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ARTICLE

The chemokine fragment CXCL9(74–103) diminishes neutrophil recruitment and joint inflammation in antigen-induced arthritis

Daiane Boff[1,2] | **Helena Crijns**[1] | **Rik Janssens**[1] | **Vincent Vanheule**[1] | **Gustavo B. Menezes**[3] | **Soraia Macari**[4] | **Tarcilia A. Silva**[4] | **Flavio A. Amaral**[2] | **Paul Proost**[1]

[1]Laboratory of Molecular Immunology, Department of Microbiology and Immunology, Rega Institute, KU Leuven, Leuven, Belgium

[2]Departamento de Bioquimica e Imunologia, Instituto de Ciencias Biologicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

[3]Centro de Biologia Gastrointestinal, Departamento de Morfologia, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

[4]Departmento de Clínica, Patologia e Cirurgias Odontológicas, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

Correspondence
Paul Proost, Rega Institute, Herestraat 49, box 1042, 3000 Leuven, Belgium.
Email: paul.proost@kuleuven.be

Flavio A. Amaral and Paul Proost contributed equally to this work.

Abstract

This study investigates if treatment with a peptide corresponding to the 30 C-terminal amino acids of CXCL9, CXCL9(74–103), ameliorates joint inflammation in a murine model of antigen-induced arthritis (AIA). AIA was induced in male C57BL/6J mice. Intravenous injection of CXCL9(74–103), simultaneously performed with a tibiofemoral challenge with methylated BSA (mBSA) as antigen in mice immunized with mBSA, diminished the accumulation of leukocytes, in particular neutrophils, in the synovial cavity. The levels of the chemokines CXCL1, CXCL2, and CXCL6 and of the cytokine IL-6 were decreased in inflamed periarticular tissue of mice treated with the CXCL9-derived peptide compared to non-treated AIA mice. In addition, CXCL9(74–103) treatment substantially reduced joint and cartilage damage. CXCL9(74-103) competes with CXCL6 and CCL3 for binding to the glycosaminoglycans heparan sulfate and chondroitin sulfate in vitro. In vivo, CXCL9(74-103) quickly binds to blood vessels in joints as observed by confocal microscopy. Next, we evaluated if later treatment with CXCL9(74–103) had a beneficial impact on joint inflammation. CXCL9(74-103) injection 6 h after mBSA challenge still reduced neutrophil accumulation in the joint, although it did not reduce chemokine and IL-6 concentrations. However, a delay of treatment until 12 h after challenge had no effect on cell recruitment and chemokine and IL-6 levels. Taken together, we demonstrated that treatment with a peptide, which interferes with the interaction between chemokines and glycosaminoglycans, from the beginning of the disease controlled the massive accumulation of neutrophils in the joint of AIA mice, greatly impacting on joint inflammation and tissue damage.

KEYWORDS

chondroitin sulfate, glycosaminoglycan, heparan sulfate, joint damage, proteoglycan

1 | INTRODUCTION

Arthritis is the major category of rheumatic diseases and can be classified into autoimmune, autoinflammatory, and infectious arthritis.[1] Patients that develop arthritis present with pain and permanent articular damage as major symptoms.[2] The inflammatory process during the disease is characterized by the recruitment of leukocytes to the joints. Neutrophil recruitment can cause tissue damage since once these cells are activated, they release reactive oxygen species (ROS) and lytic enzymes upon degranulation and also produce pain-associated mediators.[3] This neutrophil recruitment is highly regulated by chemotactic gradients produced by chemokines.

Chemokines are small proteins with a molecular mass of ~7–12 kDa that belong to the family of chemotactic cytokines. Chemokines were named based on their chemoattractant properties, described first in 1987 when IL-8 or CXCL8 was shown to be a major neutrophil

Abbreviations: AIA, antigen-induced arthritis; DMARD, disease-modifying anti-rheumatic drug; GAG, glycosaminoglycan; GPCR, G protein-coupled receptor; i.a., intra-articular; mBSA, methylated BSA; NET, neutrophil extracellular trap; ROS, reactive oxygen species; TMB, 3,3′,5,5′-tetramethylbenzidine

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attractant in vitro.[4] Additionally, chemokines were described to be involved in other processes, such as embryogenesis, homeostasis, angiogenesis, and inflammation.[5,6] Chemokines can be divided into 4 subfamilies according to the position of the two cysteine residues in their N-terminal amino acid sequence[7,8]: (1) CC chemokines have 2 adjacent cysteines; (2) CXC chemokines present with one amino acid between the two cysteines; (3) the CX3C chemokine has 3 amino acids between the cysteines; and (4) C chemokines lack one of the two N-terminal cysteines.[7] The CXC chemokines can be further divided into two subgroups as follows: (1) ELR$^+$ CXC chemokines that have a specific amino acid sequence (or motif) of glutamic acid-leucine-arginine (ELR) immediately before the first cysteine of the CXC motif and are associated with neutrophil recruitment and (2) those without an ELR motif that mainly attract lymphocytes.

Chemokines can exert their chemoattractant function by binding to their G protein-coupled receptors (GPCRs), mainly expressed on leukocytes.[8] Since almost all chemokines are basic proteins, often with an isoelectric point of 10 or higher, glycosaminoglycans (GAGs) bind to these positively charged proteins. GAGs are linear carbohydrate structures, consisting of repeating disaccharide units, that comprise a hexuronic acid linked to an N-acetyl-hexosamine, which can be sulfated at different positions. GAGs are negatively charged and have a molecular weight around 10–100 kDa.[9] They can be divided into the following 6 groups: heparan sulfate, heparin, chondroitin sulfate, dermatan sulfate, keratan sulfate, and hyaluronic acid.[10] Each tissue produces specific GAG repertoires and cells can alter their GAG expression in response to specific stimuli or in pathologic states.[11] Major consensus epitopes for GAG binding in chemokines are described to be BBXB and BBBXXB motifs, where B represents a basic amino acid.[12] It has been shown that some chemokines act as monomers, whereas many chemokines can oligomerize and form diverse quaternary structures, including dimers, tetramers, and polymers, increasing the number of epitopes that bind to GAGs.[10–14] Oligomerization increases the affinity of chemokines for GAGs through an avidity effect and this interaction also stabilizes the chemokine oligomers.[13] The binding of chemokines to GAGs can generate an immobilized chemokine gradient that directs cell migration.[8] Cell surface immobilization of chemokines enables them to act locally rather than as paracrine molecules, and likely prevents inappropriate activation and desensitization of receptors on cells outside the region of interest for a given physiological situation.[14]

