A requirement for neutrophil glycosaminoglycans in the navigation of chemokine gradients is revealed by the streptococcal protease SpyCEP

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Neutrophils provide a first line of defense against bacteria and are guided to infected tissues by chemoattractants, notably the chemokine CXCL8. To evade the immune system, the bacterium *Streptococcus pyogenes* produces SpyCEP, an enzyme that cleaves the C-terminus of CXCL8, rendering it impotent. The basis for chemokine inactivation by SpyCEP is poorly understood.

We examined the *in vitro* migration of neutrophils and observed that their ability to efficiently navigate a CXCL8 gradient was compromised following CXCL8 cleavage by SpyCEP. Despite possessing an intact N-terminus, SpyCEP-cleaved CXCL8 had impaired binding not only to the chemokine receptors CXCR1 and CXCR2 but also to the glycosaminoglycan heparin. Enzymatic removal of neutrophil glycosaminoglycans similarly impaired navigation of a CXCL8 gradient, but not an fMLP gradient. We conclude that during cell migration, neutrophil glycosaminoglycans sample environmental chemokines, promoting interactions with chemokine receptors, a process that is necessary for productive chemokine:receptor interactions. This may inform strategies to inhibit SpyCEP, thereby promoting bacterial clearance by the host immune system.

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Introduction

Chemokines and their receptors form part of a complex network, noted for their roles in positioning leukocytes and other cells via the process of chemotaxis or directed migration (1, 2). Neutrophils play a prominent part in responses of the innate immune system and are guided to sites of microbial infection by members of the ELR$^+$ subgroup of CXC chemokines, which contain a Glutamate-Leucine-Arginine motif at their N-terminus. Chief amongst the ELR$^+$ chemokines is CXCL8/Interleukin-8 which interacts with two principal receptors on the neutrophil surface known as CXCR1 (3) and CXCR2 (4). CXCL8 is expressed and secreted by tissue macrophages and other cells, for example, epithelial cells following bacterial infection (5) and serves to recruit neutrophils from the circulation to deal with the invading pathogen. Mice deficient in CXCR2, the major receptor for murine ELR$^+$ chemokines such as KC and MIP-2, exhibit profound defects in neutrophil emigration to sites of both microbial (6) and sterile-induced inflammation (7) supporting the notion that gradients of ELR$^+$ chemokines direct the chemotaxis of neutrophils *in vivo*. In a variety of inflammatory diseases, inadvertent or overexpression of CXCL8 has been associated with pathological consequences (8) and consequently, much effort has been put into the discovery of small molecule antagonists of CXCL8 receptors (9).

The importance of chemokines in coordinating leukocyte migration in host defense has not escaped the attention of microbes. Several pathogens have evolved ways to subvert the chemokine system and hence evade clearance by leukocytes. These include the synthesis of chemokine binding proteins by poxviruses which neutralize the *in vivo* activity of chemokines (10, 11) and the production of enzymes by hookworms which specifically degrade chemokines involved in eosinophil recruitment (12). *Streptococcus pyogenes* (group A streptococcus or GAS) is known to cause a spectrum of infections, ranging from pharyngitis and impetigo to more invasive life-threatening diseases, such as necrotizing fasciitis. Invasive *S.pyogenes* infection is associated with the upregulation of several genes encoding virulence factors, amongst which the gene cepA/SpyCEP is of particular interest (13). The cepA gene encodes for a protein known as *Streptococcus pyogenes* cell envelope protease (SpyCEP) which specifically cleaves CXCL8 within the C-terminal α-helix, resulting in truncation of CXCL8 by 13 amino acids. This cleavage results in reduced neutrophil CD62L shedding activity and neutrophil chemotaxis *in vitro* by the truncated chemokine (14). The prominence of SpyCEP among GAS virulence factors has been ably demonstrated by loss-of-function and gain-of-function analyses (15, 16). SpyCEP-deficient strains have been found to be readily cleared in murine models of necrotizing fasciitis (15–17) whilst, conversely, heterologous expression of SpyCEP in *Lactococcus lactis* reproduced many of the features of severe necrotizing *S. pyogenes* infection in a hitherto avirulent bacterium, notably an inability to be cleared and an ability to disseminate to other organs (15).

