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

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Abbreviations: Abbreviations: AKI, Acute Kidney Injury; ANOVA, Analysis of Variance; COPD, Chronic Obstructive Pulmonary Disease; ECM, Extracellular matrix; fMLP, N-formyl-L-Methionyl-L-Leucyl-Phenylalanine; FOV, Fields of View; GAG, Glycosaminoglycan; GPCR, G-protein coupled receptor; HBSS, Hank's Balanced Salt Solution; HMEC, Human Microvascular Endothelial Cells; HUVEC, Human Umbilical Vein Endothelial Cells; HS, Heparan Sulphate, IFN-γ, Interferon gamma; IL8/CXCL8, Interleukin-8; IRI, Ischaemia Reperfusion Injury; LMWH, Low Molecular Weight Heparin; LTB4, Leukotriene B4; MMP, Matrix Metalloproteinase; PDB, Protein Data Bank; PMN, Primary Neutrophils; POSAT, Prolong Organ Survival After Transplantation (project acronym); PTM, Post-translational Modification; RU, Resonance Units / Response Units; SPPS, Solid-Phase Peptide Synthesis; SPR, Surface Plasmon Resonance; TNF/TNF-α, Tumor Necrosis Factor alpha

Abstract

Leukocyte recruitment is critical during many acute and chronic inflammatory diseases. Chemokines are key mediators of leukocyte recruitment during the inflammatory response, by signaling through specific chemokine G-protein coupled receptors (GPCRs). In addition, chemokines interact with cell surface glycosaminoglycans (GAGs) to generate a chemotactic gradient. The chemokine IL8/CXCL8, a prototypical neutrophil chemoattractant, is characterised by a long, highly positively charged GAG-binding C-terminal region, absent in most other chemokines. In order to examine whether the CXCL8 C-terminal peptide has a modulatory role in GAG binding during neutrophil recruitment, we synthesised 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 signaling 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.

Introduction

Leukocyte 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 leukocyte recruitment [4]. They can be expressed in response to pro-inflammatory mediators such as the cytokines TNF, IFN-γ or IL-1β. Chemokines recruit leukocytes to a site of injury, by binding to the endothelium via glycosaminoglycans (GAGs), forming a chemokine gradient and activating integrins which allow leukocyte adhesion. In addition, chemokines are involved in many other processes such as angiogenesis, proliferation, development, and the control of leukocyte mobilization from primary or secondary lymphoid organs [5-9]. Chemokine function depends, amongst many other factors, on their signaling 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 acute and chronic organ damage after ischaemia reperfusion injury (IRI) following transplantation [14, 15].

Studies that have focused on the chemokine interaction with GPCRs have led to the development of several neutralising 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 (HIV) entry inhibitor, and as a hematopoietic stem cell mobiliser 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-characterised, high affinity interaction of chemokines with their specific GPCRs, chemokine activity *in vivo* also depends on their interaction with

glycosaminoglycans (GAGs), such as endothelial heparan sulphate (HS) [21, 27]. GAGs are ubiquitously present on cell surfaces and in the extracellular matrix (ECM). 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 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].

CXCL8 levels significantly increase during the inflammatory response associated with IRI [32, 33], which can lead to acute kidney injury (AKI) [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 CXCL8 haptotactic gradient might have potential in ameliorating the IR injury and therefore improve organ function [30, 32, 34]. Therapeutic targeting of CXCL8 and its association with HS has been investigated in numerous neutrophil driven inflammatory diseases such as chronic obstructive pulmonary disease (COPD), 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 COPD or cystic fibrosis [41].

The C-terminal alpha-helical region of CXCL8 is known to be critical for GAG binding (Figure 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 Figure 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 synthesised the CXCL8 wild type C-terminal region (54-72) (WT peptide, Peptide 1), a peptide with substitution of glutamic acid (E) 70 with lysine (K), in order to increase the peptide positive charge thus its GAG binding potential (Peptide 2), and a scrambled peptide containing the wild type amino acids (Peptide 3) (Figure 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 & Methods

Human Neutrophil Isolation

Primary neutrophils (PMN) 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].

