid were purchased from Sigma-Aldrich. LPS from *Escherichia coli* 0111:B4 was obtained from Difco Laboratories. Recombinant human PAD2 and PAD4 and the antibody-based assay for PAD activity (ABAP kit) were purchased from MediQuest Research. Endotoxin concentrations were evaluated with the Limulus amoebocyte lysate test (Cambrex). Human embryonic kidney (HEK)293 cells transfected with CXCR1 or CXCR2 were a gift of J.M. Wang (National Cancer Institute, Frederick, MD) (75). PBMCs and granulocytes were purified from fresh human buffy coats (25). Granulocytes from individual donors were used for chemotaxis and calcium signaling experiments.

Purification and identification of natural chemokines. Natural human CXCL8 was purified using a four-step purification procedure (25). Proteins were eluted from the final reversed phase (RP-)HPLC column (2.1 × 220 mm Brownlee C-8 Aquapore RP-300 column; PerkinElmer) with an acetonitrile gradient in 0.1% TFA, detected at 214 nm, and 1/150 part of the column flow was split online to an electrospray ion trap mass spectrometer (Esquire LC). Averaged profile spectra were calculated over the CXCL8 containing fractions as measured by ELISA (76). The N-terminal sequence of proteins was determined on a 491 Procise cLC protein sequencer (Applied Biosystems).

In vitro citrullination and truncation of CXCL8. Proteins were incubated with PAD in 40 mM Tris-HCl, pH 7.4, and 2 mM $CaCl_2$ at 37°C. Deimination was stopped with 0.1% TFA and samples were desalted on C4 or C18 ZipTip (Millipore) before mass spectrometry or spotted on PVDF membranes (ProSorb; Applied Biosystems) before Edman degradation. For use in bioassays, citrullinated proteins were purified on a C8 Aquapore RP-300 HPLC column (1 × 50 mm).

Alternatively, CXCL8 was incubated with thrombin in PBS containing 1 mM $CaCl_2$ or with plasmin in 50 mM Tris, pH 7.4, at 37°C. Proteolysis was stopped by adding 0.1% TFA before Edman degradation on PVDF membranes or with 50 mM Tris-HCl, pH 6.8, 4% SDS, 12% glycerol, 2% 2-mercaptoethanol, and 0.019% Brilliant blue G, followed by heating for 5 min at 95°C before SDS-PAGE (25).

In vitro chemotaxis and signaling assays. Neutrophil chemotaxis was performed in Boyden microchambers (Neuro Probe) and the chemotactic index was calculated (25). Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were measured with the ratiometric fluorescent dye Fura-2/AM (Invitrogen) (77). Phosphorylation of ERK1/2 was determined using a specific ELISA for phospho-ERK1 and phospho-ERK2 (R&D Systems) (76).

Receptor and GAG binding assays. Competition for ^{125}I-labeled CXCL8 binding was measured on HEK293 cells transfected with CXCR1 or CXCR2 in binding buffer (50 mM Hepes, pH 7.2, containing 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.1% [wt/vol] bovine serum albumin). In brief, 2 × 10^6 cells were incubated for 2 h at 4°C with ^{125}I-CXCL8 (PerkinElmer) and unlabeled chemokine. Cells were centrifuged and washed three times with 2 ml of binding buffer supplemented with 0.5 M NaCl and the radioactivity was measured in a gamma counter (Hidex; Finland).

GAG binding was evaluated by immobilizing low molecular weight heparin or heparan sulfate (Sigma-Aldrich) on EpranEx plates (Plasso Technology, Ltd.) (78). In brief, 25 μg/ml of GAG diluted in PBS was coated overnight at room temperature on 96-well plates. Plates were washed three

times with assay buffer (100 mM NaCl, 50 mM NaAc, pH 7.2, and 0.2% [vol/vol] Tween-20) and blocked at 37°C with assay buffer enriched with 0.2% (wt/vol) gelatin or 1% (wt/vol) BSA for heparin and heparan sulfate binding, respectively. The captured CXCL8 was detected with biotinylated anti–human CXCL8 (PeproTech) and consecutively by peroxidase-conjugated streptavidin. Peroxidase activity was quantified by measuring the conversion of 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich) at 450 nm. Polyclonal biotinylated anti–human CXCL8 recognized all CXCL8 isoforms with equal affinity.

In vivo assays. Chemokine-containing Hydron pellets were implanted in corneal micropockets in rabbits (79). Maximal neovascularization obtained between days 5 and 7 after implantation was used for comparison.

Neutrophil mobilization into the peritoneal cavity was determined in NMRI female mice (Elevage Janvier) by i.p. injection (200 µl in saline) of pyrogen-free CXCL8 isoforms or saline (0.9% NaCl). After 2 or 4 h, the mice were killed and the peritoneal cavity was washed with 5 ml of saline enriched with 2% FBS and 20 U/ml heparin. The total amount of leukocytes in the peritoneal lavage was determined in duplicate. Cytospins were stained with Hemacolor solutions (Merck) for evaluation of the percentage of neutrophils by differential 100-cell counts in triplicate (80).

All animal studies were approved by the review board of the ethical committee of the K.U.Leuven, and experiments were performed according to Belgian and European legislation, including the Helsinki declaration.

Online supplemental material. Detailed information on the purification of natural PBMC-derived CXCL8 isoforms is included in Table S1. Mass spectra of CXCL8(1–77) and CXCL8(1–77)Cit$_5$ at early and later time points after treatment with thrombin or plasmin are shown in Fig. S1 and Fig. S2, respectively.

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