Of interest, is the mechanism of action of SpyCEP, namely why C-terminal truncation of CXCL8 should result in a re-

duction of biological activity. Earlier structure:function studies of CXCL8 and the receptors CXCR1 and CXCR2 suggested major roles for the CXCL8 N-terminus in receptor activation following ligand binding (18–20) and for the receptor N-termini in ligand recognition (21, 22). In contrast, the C-terminus of CXCL8 has been implicated in binding to glycosaminoglycans (GAGs) via a cluster of lysine residues (23, 24). Translocation of CXCL8 to the luminal surface of the endothelium is known to require interactions between the chemokine C-terminus and endothelial GAGs (25) and binding to GAGs on the surface of vascular endothelial cells allows chemokines to form stable or haptotactic gradients, encountered by leukocytes rolling along the surface under the control of endothelial-expressed selectins (26). GAG binding is crucial for the *in vivo* activity of several chemokines, notably CCL5, as mutants that are unable to bind to GAGs have been shown to retain the ability to induce leukocyte chemotaxis *in vitro*, but not recruitment *in vivo* when introduced into the peritoneum of mice (27). Although reduced interaction with endothelial GAGs may explain some of the effects of SpyCEP observed *in vivo*, it cannot explain the wider effects of SpyCEP on neutrophils that are evident *in vitro*, such as reduced bacterial killing. If N-terminal mediated receptor binding of SpyCEP-truncated CXCL8 were preserved, and this were independent of GAG binding, some CXCR1 and CXCR2 function might be expected to be preserved.

In this study we examined the effects of SpyCEP cleavage upon neutrophil migration and the interaction of CXCL8 with the receptors CXCR1 and CXCR2 and cell surface GAGs. We used a combination of cell transfectants and freshly isolated human neutrophils. We highlight a previously unappreciated role for the C-terminal α-helix of CXCL8 in chemokine receptor binding and signaling, that is explained by a requirement for GAG binding. We put forward a model in which neutrophil GAGs play a key role in the navigation of CXCL8 gradients by sampling the neutrophil environment. We suggest that loss of migration of neutrophils in the presence of SpyCEP is not due to an inability to generate a trans-endothelial chemokine gradient, but that once cleaved by SpyCEP, the neutrophils are unable to "sense" that CXCL8 is present.

Results

Truncation of CXCL8 by SpyCEP results in impaired navigation of a chemokine gradient. The TAXIScan instrument was employed to assess real-time neutrophil migratory responses to CXCL8 following cleavage with SpyCEP. In this system, leukocytes are aligned at one end of a 260 µm long terrace, with a ceiling of 5 µm under which the cells must actively crawl (28). Using this system, the migration of freshly isolated neutrophils along a gradient of intact CXCL8 or SpyCEP-cleaved CXCL8 was assessed over a 1 hr period. SpyCEP-cleaved CXCL8 ($CXCL8^{1\text{-}59}$) was derived by overnight incubation of recombinant CXCL8 with supernatant from SpyCEP-positive *S.pyogenes* (H292) while, as a control, intact CXCL8 ($CXCL8^{1\text{-}72}$) was subject to incubation with supernatant from an isogenic SpyCEP-negative (H575) strain. Intact CXCL8 was seen to elicit rapid neutrophil migration with the majority of cells traversing the terrace in under 30 minutes in a direct fashion (Figure 1A, Supplementary video 1). SpyCEP-cleaved CXCL8 induced the migration of far fewer neutrophils which lacked focus and appeared hesitant, making more turns and correspondingly taking much longer to traverse the terrace (Figure 1B, Supplementary video 2). Basal neutrophil migration in the absence of stimulus was minimal (Fig. 1C). Our observations were confirmed by single-cell tracking analysis with SpyCEP-cleaved CXCL8 inducing significantly slower migration than intact CXCL8 (Fig. 1D), accompanied by a significantly lower directionality component (Fig. 1E). When the forward migration indices parallel to the chemokine gradient were calculated ($FMI^{\parallel}$), SpyCEP-cleavage of CXCL8 was also seen to significantly impair the extent of migration compared to intact CXCL8 (Fig. 1F). Thus, SpyCEP cleavage of CXCL8 significantly impairs migration along the chemokine gradient, resulting in slower, less directed neutrophil responses. Similar data sets were also obtained with the N-terminally extended form of CXCL8 ($CXCL8^{1\text{-}77}$) which is expressed by endothelial cells (Figure 2A-F). Thus SpyCEP is able to cleave both biologically active forms of CXCL8 rendering them less efficacious in terms of leukocyte recruitment.