Interaction with GAGs also reduces the susceptibility of chemokines to proteolytic modification.[15–17] Indeed, chemokine activity can also be regulated by proteolytic cleavage, truncation, and nitration resulting in loss or gain of function.[18] The enhanced knowledge about the chemokine system in the context of inflammatory diseases has encouraged the development of different compounds that target chemokines and their receptors.[19] Recently, the use of modified chemokines, such as a truncated form of the CC chemokine CCL2, was explored as an antagonist of CCR2. It protected against tissue damage in a model of spontaneous arthritis.[20] Likewise, the inhibition of neutrophil recruitment to the joint in arthritis can be an efficient therapy to avoid tissue damage. Targeting the chemokines and their receptors was effective in some animal models of arthritis, but clinical trials in humans were not that successful.[1] The CXC chemokine CXCL9, also known as monokine induced by IFN-γ (MIG), consists of 103 amino acids and binds to the receptor CXCR3.[21–24] CXCL9 can recruit activated Th1 lymphocytes and NK cells and is produced by a variety of cells following stimulation with IFN-γ.[21–25] We previously demonstrated that a fragment of CXCL9 consisting of its 30 C-terminal amino acids [CXCL9(74–103)] competes with chemokines for the binding to GAGs, inhibits CXCL8-induced neutrophil migration, and reduces the recruitment of neutrophils to the joint in a murine model of gout.[26,27]

Here, we investigated the potential of CXCL9(74–103) as an inhibitor of an established murine model of rheumatoid arthritis, i.e., antigen-induced arthritis (AIA). We demonstrated in vitro and in vivo that CXCL9(74–103) binds to GAGs and competes with murine chemokines for GAG binding. Systemic treatment of mice with CXCL9(74–103) inhibits neutrophil recruitment and joint damage in antigen-induced arthritis.

2 | MATERIALS AND METHODS

2.1 | Mice

Eight-to-ten-week-old male C57BL/6J mice were purchased from the Centro de Bioterismo of the Universidade Federal de Minas Gerais. All animals were maintained with filtered water and food ad libitum and kept in a controlled environment. Experiments received prior approval by the animal ethics committee of the UFMG (CEUA 86/2014).

2.2 | Solid-phase synthesis of the C-terminal CXCL9-derived peptide

The C-terminal peptide of CXCL9, CXCL9(74–103), was chemically synthesized with fluorenyl methoxycarbonyl (Fmoc) chemistry using an Activo-P11 automated synthesizer (Activotec, Cambridge, UK), as previously described.[28] Part of the material was fluorescently labeled at the N-terminus using TAMRA (Merck Millipore, Darmstadt, Germany).[26] After synthesis, intact synthetic peptides were purified by RP-HPLC and identified by mass spectrometry (Amazon SL or Amazon Speed ETD ion trap mass spectrometers, Bruker, Bremen, Germany).

2.3 | Antigen-induced arthritis

The mice were anesthetized (60:15 mg/kg ketamine:xylazine [Synthec, Brazil]; i.p.) and were immunized intradermally at the base of the tail with 500 μg of methylated BSA (mBSA; Sigma, St. Louis, MO) in 100 μL of an emulsion of saline and an equal volume of Freund's complete adjuvant (CFA; Sigma) on day 0.[29] After 14 days, antigen challenge was performed in anesthetized mice with a tibiofemoral injection of 10 μg of mBSA in 10 μL of phosphate-buffered saline (PBS). Non-immunized mice were challenged with PBS or mBSA, representing the negative controls. Groups of mice were treated with CXCL9(74–103) 100 μg/100 μL i.v. at the same time and/or 6 or 12 h after the challenge. At 24 h after the challenge, mice were killed by anesthetic overdose

followed by cervical dislocation and the articular cavity was washed with PBS containing 3% BSA for cell counts. The number of leukocytes in the articular cavity was determined in a Neubauer chamber, after staining the cells with Turk's solution. Differential counts were performed on Cytospin (Shandon III) preparations by evaluating the percentage of each leukocyte type on a slide stained with May-Grunwald-Giemsa. Periarticular tissue was removed from the joints for evaluation of cytokine and chemokine levels.

2.4 | Measurement of chemokines and cytokines

Periarticular tissue was collected and homogenized in PBS containing protease inhibitors and samples were stored at −20°C until further analysis.[29] Samples were processed and the supernatant was evaluated in specific cytokine and chemokine ELISAs in accordance with the manufacturer's instructions (R&D Systems).

2.5 | Histopathological analysis

The whole tibiofemoral joints were fixed in 10% buffered formalin (pH 7.4), decalcified for 30 days in 14% EDTA, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Two sections of knee joints were microscopically examined by a single pathologist, and scored in a blinded manner. The histological score was adapted from an arthritis index as described previously.[30] The parameters evaluated were as follows: severity of synovial hyperplasia, intensity and extension of the inflammatory infiltrate, vascular hyperemia, presence of inflammatory cells in the synovial cavity, and changes in tissue architecture. These criteria result in a maximal score of 9 points. Toluidine blue (TB)-stained slides were used to estimate joint proteoglycan content, as described previously.[31] Images of the joint surface of each sample were digitalized and evaluated using Image J software (National Institutes of Health, Bethesda, MD). Cartilage proteoglycan content is reported as the percentage of the TB-stained area in relation to the total evaluated cartilage surface.

