

ORIGINAL ARTICLE

Experimental Models of Allergic Disease

Anti-inflammatory effects of the GAG-binding CXCL9(74-103) peptide in dinitrofluorobenzene-induced contact hypersensitivity in mice

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Funding information

Fonds Wetenschappelijk Onderzoek, Grant/Award Number: G.0764.14, G.0D25.17N; Hercules Foundation Flanders, Grant/Award Number: AKUL/11/31; Federaal Wetenschapsbeleid, Grant/Award Number: P7/40; KU Leuven, Grant/Award Number: C16/17/010, GOA/13/014

Summary

Background: To recruit leucocytes to an inflammatory site, chemokine binding to glycosaminoglycans (GAGs) is critical. Therefore, strategies to interfere with this interaction, aiming at the production of anti-inflammatory agents, were developed. These include production of modified chemokines without affinity for G protein-coupled receptors but with enhanced affinity for GAGs. Such modified chemokines compete with functional chemokines for GAG binding, prevent chemokine immobilization and presentation, and inhibit leucocyte migration. In addition to modified chemokines, a GAG-binding peptide consisting of the 30 COOH-terminal residues of CXCL9, that is CXCL9(74-103), inhibited CXCL8- and monosodium urate crystal-induced neutrophil migration.

Objective: We wanted to explore whether interference with chemokine-GAG interactions by CXCL9(74-103) reduces inflammation in neutrophil-dependent dinitrofluorobenzene-induced contact hypersensitivity.

Methods: For this study, we evaluated several inflammatory parameters, including ear swelling and the levels of chemokines, cytokines, proteases and neutrophils in the ears of dinitrofluorobenzene-induced mice treated with CXCL9(74-103) or buffer.

Results: One intravenous injection of CXCL9(74-103), just before painting with dinitrofluorobenzene on the ear, did not affect protein levels of the major murine neutrophil attractant, that is CXCL6, in this contact hypersensitivity model. However, IL-6, CXCL1, CCL2 and matrix metalloproteinase-9 (MMP-9) protein concentrations and peroxidase activity in challenged ears were reduced. In addition, intravenous injection of the CXCL9-derived peptide led to a reduced ear swelling response, indicating that the locally produced chemokines were hindered to attract leucocytes. The inhibiting potential of CXCL9(74-103) was explained by its competition for GAG binding with CXCL1, CXCL6 and CCL3 and inhibition of transendothelial migration of neutrophils to CXCL6.

Conclusions and Clinical Relevance: The CXCL9(74-103) peptide inhibited dinitrofluorobenzene-induced infiltration of neutrophils and neutrophil-dependent

inflammation in ears. Therefore, CXCL9(74-103) may be a lead molecule for the development of therapeutic peptides or peptide derivatives that compete with functional chemokines for GAG binding.

KEYWORDS

allergic contact dermatitis, animal models, basic immunology, basic mechanisms, chemokines

1 | INTRODUCTION

Glycosaminoglycans (GAGs) are linear, negatively charged polysaccharides consisting of repeating disaccharide subunits with molecular weights of 10-100 kDa.¹⁻³ Depending on the disaccharide subunit and the glycosidic linkage, GAGs are classified into six major classes including heparin, heparan sulphate and chondroitin sulphate. After synthesis, the polysaccharides are enzymatically modified resulting in the generation of mature and functional GAGs that exert biological functions dependent on their specific structure. An additional level of complexity is formed by the variety in GAG length and structure in different tissues.⁴ GAGs are often coupled to core proteins, forming proteoglycans, which are located at the surface of almost all eukaryotic cells. Therefore, GAGs play a major role in cell signalling and development, cell proliferation, angiogenesis, tumour progression, metastasis and anticoagulation.⁵⁻⁷ In addition, GAGs are believed to play a critical role in the migration of leucocytes from blood vessels to sites of inflammation.^{8,9} During inflammation, GAGs expressed on the endothelium interact with locally secreted chemotactic cytokines or chemokines, which are a class of proteins that play an important role in directional cell migration.¹⁰⁻¹³ This chemokine-GAG interaction is thought to occur between, for example, sulphated domains of GAGs and basic amino acid motifs (GAG-binding motifs) of chemokines to ensure a high local concentration of the produced chemokines and to prevent chemokine diffusion and degradation, thereby creating a haptotactic gradient on the vascular endothelial cells at the site of inflammation.¹⁴⁻¹⁹ In addition, chemokine secretion by tumour cells has been reported to depend on GAGs.²⁰ Recently, it was shown that both CXCL8 (in zebrafish) and CCL21 (in mice) form a GAG-dependent extracellular gradient in tissue. Destruction of these gradients abolished directional leucocyte migration.^{21,22} In addition, it has been proven that chemokine-GAG interactions are critical for chemokine activity in vivo.²³ Chemokine variants with mutations in their GAG-binding motifs show abrogated GAG binding and abolished chemotactic activity in vivo, although receptor binding and chemotactic activity in vitro are seldom affected.²³⁻²⁷ In addition, chemokine variants that lost receptor binding properties and retained GAG binding competed with active chemokines for GAG binding and inhibited leucocyte migration.²⁸⁻³¹ Finally, mutant mice with abrogated heparan sulphate synthesis in endothelial cells showed impaired neutrophil inflammation in various inflammation models.³²

Contact hypersensitivity (CHS), an animal model for human contact dermatitis, is an inflammatory skin disease characterized by an

influx of nonspecific inflammatory cells and the secretion of cytokines that induce a localized inflammatory reaction called delayed-type hypersensitivity (DTH).³³ In mice, CHS is triggered by repeated exposure to allergens such as dinitrofluorobenzene (DNFB) or trinitrochlorobenzene.³⁴ After primary contact with a contact allergen, the DTH response begins with an initial sensitization phase. During this period, antigen-presenting cells, such as Langerhans cells and macrophages, are thought to pick up the antigen and transport it to regional lymph nodes, where especially CD4⁺ T_H1 cells are activated. A subsequent exposure to the antigen induces the elicitation phase, in which T_H1 cells secrete a variety of cytokines that recruit and activate macrophages and other nonspecific inflammatory cells. Although the DTH response does not become apparent until on average 24 hours after the second exposure to the antigen, mouse neutrophils already infiltrate after 6 hours.³⁵ Recently, it was even shown that neutrophils are required for both the sensitization and the elicitation phase of CHS.^{36,37} Both depletion and genetic deficiency of neutrophils in mice abrogate the CHS response. In addition, blocking of granulocyte-colony-stimulating factor (G-CSF) results in reduced numbers of neutrophils as well as suppressed ear swelling responses.³⁷ These results indicate that neutrophils play critical roles in various phases of allergic skin inflammation, making those cells attractive targets for the development of future therapeutic strategies.

Because of their central role in leucocyte migration, chemokines became potential targets for the inhibition of inflammation. In recent years, several strategies to inhibit chemokine activity were developed. For instance, it was demonstrated that the human CXCL9-derived COOH-terminal peptide CXCL9(74-103) binds with high affinity to GAGs.³⁸ CXCL9(74-103) was able to compete with active chemokines for GAG binding, thereby inhibiting CXCL8- and monosodium urate crystal (MSU)-induced neutrophil migration after 2-3 hours in vivo. Here, we report the anti-inflammatory effect of CXCL9(74-103) in the CHS model. We show that a single intravenous injection of CXCL9(74-103) inhibited a variety of skin inflammation parameters including ear swelling, peroxidase activity and the infiltration of neutrophils. In addition, by this treatment, matrix metalloproteinase (MMP)-9, cytokine and chemokine levels were decreased in the affected tissue.

