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A C-terminal CXCL8 peptide based on chemokine–glycosaminoglycan interactions reduces neutrophil adhesion and migration during inflammation

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Summary

Leucocyte recruitment is critical during many acute and chronic inflammatory diseases. Chemokines are key mediators of leucocyte recruitment during the inflammatory response, by signalling through specific chemokine G-protein-coupled receptors (GPCRs). In addition, chemokines interact with cell-surface glycosaminoglycans (GAGs) to generate a chemotactic gradient. The chemokine interleukin-8/CXCL8, a prototypical neutrophil chemoattractant, is characterized by a long, highly positively charged GAG-binding C-terminal region, absent in most other chemokines. To examine whether the CXCL8 C-terminal peptide has a modulatory role in GAG binding during neutrophil recruitment, we synthesized the wild-type CXCL8 C-terminal [CXCL8 (54–72)] (Peptide 1), a peptide with a substitution of glutamic acid (E) 70 with lysine (K) (Peptide 2) to increase positive charge; and also, a scrambled sequence peptide (Peptide 3). Surface plasmon resonance showed that Peptide 1, corresponding to the core CXCL8 GAG-binding region, binds to GAG but Peptide 2 binding was detected at lower concentrations. In the absence of cellular GAG, the peptides did not affect CXCL8-induced calcium signalling or neutrophil chemotaxis along a diffusion gradient, suggesting no effect on GPCR binding. All peptides equally inhibited neutrophil adhesion to endothelial cells under physiological flow conditions. Peptide 2, with its greater positive charge and binding to polyanionic GAG, inhibited CXCL8-induced neutrophil transendothelial migration. Our studies suggest that the E70K CXCL8 peptide, may serve as a lead molecule for further development of therapeutic inhibitors of neutrophil-mediated inflammation based on modulation of chemokine–GAG binding.

Keywords: chemokine; CXCL8; glycosaminoglycan; inflammation; neutrophil migration; structure–function; synthetic chemistry.

Introduction

Leucocyte recruitment, a hallmark of the inflammatory response, is a crucial component of many acute and chronic inflammatory situations.[1–3] Chemokines are small, soluble chemotactic proteins that co-ordinate leucocyte recruitment.[4] They can be expressed in response to pro-inflammatory mediators such as the

Abbreviations: BSA, bovine serum albumin; GAG, glycosaminoglycan; GPCR, G-protein-coupled receptor; HBSS, Hank's balanced salt solution; HMECs, human microvascular endothelial cells; HUVECs, human umbilical vein endothelial cells; ICAM, intercellular adhesion molecule type 1; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; POSAT, Prolong Organ Survival After Transplantation (project acronym); PTM, post-translational modification; RP-HPLC, reverse-phase high-performance liquid chromatography; RU, resonance units/response units; SA, streptavidin; SPR, surface plasmon resonance; TNF-α, tumour necrosis factor-α

cytokines tumour necrosis factor (TNF), interferon-γ or interleukin-1β. Chemokines recruit leucocytes to a site of injury, by binding to the endothelium via glycosaminoglycans (GAGs), forming a chemokine gradient and activating integrins, which allow leucocyte adhesion. In addition, chemokines are involved in many other processes such as angiogenesis, proliferation, development and the control of leucocyte mobilization from primary or secondary lymphoid organs.[5–9] Chemokine function depends, among many other factors, on their signalling via specific chemokine G-protein-coupled receptors (GPCRs). The interaction between a chemokine and its receptor is an attractive therapeutic target in many diseases, including rheumatoid arthritis,[10–12] psoriasis,[13] or in acute and chronic organ damage after ischaemia reperfusion injury following transplantation.[14,15]

Studies that have focused on the chemokine interaction with GPCRs have led to the development of several neutralizing antibodies, modified chemokines and antagonists.[16–21] However, to date, only two chemokine receptor antagonists have been fully validated and marketed as therapeutics: Maraviroc (a CCR5 antagonist) and AMD3100 (a CXCR4 antagonist).[22–24] These two antagonists are not used as anti-inflammatory drugs, but rather as a human immunodeficiency virus entry inhibitor, and as a haematopoietic stem cell mobilizer during transplantation, respectively. The challenge of targeting chemokines in anti-inflammatory therapy arises primarily from the apparent redundancy within the human chemokine system.[25,26]

In addition to the well-characterized, high-affinity interaction of chemokines with their specific GPCRs, chemokine activity *in vivo* also depends on their interaction with GAGs, such as endothelial heparan sulphate.[21,27] GAGs are ubiquitously present on cell surfaces and in the extracellular matrix. They are thought to inhibit chemokine diffusion, recruiting chemokines at high concentration forming a gradient towards the site of injury.[28–30] The highly sulphated and acidic GAGs bind to basic residues within chemokines largely through electrostatic forces, but also through Van der Waals interactions and hydrogen bonding. This usually involves residues such as arginine, lysine or histidine, which typically form the BBXB or (B)BXX(X/B)BXXB(B) peptide sequence signature, where B is a basic amino acid residue and X is a non-conserved amino acid, which is present in virtually all chemokines.[27] The importance of the chemokine–GAG interaction is highlighted by studies that have selectively targeted either GAG or GPCR binding domains. For example, chemokines with increased GAG binding but decreased GPCR binding, show anti-inflammatory activity in *in vivo* models of CXCL8/neutrophil-driven inflammation presumably by disrupting the natural chemokine gradient.[31]

Levels of CXCL8 significantly increase during the inflammatory response associated with ischaemia reperfusion injury,[32,33] which can lead to acute kidney injury[34,35] and transplant rejection.[36–38] CXCL8 expressed at high concentrations on the endothelial GAG surface at the site of injury contributes to neutrophil firm arrest, by activation of integrins.[39] Therefore, modulation of a CXCL8 haptotactic gradient might have potential in ameliorating the ischaemia reperfusion injury and therefore improve organ function.[30,32,34] Therapeutic targeting of CXCL8 and its association with heparan sulphate has been investigated in numerous neutrophil-driven inflammatory diseases such as chronic obstructive pulmonary disease, Crohn's disease and psoriasis.[40] A CXCL8-based decoy protein named PA401, with decreased GPCR binding and increased GAG binding, decreased CXCL8-mediated neutrophil recruitment in *in vivo* studies, suggesting its translational potential for the treatment of respiratory diseases such as chronic obstructive pulmonary disease or cystic fibrosis.[41]

The C-terminal α-helical region of CXCL8 is known to be critical for GAG binding (Fig. 1), largely due to its positive electrostatic charge giving it micromolar affinity for negatively charged GAGs.[29,42–44] This binding is mediated by core residues H18, K20, R60, K64, K67 and R68, as shown in Fig. 1, where known CXCL8-receptor binding residues are also highlighted.