Synthesis of chemokine peptides

The chemokine C-terminal peptides (Peptides 1-3) were synthesised on Rink Amide resin using Fmoc Solid-Phase Peptide Synthesis (SPPS) on a CEM Liberty 1 single-channel microwave peptide synthesizer equipped with a Discover microwave unit, as earlier described [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 characterised by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) using an Autoflex II ToF/ToF mass spectrometer (Bruker Daltonik GmBH, Germany), and using the Pep-Calc calculator to analyse the sequence [47] and the obtained MS spectra. Following analytical RP-HPLC examined the pure peptide. Chemokine peptides were initially synthesised at Durham University Chemistry Department UK, and further synthesised by ISCA Biochemicals, UK (>95% purity).

Circular Dichroism Spectroscopy

Far-UV circular dichroism (CD) spectroscopy was conducted using a Jasco J-810 spectropolarimeter (Jasco, GmbH, Germany) in the range of (240-197) nm wavelength, with a 1mm path length and a 500 µL quartz cuvette. Peptide samples (Peptide 1, Peptide 2 or Peptide 3) were diluted (5-100) µM in a phosphate buffered solution (PBS). 300 µL peptide solution was transferred to a cuvette for the measurements. All data collection was taken at room temperature, and the mean spectrum derived from 5-10 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, USA), spectrum was also corrected by subtraction of heparin blank. Scans were conducted at 50 nm/min, 1 nm data pitch, 5 mdeg sensitivity and a 2s response [49].

Surface Plasmon Resonance

Surface plasmon resonance was performed using a BIAcore X100 as previously described [50]. The running buffer used was HBS-P (10mM HEPES pH7.4, 150mM NaCl, 0.005% Tween 20). Unless otherwise stated all reagents were from GE Healthcare (Uppsala, Sweden). To allow immobilisation onto the streptavidin-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 immobilised. 5-20µg/ml biotinylated heparin in 300mM NaCl were injected at 10µl/min for 30sec followed by a 2M NaCl wash to remove unbound heparin. Injections were repeated until a total RU of 200 was achieved. Following preparation of the chip surface, SPR assays assessed the GAG binding properties of CXCL8; and synthesised 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 5µl/min for 5mins followed by a 500sec dissociation phase and their resonance units (RU) were measured. Same conditions were applied to the peptides analysed at concentrations (2500nM - 10000µM). After every chemokine or chemokine peptide measurement, regeneration buffer was used to remove sample from the chip surface (10mM HEPES, 2M NaCl, 50mM EDTA, 0.005% Tween 20). Binding was calculated by subtraction of the RU of the SA flow cell from the RU of the GAG-SA flow cell. Data analysis was performed using BIAevaluation 4.1.

Solute diffusion gradient chemotaxis and transendothelial chemotaxis of neutrophils

Chemotaxis experiments were done using a Transwell system (BD Falcon, USA), as previously reported [53]. First 24 well companion plates (BD Falcon, USA) were blocked with 1ml 1% BSA/RPMI (Sigma-Aldrich, St Louis, MO, USA) (Lonza, Wokingham, UK) per well for 1hour before the assay to prevent chemokine binding and consequent decreased chemokine concentration. Then, 800µL of 10nM chemokine, after optimization (data not shown) and as earlier described [54-56], or chemokine peptide at range of (0.1-10000) nM in 1% BSA/RPMI were added to each well. 3µm-pore size cell culture inserts (BD Falcon, USA) formed the transwell upper chamber where 500µL of $3x10^5$ PMN in 1% BSA/RPMI were added. Wells containing 1% BSA/RPMI only on the transwell bottom chamber were used as a negative control. Plate was then incubated at 37°C for 90 minutes. After incubation, cells that had fully migrated to the transwell lower chamber were counted by flow cytometry as a ratio to known number of counting beads. For transendothelial chemotaxis, three days before the assay Human Microvascular Endothelial Cells (HMECs) (ATCC CRL-3243) [57, 58] were seeded onto the transwell upper chamber using 500µL of $2x10^5$ HMECs per insert in MCDB-131 media (10372019) (Thermo Fisher, Waltham, MA, USA) with 10% FBS as earlier described [59, 60]. MCDB-131 media was then carefully aspirated before the assay. Anti-ICAM-

