ORIGINAL ARTICLES

Citrullination of CXCL8 increases this chemokine's ability to mobilize neutrophils into the blood circulation

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ABSTRACT

Background
During the first line defense of an infected host, circulating neutrophils invade the inflamed tissue, whereas mature neutrophils from the bone marrow pool migrate into the blood circulation and from there reinforce tissue infiltration. The CXC chemokine CXCL8, also know as interleukin-8, is a potent attractant of neutrophils. Recently, we discovered a new natural post-translational modification of CXCL8, i.e. the deimination of arginine into citrulline by peptidylarginine deiminases.

Design and Methods
The ability to provoke leukocytosis was assessed by intravenous administration of citrullinated CXCL8 in rabbits. Adsorption of citrullinated CXCL8 to the Duffy antigen/receptor for chemokines on human or rabbit erythrocytes was evaluated using a competitive binding assay. Finally, surface expression of adhesion molecules was studied after stimulating neutrophils with citrullinated CXCL8.

Results
Citrullination of CXCL8 significantly increased this chemokine's ability to recruit neutrophils into the blood circulation. In addition, the competitive binding properties of CXCL8 for the Duffy antigen/receptor for chemokines were impaired upon citrullination. Since the Duffy antigen/receptor for chemokines is an important scavenging receptor for CXCL8 in the blood stream, citrullination may delay CXCL8 clearance from the circulation. Furthermore, the shedding of CD62L (L-selectin) and the upregulation of CD11b (β_2-integrin) protein expression on CXCL8-induced neutrophils were improved by deimination of CXCL8, possibly contributing to the neutrophil egress from the bone marrow. Conversely, surface expression of CD15, the neutrophilic ligand of endothelial selectins, was equally well upregulated by intact and citrullinated CXCL8.

Conclusions
These data show that citrullination of CXCL8 enhances leukocytosis, possibly through impaired chemokine clearance from the blood circulation and prolonged presentation to the bone marrow.

Key words: chemokine, peptidylarginine deiminase, CXCL8, leukocytosis, neutrophil.

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Introduction

Chemokines are small chemotactic cytokines involved in inflammation-induced leukocyte recruitment, tumor development, angiogenesis as well as homeostasis.[1] Based on the conserved pattern of Cys residues, chemokines are classified into four groups, i.e. C, CC, CXC and CX_3C chemokines.[2,3] CXCL8 (also known as interleukin-8) is an inflammatory CXC chemokine that contains an ELR motif (Glu-Leu-Arg) in front of the most NH_2-terminal Cys residue. CXCL8 preferentially targets neutrophils via two CXCL8 receptors, CXC chemokine receptor (CXCR) 1 and CXCR2, both members of a seven transmembrane spanning G protein-coupled receptor superfamily.[4] Presentation of chemokines on the surface of endothelial cell layers to neutrophils in blood is achieved through the interaction with glycosaminoglycans (GAG).[5] In addition, CXCL8 can be adsorbed by the Duffy blood group antigen/receptor for chemokines (DARC) present on erythrocytes and vascular endothelium, restricting its capacity to stimulate neutrophils in the blood flow.[6-8]

Post-translational modifications of chemokines constitute an important regulatory pathway in the immune response, since structural alterations can profoundly affect the biological characteristics of chemokines.[9] Partial NH_2-terminal proteolysis of CXCL8 into CXCL8(6-77) potentiates its *in vitro* and *in vivo* neutrophil chemotactic properties 3- to 10-fold, whereas truncation in or beyond the ELR (Glu-Leu-Arg) motif abolishes its biological functions.[10-14] Recently, our group discovered a new post-translational modification of chemokines, i.e. the deimination of arginine (Arg) into citrulline (Cit).[14,15] Indeed, a naturally citrullinated CXCL8 isoform was isolated and identified in which Arg at position 5 was converted into Cit, designated CXCL8(1-77)Cit_5. Peptidylarginine deiminase (PAD) 2 and 4 efficiently and specifically modify Arg_5 in CXCL8. CXCL8 citrullination only moderately alters its *in vitro* activities, but resulted in a considerable reduction of glycosaminoglycan binding properties. In addition, citrullination of CXCL8 prevented proteolysis by plasmin or thrombin into CXCL8(6-77). Furthermore, this modification of CXCL8 abrogated the chemokine's *in vivo* capacity to recruit neutrophils into the peritoneal cavity, whereas angiogenic properties were retained.[14]