In this study, we aimed to assess whether the CXCL8 C-terminal peptide (54–72) could modulate CXCL8 function. We synthesized the CXCL8 wild-type C-terminal region (54–72) (wild-type peptide, Peptide 1), a peptide with substitution of glutamic acid (E) 70 with lysine (K), in order to increase the peptide positive charge, and hence its GAG-binding potential (Peptide 2), and a scrambled peptide containing the wild-type amino acids (Peptide 3; Fig. 1). The biophysical properties of the peptides and their potential biological functions, using *in vitro* cytokine-mediated neutrophil flow-based adhesion and transendothelial migration studies, were investigated.

Materials and methods

Human neutrophil isolation

Primary neutrophils were isolated from whole blood of healthy volunteers. Ethical approval to obtain blood from healthy volunteers was granted by the County Durham and Tees Valley Research Ethics Committee (12/NE/0121). Primary neutrophils were isolated by dextran sedimentation (Dextran T500; Pharmacosmos, Holbaek, Denmark) and centrifugation on Percoll (GE Healthcare, Buckinghamshire, UK) density gradients as previously described.[45]

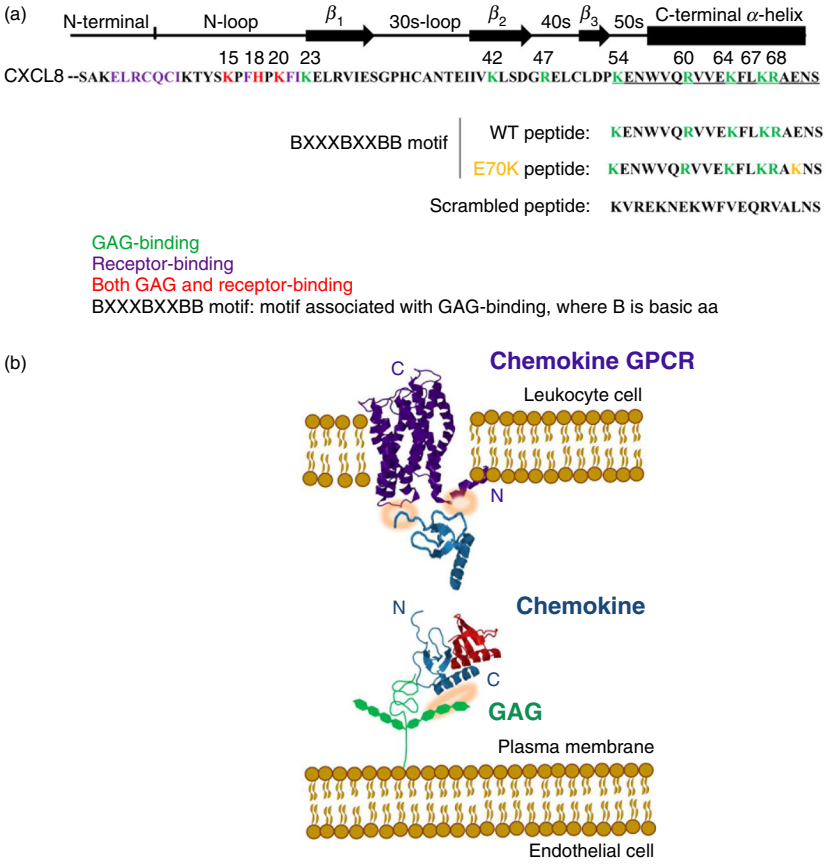

Figure 1. (a) CXCL8 active sequence. (b) Schematic representation of the chemokine binding to the endothelial glycosaminoglycan (GAG) and to the leucocyte chemokine G-protein coupled receptor (GPCR). (a) Sequence of the most common active CXCL8 form (amino acids 28–99), with 72 amino acids. Green: GAG-binding residues. Purple: GPCR-binding residues. Red: residues involved in both GAG- and receptor-binding. Underlined amino acids: C-terminal α-helix region selected for chemical synthesis. (b) Schematic representation of chemokine (Protein Data Bank ID 1IL-8/CXCL8) interaction with endothelial surface through GAG (residues involved highlighted in orange), which enables subsequent high-affinity chemokine binding to leucocyte CXCR1/2 GPCR receptor (Protein Data Bank ID 2LNL; also highlighted in orange). Chemokine monomer is shown in blue and the dimer is depicted with one molecule in blue and the other in red. Note that illustration shows one potential scenario of chemokine binding.

Synthesis of chemokine peptides

The chemokine C-terminal peptides (Peptides 1–3) were synthesized on Rink Amide resin using Fmoc solid-phase peptide synthesis on a CEM Liberty 1 single-channel microwave peptide synthesizer equipped with a Discover microwave unit, as described earlier.[46] After synthesis, peptides were acetylated at the N-terminal (20% acetic anhydride), having amide at the C-terminal. They were then cleaved from the resin, and crude peptides were purified by semi-prep reverse-phase high-performance liquid chromatography (RP-HPLC). Then peptides were characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) using an Autoflex II ToF/ToF mass spectrometer (Bruker Daltonik GmbH, Coventry, UK), and using the Pep-Calc calculator to analyse the sequence[47] and the obtained mass spectrometry spectra. Following this, analytical RP-HPLC was used to examine the pure peptide. Chemokine peptides were initially synthesized at Durham University Chemistry Department (Durham, UK), and further synthesized by ISCA Biochemicals (Exeter, UK) (>95% purity).

Circular dichroism spectroscopy

Far-UV circular dichroism spectroscopy was conducted using a Jasco J-810 spectropolarimeter (Jasco GmbH, Gross-Umstadt, Germany) in the range of 240–197 nm wavelength, with a 1-mm path length and a 500-μl quartz cuvette. Peptide samples (Peptide 1, Peptide 2 or Peptide 3) were diluted 5–100 μM in phosphate-buffered saline. For the measurements, 300 μl peptide solution was transferred to a cuvette. All data collection was taken at room temperature, and the mean spectrum derived from five to ten scans was corrected by subtraction of the buffer blank, as previously reported.[48] For samples of peptide combined with heparin (Sigma-Aldrich, St Louis, MO), the spectrum was also corrected by subtraction of a heparin blank. Scans were conducted at 50 nm/min, 1 nm data pitch, 5 mdeg sensitivity and with a 2-second response.[49]

Surface plasmon resonance

Surface plasmon resonance (SPR) was performed using a BIAcore X100 as previously described.[50] The running buffer used was HBS-P (10 mM HEPES pH 7·4, 150 mM NaCl, 0·005% Tween-20). Unless otherwise stated all reagents were from GE Healthcare (Uppsala, Sweden). To allow immobilization onto the streptavidin (SA)-coated chip, biotinylated GAG heparin was obtained as previously described[50–52] (generously provided by Prof. Hughes Lortat-Jacob's Laboratory, Institute of Structural