1 blocking monoclonal antibody (HA58) (eBioscience; Thermo Fisher, Waltham, MA, USA) and IgG1 κ isotype control (MOPC-21) (BD Biosciences, San Jose, CA, USA) were used to treat the HMEC layer at 20μg/mL in 0.5% BSA/PBS for 30 mins at room temperature.

Calcium signaling

Intracellular calcium (Ca^{2+}) was measured loading cells with Indo-1, AM. For each tube, three million neutrophils were used. Freshly isolated neutrophils were first left to rest in incubator for about 15 minutes, and then used for the experiment. Cells were washed in HBSS (Sigma-Aldrich, St Louis, MO, USA) and resuspended at 10 million cells per mL. Then, cells were washed in HBSS supplemented with 1mM $CaCl_2$, 1mM $MgCl_2$, 1% FBS (v/v). Once cells were washed, they were loaded with 3μM indo-1, AM, and incubated for 30 minutes at 37°C covered in foil. After the 30 minutes of indo-1, AM incubation, cells were washed with supplemented HBSS at 400xg for 5 minutes, then resuspended at 3 million cells per 1.5mL in their corresponding FACS tube and left to rest for 30 minutes at 37°C 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 minute at medium flow. Then 1μL HBSS or chemokine was added for 4 minutes, and then 8μL ionomycin (I0634) (Sigma-Aldrich, St Louis, MO, USA) were added for 2 minutes. Cells were studied for the effect of CXCL8 on calcium flux and compared to 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 nm and 380 nm, was done using the equation [calcium (nmol/L)] = Kd x (R - Rmin) / (Rmax - R), where Kd (844 nmol/L) is the dissociation constant of calcium bound to the fluorochrome [61] 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, similarly to previous studies [62-64]. To accommodate an endothelial layer on the biochip platform for neutrophil perfusion, Vena8 Endothelial+ chip was initially coated with 10µL 100µg/mL fibronectin (Sigma-Aldrich, St Louis, MO, USA). Coated biochip was stored in a closed humidified chamber O/N at 4°C. On the first day, Human Umbilical Vein Endothelial Cells (HUVECs) (C-12203) (PromoCell, Heidelberg, Germany) were treated on 75cm^2 flask with 1 ng/mL TNF (210-TA-010) (R&D Systems, MN, USA) O/N at 37°C [65]. Next day, fibronectin-coated Vena8 Endo+ biochip was seeded with 10µL of 1.5million HUVECs per 100µL, used as negative control, or with TNF-stimulated HUVECs, as positive control. HUVEC layer was generated within 1-1.5 hour of seeding. For it, addition of 40µL of extra culture media to each channel reservoir was required 10-15 minutes right after HUVEC seeding to humidify channel and generate the endothelial layer. Afterwards, chemokine treatment was done. Seeded biochip channel was treated with chemokine (20nM), chemokine peptide (50nM) (Peptide 1, Peptide 2 or Peptide 3); or Low Molecular Weight Heparin (LMWH) tinzaparin (50nM) (Leo Pharmaceuticals, Ballerup, Denmark) to analyse their potential role in displacing the chemokine from GAG [66]. In parallel, different CXCR1/2 antagonists (CXCR1/2 antagonists repertaxin (Cayman Chemical, Cambridge, UK) and SB225002 (SML0716) (Sigma-Aldrich, St Louis, MO, USA)); and CXCR2 antagonist SB265610 (SML0421) (Sigma-Aldrich, St Louis, MO, USA) at 50nM, to analyse their role in displacing the chemokine from GPCR [67], were used to treat neutrophils before the assay.