Chemokines (e.g. CXCL8) influence mobilization of leukocytes from the bone marrow into blood.[16-20] In complementation of our recent studies, we evaluated the effect of CXCL8 citrullination on the recruitment of neutrophils into the blood vessels by intravenous administration of different CXCL8 isoforms. In addition, further explanation is provided on the previously documented decreased intraperitoneal recruiting potency of citrullinated CXCL8.[14] Finally, the effect of chemokine citrullination on the regulated expression of adhesion molecules was assessed.

Design and Methods

Citrullination of chemokines

Human recombinant CXCL8(1-77) was purchased from PeproTech Inc. (Rocky Hill, NJ, USA). Citrullinated CXCL8 was obtained by incubation of CXCL8(1-77) with rabbit skeletal muscle PAD (Sigma-Adrich, St. Louis, MO, USA) in 40 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$ at 37°C for 90 min at an enzyme-substrate ratio of 1:200.[14] Deimination was stopped with 0.1% TFA and citrullinated protein was purified on a C8 Aquapore RP-300 HPLC column (1×50 mm, PerkinElmer, Norwalk, CT, USA). Endotoxin concentrations were evaluated with the Limulus amoebocyte lysate test (Cambrex, East Rutherford, NJ, USA).

Leukocytosis assays

Induction of leukocytosis was measured in New Zealand white rabbits by i.v. injection (1 mL) of 10 μg of chemokine in phosphate-buffered saline (PBS).[19] Blood samples were collected from a peripheral ear vein into potassium EDTA-coated tubes at several time intervals before and after treatment. Total leukocytes were counted in triplicate and the percentage of granulocytes was determined in quadruplicate by 100-cell differential cell counts on blood smears stained with Hemacolor solutions. 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. Statistical analyses were performed using the Mann-Whitney test on paired values.

Isolation of neutrophils and erythrocytes

Freshly isolated buffy coats (Blood Transfusion Center, Red Cross Leuven, Belgium) were subjected to sedimentation in hydroxyethyl-starch (Plasmasteril, Fresenius, Bad Homburg, Germany) and subsequently to gradient centrifugation using Ficoll-sodium metrizoate (Lymphoprep, Nycomed, Oslo, Norway).[21] Alternatively, rabbit blood, collected from ear veins, was subjected to gradient centrifugation using Nycodenz solution (NycoPrep, Nycomed).[22] Neutrophils recovered from the pellet were subjected to hypotonic shock and washed twice in PBS before use in binding assays. Alternatively, human blood withdrawn from a healthy donor or rabbit blood from ear veins was centrifuged at 125 g at 4°C for 10 min. Plasma and buffy coat were removed by aspiration, and erythrocytes were washed twice with PBS.

Binding competition assay

Competition for ^{125}I-labeled CXCL8(6-77) binding was measured on freshly isolated neutrophils or erythrocytes in binding buffer [50 mM HEPES pH 7.2 containing 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.1% (w/v) bovine serum albumin]. Briefly, 2×10^6 neutrophils or 2×10^8 erythrocytes were incubated for 2 h at 4 °C with ^{125}I-CXCL8(6-77) (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 γ counter (Triathler

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Multilabel Tester, Hidex, Finland). Statistical analyses were performed using the Mann-Whitney test on paired values. IC_{50} values were calculated by performing linear regression on the descending part of the curves of individual experiments.