Biology, Grenoble, France). Mono-biotinylation at the reducing end of the GAG is important for correct presentation when immobilized. Between 5 and 20 μg/ml biotinylated heparin in 300 mM NaCl was injected at 10 μl/min for 30 seconds followed by a 2 M NaCl wash to remove unbound heparin. Injections were repeated until a total resonance units of 200 was achieved. Following preparation of the chip surface, SPR assays assessed the GAG-binding properties of CXCL8; and synthesized peptides (Peptide 1, Peptide 2 and Peptide 3). A range of CXCL8 concentrations (50–1000 nM; CN-09; Almac, Edinburgh, UK) were flowed across the chip at 5 μl/min for 5 min followed by a 500-second dissociation phase and their resonance units were measured. The same conditions were applied to the peptides analysed at concentrations from 2500 nM to 10 000 μM. After every chemokine or chemokine peptide measurement, regeneration buffer was used to remove sample from the chip surface (10 mM HEPES, 2 M NaCl, 50 mM EDTA, 0·005% Tween-20). Binding was calculated by subtraction of the resonance units of the SA flow cell from the resonance units of the GAG-SA flow cell. Data analysis was performed using BIA EVALUATION 4.1.

Solute diffusion gradient chemotaxis and transendothelial chemotaxis of neutrophils

Chemotaxis experiments were performed using a Transwell system (Falcon, BD Biosciences, Oxford, UK), as previously reported.[53] First, 24-well companion plates (Falcon, BD Biosciences) were blocked with 1 ml 1% bovine serum albumin (BSA) (Sigma-Aldrich)/RPMI (Lonza, Wokingham, UK) per well for 1 hr before the assay to prevent chemokine binding and consequent decreased chemokine concentration. Then, 800 μl of 10 nM chemokine, after optimization (data not shown) and as earlier described,[54–56] or chemokine peptide in a range of 0·1–10 000 nM in 1% BSA/RPMI was added to each well. Cell culture inserts (3-μm pore size; Falcon, BD Biosciences) formed the transwell upper chamber where 500 μl of 3×10^5 primary neutrophils in 1% BSA/RPMI were added. Wells containing 1% BSA/RPMI only on the transwell bottom chamber were used as a negative control. The plate was then incubated at 37° for 90 min. After incubation, cells that had fully migrated to the transwell lower chamber were counted by flow cytometry as a ratio to the known number of counting beads. For transendothelial chemotaxis, 3 days before the assay human microvascular endothelial cells (HMECs; ATCC CRL-3243)[57,58] were seeded onto the transwell upper chamber using 500 μl of 2×10^5 HMECs per insert in MCDB-131 media (10372019) (Thermo Fisher, Waltham, MA) with 10% fetal bovine serum as earlier described.[59,60] MCDB-131 medium was then carefully aspirated before the assay. Anti-intercellular adhesion molecule type 1 (ICAM-1) blocking monoclonal antibody (HA58) (eBioscience; Thermo Fisher, Waltham, MA) and IgG1 κ isotype control (MOPC-21) (BD Biosciences, San Jose, CA) were used to treat the HMEC layer at 20 μg/ml in 0·5% BSA/phosphate-buffered saline for 30 min at room temperature.

Calcium signalling

Intracellular calcium was measured loading cells with Indo-1, AM (Thermo Fisher, Waltham, MA). For each tube, 3 million neutrophils were used. Freshly isolated neutrophils were first left to rest in an incubator for about 15 min, and then used for the experiment. Cells were washed in Hank's balanced salt solution (HBSS; Sigma-Aldrich) and resuspended at 10 million cells/ml. Then, cells were washed in HBSS supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% fetal bovine serum (volume/volume). Once cells were washed, they were loaded with 3 μM indo-1, AM, and incubated for 30 min at 37° covered in foil. After the 30 min of indo-1, AM incubation, cells were washed with supplemented HBSS at 400 ***g*** for 5 min, then resuspended at 3 million cells per 1·5 ml in their corresponding FACS tube and left to rest for 30 min at 37° before analysis. Calcium flux was measured by FACS-Fortessa flow cytometry, using UV filter 530/30. Once settings were adjusted with unstained cells at low flow rate, the stained cells were run. As baseline, stained untreated cells (HBSS only) were first run for 1 min at medium flow. Then 1 μl HBSS or chemokine was added for 4 min, and then 8 μl ionomycin (I0634) (Sigma-Aldrich) was added for 2 min. Cells were studied for the effect of CXCL8 on calcium flux and compared with the effect of CXCL8 combined with Peptide 1, Peptide 2 or Peptide 3. Calculation of intracellular calcium concentrations, measured in terms of the light emission as a ratio of fluorescence intensities at 340 and 380 nm, was carried out using the equation $[\text{calcium (nmol/l)}] = K_d \times (R - R_{\min})/(R_{\max} - R)$, where K_d (844 nmol/l) is the dissociation constant of calcium bound to the fluorochrome[61]

Table 1. Summary of yield and purity obtained for each synthesized peptide

Peptide	Chemokine region	Yield[1]	Purity[2]
WT (Peptide 1)	WT C-terminal	60·4%	Approx. 95%
E70K (Peptide 2)	E70K C-terminal	10·4%	Approx. 95%
Scrambled (Peptide 3)	Scrambled from C-terminal	12·7%	Approx. 95%

[1]Yield is calculated comparing the dry mass of pure peptide with the mass of crude peptide [theoretical mass at 100% yield based on the 0·1 mmol resin (0·1 mmol peptide) = 100% peptide = mass of peptide (mg)].

[2]Purity is obtained from analytical high performance liquid chromatography.

and R is the peak intracellular calcium flux in response to the additive (chemokine or chemokine peptide). The basal concentration (HBSS, negative control) was subtracted to calculate the values.