2.6 | Competition of CXCL9(74–103) with CCL3 and CXCL6 for binding to GAGs

The ability of the CXCL9(74–103) to compete for GAG binding with the inflammatory chemokines CXCL6 and CCL3 was evaluated on 96-well GAG-binding plates (Galen Laboratory Supplies, North Haven, CT). In brief, plates were coated overnight with 25 μg/mL GAGs (heparan sulfate or chondroitin sulfate; Iduron, Cheshire, UK) at room temperature.[26] Dilutions of the highly potent form of recombinant murine CXCL6, i.e., CXCL6(9–78),[32] or recombinant CCL3 (Peprotech, Rocky Hill, NJ) with or without CXCL9(74–103) peptide were added and incubated for 2 h at 37°C. Subsequently, bound CXCL6 or CCL3 was detected with biotinylated anti-murine CXCL6 or CCL3 antibodies (Peprotech) and HRP-labeled streptavidin. A chromogenic substrate for HRP, 3,3′,5,5′-tetramethylbenzidine (TMB), was added. Finally, the conversion of TMB was measured at 450 nm in order to quantify the peroxidase activity.

2.7 | Confocal microscopy

For in vivo confocal imaging, mice were anesthetized (60:15 mg/kg ketamine:xylazine injected intraperitoneally) and the joint was exposed for imaging. The TAMRA-labeled CXCL9(74–103) was diluted in sterile saline (100 μg/100 μL) and injected i.v. before imaging. Mice were imaged using a Nikon Eclipse Ti microscope with a C2 confocal head equipped with three different lasers (excitation at three wavelengths: 405 nm, 488 nm, and 543 nm) and emission bandpass filters at 450/50, 515/30, and 584/50 nm. The *z*-position was controlled by an automated device and 10× objectives were used on the required resolution. The analysis was performed using Volocity 6.3 software (PerkinElmer).

2.8 | Statistical analyses

Data were expressed as mean ± SEM or median and analysis was performed using the statistical software GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Differences between means were evaluated using ANOVA test, followed by Newman–Keuls and *t*-test followed by unpaired test. For the competition assays with GAGs, a Wilcoxon matched-pairs signed rank test was used in Graphpad. Results with $P < 0.05$ were considered significant.

3 | RESULTS

3.1 | The treatment with CXCL9(74–103) decreased neutrophil recruitment in antigen-induced arthritis

Neutrophils play an important role in the pathogenesis of experimental antigen-induced arthritis and their presence is related with articular damage and hypernociception.[33,34] Additionally, we previously demonstrated that systemic treatment with CXCL9(74–103) decreased the recruitment of neutrophils in two different murine models of joint inflammation: local injection of CXCL8 and gout.[26,27] In order to check if CXCL9(74-103) also reduces neutrophil recruitment in an antigen-induced arthritis, we immunized mice with mBSA and CFA as described before.[29] Fourteen days after of the immunization, the mice were challenged with mBSA (intra-articular, i.a.). Simultaneously with the challenge, the mice were treated with CXCL9(74-103) 100 μg/100 μL i.v. or PBS as vehicle and euthanized 24 h after the challenge. The treatment with CXCL9(74-103) decreased the amount of total cells accumulated in the joint, compared with vehicle-treated mice (Fig. 1A). In accordance, the treatment with the CXCL9-derived peptide also reduced the number of neutrophils recovered from the joint (Fig. 1B), but did not affect the infiltration of mononuclear cells (Fig. 1C).

3.2 | The treatment with CXCL9(74–103) decreased chemokine and cytokine levels in antigen-induced arthritis

In order to check if the decrease in cell recruitment in mice treated with CXCL9(74–103) also affects the production of chemokines and

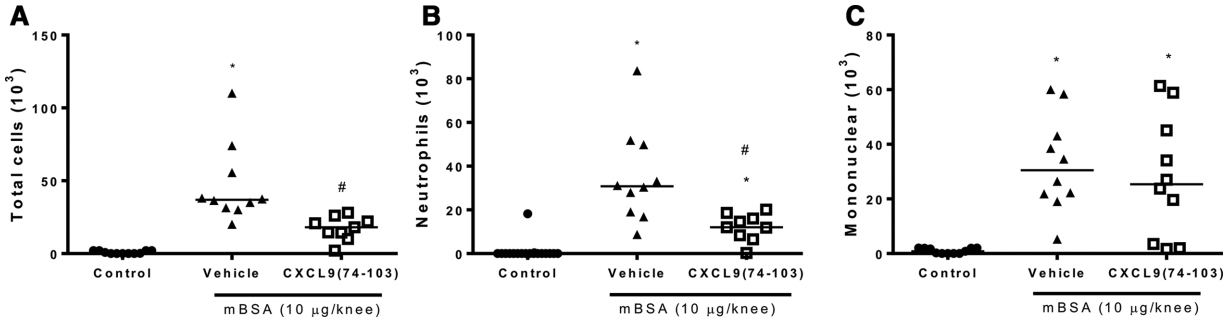

FIGURE 1 **The treatment with CXCL9(74–103) decreases the influx of neutrophils in the joints in antigen-induced arthritis.** Mice were immunized with mBSA and CFA and were challenged i.a. with mBSA 14 days later. Simultaneously with the challenge, the mice were treated i.v. with CXCL9(74–103) 100 μg/100 μL or vehicle (PBS). The mice were euthanized 24 h after the challenge, the amount of total cells (A), neutrophils (B), and mononuclear cells (C) was evaluated in the articular lavage. Data are shown as median from one representative out of two independent experiments. $^{*}P < 0.05$ when compared with the control group; $^{\#}P < 0.05$ when compared with vehicle control. $N = 5–10$ mice per group