Adhesion molecule staining in peripheral blood

Blood samples from healthy volunteers were collected by venipuncture, using a 21 G needle, into lithium heparin-treated tubes (Vacutainer, BD Biosciences, San Jose, CA, USA). Blood samples were handled with care in order not to activate the cells and immediately diluted in warm PBS (37°C) to 10^6 leukocytes/mL in sterile tubes containing prewarmed dilutions of chemokines or fMLP (Sigma-Aldrich.). After incubation for 10 min at 37°C in the presence of 5% CO_2, the samples were immediately placed on ice and three volumes of ice-cold PBS were added to the tubes to stop the signal transduction cascades at once. The Fc-receptors were blocked by storing the samples in ice-cold PBS containing 2% fetal calf serum (FCS) for 15 min, prior to staining for flow-assisted cell sorting (FACS). Antibodies used for staining were purchased from BD Biosciences (anti-human CD16 labeled with phycoerythrin, CD11b labeled with CyChrome) or from eBioscience (San Diego, CA, USA) (CD15 labeled with fluorescein isothiocyanate, CD62L labeled with allophycocyanin). Subsequently, red blood cells were lysed after washing the cells three times with PBS enriched with 2% FCS and 15 min of fixation with PBS containing 2% FCS and 2% formaldehyde. FACS analysis and data acquisition were performed with a BD FACSCalibur cytometer (BD Biosciences) using CellQuest software (BD Biosciences). Neutrophils were first selected by side/forward scatter and subsequently by CD16 positivity. Statistical analyses were performed using the Mann-Whitney test on paired values.

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Results

Citrullinated CXCL8 has enhanced granulocytosis-inducing capacity

Intravenous administration of CXCL8 is known to result in a rapid decrease of the number of circulating granulocytes followed by granulocytosis.[19,20] The effects of recombinant CXCL8(1-77) and its citrullinated isoform $CXCL8(1\text{-}77)Cit_5$ were compared following i.v. injections into rabbits at doses of 3 μg/kg. The numbers of circulating granulocytes and total leukocytes were determined at different time points (Figure 1). Interestingly, at identical doses $CXCL8(1\text{-}77)Cit_5$ induced a significantly more profound granulocytosis than CXCL8(1-77). Although injection of $CXCL8(1\text{-}77)Cit_5$ provoked a comparable drop in granulocyte concentration after 15 min, the increase of circulating granulocytes at 2 h post-injection was significantly greater with $CXCL8(1\text{-}77)Cit_5$ than with CXCL8(1-77). Moreover, this enhanced granulocytosis induced by $CXCL8(1\text{-}77)Cit_5$ remained significantly more pronounced until at least 8 h post-injection compared to that produced by CXCL8(1-77). After 24 h, the number of circulating granulocytes returned to baseline levels for both CXCL8(1-77) and $CXCL8(1\text{-}77)Cit_5$.

Citrullination reduces scavenging of CXCL8 by erythrocytes

In order to better understand the differences between intact and citrullinated CXCL8 at inducing granulocytosis *in vivo*, the cell-binding capacities of these two CXCL8 isoforms were compared *in vitro*. Freshly isolated human neutrophil granulocytes were used to assess competitive binding with ^{125}I-CXCL8. Interestingly, compared to intact CXCL8, citrullinated CXCL8 showed a modest but significant decrease in competition for ^{125}I-CXCL8 for chemokine receptor binding on human neutrophils (Figure 2A). Since the leukocytosis experiments were carried out in rabbits, binding experiments were also performed on freshly isolated rabbit neutrophils. Again, citrullination of CXCL8 moderately reduced the chemokine's competition for neutrophil receptors (Figure 2B).