2 | MATERIALS AND METHODS

2.1 | Solid-phase synthesis

The COOH-terminal fragment of CXCL9, namely CXCL9(74-103), was synthesized using fluorenyl methoxycarbonyl (Fmoc) chemistry

using an Activo-P11 automated peptide synthesizer (Activotec, Cambridge, UK), as previously described.³⁸ Intact synthetic peptide was purified by reversed-phase high-performance liquid chromatography (RP-HPLC) and identified by mass spectrometry (Amazon Speed ETD ion trap, Bruker, Bremen, Germany).

2.2 | Mice and ethics statement

Female C57Bl/6 mice (8–10 weeks old) were purchased from Janvier Labs (Le Genest Saint Isle, France) and were used to induce CHS. Mice were group-housed at room temperature and received food and water ad libitum. Mice were killed by intraperitoneal injection of an overdose of Nembutal prior to the collection of ears.

All animal experimentation was in adherence to the international guidelines for animal ethics and welfare and was reviewed and approved by the Ethical Committee for Animal Care and Use of the KU Leuven (license P019/2015).

2.3 | DNFB-induced CHS in mice

Mice were sensitized on day 0 by applying 20 μ L of 0.5% 2,4-dinitrofluorobenzene (DNFB; Sigma, St Louis, MO, USA) dissolved in 4:1 acetone/liquid paraffin on the shaved abdominal skin.³⁹ As a vehicle, 20 μ L of 4:1 acetone/liquid paraffin was used. Five days later, the baseline ear thickness on the right ear was measured with a dial thickness gauge (Baty, Albany Court, UK), after which it was challenged by epicutaneous application of 20 μ L 0.5% DNFB. Simultaneously, mice were treated with an injection of 100 μ g CXCL9(74-103) in 100 μ L phosphate-buffered saline (PBS) or 100 μ L PBS in the tail vein. After 24 hours, the ear thickness was measured and mice were killed.

2.4 | Digestion of skin samples and chemokine measurements

Ears were removed 24 hours after challenge, weighed and placed on ice in 0.5 mL buffer containing 20 mmol/L Tris, 0.15 mol/L NaCl, 10 mmol/L CaCl₂, 1% Triton X-100 and 1 protease inhibitor cocktail tablet (1 tablet per 10 mL extraction buffer; Roche Diagnostics, Vilvoorde, Belgium). The biopsies were subsequently homogenized with a Precellys tissue homogenizer (Bertin Instruments, Montigny-le Bretonneux, France). Homogenates were centrifuged (10 000 g at 4°C) for 15 minutes and supernatants were kept at –20°C for further use.

Total protein concentrations were measured by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Supernatants were analysed with DuoSet ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions for the following murine chemokines and cytokines: CXCL1 (KC), CXCL2 (MIP-2), CCL2 (MCP-1), CCL3 (MIP-1 α), CXCL6 (GCP-2), CXCL10 (IP-10), tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ).

2.5 | Determination of peroxidase activity

Peroxidase activity, as a parameter of total granulocyte infiltration, was measured in homogenate supernatants with tetramethylbenzidine (TMB) as a sensitive substrate for peroxidase. The conversion of TMB supplemented with 0.004% (v/v) H₂O₂ was measured at 450 nm using a spectrophotometer.

2.6 | In-gel zymography

Supernatants of ear homogenates were prepurified on gelatin-Sepharose beads (GE Healthcare, Little Chalfont, UK) and analysed on 7.5% polyacrylamide gels containing 0.1% gelatin (Sigma).⁴⁰ After electrophoresis, the gels were washed two times for 20 minutes in 2.5% Triton X-100 followed by overnight incubation in 50 mmol/L Tris, pH 7.5, 10 mmol/L CaCl₂ at 37°C. The gels were stained with 0.2% Coomassie Blue and areas of gelatinolysis were visualized as transparent lysis zones. Quantification of zymographic bands (mouse pro-MMP-9 at 105 kDa, activated MMP-9 at 92 kDa and MMP-2 at 72 kDa) was done by densitometry using ImageQuant TL software (GE Healthcare).⁴⁰

2.7 | Affinity measurement of CXCL9(74-103) for heparin by the Octet K2 system

All measurements were performed on an Octet K2 system (Pall FortéBio, Fremont, CA, USA) with an operating temperature of 30°C. Samples or buffer were dispensed into 96-well microtitre plates at a volume of 200 μ L per well. Streptavidin-coated biosensor tips (Pall FortéBio) were prewetted with running buffer (PBS + 0.005% Tween-20) in order to establish a baseline prior to immobilization with biotinylated low-molecular-weight heparin (LMWH; Iduron, Manchester, UK) at a concentration of 5 μ g/mL for 120 seconds at 1000 rpm. Subsequently, biosensor tips were consecutively dipped into a serial dilution of CXCL9(74-103) (association) and running buffer (dissociation) for 300 seconds each. Afterwards, the biosensor tips were regenerated for 15 minutes in 0.1 mol/L Glycine (pH 2). Data were generated automatically by the Octet User software using steady state analysis. The binding profile of each sample was summarized as a nm shift, which represented the difference between the start and end of the 300 seconds sample association and dissociation step. Affinity constants were assessed by a simple 1:1 equilibrium binding model.

2.8 | Competition of CXCL9(74-103) for murine CXCL1, CXCL6 and CCL3 binding to heparin-coated plates

The ability of CXCL9(74-103) to compete for GAG binding with either CXCL1, CXCL6 or CCL3 was evaluated on heparin-binding plates (BD Biosciences, Erembodegem, Belgium) as recently described.³⁸ In brief, heparin (Iduron; 25 μ g/mL) was coated overnight at room temperature. Dilutions of CXCL9(74-103) combined

with recombinant murine CXCL1, CXCL6(9-78) or CCL3 (Peprotech, Rocky Hill, NJ, USA) were added in duplicate and incubated for 2 hours at 37°C. Subsequently, bound chemokine was detected with either biotinylated polyclonal rabbit antimurine CXCL6 antibody (Peprotech), biotinylated polyclonal goat antimurine CXCL1 or CCL3 antibody (R&D Systems) and horseradish peroxidase-labelled streptavidin. Finally, the peroxidase activity was quantified at 450 nm. The percentage inhibition of chemokine binding to GAG was calculated as follows.

Percentage inhibition

$$= \left(1 - \frac{\left(\frac{\text{OD (x nmol/L chemokine + y nmol/L CXCL9 peptide)}}{\text{OD (x nmol/L chemokine) - OD (control sample)}} \right)}{\left(\frac{\text{OD (control sample)}}{\text{OD (x nmol/L chemokine) - OD (control sample)}} \right)} \right) * 100$$

This binding assay was performed 2-6 times in duplicate.

2.9 | Transendothelial migration assay

Transwell plates (HTS Transwell-96 well plate, polycarbonate membrane with 5 µm pore size, Corning, New York, NY, USA) were coated with 1.6 µg collagen in 40 µL 0.1% acetic acid per well in the upper compartment and incubated for 2 hours at 37°C. After incubation, wells were washed with HBSS-/- (120 µL/well; Thermo Fisher Scientific) and air-dried. Human EA.hy926 (ATCC, Manassas, VA, USA) endothelial cells and mouse bEND3 cells (ATCC) were seeded to a final concentration of 35 000 cells in 100 µL medium [consisting of DMEM, 10% FBS, 4 mmol/L L-Glutamine and 1% Penicillin/Streptomycin 1:100 (all Thermo Fisher Scientific)] for the human and mouse transwell assay, respectively. The endothelial cells were grown in the upper wells for 2 days at 37°C with 5% CO₂ with 220 µL medium in the lower wells of the transwell compartment.