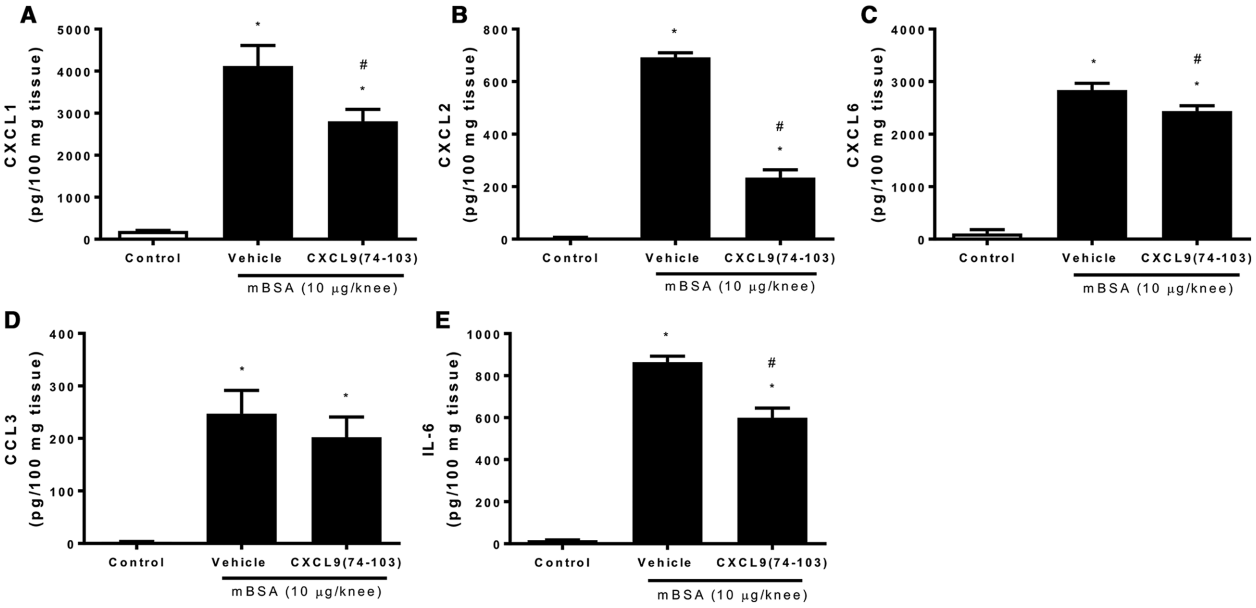

FIGURE 2 **Treatment with CXCL9(74-103) decreases chemokine and cytokine levels in antigen-induced arthritis.** Mice were immunized with mBSA and CFA and were challenged i.a. with mBSA 14 days later. Simultaneously with the challenge, the mice were treated i.v. with CXCL9(74-103) 100 μg/100 μL or vehicle (PBS). The mice were euthanized 24 h after the challenge and the periarticular tissue was processed for quantification by ELISA of CXCL1 (A), CXCL2 (B), CXCL6 (C), CCL3 (D), and IL-6 (E). Data show the mean ± SEM from one representative out of two independent experiments. $^{*}P < 0.05$ when compared with the control group; $^{\#}P < 0.05$ when compared with vehicle. $N = 5–10$ mice per group

cytokines, we measured the protein levels of these mediators in the periarticular tissue. Two main chemokines related to recruitment of neutrophils, murine CXCL1 and CXCL6, were more abundantly produced in the tissue compared to other neutrophil attractants, such as CXCL2 and CCL3 (Fig. 2A–D). Also, IL-6 production was significantly enhanced in the periarticular tissue. The treatment with CXCL9(74–103) significantly decreased the levels of CXCL1, CXCL2, and CXCL6 compared to vehicle (Fig. 2A–C, respectively). However, the levels of CXCL1 and CXCL6 remained high despite the clear reduction in neutrophil influx (Fig. 1B). In particular, the production of CXCL2 dropped to about one third. The treatment also moderately, but significantly reduced the levels of the cytokine IL-6 (Fig. 2E). Production of CCL3, which may also recruit neutrophils in mice, but is primarily an attractant of mononuclear cells, was not affected by the treatment.

3.3 | CXCL9(74-103) competes with murine chemokines for GAG binding and binds to GAGs in vivo

Proteoglycans are the main component present in cartilage and are formed by addition of a single GAG chain or can have over 100 GAG chains, as in case of aggrecan, the predominant GAG in cartilage.[35] Heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate can be present in these proteoglycans and also on the endothelium of blood vessels present in joints.[36,37] Although all chemokines are known to interact with GAGs, plates coated with heparan sulfate or chondroitin sulfate were rapidly saturated with CCL3 (Fig. 3A). Indeed, addition of more than 30 nM CCL3 to the GAG-coated plates did not result in additional binding of CCL3. CXCL6 binding to plates coated with heparan sulfate or chondroitin sulfate increased up to