An alternative pathway for chemokine clearance from the blood circulation is scavenging by chemokine interceptors.[7] Indeed, the majority of circulating CXCL8 is adsorbed by DARC present on erythrocytes, affecting the chemokine's capacity to stimulate neutrophils in the circulation.[6,7] To examine this option, the two CXCL8 isoforms were subjected to a receptor competition assay with ^{125}I-CXCL8 on freshly isolated human erythrocytes. Remarkably, over a wide range of concentrations citrullinated $CXCL8(1\text{-}77)Cit_5$ was significantly less effective than CXCL8(1-77) on erythrocytes at displacing labeled CXCL8 (Figure 2C). These results suggest that citrullination reduces binding of CXCL8 to DARC. As a positive control, monocyte chemotactic protein-1 (MCP-1/CCL2) efficiently displaced ^{125}I-CXCL8 from

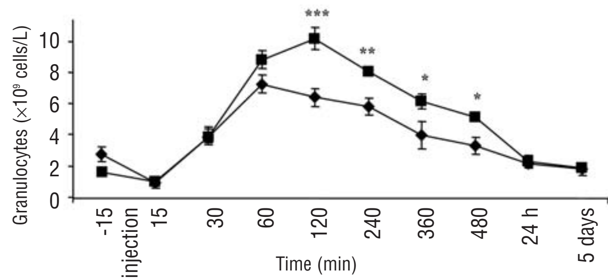

Figure 1. Effect of citrullination on granulocytosis induced by CXCL8 *in vivo*. Induction of leukocytosis was measured in New Zealand white rabbits after i.v. injection of 10 μg of CXCL8(1-77) (◆) or $CXCL8(1\text{-}77)Cit_5$ (■) (1 mL in PBS). Blood samples were collected from a peripheral ear vein into potassium EDTA-coated tubes. Total and differential leukocyte concentrations were determined 15 min pre-injection and at different time points following the injection. Results represent the mean (± SEM) number of circulating granulocytes measured by counting the total amount of leukocytes and determining the percentage of granulocytes by 100-cell differential cell counts on blood smears, both determined double-blind by four researchers. Independent experiments were carried out in three rabbits. [*$p<0.05$, **$p<0.01$, ***$p<0.001$ for the comparison with CXCL8(1-77)]

red blood cells (Figure 2C and 2D).[23,24] In addition, citrullination of CXCL8 also significantly diminished its potency to compete for DARC on rabbit erythrocytes (Figure 2D).

Effect of citrullination on the expression of adhesion molecules on human neutrophils in peripheral blood

Transcellular and intercellular migration of neutrophils across the endothelium requires selectin and integrin activation.[25-27] The effects of CXCL8(1-77) and CXCL8(1-77)Cit$_5$ on the neutrophilic expression of adhesion molecules were determined after whole blood stimulation (Figures 3-5).

First, the surface expression of sialyl Lewis X/CD15, the moiety that interacts with endothelial selectins, was evaluated. Both CXCL8(1-77) and CXCL8(1-77)Cit$_5$ caused an upregulation of the neutrophilic expression of CD15 (Figure 3). However, no significant difference in CD15 expression levels on neutrophils was observed between the two CXCL8 isoforms.

Next, the shedding of L-selectin (CD62L) and upregulation of integrins (CD11b) on CXCL8-activated neutrophils were investigated. In contrast with CD15 upregulation, these processes were improved by citrullination of CXCL8 (Figures 4 and 5). Although the CXCL8 isoforms were less efficient than bacterial formyl peptide fMLP, 100 nM CXCL8(1-77) provoked a significant decrease of CD62L expression (Figure 4). CXCL8(1-77)Cit$_5$ already significantly enhanced shedding of CD62L on neutrophils at a concentration of 30 nM. Besides, 3 nM CXCL8(1-77)Cit$_5$ notably increased the CD16/CD11b$^{+/+}$ population, whereas CXCL8(1-77) only induced a significant rise of CD11b expression from concentrations of 30 nM upwards (Figure 5). These results indicate that citrullinated CXCL8 is 3 to 10 times more potent than intact CXCL8 in causing CD62L shedding and CD11b upregulation.