On the day of the assay, endothelial monolayers were stimulated with 50 ng/mL human TNF-α or mouse TNF-α (Peprotech) for 4 hours at 37°C. Either human (for the assay with CXCL8) or murine (for the assay with murine CXCL6) neutrophils were isolated from fresh whole blood samples from healthy donors and from pooled mice blood, respectively, via density gradient centrifugation (30 minutes, 500 g) using Ficoll-Paque™ PLUS (GE Healthcare), as previously described.⁴¹ After labelling of the neutrophils with 2 µmol/L calcein acetoxymethyl ester (AM, Sigma 17783) for 30 minutes at 37°C, cells were washed with HBSS-/- and resuspended in HBSS+/+ (Thermo Fisher Scientific) with 20 mmol/L HEPES (Sigma) to a final concentration of 2.10⁵ cells/40 µL (in each well). Dilutions of CXCL8 and murine CXCL6 were prepared and added to the lower compartment and the diluted competitor CXCL9(74-103) was added to the upper compartment. The transmigration assay was run for 2 hours at 37°C and 5% CO₂. The fluorescence intensity of migrated cells in the lower wells was measured at 496 nm (excitation)/516 nm (emission) using the SpectraMax M3 Plate reader (Molecular Devices, San Jose, CA, USA). Data were normalized using GraphPad software with

spontaneous migration in response to saline as 0% and maximal migration in response to 20 nmol/L CXCL8 or CXCL6 as 100%.

2.10 | Immunofluorescence microscopy analysis

Study of histological changes was performed on 4-µm acetone-fixed frozen sections. To investigate neutrophil infiltration and to determine the effect of CXCL9(74-103) in inflamed ears, immunofluorescent double labelling was performed using FITC-labelled rat anti-mouse CD11b antibody (Clone M1/70, eBioscience, San Diego, CA, USA) and PE-labelled rat anti-mouse Ly6G antibody (Cat:551461, BD Bioscience). Quantification of Ly6G⁺CD11b⁺ neutrophils was performed on micrographs of ear sections of different mice (n ≥ 4/group) using ImageJ software.

To examine the expression of MMP-9, the sections were stained with goat anti-mouse MMP-9 antibody (R&D Systems) and Alexa 647-labelled donkey anti-goat antibody (Life Technologies, Eugene, OR, USA) as primary and secondary antibodies, respectively. DAPI was used for nuclear counterstaining, and sections were coverslipped with Prolong Gold antifade reagent (Life Technologies). Images were taken with a Zeiss Axiovert 200M and AxioVision Rel 4.8 acquisition software (Carl Zeiss AG, Oberkochen, Germany).

2.11 | Statistical analysis

Results are shown as mean ± SEM or median ± SEM. A standard one-way ANOVA test (GraphPad Prism 7, GraphPad Software, La Jolla, CA, USA) was used to compare means from independent groups followed by multiple comparison analysis. *P* ≤ 0.05 was considered statistically significant (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001).

3 | RESULTS

3.1 | Both immunization and challenge of mice with DNFB are necessary for ear swelling

To assess the anti-inflammatory effects of CXCL9(74-103) in a model of contact hypersensitivity, we first evaluated ear swelling of mice immunized (on the shaved abdominal skin) and/or challenged (epicutaneous application on one ear) with DNFB and/or vehicle. Exposure of nonimmunized mice to DNFB challenge resulted in a limited, non-significant increase in ear thickness (increase of 121 ± 15 µm), while re-exposure of immunized mice led to a significant ear swelling response with an increase in ear thickness of 423 ± 47 µm 24 hours after challenge (Figure 1A).

3.2 | CXCL9(74-103) reduces ear swelling and granulocyte infiltration in CHS

A variety of skin inflammation parameters were compared 24 hours after the initiation of the acute DNFB-induced CHS. The ear thickness of mice treated with DNFB (immunized and challenged) was