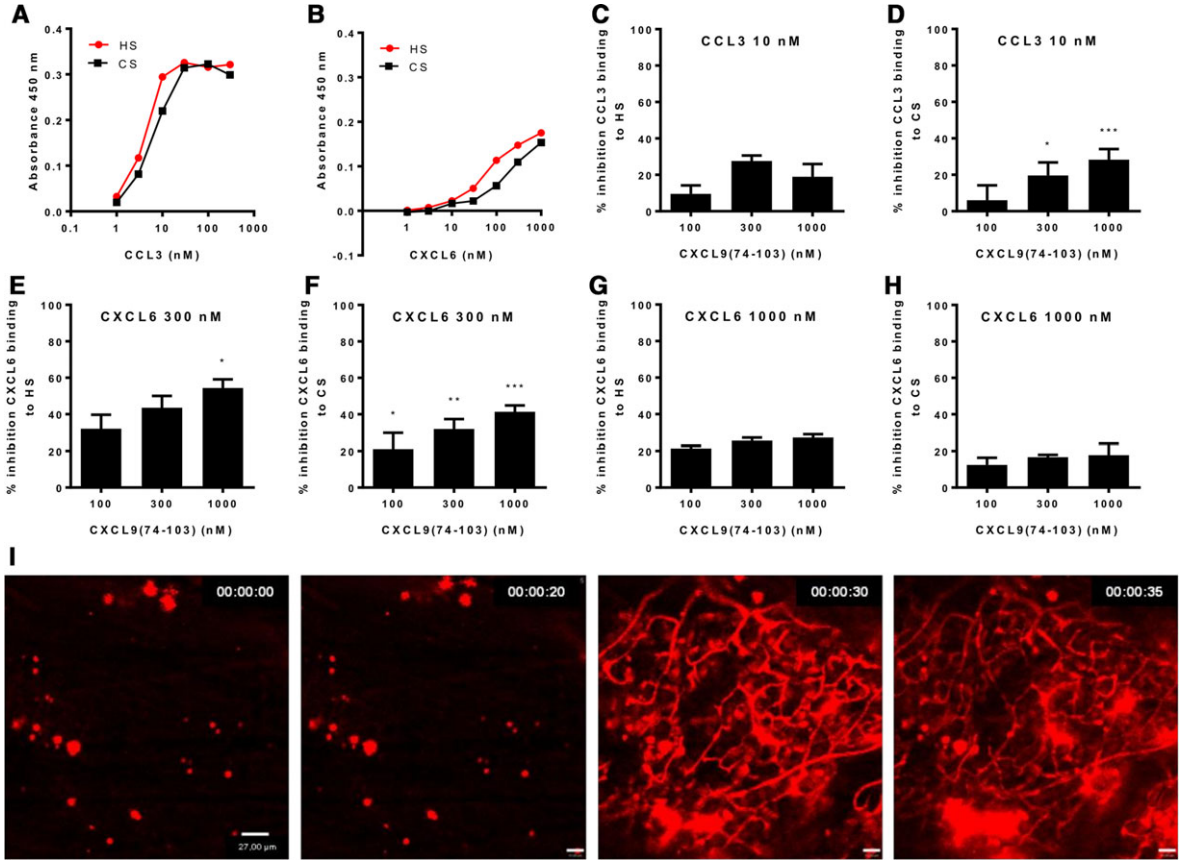

FIGURE 3 CXCL9(74-103) competes with CXCL6 and CCL3 for GAG binding and binds to GAGs in the joint. Different doses of murine CCL3 (panel A) or CXCL6 (panel B) were bound to plates coated with heparan sulfate (red line) or chondroitin sulfate (black line) and detected with specific anti-CXCL6 or anti-CCL3 antibodies. To investigate competition for GAG binding, heparan sulfate (panel C, E, and G) or chondroitin sulfate (panel D, F, and H) coated plates were treated with 10 nM CCL3 (panel C and D), 300 nM CXCL6 (panel E and F) or 1000 nM CXCL6 (panel G and H) in the absence or presence of different doses of CXCL9(74-103). The percentage inhibition of GAG binding was averaged over $n = 3–14$ experiments. Error bars represent the standard error of the mean. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ when compared with GAG binding of CCL3 or CXCL6 in the absence of CXCL9(74–103). To show in vivo binding of CXCL9(74–103), mice were anesthetized and the joint cavity was exposed for confocal microscopy images. TAMRA-labeled CXCL9(74–103) (100 μg/100 μL) was injected i.v. and images were collected for 1 min. Panel I shows images of TAMRA-labeled CXCL9(74-103) (in red) binding to vessels. Time is represented in seconds

addition of 1000 nM chemokine and a minimal concentration of 10 nM CXCL6 was needed to show significant binding (Fig. 3B). We previously demonstrated that CXCL9(74–103) can compete with human CXCL8(1–77) and murine CXCL1(1–72) (KC Keratinocyte Chemoattractant) for binding to GAGs including heparan sulfate and chondroitin sulfate.[26,27] Next, we evaluated if CXCL9(74–103) was able to inhibit the interaction between 10 nM CCL3 (or MIP-1α), 300 nM CXCL6 (or GCP-2), or 1000 nM CXCL6 with heparan sulfate or chondroitin sulfate coated plates. At 300 or 1000 nM, CXCL9(74–103) inhibited the interaction of CCL3 with both GAGs by 20–30% (Fig. 3C and D). Even lower concentrations of CXCL9(74–103) were able to inhibit the interaction of 300 nM murine CXCL6 with heparan sulfate or chondroitin sulfate (Fig. 3E and F). At a concentration of 1000 nM murine CXCL6, CXCL9(74–103) showed the ability to inhibit the interaction between the chemokine and both GAGs, however this inhibition was less pronounced (Fig. 3G and H). To show that CXCL9(74–103) also interacts with GAGs on the blood vessel wall in joints, we injected mice with N-terminally TAMRA-labeled CXCL9(74–103) and detected the peptide by in vivo imaging confocal microscopy. The site-specific labeling of the peptide prevents modification of lysines, which are important for the interaction with GAGs. As demonstrated in Fig. 3I, 30 s after the injection of TAMRA-labeled CXCL9(74-103), fluorescent labeling of the blood vessels is observed. Taken together, these results demonstrate that CXCL9(74–103) binds to GAGs in the joint and competes with CXCL6(9–78) and CCL3 for the binding to heparan sulfate and chondroitin sulfate.

3.4 | The treatment with CXCL9(74–103) decreases articular and cartilage damage in antigen-induced arthritis

Since we observed that treatment with CXCL9(74–103) simultaneously with the challenge decreased the number of neutrophils and levels of cytokines and chemokines, we next evaluated the articular and cartilage damage in those mice. The histopathological scores were evaluated using the following parameters: severity of synovial hyperplasia, intensity and extension of the inflammatory infiltrate, vascular hyperemia, presence of inflammatory cells in the synovial cavity, and changes in tissue architecture. The mice treated with CXCL9(74–103) presented a decrease in histopathological score