Discussion

The bone marrow is a source of hematopoietic stem and progenitor cells able to differentiate into myeloid and lymphoid cell lineages. Neutrophil granulocytes are not only produced in the bone marrow, but are also stored there in a mature form known as the bone marrow pool. Upon inflammation, these mature granulocytes are rapidly mobilized into the blood stream to reinforce the first line defense of circulating neutrophils, a process termed leukocytosis. Therefore, the clearance of chemokines from the blood circulation and the rate of neutrophil intravasation over the bone marrow endothelium are essential factors affecting the number of circulating neutrophils.[28,29] Furthermore, the potency of bone marrow neutrophils in responding to chemoattractants, phagocytosis and bacterial eradication is greater than that of circulating neutrophils.[30] Strong granulocytosis is provoked within hours by most CXC chemokines, including CXCL8.[16-20] Here, an even higher neutrophil mobilization into the blood stream was evidenced upon i.v. administration of citrullinated CXCL8 in rabbits. In addition, we observed that erythrocyte binding was severely decreased for CXCL8(1-77)Cit$_5$, in both humans and rabbits. Since the clearance of CXCL8 from the blood circulation is predominantly due to binding to DARC on erythrocytes,[6,7] this impairment could result in a longer presentation of the chemokine to the bone marrow and, hence, enhanced neutrophilia. In previously reported studies, we observed that citrullination of CXCL8 at Arg$_5$ prevents the chemokine's truncation by

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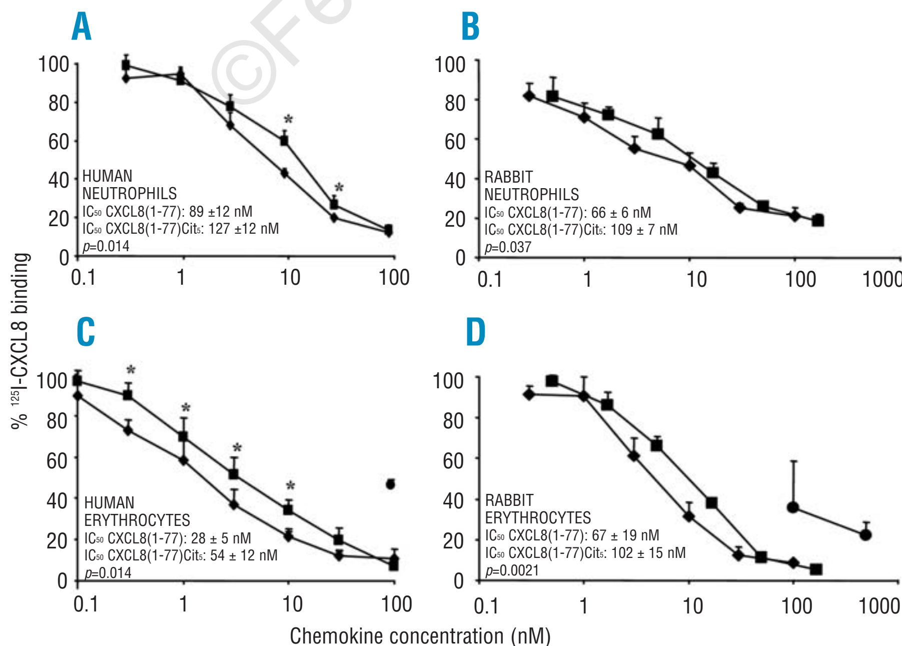

Figure 2. Neutrophil and erythrocyte binding properties of citrullinated CXCL8. Receptor binding properties of human CXCL8(1-77) (◆), CXCL8(1-77)Cit$_5$ (■) or CCL2 (●) were evaluated in receptor binding competition assays. Increasing concentrations of unlabeled chemokine were added together with ^{125}I-CXCL8(6-77) to freshly isolated human neutrophils (A), rabbit neutrophils (B), human erythrocytes (C) or rabbit erythrocytes (D). Results represent the mean % (± SEM) of remaining specific ^{125}I-CXCL8 binding. [n=3 to 6; *p<0.05 for the comparison with CXCL8(1-77) at the same concentration] IC$_{50}$ values (± SEM) and p values for the comparison of CXCL8(1-77) with CXCL8(1-77)Cit$_5$ binding curves are shown in the left corner.