Flow-based neutrophil adhesion

In order to evaluate the neutrophil adhesion in response to chemokine or chemokine peptide under physiological *in vitro* conditions, the Venaflux platform (Cellix Ltd., Dublin, Ireland) was used, in a similar way to previous studies.[62–64] To accommodate an endothelial layer on the biochip platform for neutrophil perfusion, the Vena8 Endothelial$^+$ chip was initially coated with 10 µl 100 µg/ml fibronectin (Sigma-Aldrich). Coated biochip was stored in a closed humidified chamber overnight at 4°. On the first day, Human Umbilical Vein Endothelial Cells (HUVECs; C-12203; PromoCell, Heidelberg, Germany) were treated in a 75-cm^2 flask with 1 ng/ml TNF (210-TA-010; R&D Systems, Minneapolis, MN) overnight at 37°.[65] Next day, the fibronectin-coated Vena8 Endo$^+$ biochip was seeded with 10 µl of HUVECs (at 1·5 million per 100 µl), used as

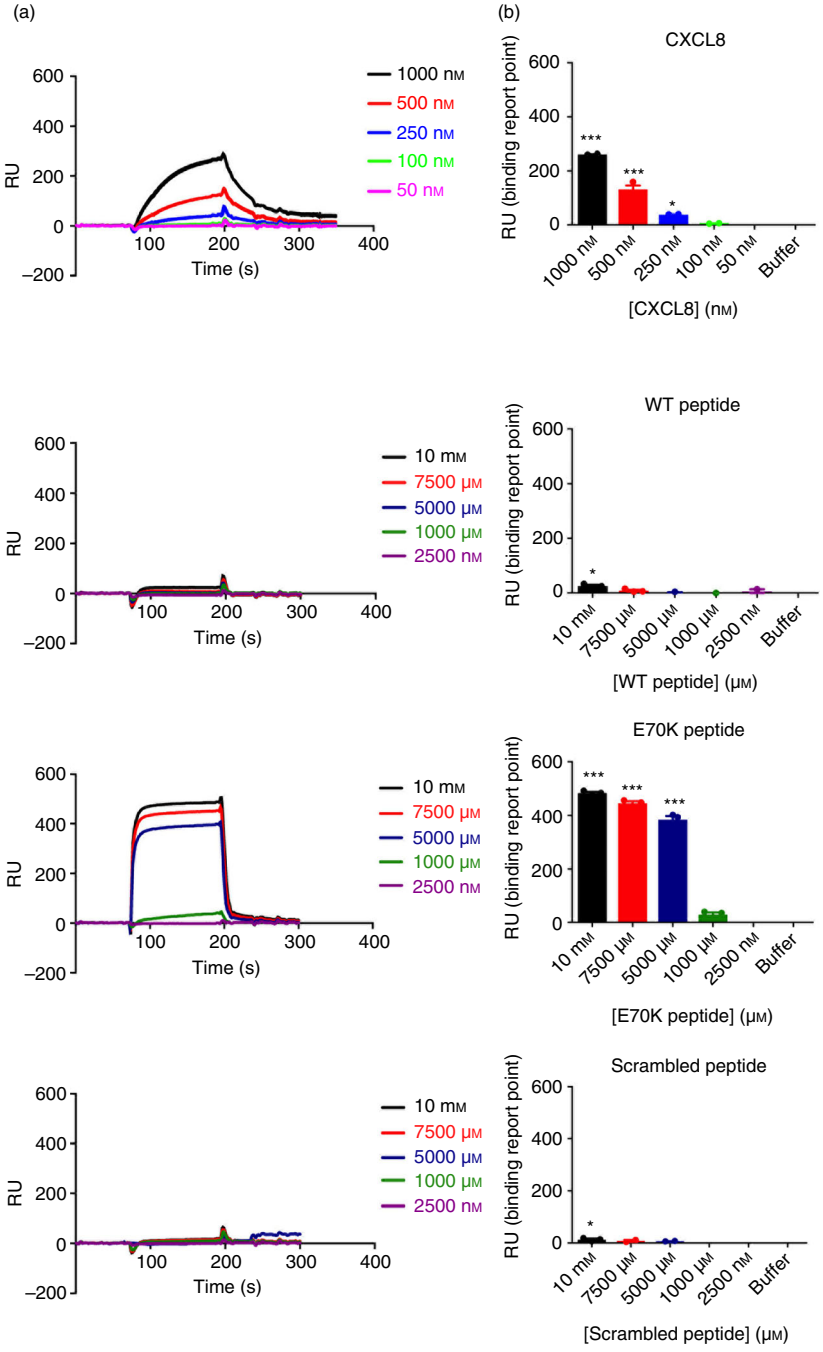

Figure 2. Surface plasmon resonance of CXCL8 peptide–heparin binding. (a) Surface plasmon resonance sensorgram shows heparin–CXCL8 binding in the range of 50–1000 nM CXCL8, and heparin–CXCL8 peptide binding in the range of 2·5–10 000 µM peptide. Chemokine or peptide were flowed at 5 µl/min over the chip. (b) Binding shown for each chemokine or peptide concentration. Sensorgram with magnified y-axis of binding of wild-type (WT) peptide, and scrambled peptide is shown in Supplementary material (Fig. S3). Data were analysed by one-way analysis of variance ($P < 0·0001$) followed by Bonferroni *post-hoc* test. *$P < 0·05$, ***$P < 0·001$. Data are representative of three independent experiments over a single heparin-coated SA chip.

negative control, or with TNF-stimulated HUVECs, as positive control. A HUVEC layer was generated within 1–1·5 hr of seeding. For this, the addition of 40 μl of extra culture medium to each channel reservoir was required 10–15 min after HUVEC seeding to humidify the channel and generate the endothelial layer. Afterwards, chemokine treatment was carried out. The seeded biochip channel was treated with chemokine (20 nM), chemokine peptide (50 nM; Peptide 1, Peptide 2 or Peptide 3); or low-molecular-weight heparin, tinzaparin (50 nM; Leo Pharmaceuticals, Ballerup, Denmark), to analyse their potential role in displacing the chemokine from GAG.[66] In parallel, different CXCR1/2 antagonists [repertaxin (Cayman Chemical, Cambridge, UK) and SB225002 (SML0716; Sigma-Aldrich)], and CXCR2 antagonist SB265610 (SML0421; Sigma-Aldrich) were used at 50 nM – to analyse their role in displacing the chemokine from GPCR[67] – treating neutrophils before the assay. A 10-μl treatment was inserted into each channel, followed by careful addition of 40 μl of the treatment on to each channel reservoir. The effect of each treatment on the neutrophil flow-based adhesion was evaluated using the Venaflux platform; 3×10^5 primary neutrophils were flowed per ml through each biochip channel and analysed. Cell adhesion analysis was performed using ImageJ Analysis Software. Cell adhesion count for each treatment was calculated from the average of five standard fields of view of adhered neutrophils.