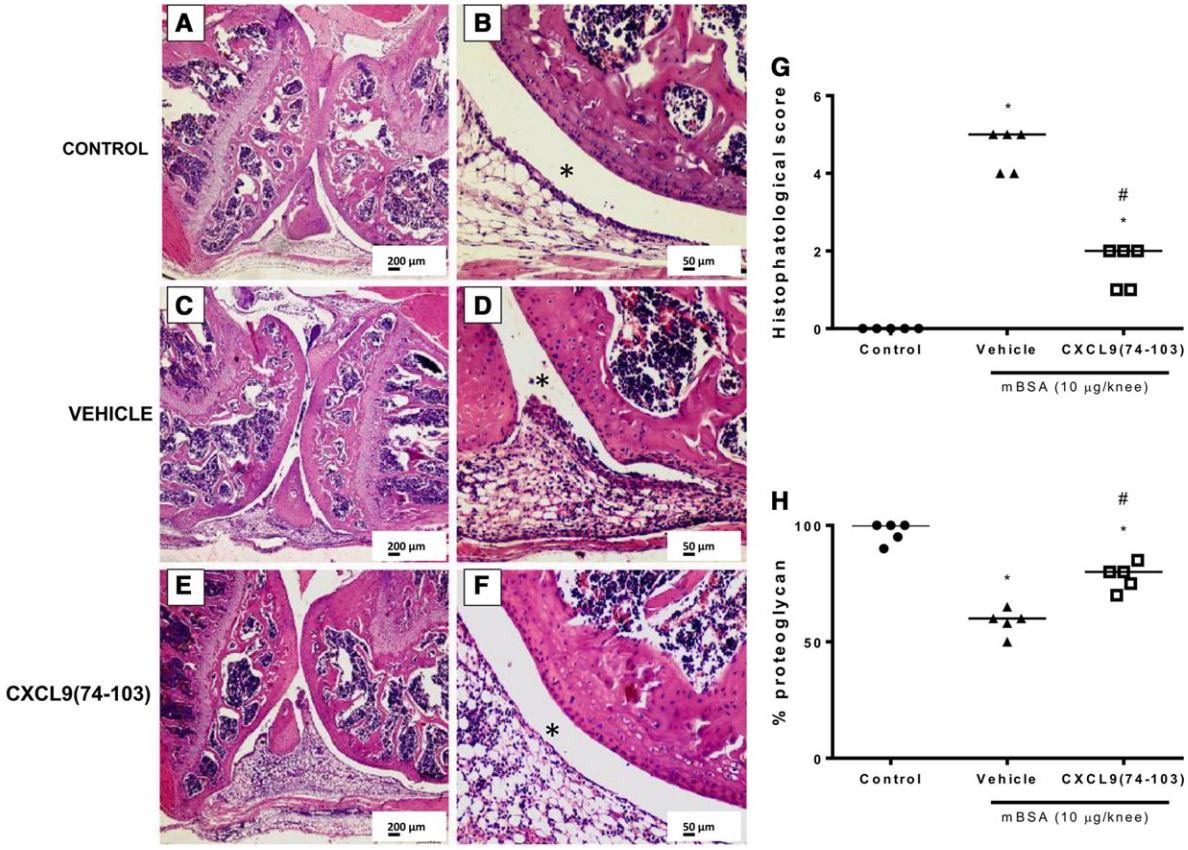

FIGURE 4 **The treatment with CXCL9(74-103) decreased the articular and cartilage damage in antigen-induced arthritis.** Mice were immunized with mBSA and CFA and were challenged i.a. with mBSA 14 days later. Simultaneously with the challenge, the mice were treated i.v. with CXCL9(74–103) 100 μg/100 μL or vehicle (PBS). The mice were euthanized 24 h after the challenge and whole joints were removed and processed for histopathology (G) and analyses of proteoglycan content (H). (A–F) Representative images of histopathological score. (A), (C), and (E) are shown at 200 μm scale and (B), (D), and (F) at 50 μm scale. Data shown represent individual mice and bars show the median from 1 representative out of 2 independent experiments. $^{*}P < 0.05$ when compared with the negative control group; $^{\#}P < 0.05$ when compared with vehicle-treated mice. $N = 4–5$ mice per group

compared to vehicle-treated mice (Fig. 4A–G). We also evaluated cartilage damage by measuring the content of proteoglycans, the main biomolecule in the cartilage. The mice treated with CXCL9(74–103) have preserved the proteoglycan content in the cartilage compared with the vehicle-treated mice (Fig. 4H). Taken together, these results show that the treatment with CXCL9(74–103) simultaneously with the challenge with mBSA prevents articular and cartilage damage in the antigen-induced arthritis model.

3.5 | The treatment with CXCL9(74–103) at a later time point has little or no effect on cell recruitment in antigen-induced arthritis

We demonstrated that treatment of mice with CXCL9(74–103) simultaneously with the challenge decreased neutrophil recruitment to the joint. In order to check if the treatment at later time points was able to reduce neutrophil recruitment, we treated the mice with CXCL9(74–103) simultaneously with the challenge 6 or 12 h after the challenge. Only the treatment at 0 h was able to reduce the total number of cells recruited to the joint compared to the vehicle (Fig. 5A). Both the treatments at time points 0 and 6 h were able to decrease the number of neutrophils, although with the treatment at 0h, the number of neutrophils was reduced more than with treatment at 6 h (Fig. 5B). Treatment after 12 h failed to inhibit neutrophil infiltration into the joint. The number of mononuclear cells was similar in all groups compared to the positive vehicle control mice (Fig. 5C).

Next, we investigated whether treatment with CXCL9(74–103) at later time points could decrease the level of chemokines and cytokines (Fig. 6). The treatment at time of challenge (0 h) decreased the levels of CXCL1, CXCL2, CXCL6, and IL-6 whereas treatment at later time points (after 6 or 12 h) had no effect on the levels of these inflammatory mediators. CCL3 was present at the same levels in all treatment schedules compared to the positive vehicle control. Taken together, these data show that the treatment at 0 and 6 h can decrease the number of neutrophils. On the other hand, the treatment at 12 h has no effect on cell recruitment. Additionally, the treatments at 6 and 12 h had no effect on the levels of chemokines and IL-6.

4 | DISCUSSION

Rheumatic diseases are considered a common cause of disability and represent high costs to public health in Brazil and the United States.[38,39] Nonsteroidal anti-inflammatory drugs are used in the

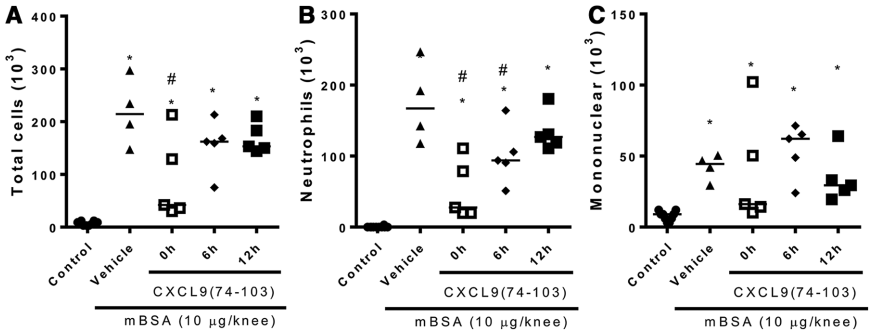

FIGURE 5 **Treatment with CXCL9(74-103) at late time points has no effect on cell recruitment to the joint in antigen-induced arthritis.** Mice were immunized with mBSA and CFA and were challenged i.a. with mBSA 14 days later. Simultaneously with the challenge (0 h), the mice were treated i.v. with vehicle (PBS) or CXCL9(74-103) 100 μg/100 μL. Alternatively, mice were treated 6 or 12 h after challenge. The mice were euthanized 24 h after the challenge and the amount of total cells (A), neutrophils (B), and mononuclear cells (C) was evaluated in the joint lavage. Data are shown as median from 1 representative of 2 independent experiments. $^{*}P < 0.05$ when compared with the control group; $^{\#}P < 0.05$ when compared with vehicle $N = 4–5$ mice per group