plasmin or thrombin into the more potent CXCL8(6-77) isoform.[14] Furthermore, the reduced GAG binding capacity of citrullinated CXCL8 may impair its immobilization on the vessel wall, which is necessary for transcytosis of leukocytes to the luminal side of endothelial cells.[31]

In conclusion, all these data suggest that citrullination of CXCL8 reduces the clearance of this chemokine from the blood circulation. These results may provide an explanation for the previously documented deficient recruitment of neutrophils from the blood circulation towards inflamed tissue.[14,15] Indeed, intra-peritoneal administration of CXCL8(1-77)Cit5 in mice did not result in an infiltration of the peritoneal cavity by neu-

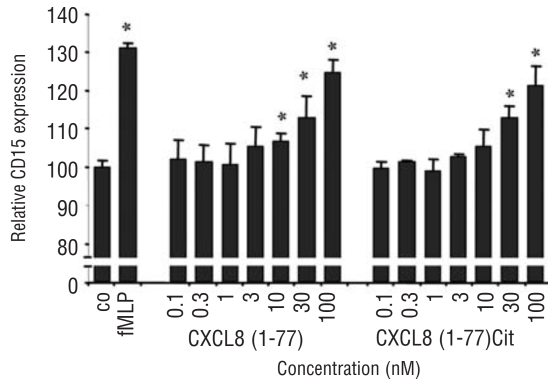

Figure 3. **Effect of citrullination on expression of CD15. Expression patterns of the selectin ligand CD15 were evaluated following activation of neutrophils for 10 min with 0.1 to 100 nM human CXCL8(1-77), CXCL8(1-77)Cit5, 10^{-7} M fMLP or PBS as a control (co). Results (± SEM) are presented as the acquired mean fluorescence intensity (MFI) on the CD16+ neutrophils relative to the MFI obtained in PBS-treated neutrophils (set at 100%). [n=3 to 16; *p<0.05 for the comparison of chemoattractant with buffer].**

Figure 4. **CXCL8-induced downregulation of CD62L is altered on neutrophils by chemokine citrullination. Neutrophils in whole blood were stimulated for 10 min with 0.1 to 100 nM human CXCL8(1-77), CXCL8(1-77)Cit5, 10^{-7} M fMLP or PBS as a control (co) and subsequently stained with fluorescently labeled anti-CD16 and anti-CD62L. (A) The figures illustrate a representative experiment on the acquired CD62L fluorescence on CD16+ neutrophils (gate R1). The percentage of CD16+ neutrophils that undergoes downregulation of CD62L is depicted in the lower right quadrant. (B) Results (± SEM) are presented as the acquired mean fluorescence intensity (MFI) on the CD16+ neutrophils relative to the MFI obtained in PBS-treated neutrophils (set as 100%, dashed line). [n=3 to 16; ***p<0.001 or *p<0.05 for the comparison of chemoattractant with buffer; or §§§p<0.001 or §p<0.05 for the comparison of CXCL8(1-77) with CXCL8(1-77)Cit5].**

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trophils, whereas intact CXCL8(1-77) and truncated CXCL8(6-77) were highly potent at mobilizing neutrophils from the blood circulation. This insufficient pro-inflammatory response after citrullination indicates an inherent protective role for PAD during local inflammation but may also guarantee a constant mobilization of neutrophils from the bone marrow to keep cell numbers equivalent in the circulation after extravasation into inflamed tissue.