10μL treatment were inserted into each channel, followed by careful addition of 40μL treatment on to each channel reservoir. Effect of each treatment on the neutrophil flow-based adhesion was evaluated using the Venaflux platform. $3x10^5$ primary neutrophils were flowed per mL through each biochip channel and analysed. Cell adhesion analysis was done using ImageJ Analysis Software. Cell adhesion count for each treatment was calculated from the average of five standard fields of view (FOV) of adhered neutrophils.

Data analysis

Data were analysed using Prism7c software (GraphPad Software Inc, La Jolla, CA, USA). Each graph column denotes mean (M) and each bar indicates standard error of the mean (SEM). P values were calculated using one-way statistical analysis of variance (ANOVA) followed by Bonferroni's post hoc test, with significant differences when $p<0.05$ (*), highly significant when $p<0.01$ (**), and extremely significant when $p<0.001$ (***) or $p<0.0001$ (****).

Results

Design, Synthesis and Biophysical Characterisation 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 synthesised using Fmoc-SPPS on Rink Amide resin (Figure S1). The purified peptides were characterised by MALDI-TOF and analytical RP-HPLC. A summary of yields and purity for the three peptides is shown in Table 1. Circular Dichroism (CD) was used to determine the structure of synthesised 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 (Figure S2).

Binding of CXCL8 C-terminal Peptides to GAG-Heparin

To assess the GAG-binding ability of synthesised 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 synthesised peptide was studied, in order to evaluate affinity for heparin. Heparin-CXCL8 SPR confirmed binding [68, 69] as shown in Figure 2. Peptide binding was only detectable at much higher concentrations of peptide 1 and 3 (10mM), $>10^4$-fold higher than with full length CXCL8 (Sensorgram with magnified y-axis of binding of peptides 1 and 3 is in Supporting Information (Figure S3)). The E70K peptide (Peptide 2) (charge +4), showed significant binding at lower concentrations (5mM) than the other peptides (charge +2), but this was still a much higher concentration than full length CXCL8 (Figure 2).

CXCL8 C-terminal Peptides do not Interfere with GPCR-mediated Signaling

The peptides were predicted to bind endothelial GAGs. In order 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 signaling. The peptides had no significant effect on CXCL8-diffusion gradient chemotaxis (Figure 3). Data on CXCL8-mediated neutrophil calcium signaling 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 synthesised peptides did not affect calcium flux compared with CXCL8 alone (Figure 4). Thus, 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 leukocyte flow-based adhesion is shown in Figure 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 50nM of all 3 peptides (WT peptide and scrambled peptide $P<0.01$; E70K peptide $P<0.001$). Similarity 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 (Figure 5).

Further studies performed with the low molecular weight heparin (LMWH) tinzaparin showed significant chemokine displacement and inhibition of flow-based chemokine-mediated neutrophil adhesion ($p<0.0001$).

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 ($p<0.0001$).

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 ($p<0.001$) (Figure 6) (Figure S4). Primary neutrophils express several cell surface proteins involved in endothelial adhesion, in addition to high levels of the CXCL8 receptors, CXCR1 and CXCR2 (Figure S5). This may partly explain why CXCL8-displacing peptides do not fully inhibit neutrophil migration. To determine whether blocking the function of other 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 (Figure 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 response 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, in order to better understand the regulation of chemokine function by GAG binding, chemokine-derived peptides were synthesised. All peptides showed low-affinity but significant GAG binding, in a charge-dependent manner presumably via electrostatic interactions. Chemotaxis and calcium signaling 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-endothelial 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 3D 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 WT 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 (Figure 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 IRI during transplantation, or in rheumatoid arthritis [79].

Mice express only CXCL8 homologues, KC and MIP-2. Human CXCL8 C-terminal peptide used (54-72aa) shares 32% identity and 21% identity with murine homologs (within KC/CXCL1 & 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 optimised earlier 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 inhibitory effect only in a specific environment. Alternative animal models such as humanized 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 (f-MLP), leukotriene B4 (LTB4), 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 the 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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Authors Contributions

B.M.-B. - performed research, analysed the data and wrote the manuscript. S.A., N.S.S., J.A.K., T.P., D.K., E.P. and S.L.C. - provided intellectual in-put in the design of study. S.A., N.S.S., E.P. and S.L.C. - helped with the writing of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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Legends

Figure 1. A) CXCL8 active sequence. B) Schematic representation of the chemokine binding to the endothelial GAG and to the leukocyte chemokine GPCR.