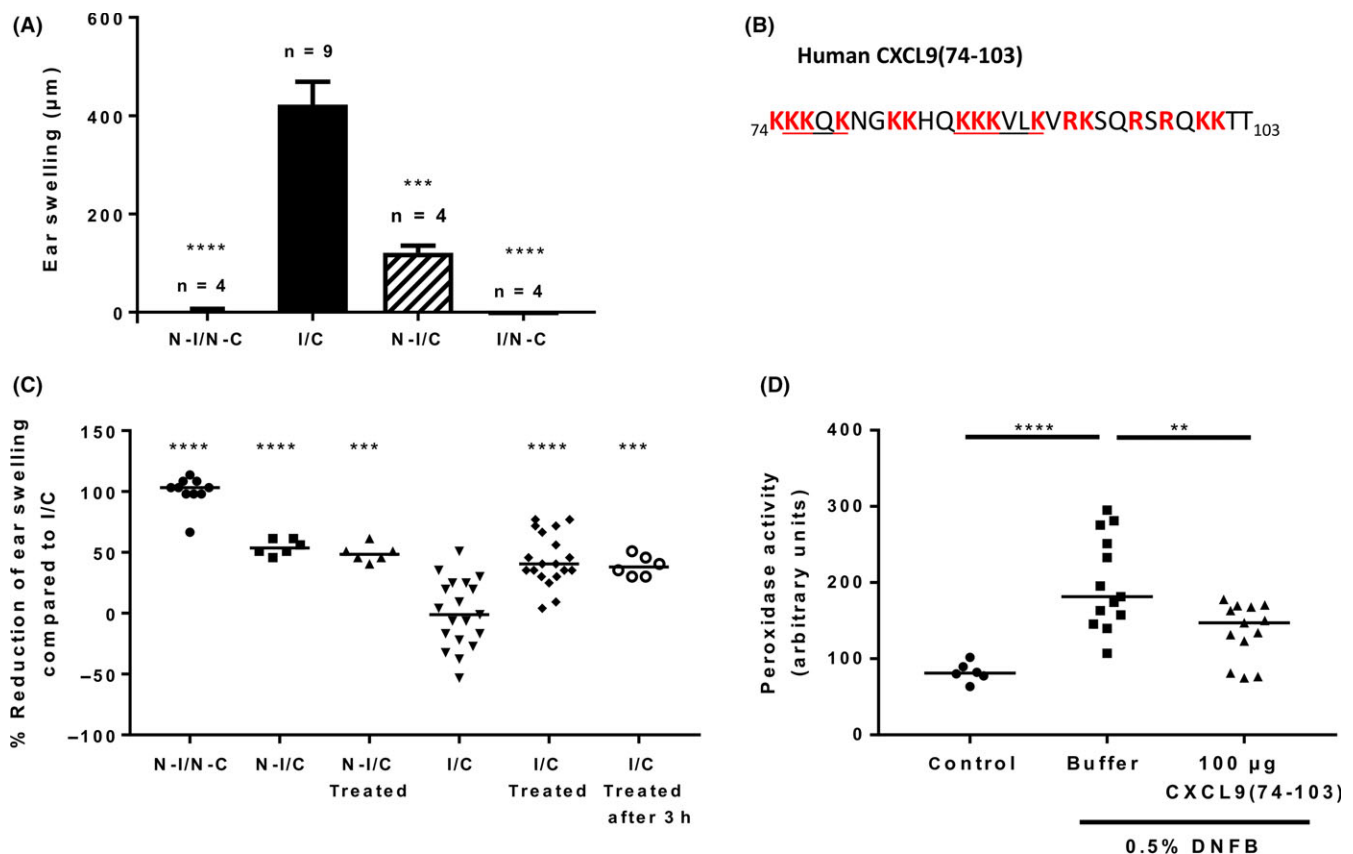


FIGURE 1 Ear swelling during CHS response and effect of treatment with CXCL9(74-103). A, Mice were sensitized with 0.5% DNFB in 4:1 acetone/paraffin (I) or 4:1 acetone/paraffin (N-I) and were challenged with 0.5% DNFB in 4:1 acetone/paraffin (C) or 4:1 acetone/paraffin (N-C) 5 d after sensitization. The ear swelling in μm 24 h after challenge is depicted and compared with the I/C group. The graph shows the mean ± SEM from 4 to 9 individual mice per group of two independent experiments. B, The amino acid sequence of the human CXCL9-derived COOH-terminal peptide CXCL9(74-103) is shown. For clarity, basic amino acids were marked in red, and GAG-binding motifs were underlined. C, Mice were intravenously treated with 100 μg CXCL9(74-103) or PBS. Treatment with the peptide was given either simultaneously with the challenge (treated) or 3 h after the challenge (treated after 3 h). The percentage median reduction in ear swelling compared to the I/C group is shown. Each dot represents one individual mouse (n ≥ 6) of two to four independent experiments. D, Mice were sensitized with 0.5% DNFB in 4:1 acetone/paraffin or 4:1 acetone/paraffin (control) and were challenged with 0.5% DNFB in 4:1 acetone/paraffin or 4:1 acetone/paraffin (control) 5 d after sensitization. Simultaneously with the challenge, mice were injected intravenously with 100 μg of CXCL9(74-103) or PBS (buffer). The median peroxidase activity in arbitrary units 24 h after challenge is depicted. Each dot represents one individual mouse (n ≥ 6) of 4 independent experiments. I, immunized; C, challenged; N-I, nonimmunized; N-C, nonchallenged; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$

significantly increased compared to vehicle-treated mice (nonimmunized and nonchallenged) 24 hours after challenge (Figure 1A). Intravenous injection of 100 μg of CXCL9(74-103) (Figure 1B), just before the challenge, significantly reduced ear swelling in immunized and challenged mice (median reduction 41%) compared to non-treated immunized and challenged mice (Figure 1C). Even when CXCL9(74-103) was administered 3 hours after challenge, a significant decrease in ear swelling was observed (median reduction 38%). Treatment with CXCL9(74-103) did not influence ear swelling in mice that were nonimmunized and challenged. To assess the infiltration of granulocytes in the ear, the peroxidase activity in ear homogenates was measured. Re-exposure of immunized mice to DNFB challenge resulted in a significant increase in peroxidase activity (Figure 1D). As most of the granulocytes are neutrophils, it can be suggested that this peroxidase activity was predominantly the result of the infiltration of neutrophils. Intravenous injection of CXCL9(74-

103) inhibited the infiltration of granulocytes (decrease in peroxidase activity) (Figure 1D). Treatment with CXCL9(74-103) did not affect ear thickness or peroxidase activity in healthy mice (data not shown).

3.3 | Reduced in vivo infiltration of neutrophils upon treatment with CXCL9(74-103)

The infiltration of neutrophils was visualized with the use of immunofluorescence microscopy (Figure 2A-C). Staining for CD11b and Ly6G revealed that ears immunized and challenged with DNFB are indeed characterized by an infiltration of CD11b⁺Ly6G⁺ neutrophils (indicated by arrowheads). This neutrophil infiltration was significantly reduced by a single intravenous injection of 100 μg of CXCL9(74-103) from 132 ± 22 to 2.4 ± 5.7 neutrophils/mm² (mean ± SEM) (Figure 2D).

3.4 | The tissue levels of MMP-9 are reduced upon treatment with CXCL9(74-103) in CHS

An important consequence of a local influx of neutrophils is the release of proteolytic enzymes, such as MMPs. MMPs are involved in the remodelling of the extracellular matrix and possibly contribute to tissue damage and the CHS response.⁴² In-gel zymography of tissue extracts revealed up-regulation of gelatinase B (MMP-9) in the affected skin of DNFB-treated mice, which was strongly decreased upon intravenous injection of 100 µg of CXCL9(74-103) (Figure 3A, B). Correspondingly, immunofluorescent staining showed more MMP-9 in ear sections upon re-exposure to DNFB (Figure 3C). Again, simultaneous treatment with CXCL9(74-103) at the DNFB re-exposure reduced this MMP-9 expression. In contrast, no significant differences in the production of gelatinase A (MMP-2) were observed (Figure 3A,B).

3.5 | Differential impact on inflammatory cytokines and chemokines

Upon treatment with CXCL9(74-103), a clear difference in the ear swelling response was observed. Therefore, the local inflammatory reaction in the tissue was evaluated in further detail. Homogenates of ear tissue were analysed for their content of selected cytokines and chemokines important in CHS at 24 hours after local challenge (Figure 4). Corresponding to the increase in ear thickness and the infiltration of granulocytes, the total protein concentration was elevated significantly 24 hours after re-exposure to DNFB (Figure 4A). IFN-γ, IL-1β and CXCL10 in ear homogenates were below the detection limits of the ELISAs (detection limits were 31.5, 15.6 and 62.5 pg/mL, respectively) (data not shown). No change in TNF-α protein concentration was detected (Figure 4B). An increase in the production of IL-6, CXCL1, CXCL6, CCL2 and CCL3 protein was observed when mice were re-exposed to DNFB (Figure 4C,D,F,G,H). Interestingly, the level of the neutrophil chemoattractant CXCL6 was increased to a much higher extent (19.7-fold change) than the two other murine neutrophil attractants (less than 4-fold change for CXCL1 and CXCL2) in the immunized and challenged ears, suggesting an important role for CXCL6 in DNFB-induced CHS. In contrast, no increased production of CXCL2 was observed by ELISA (Figure 4E). Simultaneous injection of CXCL9(74-103) significantly reduced the expression of IL-6, CXCL1 and CCL2 and resulted in a decrease in total protein (Figure 4A,C,D, G). Although the production of CCL3 and CXCL6 was elevated after re-exposure to DNFB, the effect of CXCL9(74-103) on these chemokines was not significant at 24 hours.