Although PAD is normally expressed in the cytoplasm and occasionally in the nucleus, extracellular localization of PAD2 and PAD4 from infiltrated leukocytes has been detected in inflamed synovial tissue.[32-35] When the citrullinated chemokines appear in the blood flow, they may contribute to the development of generalized inflammation.

Leukocyte mobilization into the circulation and recruitment into tissues requires upregulation and activation of adhesion molecules.[25-27] Neutrophils express CD15 or Lewis-X sialic acid, which binds selectins on

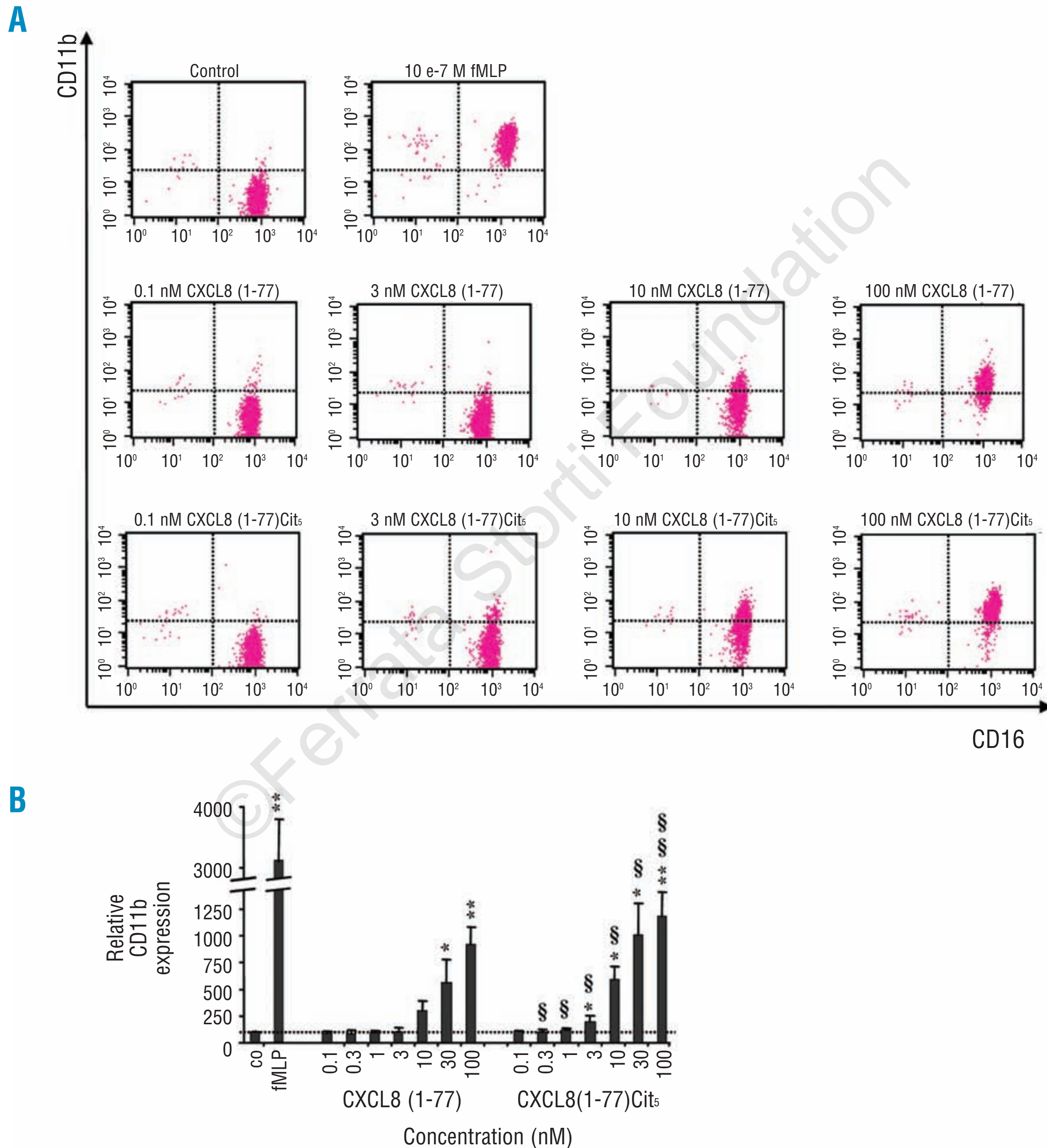

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Figure 5. Citrullination of CXCL8 enhances upregulation of CD11b on neutrophils. Whole blood neutrophils were activated for 10 min with 0.1 to 100 nM human CXCL8(1-77), CXCL8(1-77)Cit5, 10^{-7} M fMLP or PBS as a control (co) and subsequently stained with fluorescently labeled anti-CD16 and anti-CD11b. (A) The figures show a representative experiment of the acquired CD11b fluorescence data on $CD16^{+}$ neutrophils (gate R1). The percentage $CD16^{+}$ neutrophils upregulating CD11b is displayed in the upper right quadrant. (B) Results (± SEM) are presented as the acquired mean fluorescence intensity (MFI) on the $CD16^{+}$ neutrophils relative to the MFI obtained in PBS-treated neutrophils (set at 100%, dashed line). [n=3 to 16; ** $p<0.01$ or * $p<0.05$ for the comparison of chemoattractant with buffer; §§ $p<0.01$ or § $p<0.05$ for the comparison of CXCL8(1-77) with CXCL8(1-77)Cit5]

endothelial cells. In this study, no increase in the upregulation of CD15 was detected after citrullination of CXCL8. In contrast to extravasation from blood vessels into the interstitium that occurs through cell-cell junctions, mobilization from the bone marrow seems to involve transcytosis through pores in the endothelial cells.[36] Bone marrow neutrophils do not appear to require tethering on the endothelium, although L-selectin/CD62L is significantly downregulated upon inflammation, suggesting that CD62L acts as a retention factor, in analogy with its lymphocyte homing function in lymph nodes.