A) Sequence of the most common active CXCL8 form (aa 28-99), with 72 aa. Green: GAG-binding residues. Purple: GPCR receptor-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 (PDB ID 1IL8/CXCL8) interaction with endothelial surface through GAG (residues involved highlighted in orange), which enables subsequent high-affinity chemokine binding to leukocyte CXCR1/2 GPCR receptor (PDB 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.

Figure 2. Surface Plasmon Resonance of CXCL8 peptide-heparin binding.

A) SPR sensorgram shows heparin-CXCL8 binding in the range of (50-1000) nM CXCL8, and heparin-CXCL8 peptide binding in the range of (2.5-10000) 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 WT peptide, and scrambled peptide is in Supporting Information (Figure S3). Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. *$P<0.05$, ***$P<0.001$. Data is representative of three independent experiments over a single heparin-coated SA chip.

Figure 3. Diffusion gradient migration in response to CXCL8 combined with each peptide.

10nM CXCL8 were used (positive control). Synthesised CXCL8 C-terminal peptides (10, 100) nM showed no interference with neutrophil migration in absence of endothelial GAG surface, which suggests no binding to CXCR1/2 receptors. 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 nonspecifically and was calculated for each treatment. Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. ***$P < 0.001$ shows significant migration in response to CXCL8 compared to negative control. ns: no significant. Representative data of three independent experiments (n=3), each performed in triplicate.

Figure 4. Calcium flux in response to CXCL8 combined with each peptide.

Intracellular calcium ([Ca2+] i) was measured in response to CXCL8, or CXCL8 combined with each peptide (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 HBSS only (negative control), 10nM CXCL8 (positive control) or CXCL8 combined with each peptide at 50nM, within range of (10-100) nM. Data was analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. **$P < 0.01$ shows significant calcium flux in response to CXCL8 compared to the negative control. ns: no significant. Data is representative of three independent experiments (n=3).

Figure 5. Schematic representation of leukocyte perfusion and adhesion over primary HUVECs.

A) A. First, HUVEC endothelial cells were seeded over the fibronectin-coated biochip. B. Next, leukocytes were loaded onto the endothelial layered chip and initially perfused at high flow rate, -10 dynes/cm2 for 10 seconds, to allow leukocyte circulation over the chip (negative flow, towards pump). C. Leukocyte adhesion was then analysed at more physiological flow rate, -0.5 dynes/cm2 for 3 minutes. Leukocytes were fluorescently labelled using 1μM $(DIOC_6)_3$.

B) Flow-based adhesion of primary neutrophils in presence of different modulators. Negative control is untreated HUVECs (fibronectin only). Positive control is TNF-stimulated HUVECs with 20nM CXCL8 (100μg/mL fibronectin, 1ng/mL TNF/TNF-α). CXCL8 (20nM) and CXCL8 peptide (50nM) were added over TNF-stimulated HUVECs and neutrophil adhesion was analysed after 1hour treatment. HUVECs were treated with LMWH tinzaparin at 50nM for 1hour before performing the assay. Neutrophils were treated with each CXCR1&2 antagonist (Repertaxin (R); or SB225002 (S1)) or CXCR2 antagonist (SB265610) (S2) at 50nM for 1hour before the assay. Adherence ratio, obtained from the average of 5 fields of view (FOV) per channel of chip, is the ratio between the total number of adhered neutrophils and the number of neutrophils that adhered nonspecifically. WT/Peptide 1 (P1) is KENWVQRVVEKFLKRAENS; E70K/Peptide 2 (P2) is KENWVQRVVEKFLKRAKNS; scrambled/Peptide 3 (P3) is KVREKNEKWFVEQRVALNS. Data was analysed by one-way ANOVA ($P < 0.0001$) 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 (10nM), or to CXCL8 combined with each peptide, at (1-1000) nM (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 nonspecifically. Further titration of peptides is shown in Supporting Information (Figure S4). Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. ***$P < 0.001$ on black column indicates significant migration in response to CXCL8 compared to negative control. Data is representative of two independent experiments (n=2) from different primary neutrophil preparations, each performed in triplicate.