3.6 | Mechanism of action of CXCL9(74-103) in inhibiting neutrophil migration

CXCL9(74-103) was recently shown to interact with high affinity with GAGs and to compete with human chemokines for GAG binding.³⁸ Using the Octet K2 system, the affinity (K_D value) of CXCL9(74-103) for low molecular weight heparin (LMWH) was determined to be

970 ± 205 nmol/L (Figure 5A). In view of the high production levels of murine CXCL6 in DNFB-treated mice (Figure 4), the ability of the CXCL9-derived peptide to compete with this neutrophil chemoattractant for GAG binding was evaluated. As evidenced in Figure 5B, CXCL9(74-103) clearly competed for binding to heparin with CXCL6 (9-78), a highly potent natural isoform of murine CXCL6.⁴³ A 2-fold molar excess of the CXCL9 peptide compared to CXCL6(9-78) already resulted in a 50% reduction of CXCL6(9-78) binding to heparin. If the molar concentration of CXCL9(74-103) exceeded the concentration of CXCL6(9-78) by a factor 20, CXCL6(9-78) binding to heparin was reduced by more than 80%. Furthermore, CXCL9(74-103) competed with murine CXCL1 for binding to heparin as well (Figure 5C). A 60% reduction of CXCL1 binding to heparin was observed at a 10-fold molar excess of the CXCL9-derived peptide. A 100-fold molar excess of CXCL9(74-103) resulted in an 80% decrease of binding of CXCL1 to heparin. In addition, competition of CXCL9(74-103) with murine CCL3 for binding to heparin was detected (Figure 5D). A 100-fold molar excess of the peptide led to a reduction of 50% of CCL3 binding to heparin. In addition, the effect of the CXCL9-derived peptide on the transendothelial migration of neutrophils in response to CXCL6(9-78) was investigated. Both addition of a 3- and 0.3-fold molar excess of CXCL9(74-103) compared to CXCL6 to the neutrophils resulted in a significant inhibition of neutrophil migration across the endothelial layer in response to 20 nmol/L CXCL6 (Figure 6A). As CXCL8, which does not exist in mice, is as CXCL6 in mice the most potent human neutrophil attractant, the effect of CXCL9(74-103) on the transendothelial migration of neutrophils in response to CXCL8 was assessed. A three times lower molar amount of CXCL9(74-103) (6 nmol/L) significantly inhibited the migration of neutrophils across the endothelium in response to 20 nmol/L CXCL8 (Figure 6B). In contrast, the lower doses tested [i.e. 2 nmol/L (0.1-fold molar excess) and 0.6 nmol/L (0.03-fold molar excess)] were not able to significantly inhibit the CXCL8-induced transendothelial migration of neutrophils.

4 | DISCUSSION

CXCL9(74-103) has recently been shown to be an effective inhibitor of MSU crystal-induced gout in mice. In this inflammation model, the named peptide was able to reduce CXCR2-dependent neutrophil migration to the knee joint 3 hours after injection of the MSU crystals.³⁸ As the CXCL9-derived peptide binds with high affinity to GAGs, competes with active chemokines for GAG binding *in vitro* and does not signal through CXCR3 (the GPCR for intact CXCL9), it can be stated that the inhibitory effect is GAG- and not receptor-mediated. This strategy for the inhibition of inflammation is comparable to the recently developed CellJammer and NOX A12 aptamer approaches and is an alternative method for small molecule chemokine receptor antagonists to inhibit chemokine function.^{41,44-50} In the CellJammer and NOX A12 approaches, chemokine mutants with increased GAG-binding affinity and abrogated GPCR signalling or aptamers that mask the GAG-binding site of a chemokine are used, respectively. This results in antagonists which potentially compete with active chemokines

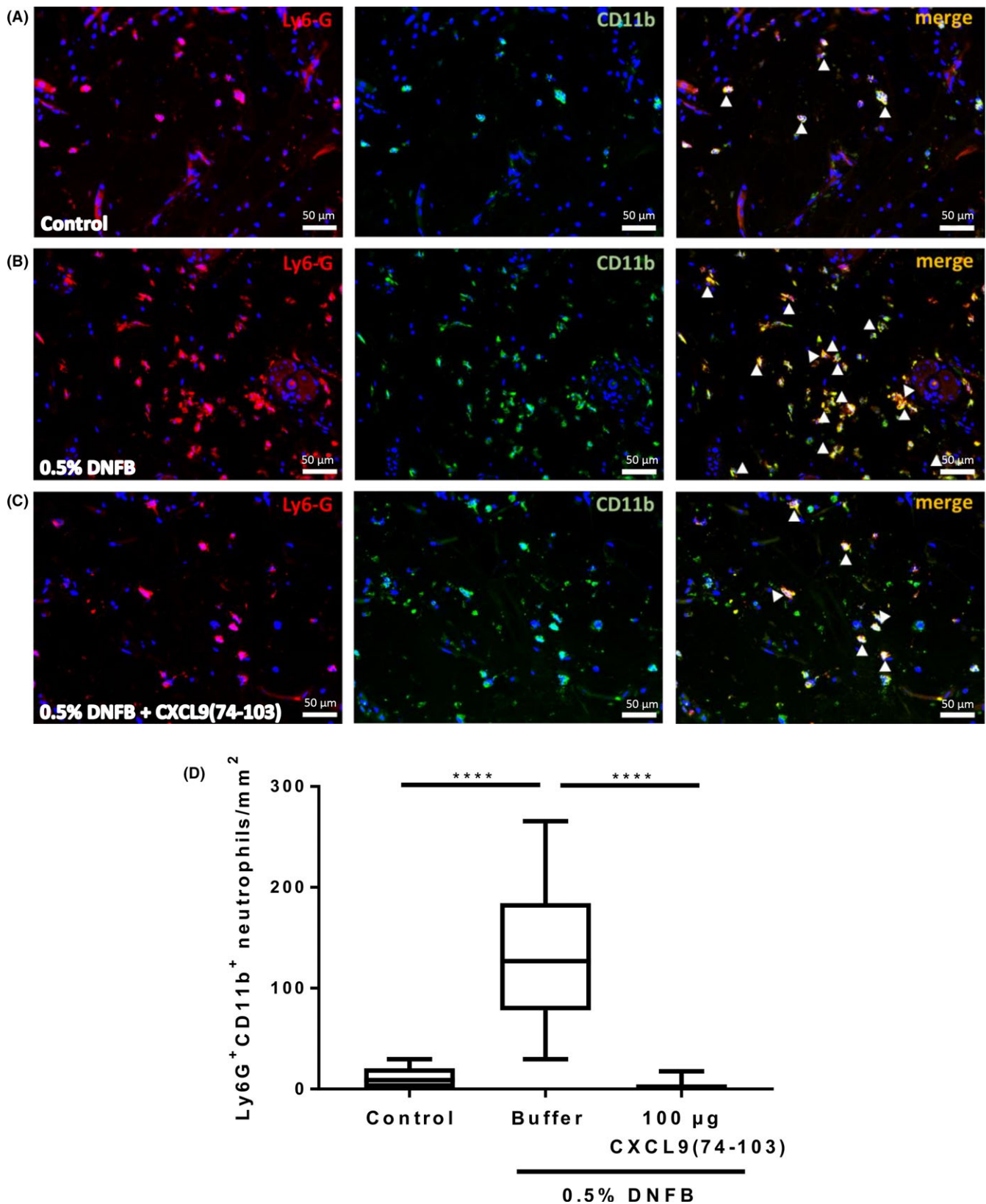


FIGURE 2 Immunofluorescent staining and quantification of neutrophils in ear sections. A, B, C Representative photomicrographs of CD11b and Ly6G stained ear sections of control (A) and DNFB-challenged mice treated with buffer (B) or CXCL9(74-103) (C) were shown ($\times 20$). Arrowheads indicate CD11b⁺Ly6G⁺ neutrophils in the merged figures. Scale bar = 50 μ m. (D) Ly6G⁺CD11b⁺ cells were quantified on photomicrographs ($\times 20$) of CD11b and Ly6G stained ear sections of control or DNFB-challenged mice treated with buffer or CXCL9(74-103). For each group, micrographs were taken of ear sections of different mice ($n \geq 4$ mice/group). The box plot shows minimum, first quartile, median, third quartile and maximum. ****, $P < 0.0001$