[37,38] In this study, it was found that CD62L was shed when neutrophils were stimulated with citrullinated CXCL8. In fact, citrullination converted CXCL8(1-77) into a significantly more potent downmodulator of CD62L. After binding of the chemokines to their appropriate receptors, several intracellular cascades are activated, including upregulation and activation of integrins, which are implicated in the immobilization of leukocytes on the vessel wall.[39] The expression of β_2-integrin (CD11b) on neutrophils was found to be improved after citrullination of CXCL8(1-77). Thus, citrullination of CXCL8 prolongs the presence of the chemokine in the circulation and increases the shedding of CD62L and upregulation of CD11b expression. Such processes may contribute to the sustained intravasation of neutrophils from the bone marrow through the sinusoidal endothelium into the circulation.

Overall, these data shed light on the greater capacity of CXCL8(1-77)Cit_5 to recruit neutrophils from the bone marrow. Indeed, it may be valuable to explore the use of citrullinated chemokines as an additional clinical approach to mobilize progenitor cells into the blood. Growth-related oncogen-β (GROβ/CXCL2), another CXCR2 agonist, synergizes with granulocyte colony-stimulating factor in mobilizing hematopoietic progenitor cells from the bone marrow.[40-43] Interestingly, citrullination is a natural and irreversible reaction and we showed that it protects CXCL8 against proteolysis.[14,44] Furthermore, significantly elevated leukocytosis was obtained by citrullinating CXCL8. Thus, these data suggest that citrullination of CXCL8 or other chemokines could be a potential mechanism to assist granulocyte colony-stimulating factor in the recruitment of hematopoietic cells from the bone marrow into the blood.

Authorship and Disclosures

TL, GO and PP performed the experiments; TL and PP collected and analyzed the data; TL, GO, JVD and PP designed the research and wrote the manuscript. All authors approved the final version of the manuscript and declare that they have no conflicts of interest.

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