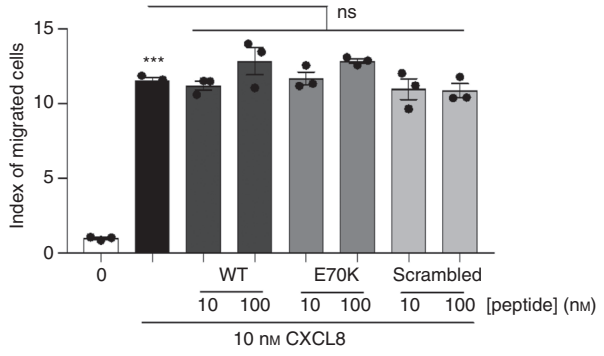

Figure 3. Diffusion gradient migration in response to CXCL8 combined with each peptide. For positive control, 10 nM CXCL8 was used. Synthesized CXCL8 C-terminal peptides (10 or 100 nM) showed no interference with neutrophil migration in the absence of endothelial glycosaminoglycan (GAG) surface, which suggests no binding to CXCR1/2 receptors. Wild-type (WT)/Peptide 1 (KENWVQRVVEKFLKRAENS); E70K/Peptide 2 (KENWVQRVVEKFLKRAKNS); or scrambled/Peptide 3 (KVREKNEKWFVEQRVALNS) were studied. Index of migrated cells or chemotaxis index (CI) is the ratio between the total number of migrated neutrophils and the number of neutrophils that migrated non-specifically, and was calculated for each treatment. Data were analysed by one-way analysis of variance ($P < 0{\cdot}0001$) followed by Bonferroni *post-hoc* test. ***$P < 0{\cdot}001$ shows significant migration in response to CXCL8 compared with negative control; ns, not significant. Representative data of three independent experiments ($n = 3$), each performed in triplicate.

Data analysis

Data were analysed using Prism7c software (GraphPad Software Inc., La Jolla, CA). Each graph column denotes mean and each bar indicates standard error of the mean (SEM). *P* values were calculated using one-way statistical analysis of variance followed by Bonferroni's *post hoc* test, with significant differences when $P < 0{\cdot}05$, highly significant when $P < 0{\cdot}01$, and extremely significant when $P < 0{\cdot}001$ or $P < 0{\cdot}0001$.

Results

Design, synthesis and biophysical characterization of CXCL8 C-terminal peptide

The wild-type C-terminal region of CXCL8 [CXCL8 (54–72)] (Peptide 1), the E70K peptide (Peptide 2), and a scrambled peptide with the same amino acids as the wild-type peptide in a random order, (Peptide 3), were synthesized using Fmoc solid-phase peptide synthesis on Rink Amide resin (see Supplementary material, Fig. S1). The

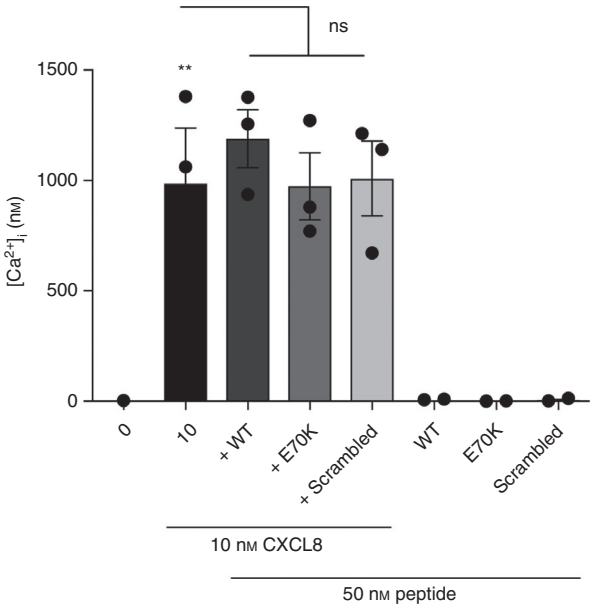

Figure 4. Calcium flux in response to CXCL8 combined with each peptide. Intracellular calcium ($[Ca^{2+}]_i$) was measured in response to CXCL8, or CXCL8 combined with each peptide [wild-type (WT)/Peptide 1: KENWVQRVVEKFLKRAENS; E70K/Peptide 2: KENWVQRVVEKFLKRAKNS; or scrambled/Peptide 3: KVREKNEKWFVEQRVALNS]. Primary blood neutrophils were labelled with Indo-1, AM. Then, cells were analysed in response to Hank's balanced salt solution (HBSS) only (negative control), 10 nM CXCL8 (positive control) or CXCL8 combined with each peptide at 50 nM, within the range of 10–100 nM. Data were analysed by one-way analysis of variance ($P < 0{\cdot}0001$) followed by Bonferroni *post-hoc* test. **$P < 0{\cdot}01$ shows significant calcium flux in response to CXCL8 compared with the negative control. ns, no significant. Data are representative of three independent experiments ($n = 3$).

purified peptides were characterized by MALDI-TOF and analytical RP-HPLC. A summary of yields and purity for the three peptides is shown in Table 1. Circular dichroism was used to determine the structure of synthesized peptides alone and in comparison with peptides combined with heparin. All peptides showed an extended, non-helical or random coil structure, different to the α-helix structure of this region within full-length CXCL8. However, Peptide 1 and Peptide 2 in solution with heparin showed a minor change in structure, not seen with Peptide 3, indicating a potential interaction between CXCL8-derived peptide and heparin (see Supplementary material, Fig. S2).

Binding of CXCL8 C-terminal peptides to GAG-heparin

To assess the GAG-binding ability of synthesized C-terminal peptides, SPR binding studies were performed. We first evaluated the binding of CXCL8 to a heparin-coated chip following established protocols.[68] Then, binding of each synthesized peptide was studied, to evaluate affinity for heparin. Heparin-CXCL8 SPR confirmed binding[68,69] as shown in Fig. 2. Peptide binding was only detectable at much higher concentrations of Peptides 1 and 3 (10 mM), $>10^4$-fold higher than with full-length CXCL8 (the sensorgram with magnified *y*-axis of binding of Peptides 1 and 3 is shown in the Supplementary material, Fig. S3). The E70K peptide (Peptide 2; charge +4), showed significant binding at lower concentrations (5 mM) than the other peptides (charge +2), but this was still a much higher concentration than full-length CXCL8 (Fig. 2).

CXCL8 C-terminal peptides do not interfere with GPCR-mediated signalling

The peptides were predicted to bind endothelial GAGs. To determine whether the peptides also had a role in GPCR-binding, all three peptides were evaluated by CXCL8-diffusion gradient chemotaxis and CXCL8-mediated calcium signalling. The peptides had no significant effect on CXCL8-diffusion gradient chemotaxis (Fig. 3).