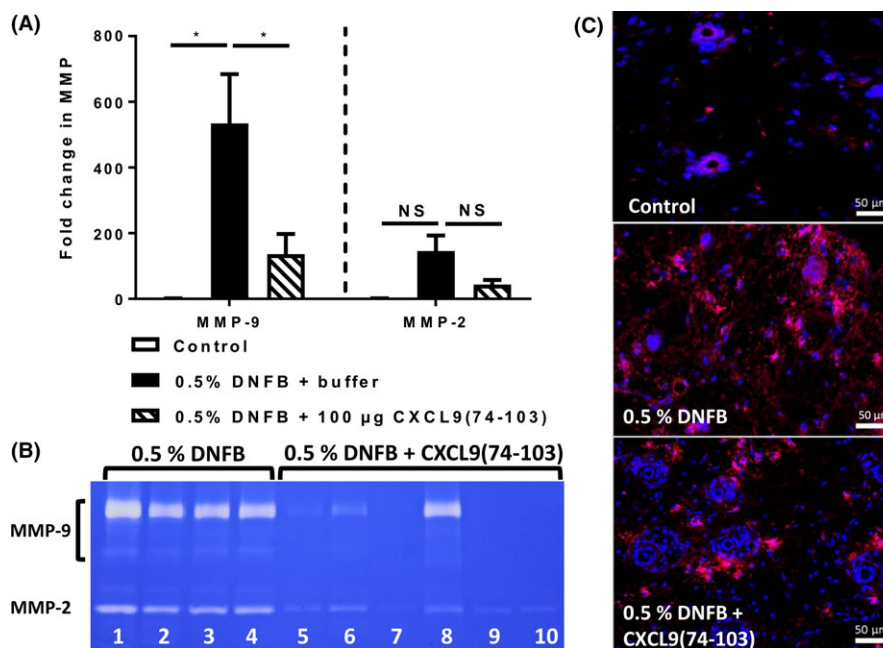


FIGURE 3 Reduced MMP-9 levels in ears from mice treated with CXCL9(74-103). A Ear homogenates of mice immunized and challenged with 0.5% DNFB in 4:1 acetone/paraffin or 4:1 acetone/paraffin (control) were analysed on polyacrylamide gels containing gelatin. Mice were treated with CXCL9(74-103) or buffer at the time of challenge and were killed 24 h later. The fold change of MMP-9 and MMP-2 was calculated by quantification of zymographic bands on a densitometer.⁴⁰ Bar graphs show median $\pm$ SEM of 4 independent experiments. B, A representative image from a zymography gel illustrates gelatinolysis as transparent lysis zones. Each lane represents the gelatinase levels in an individual ear homogenate [lanes 1-4: 0.5% DNFB immunized and challenged mice, lanes 5-10: 0.5% DNFB immunized and challenged mice treated with a single intravenous injection of CXCL9(74-103)]. C, Representative photomicrographs of MMP-9-immunostained ear sections of control and DNFB-challenged mice treated with CXCL9(74-103) or buffer are shown ($\times 20$). Nuclei were stained with DAPI (blue), and MMP-9 was stained with goat anti-mouse MMP-9 antibody and donkey anti-goat Alexa 647 antibody (pink). Scale bar = 50 μ m. *, $P < 0.05$

for GAG binding. Another approach which was suggested is the use of GAGs or GAG-mimetics.^{1,51} These could specifically target a chemokine of choice, inhibiting its interaction with endogenous GAGs and preventing its natural function. However, very few GAG fragments have been developed for therapeutic use, mostly because the synthesis of such fragments is difficult. We used a natural chemokine-derived peptide of 30 amino acids which contains 16 positive amino acids and two GAG-binding motifs of the type XBBXB (Lys75-Lys78) and XBBBXXB (Lys85-Lys90). B represents a basic and X represents any nonbasic amino acid.⁵² Because the synthesis of such a peptide is easier than the synthesis of GAG mimetics, we suggest that this peptide-based approach is favourable. Through competition with active chemokines for GAG binding, the peptide was able to inhibit chemokine presentation on the endothelium, thereby preventing attraction of leucocytes, such as neutrophils.³⁸ However, shortening the length of the CXCL9(74-103) peptide gradually from the NH₂-terminus was shown to decrease the affinity for heparin and the capacity to compete with CXCL8 for GAG binding. Moreover, CXCL9(86-103) was unable to inhibit chemotactic activity of CXCL8 in vivo.^{38,53} In addition, it was shown that shortening CXCL9(74-103) at the COOH-terminus increases the specificity for particular GAGs.⁵⁴ This indicates a certain degree of specificity for different GAGs due to a reduced spectrum of GAG binding.

Here, we corroborate in the CHS model that neutrophils are crucial for DTH responses.^{33,36,37,55} It seems that neutrophils, attracted

by mast cell-derived mediators, are required for the activation and migration of dendritic cells (DCs) and the subsequent priming of T cells in the lymph nodes. Inhibition of neutrophil migration could therefore lead to a reduced DTH response. Previously, it was described that both depletion and genetic deficiency of neutrophils in mice abrogate the CHS response.^{36,55} We show that a single intravenous injection of 100 μ g CXCL9(74-103), just before the challenge with the hapten DNFB on the ear of immunized mice, reduced the ear swelling, MMP-9 production and peroxidase activity after 24 hours. As neutrophils are the major source of MMP-9 and peroxidases, such as myeloperoxidase, it can be stated that less neutrophils infiltrated the ears.^{56,57} Neutrophil collagenase (MMP-8) and MMP-9 are released from neutrophil granules to degrade collagen, a major component of the basement membrane and connective tissue, and to facilitate neutrophil extravasation.⁵⁸ In addition, in humans, MMP-9 was shown to potentiate CXCL8, the most potent human neutrophil chemotactic chemokine, in a positive feedback loop.⁵⁹ In the mouse, MMP-9 also potentiates CXCL6,⁶⁰ the predominant chemokine in the present model. As with other examples of inflammatory disease,^{58,61} the constitutively expressed gelatinase A (MMP-2) remained unaffected as only a trend of increase could be observed upon re-exposure of immunized mice to DNFB challenge, whereas MMP-9 was significantly increased by the DTH reaction.