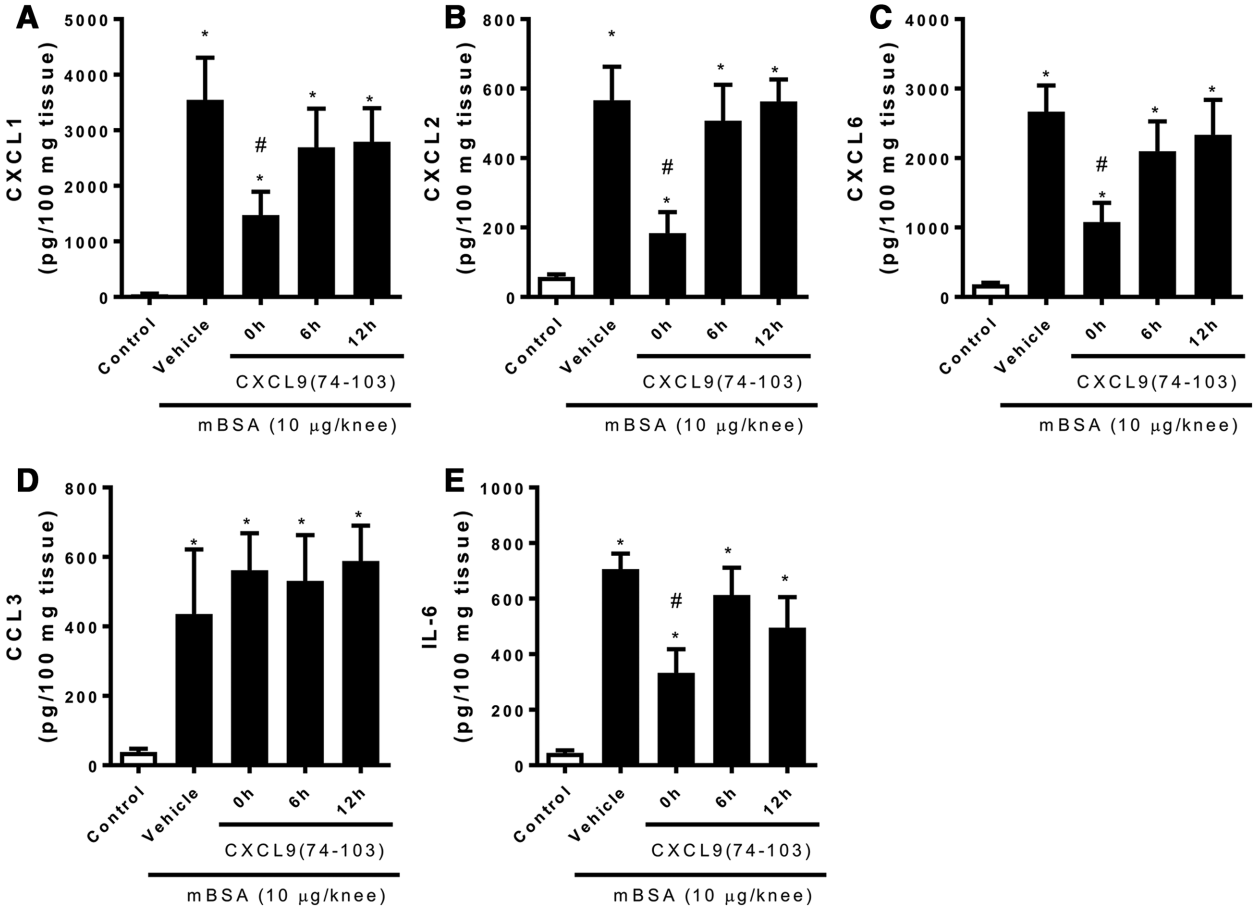

FIGURE 6 **Treatment with CXCL9(74–103) at later time points has no effect on the production of chemokines and IL-6 in antigen-induced arthritis.** Mice were immunized with mBSA and CFA and were challenged i.a. with mBSA 14 days later. Simultaneously with the challenge (0 h) the mice were treated i.v. with vehicle (PBS) or CXCL9(74–103) 100 μg/100 μL. Alternatively, mice were treated 6 or 12 h after challenge with CXCL9(74–103). The mice were euthanized 24 h after the challenge and the periarticular tissue was processed for quantification by ELISAs of CXCL1 (A), CXCL2 (B), CXCL6 (C), CCL3 (D), and IL-6 (E). Data show the mean ± SEM from 1 representative out of 2 independent experiments. $^{*}P < 0.05$ when compared with the control group; $^{\#}P < 0.05$ when compared with vehicle. $N = 4–5$ mice per group

treatment of arthritis, but in recent years biological and conventional disease-modifying anti-rheumatic drugs (DMARDs) became a new option for treatment. The biological DMARDs are inhibitors for specific cytokines, such as TNF, IL-1, and IL-6.[40,41] These inhibitors are immunosuppressors and treatment with these drugs is associated with increased risk of infection, such as pneumonia.[42] Moreover, it is well established that some chemokines and their receptors play a role in the pathogenesis of arthritis.[43,44] Consequently, targeting chemokines with inhibitors, antibodies, and antagonists as new options in the treatment of patients with arthritis was promising. The development of

antagonists of CCR1,[44,45] CCR2,[46,47] and CCR5[48] presented promising results in different murine models of arthritis, but the antagonists failed during clinical trials. Many reasons can be found to explain the failure in the development of these drugs, such as inappropriate target selection in some cases, insufficient dosing of chemokine receptor antagonists in vivo,[49] and problems related to properties and complexity of the chemokine system, including redundancy and pleiotropy.[50]