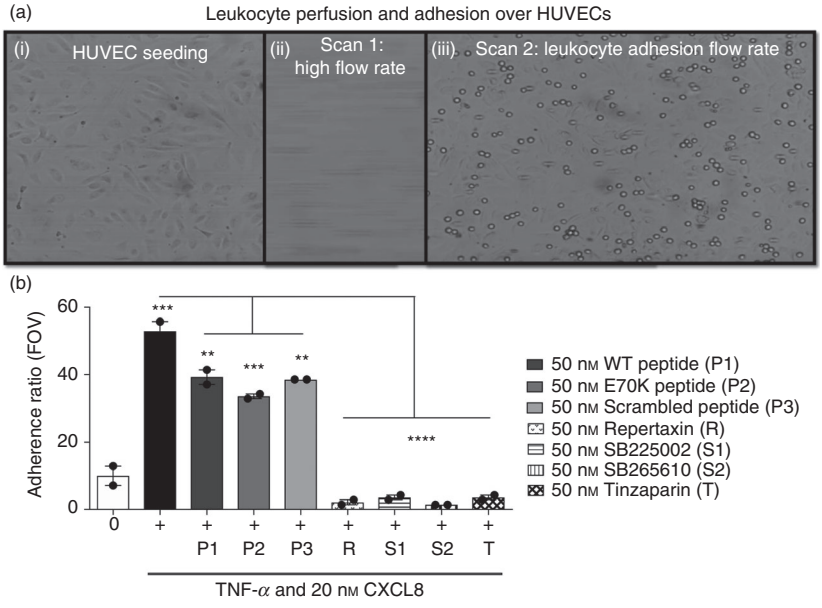

Figure 5. Schematic representation of leucocyte perfusion and adhesion over primary human umbilical vein endothelial cells (HUVECs). (a) (i) First, HUVECs were seeded over the fibronectin-coated biochip. (ii) Next, leucocytes were loaded onto the endothelial layer and initially perfused at a high flow rate, −10 dynes/cm^2 for 10 seconds, to allow leucocyte circulation over the chip (negative flow, towards pump). (iii) Leucocyte adhesion was then analysed at a more physiological flow rate, −0·5 dynes/cm^2 for 3 min. Leucocytes were fluorescently labelled using 1 μM $(DIOC_6)_3$. (b) Flow-based adhesion of primary neutrophils in the presence of different modulators. Negative control is untreated HUVECs (fibronectin only). Positive control is tumour necrosis factor (TNF) -stimulated HUVECs with 20 nM CXCL8 (100 μg/ml fibronectin, 1 ng/ml TNF/TNF-α). CXCL8 (20 nM) and CXCL8 peptide (50 nM) were added over TNF-stimulated HUVECs and neutrophil adhesion was analysed after 1 hr of treatment. HUVECs were treated with low-molecular-weight heparin (LMWH) tinzaparin at 50 nM for 1 hr before performing the assay. Neutrophils were treated with each CXCR1&2 antagonist [Repertaxin (R); or SB225002 (S1)] or CXCR2 antagonist (SB265610) (S2) at 50 nM for 1 hr before the assay. Adherence ratio, obtained from the average of five fields of view per channel of chip, is the ratio between the total number of adhered neutrophils and the number of neutrophils that adhered non-specifically. Wild-type (WT)/Peptide 1 (P1) is KENWVQRVVEKFLKRAENS; E70K/Peptide 2 (P2) is KENWVQRVVEKFLKRAKNS; scrambled/Peptide 3 (P3) is KVREKNEKWFVEQRVALNS. Data were analysed by one-way analysis of variance ($P < 0·0001$) followed by Bonferroni *post-hoc* test. $^{**}P < 0·01$, $^{***}P < 0·001$, $^{****}P < 0·0001$. Representative data of three independent experiments ($n = 3$).

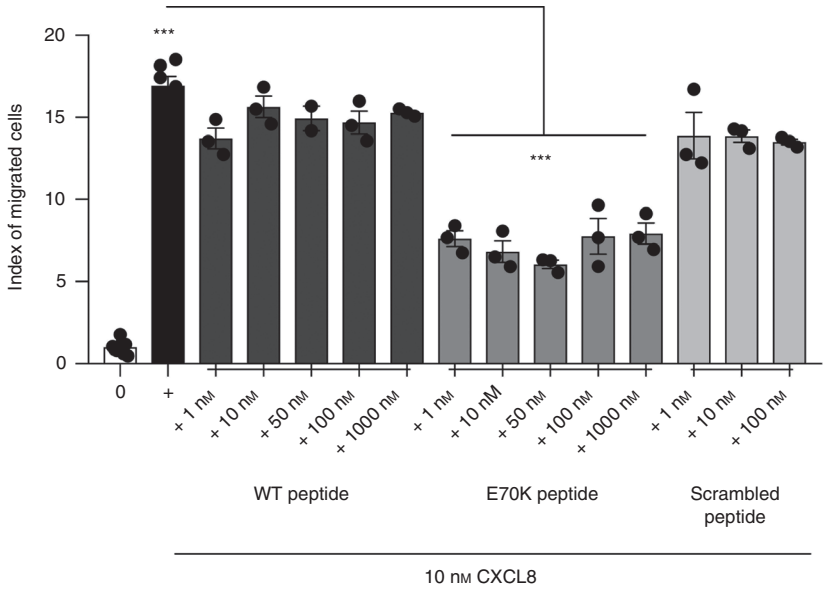

Figure 6. Neutrophil transendothelial migration directed by CXCL8 combined with peptide. Neutrophil response to CXCL8 (10 nM), or to CXCL8 combined with each peptide, at 1–1000 nM [wild-type (WT)/Peptide 1: KENWVQRVVEKFLKRAENS; E70K/Peptide 2: KENWVQRVVEKFLKRAKNS; or scrambled/Peptide 3: KVREKNEKWFVEQRVALNS] was measured. Cell counts were performed using counting beads by flow cytometry. Index of migrated cells or chemotaxis index (CI) is the ratio between the total number of migrated neutrophils and the number of neutrophils that migrated non-specifically. Further titration of peptides is shown in the Supplementary material (Fig. S4). Data were analysed by one-way analysis of variance ($P < 0{\cdot}0001$) followed by Bonferroni *post-hoc* test. ***$P < 0{\cdot}001$ on black column indicates significant migration in response to CXCL8 compared with negative control. Data are representative of two independent experiments ($n = 2$) from different primary neutrophil preparations, each performed in triplicate.

Data on CXCL8-mediated neutrophil calcium signalling was consistent with the diffusion gradient chemotaxis. Neutrophil calcium increased in response to CXCL8 stimulation, but no change was seen with the peptides alone. The combination of CXCL8 with each of the synthesized peptides did not affect calcium flux compared with CXCL8 alone (Fig. 4). Hence, data suggested that the peptides do not interfere with chemokine–GPCR binding.