No IL-1 β , IFN- γ or CXCL10 protein production was detected. This suggests that either the protein levels in the ear were too low to be

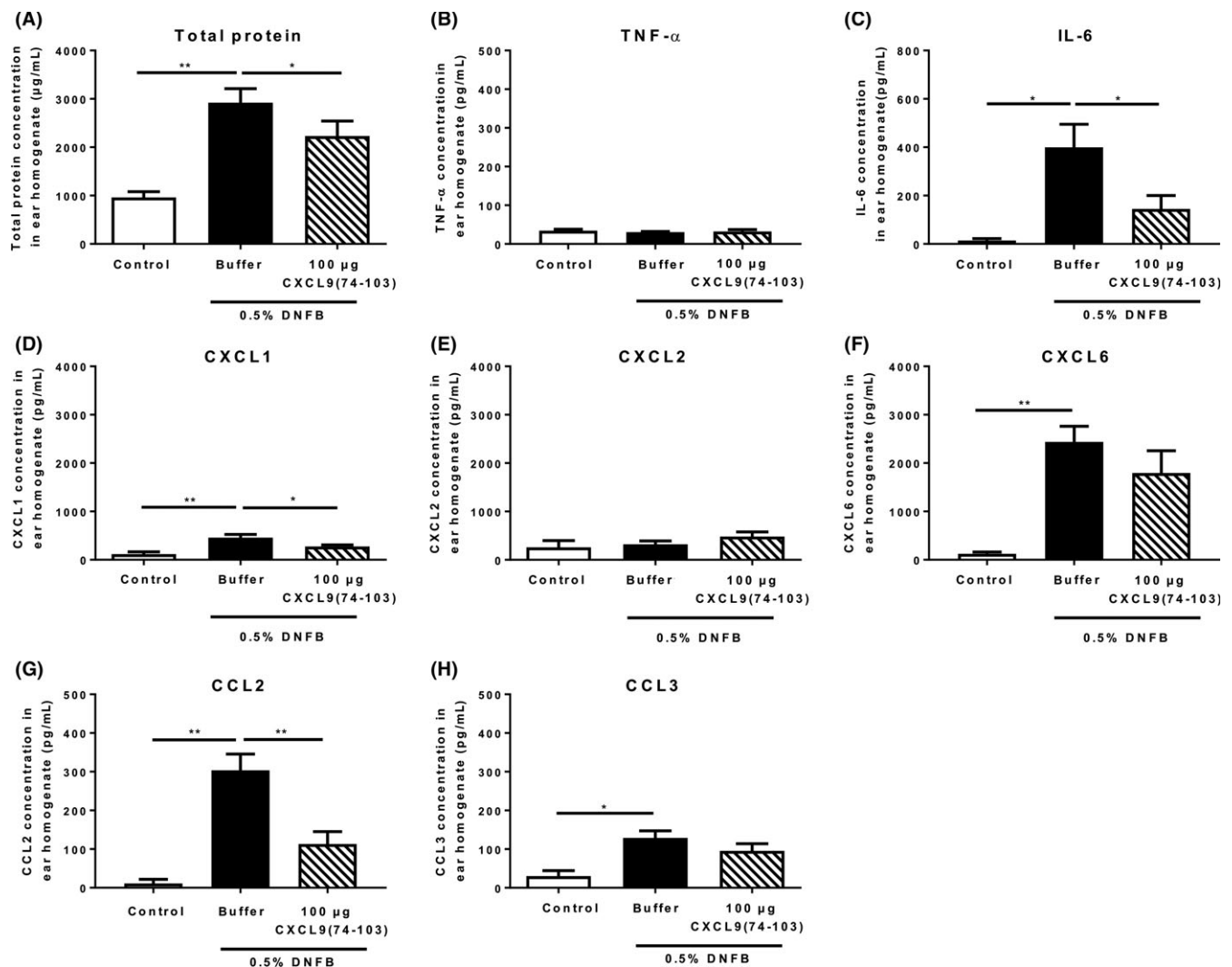


FIGURE 4 Chemokine and cytokine protein levels in ear homogenates after DNFB challenge. Mice were sensitized (day 0) and challenged (day 5) with 4:1 acetone/paraffin (control) or 0.5% DNFB in 4:1 acetone/paraffin as described. Just before epicutaneous application of DNFB solution on the right ear, mice were injected with CXCL9(74-103) or buffer (PBS). Homogenates of ear tissue were prepared 24 h after challenge and analysed by ELISA for their content of a number of selected cytokines and chemokines. Shown are the concentrations of total proteins (A), TNF-α (B), IL-6 (C), CXCL1 (D), CXCL2 (E), CXCL6 (F), CCL2 (G) and CCL3 (H). The bar graphs show the mean ($\pm$ SEM) concentration from ≥ 8 individual mice from 4 independent experiments. *, $P < 0.05$; **, $P < 0.01$

detected or that these cytokines and chemokines were not produced yet. Challenging the ears resulted in high IL-6 concentrations. In addition, a decrease in IL-6 was measured at the protein level upon treatment of mice with the CXCL9 peptide. Locally, IL-6 is predominantly produced by macrophages,⁶² confirming the importance of these cells in DTH. In contrast to the reduced IL-6 level, treatment with the CXCL9 peptide hardly affected local concentrations of the chemokine CXCL6. This was in-line with the idea that chemokines were still produced in the ears by nonleucocytic tissue cells, but bound less efficiently to GAGs due to competition with the CXCL9 peptide. In support of this mechanism of action, we showed that the CXCL9-derived peptide had high affinity for GAGs and competed for GAG binding with CXCL1, CCL3 and with the most prominent neutrophil-attracting murine chemokine in the ears, that is CXCL6. In comparison to competition with CXCL8 binding to heparin performed before,

equimolar concentrations of the CXCL9 peptide already inhibited CXCL6 binding to heparin by approximately 50% (to inhibit CXCL8 binding to heparin by 50% at least a 10-fold molar excess of the peptide was needed).³⁸ To clarify the mode of action, we showed that the CXCL9(74-103) peptide is able to inhibit the migration of neutrophils across the endothelium. In this transendothelial migration assay, GAGs are important coreceptors of chemokines, which are essential for the migration of the neutrophils across the endothelial monolayer. Reduced GAG binding of chemokines resulted in reduced migration of leucocytes to the site of inflammation and prevented additional cytokine and chemokine production by the infiltrating and activated leucocytes. This mechanism also explains why delivery of CXCL9 peptide is still beneficial 3 hours after DNFB application on the ears. Indeed, the peptide inhibits chemokine-GAG binding and thus chemokine activity, but not chemokine production.