Figure 7. Neutrophil transendothelial migration directed by CXCL8 can be inhibited by E70K peptide. Similar effect was shown when peptide was combined with ICAM-1 blocking antibody.

Neutrophil response to CXCL8 (10nM), or to CXCL8 combined with each peptide, at 50nM (WT/Peptide 1: KENWVQRVVEKFLKRAENS; E70K/Peptide 2: KENWVQRVVEKFLKRAKNS; or scrambled/Peptide 3: KVREKNEKWFVEQRVALNS) 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 nonspecifically. Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. **$P < 0.01$. ***$P < 0.001$. ***, black column indicates significant migration in response to CXCL8 compared to negative

control. Data is representative of three independent experiments (n=3) from different primary neutrophil preparations, each performed in duplicate.

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 GAG potentially interfering with the formation of the chemokine gradient.

Sup. Figure 1. Schematic representation of chemistry for WT CXCL8 C-terminal peptide.

A) MALDI of crude peptide produced by SPPS, B) Fractions separation by HPLC, peaks 1-6, C) MALDI-TOF of fractions of the pure peptide (from HLPC, peak 2) and D) Analytical HPLC chromatogram confirming percentage of purity.

Sup. Figure 2. Circular Dichroism of each peptide alone or combined with heparin.

CD spectrum of WT CXCL8/IL8 peptide (black), E70K CXCL8/IL8 peptide (dark grey) and scrambled peptide (light grey) (25μM peptide), and structure of each peptide in presence of 50μM heparin (dashed lines). Extended or non-helical structural state of CXCL8 peptides shows minor change in presence of heparin, as opposed to the scrambled. Representative data of three independent experiments (n=3).

Sup. Figure 3. Surface Plasmon Resonance of heparin-CXCL8 peptide at 5 µL/min.

A) SPR sensorgram of heparin-CXCL8 peptide binding with magnified y-axis for WT CXCL8 peptide showed no significant binding at different concentrations as opposed to B) E70K CXCL8 peptide. C) Magnified y-axis for scrambled peptide. Data is representative of three independent experiments over a single heparin-coated SA chip.

Sup. Figure 4. Neutrophil transendothelial migration directed by CXCL8 combined with peptide (extended).

Neutrophil response to CXCL8 (10nM), or to CXCL8 combined with each peptide, at (0.1-10000) nM (WT/Peptide 1: KENWVQRVVEKFLKRAENS; E70K/Peptide 2: KENWVQRVVEKFLKRAKNS; or scrambled/Peptide 3: KVREKNEKWFVEQRVALNS) was measured. Cells 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 nonspecifically. Data were analysed by one-way ANOVA ($P < 0.0001$) followed by Bonferroni post-hoc test. ***$P < 0.001$ on black column indicates significant migration in response to CXCL8 compared to negative control. Data is representative of two independent experiments (n=2) from different primary neutrophil preparations, each performed in triplicate.

Sup. Figure 5. Cell surface expression of neutrophil antigens.

Primary Neutrophils were analysed for cell surface expression of A) chemokine receptors CXCR1 and CXCR2 (blue spectrum and orange spectrum, respectively, in the graph), B) CD45, and C) adhesion molecule CD11b and D) CD66b by flow cytometry, in relation to

their respective isotype controls (red spectra). Median fluorescence intensity (MFI) is representative of two independent experiments (n=2).

PEPTIDE	CHEMOKINE REGION	YIELD [a]	PURITY [b]
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%

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

a) Yield is calculated comparing the dry mass of pure peptide to the mass of crude peptide (theoretical mass at 100% yield based on the 0.1mmol resin (0.1mmol peptide) = 100%peptide = x mg peptide)). b) Purity is obtained from analytical HPLC.