C-terminal peptides inhibit neutrophil flow-based adhesion to endothelial cells

A schematic representation of the endothelial biochip seeding, and subsequent leucocyte flow-based adhesion is shown in Fig. 5. Primary neutrophil adhesion in response to TNF-stimulated, CXCL8-treated HUVECs was used as positive control. Cytokine-mediated neutrophil flow-based adhesion was reduced in the presence of 50 nM of all three peptides (wild-type peptide and scrambled peptide $P < 0{\cdot}01$; E70K peptide $P < 0{\cdot}001$). Similarities between the peptides suggest that short positively charged peptides, all containing Lys and Arg residues, interfere non-specifically or with functional redundancy with chemokine-activated neutrophil adhesion to the endothelium under physiological flow conditions (Fig. 5).

Further studies performed with the low-molecular-weight heparin tinzaparin showed significant chemokine displacement and inhibition of flow-based chemokine-mediated neutrophil adhesion ($P < 0{\cdot}0001$).

In addition, studies using the CXCR1/2 chemokine receptor antagonists repertaxin, SB225002 or SB265610 led to significant inhibition of GPCR-chemokine binding as shown by significantly reduced neutrophil flow-based adhesion ($P < 0{\cdot}0001$).

E70K peptide inhibits neutrophil transendothelial migration

To further investigate CXCL8 C-terminal peptide binding to endothelial GAG, their potential to block CXCL8-mediated transendothelial neutrophil migration was evaluated. There was no significant effect of Peptide 1 or Peptide 3 on neutrophil transendothelial chemotaxis. Peptide 2, E70K, reduced CXCL8-mediated neutrophil transendothelial migration ($P < 0{\cdot}001$; Fig. 6; see Supplementary material, Fig. S4). Primary neutrophils express several cell-surface proteins involved in endothelial adhesion, in addition to high levels of the CXCL8 receptors, CXCR1 and CXCR2 (see Supplementary material, Fig. S5). This may partly explain why CXCL8-displacing peptides do not fully inhibit neutrophil migration. To determine whether blocking the function of other

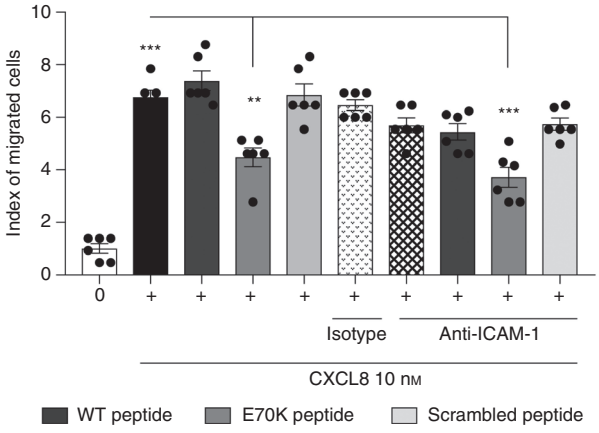

Figure 7. Neutrophil transendothelial migration directed by CXCL8 can be inhibited by the E70K peptide. A similar effect was shown when the peptide was combined with intercellular adhesion molecule type 1 (ICAM-1) blocking antibody. Neutrophil response to CXCL8 (10 nM), or to CXCL8 combined with each peptide, at 50 nM [wild-type (WT)/Peptide 1: KENWVQRVVEKFLKRAENS; E70K/Peptide 2: KENWVQRVVEKFLKRAKNS; or scrambled/Peptide 3: KVRE-KNEKWFVEQRVALNS] was measured. Human microvascular endothelial cells (HMECs) were treated with ICAM-1 blocking antibody. Cell counting was performed using a counting chamber. Index of migrated cells or chemotaxis index (CI) is the ratio between the total number of migrated neutrophils and the number of neutrophils that migrated non-specifically. Data were analysed by one-way analysis of variance ($P < 0{\cdot}0001$) followed by Bonferroni *post-hoc* test. $^{**}P < 0{\cdot}01$. $^{***}P < 0{\cdot}001$. *** in black column indicates significant migration in response to CXCL8 compared with negative control. Data are representative of three independent experiments ($n = 3$) from different primary neutrophil preparations, each performed in duplicate.

proteins involved in transendothelial migration would further interfere in the process, we combined the E70K peptide with an ICAM-1 blocking monoclonal antibody. As previously described, blocking ICAM-1 alone did not affect neutrophil transendothelial migration.[70] When ICAM-1 blockade was combined with E70K there was a significant reduction in neutrophil endothelial transmigration; however, this was not greater that E70K alone, suggesting no synergistic interaction (Fig. 7). This proposes the therapeutic potential of E70K peptide to modulate chemokine function by interfering with chemokine–GAG binding, potentially interfering with the formation of the chemokine gradient.

Discussion

Targeting chemokine–GPCR binding has been clinically approved for two indications. However, there are numerous examples in pre-clinical studies that suggest they have great potential to modify inflammatory responses during disease.[22–24,71] The regulation of chemokine function by GAG binding using chemokine peptides *in vivo* has previously been investigated,[9,41,72] but its translational potential has not been fully explored. Here, to better understand the regulation of chemokine function by GAG binding, chemokine-derived peptides were synthesized. All peptides showed low-affinity, but significant, GAG binding in a charge-dependent manner, presumably through electrostatic interactions. Chemotaxis and calcium signalling studies confirmed that peptides lacked GPCR antagonist function. The C-terminal peptides showed a significant reduction in flow-based neutrophil adhesion; however, no difference was observed between the peptides. This suggests that integrin-mediated neutrophil–endothelium adhesion, which is stimulated by cytokines, can be modulated by all the positively charged peptides tested under physiological flow rate. GAG binding of these peptides may not require a defined three-dimensional structure. Neutrophil transendothelial chemotaxis assays showed that only Peptide 2, with its higher positive charge, significantly reduced neutrophil migration. Peptide 2 has a charge of +4, which is higher than the wild-type peptide (Peptide 1) or scrambled peptide (Peptide 3; charge +2). We propose that the higher charge increases the affinity for GAG binding, and this contributes to chemokine displacement from cell surface GAGs disrupting the chemokine gradient (Fig. 8).