To mediate their activity, chemokines bind to cell surface receptors which belong to the GPCRs and in addition they interact with GAGs present on the cell surface of blood vessels.[51] The interaction of chemokines with GAGs leads to retention on the cell surface and an immobilized gradient of chemokines that provides a directional signal to guide the recruitment of leukocytes to the inflammatory site.[52,53] Chemokines can be regulated based on their binding to GAGs, by posttranslational modifications that include N-terminal and C-terminal proteolytic processing and glycosylation, leading to changes in their biological activities. We previously demonstrated that the C-terminal region of CXCL9, CXCL9(74–103), can interact with soluble GAGs and treatment with the peptide decreased neutrophil extravasation after i.a. or i.p. injection of CXCL8 and attenuated inflammation mainly by the reduction of neutrophil recruitment in a murine gout model.[26,27] Here, we demonstrated that in the antigen-induced arthritis model the treatment with CXCL9(74–103) at early time points reduced neutrophil recruitment to the joint. We demonstrated before that CXCL9(74–103) competes in vitro with human CXCL8 and mouse CXCL1 for binding to GAGs, 2 chemokines important for neutrophil recruitment. Here, we demonstrated by confocal microscopy that CXCL9(74–103) binds to blood vessels in the joint cavity. Additionally, we showed that CXCL9(74–103) competes for heparan sulfate and chondroitin sulfate binding with the highly potent truncated form of the murine neutrophil attractant CXCL6. Unfortunately, we could not evaluate competition for GAG binding with murine CXCL2 due to the high background signal we obtained with all tested anti-CXCL2 antibodies (data not shown). The binding of the peptide to GAGs in the joint and the competition with murine CXCL1 and CXCL6 can explain the reduction in neutrophil recruitment to the joint. Moreover, our data demonstrated that CXCL9(74–103) also competes with CCL3, a lymphocyte, macrophage, eosinophil, and also a neutrophil chemoattractant in mice, through the interaction with CCR1, CCR3, and CCR5. However, in the treated mice, the number of mononuclear cells, which include macrophages and lymphocytes, was similar to the vehicle-treated arthritic mice.

Neutrophils play an important role in the development of arthritis. In synovial fluid of patients with rheumatoid arthritis, a large number of activated neutrophils was found and related with tissue damage.[54] Neutrophils are activated by the recognition of immune complexes by Fc receptors which lead to the release of ROS and degranulation that cause damage to cartilage.[55,56] In addition, neutrophils also produce extracellular traps (NETs), a source of citrullinated antigens, which can lead to production of autoantibodies.[57] Several strategies have been explored to inhibit neutrophil migration to inflammatory joints. Our data demonstrate that the decrease in neutrophil recruitment by reducing chemokine—GAG interactions diminished the level of chemokines and cytokines. Accordingly, the treatment also decreased the articular and cartilage damage. Of note, although we found a higher number of neutrophils in the positive control shown in Fig. 5 compared to the positive control in Fig. 1, the levels of chemokines and IL-6 are equivalent in both sets of experiments (Figs. 6 and 2, respectively), indicating an experimental variation in vivo.

Pharmacological blockage with small molecule inhibitors of CXCR1/2, in accordance with our data, also decreased neutrophil recruitment and the severity of the disease in antigen-induced arthritis.[29] In another arthritis model in K/B × N mice, the blockage of neutrophils, using neutrophil-depleting antibodies as a third potential inhibition strategy, resulted in less inflammation as well.[58] Also, the application of modified chemokines with antagonistic properties demonstrated positive results in arthritis models. A truncated CCL2 molecule, CCL2(9–76), was used to treat MRL-lpr mice, which spontaneously develop inflammatory arthritis and was shown to be beneficial.[20] The treatment with the CCR antagonist Met-RANTES in a collagen-induced arthritis model in DBA/1 mice resulted in reduced disease severity.[59]

We also tested whether the treatment with CXCL9(74–103) was able to decrease neutrophil recruitment and inflammation at a later time point. Our data showed that the treatment 6 h after the challenge still reduced neutrophil recruitment but did not alter the production of chemokines and IL-6. This indicates that the production of those mediators is more dependent on resident cells and early migrated neutrophils. The identification of the main source of key inflammatory mediators in in vivo conditions is not a simple task. Activated neutrophils release a plethora of pro-inflammatory mediators in the tissue.[55] In addition, those molecules also activate resident and other recruited cells, creating a positive inflammatory feedback loop. When the treatment was started 12 h after the challenge, there was no inhibitory effect of the CXCL9(74–103) peptide. After 12 h, leukocytes are already recruited to the joints and therefore interference with chemokine—GAG interactions may come too late to efficiently inhibit joint inflammation. More experiments are needed to check the stability of the binding of CXCL9(74–103) to GAGs in vivo and the elimination from the circulation. In addition, potential toxicity of CXCL9(74–103) and other modified chemokines due to residual agonist activity needs to be investigated. Small peptides in general also have poor pharmacokinetics since they are rapidly cleared from the circulation.[60] However, the interaction of the peptides with GAGs in tissues and on the endothelium may significantly slow down clearance but also makes it more difficult to study the pharmacokinetics in detail.

In conclusion, the treatment with CXCL9(74–103) in the early stage of antigen-induced arthritis decreased neutrophil recruitment, production of chemokines, cytokines, and articular and cartilage damage. Interference with chemokine—GAG interactions can be a therapeutic option in the treatment of arthritis.

AUTHORSHIP

D.B., H.C., R.J., V.V., F.A.A., and P.P. performed experiments, G.B.M. assisted with and supervised in vivo imaging experiments, S.M. and T.A.S. assisted with the evaluation of pathology scores, D.B, F.A.A., and P.P. designed the study, D.B. wrote the first draft of the manuscript

together with F.A.A. and P.P. All authors corrected the final version of the manuscript.

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DISCLOSURE

The authors declare no conflict of interest.

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