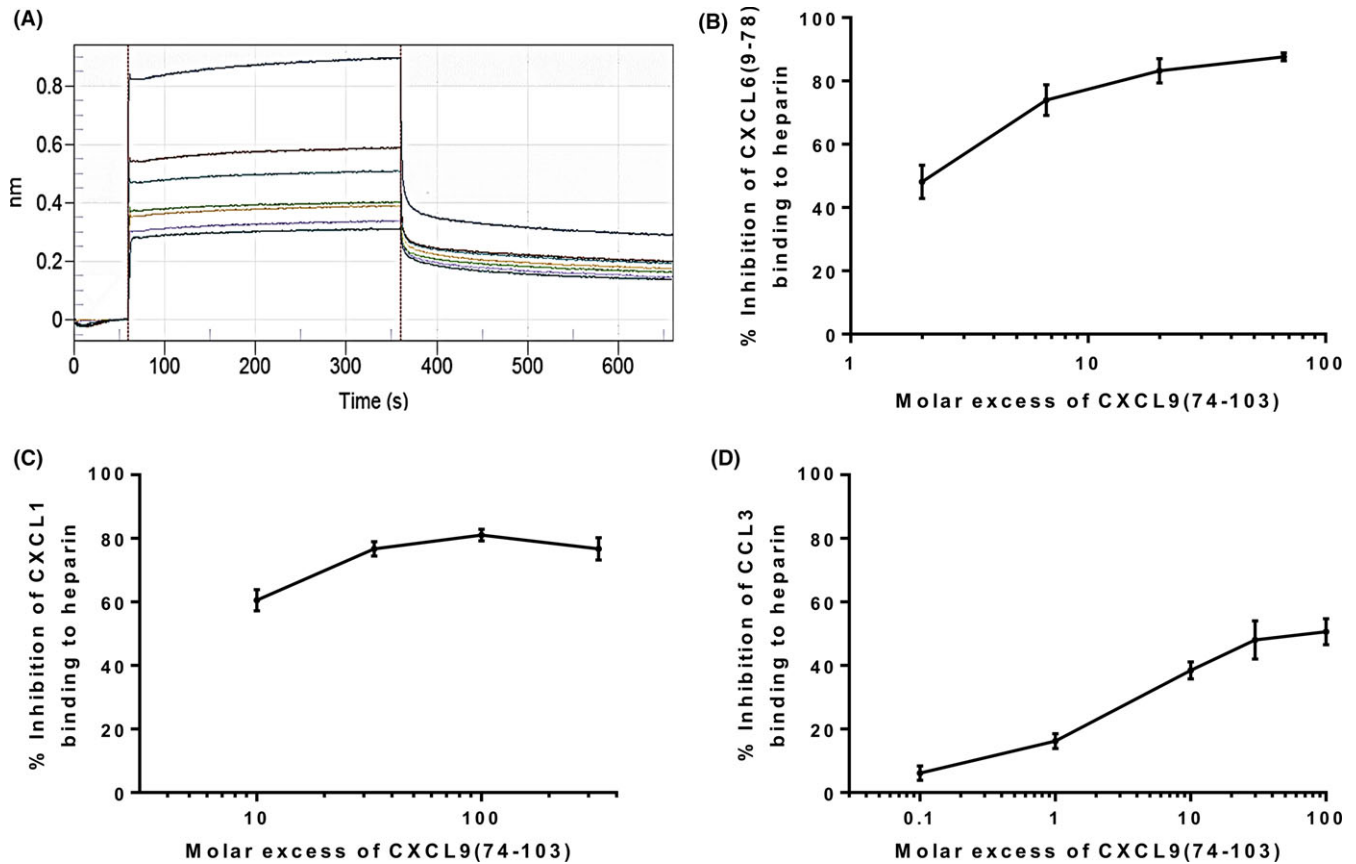


FIGURE 5 The mode of action of CXCL9(74-103) on inhibition of neutrophil migration. A, The affinity of CXCL9(74-103) for LMWH was determined by an Octet K2 system. The binding profile of each sample (from top to bottom: 200–3.125 $\mu\text{g/mL}$ in a 1:2 dilution series) was summarized as a nm shift, and affinity constants were assessed by a simple 1:1 equilibrium binding model. The baseline starts at 0 s until 60 s. The association and dissociation were measured consecutively for 300 s. B, C, D, Competition between CXCL9(74-103) and either CXCL6(9-78) (B), CXCL1 (C) or CCL3 (D) for heparin binding was performed. Dilutions of the respective chemokine were added to heparin, immobilized on 96-well plates, in the presence/absence of the indicated excess of CXCL9(74-103). Bound chemokine was detected using biotinylated antimurine CXCL6 (B), CXCL1 (C) or CCL3 (D) antibodies. The mean $\pm$ SEM percentage inhibition of binding of the respective chemokine to heparin is indicated on the y-axis [$n = 4$ (B); $n \geq 8$ (C); $n \geq 6$ (D)]

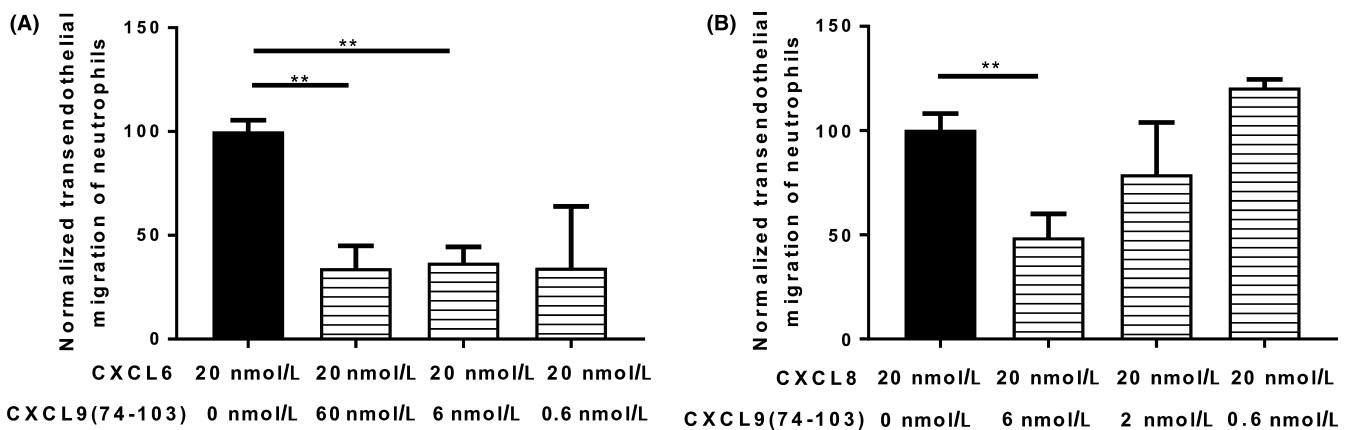


FIGURE 6 In vitro transendothelial migration of neutrophils. The migration (normalized mean $\pm$ SEM) of mouse and human neutrophils in response to CXCL6 (A) and CXCL8 (B), respectively, in the presence/absence of CXCL9(74-103), was evaluated using a transendothelial migration assay. **, $P < 0.01$

Immunofluorescent staining and quantification clearly showed reduced infiltration of neutrophils in the ear upon intravenous treatment with CXCL9(74-103). We also analysed the presence of IFN- γ -

producing T lymphocytes by immunofluorescence analysis. In correspondence with the ELISA data, no IFN- γ was detected (data not shown). Also, a reduced expression of the neutrophil attractant

CXCL1 and the monocyte attractant CCL2 in the ear homogenate was observed, which may be due to reduced production by infiltrating cells. As the CXCL9(74-103) peptide also competes with CCL3 for binding to GAGs, albeit with lower efficiency, we cannot exclude that also the influx of other leucocytes might be altered and may have an additional beneficial effect resulting in reduced ear swelling.

In view of the efficacy of the CXCL9 peptide in different models of inflammation, including CHS and gout, we conclude that CXCL9(74-103) might be a lead molecule for the development of therapeutic peptides or derivatives that compete with functional chemokines for GAG binding. Peptides are generally seen as molecules with a very short half-life. However, binding of the CXCL9(74-103) peptide to endothelial GAGs likely stabilized the molecule and allowed it to remain active during 24 hours after intravenous injection.

ACKNOWLEDGEMENTS

We thank Erik Martens for excellent technical assistance. This research was supported by the Interuniversity Attraction Poles Programme initiated by the Belgian Science Policy Office (I.A.P. Project 7/40), the Foundation for Scientific Research of Flanders (FWO-Vlaanderen) (G.0764.14 and G.0D25.17N) and the Concerted Research Actions (GOA/13/014) and a "C1" grant (C16/17/010) from KU Leuven. The Hercules foundation of the Flemish government provided funding to purchase LC-MS/MS equipment (Contract AKUL/11/31). F.P. received a postdoctoral research fellowship from FWO-Vlaanderen. H.C. received an FWO-SB PhD fellowship of FWO-Vlaanderen.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Vanheule V, Crijns H, Poosti F, et al. Anti-inflammatory effects of the GAG-binding CXCL9(74-103) peptide in dinitrofluorobenzene-induced contact hypersensitivity in mice. *Clin Exp Allergy*. 2018;48:1333-1344. <https://doi.org/10.1111/cea.13227>