Alternative approaches to enhance the peptide–GAG binding to increase its ability to displace chemokine could include further substitution of positively charged residues in the CXCL8 GAG-binding region; study of potential folding of unfolded states of the truncated chemokine region; or the development of cyclic peptides;[73,74] or stapled peptides to stabilize an α-helical structure.[75] Furthermore, the inclusion of non-standard amino acids is another strategy to increase the peptide stability against proteolytic cleavage.[76] Also, it might be of interest to study potential peptide oligomerization, as it could further increase GAG binding.[29,42,43,77,78] These strategies might facilitate the impairment of the chemokine-mediated neutrophil recruitment to ameliorate the injury associated with neutrophil-mediated inflammation, such as in ischaemia reperfusion injury during transplantation, or in rheumatoid arthritis.[79]

Mice express only CXCL8 homologues, KC/CXCL1 and MIP-2/CXCL2. The human CXCL8 C-terminal peptide used (54–72 amino acids) shares 32% identity and 21% identity with murine homologues (within KC/CXCL1 and MIP-2/CXCL2), respectively.[80] This makes targeting C-terminal domain function in mouse models more difficult. In order to study the potential role of E70K peptide *in vivo*, a murine air pouch model of inflammation was used as optimized previously by our group.[81,82] However, no significant effect was observed (data not shown), which may reflect the degree of sequence difference described above; or it might have an inhibitory effect only in a specific environment. Alternative animal models, such as a humanized

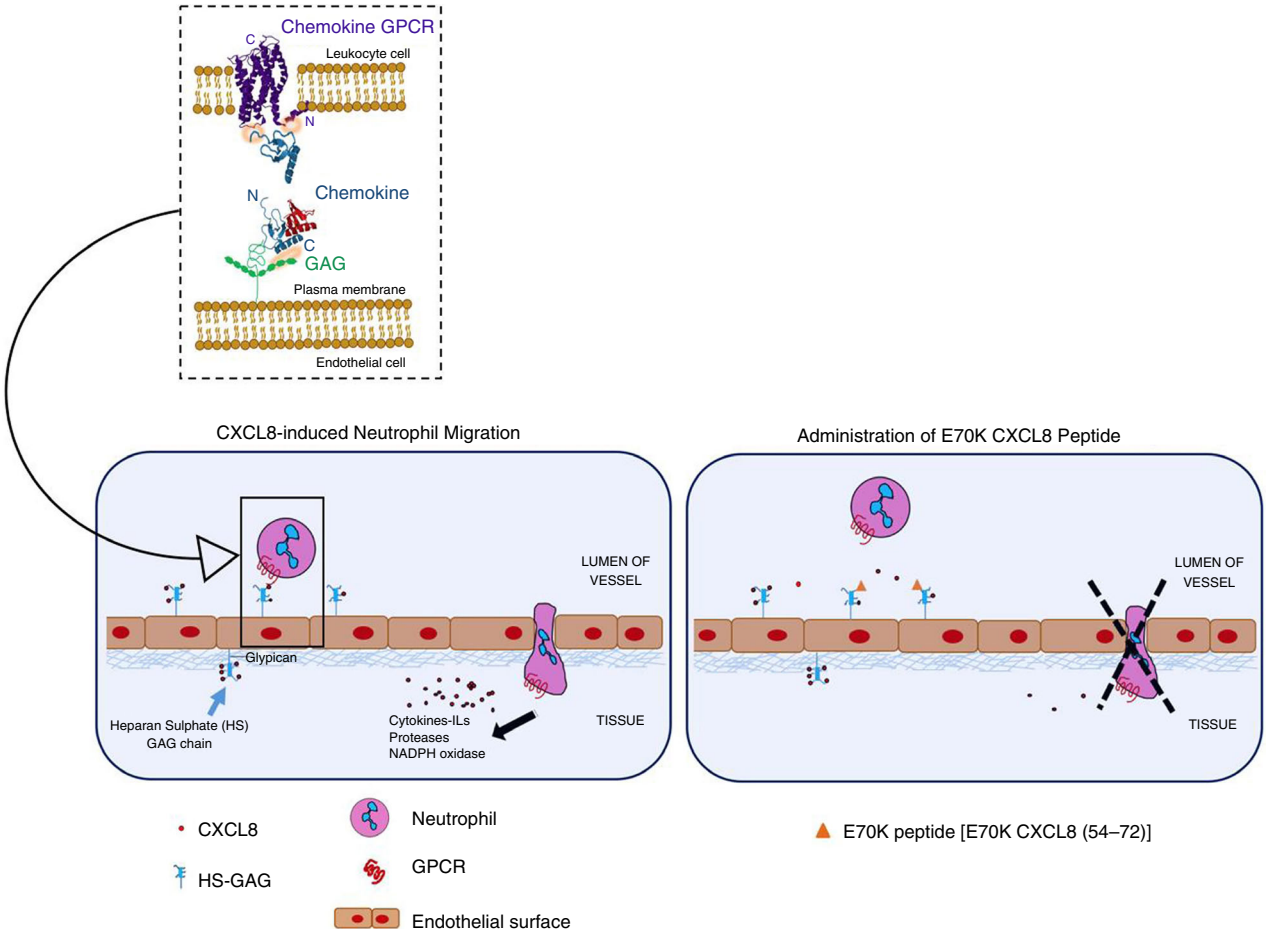

Figure 8. The proposed modulatory activity of E70K CXCL8 peptide in *in vitro* models of neutrophil flow-based adhesion and migration during inflammation. This model proposes the therapeutic potential of E70K peptide to modulate chemokine function by displacing chemokine from cell surface glycosaminoglycan, potentially interfering with the formation of the chemokine gradient.

mouse model,[83] or additional physiological studies could further probe the translational role of peptides.

Moreover, analysis of the effect of CXCL8-derived peptides on other factors such as *N*-formyl-L-methionyl-L-leucyl-phenylalanine, leukotriene B_4, C5a;[84] immunochemically related chemokines, e.g. neutrophil chemoattractant CXCL1, or CXCL9; and on other GAGs, may unravel further functionality of synthetic peptides. It is also worth noting that chemokine peptides are usually associated with favourable properties such as low toxicity and low immunogenicity, which contributes to their increasing recognition as potential candidates for novel drugs.[85,86]

Taken together, this approach shows the ability of CXCL8 (54–72) to bind GAG, and to significantly reduce chemokine-mediated neutrophil adhesion. In addition, the E70K CXCL8 peptide also showed a significant reduction in neutrophil transendothelial migration. This might be due to E70K's higher positive charge and higher binding affinity for polyanionic GAG. The ability of chemokine peptides to bind GAG and regulate chemokine function requires further work to determine if they have the potential to ameliorate acute or chronic neutrophil-driven organ damage.

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Disclosure

The authors declare no conflicts of interest.

Authors contributions

BM-B performed research, analysed the data and wrote the manuscript. SA, NSS, JAK, TP, DK, EP and SLC provided intellectual input in the design of study. SA, NSS, EP and SLC helped with the writing of the article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Schematic representation of chemistry for wild-type CXCL8 C-terminal peptide.

Figure S2. Circular dichroism of each peptide alone or combined with heparin.

Figure S3. Surface plasmon resonance of heparin-CXCL8 peptide at 5 μl/min.

Figure S4. Neutrophil transendothelial migration directed by CXCL8 combined with peptide (extended).

Figure S5. Cell-surface expression of neutrophil antigens.