Truncation of CXCL8 by SpyCEP results in reduced glycosaminoglycan binding. Chemokines have been shown to adhere with micromolar affinity to glycosaminoglycans (GAGs) which is essential for chemokine presentation on endothelial cells and the recruitment of leukocytes into tissues *in vivo* (29). The GAG binding domain of CXCL8 was shown by Kuschert *et al* to be comprised of five basic amino acids, namely K20 (within a region known as the proximal loop) and R60, K64, K67, and R68 in the C-terminal α-helix (30). Since truncation by SpyCEP at Q59 would remove four of these residues, we hypothesized that it may have deleterious effects on GAG binding. We therefore examined the ability of CXCL8 to form oligomers on heparin sepharose beads following treatment with SpyCEP containing supernatants. In agreement with previous data from Hoogewerf and co-workers (30), increasing concentrations of intact CXCL8 lead to a corresponding increase in the proportion of CXCL8 bound to the beads (Fig 3). In contrast, SpyCEP-cleaved CXCL8 was without activity in this assay, suggesting that removal of a large proportion of the GAG binding site by SpyCEP renders CXCL8 unable to bind to heparin.

Effective navigation of a CXCL8 gradient requires neutrophil GAGs. Since SpyCEP cleavage impaired both GAG binding and neutrophil recruitment *in vitro*, we postulated that binding to GAGs on the neutrophil surface is a key step in the productive activation of neutrophil CXCL8 receptors. To test this postulate, we incubated neutrophils at 37ºC in a glycanase cocktail containing heparanase and chondroitanase, since both heparan sulfate and chondroitin sulfate-decorated GAGs have been shown to bind CXCL8 *in vitro* (31). Af-

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ter incubation, neutrophils were washed once in buffer then assessed via TAXIScan for their ability to migrate along gradients of the tripeptide chemoattractant fMLP (deemed too small and uncharged to effectively bind to GAGs) or gradients of intact CXCL8. Incubation of neutrophils in buffer alone resulted in elevated basal migration which appeared to be without direction in the absence of a stimulus (Fig. 4A). The introduction of gradients of fMLP or CXCL8 induced obvious directional migration (Fig. 4B and C). Glycanase treatment resulted in an evident reduction in basal migration (Fig. 4D), although responses to fMLP remained intact (Fig. 4E). In contrast, migratory responses to CXCL8 were abolished by glycanase treatment (Fig. 4F). These findings were corroborated by single cell tracking analysis. Untreated neutrophils migrated along gradients of fMLP and CXCL8 with significantly greater velocity compared to the absence of a stimulus (Fig. 4G and H). Glycanase treatment of neutrophils reduced the velocity and directionality of the CXCL8 responses so far as they were indistinguishable from those seen in the absence of a stimulus. In contrast, despite glycanase treatment, fMLP-induced neutrophil migration remained significantly faster and more directional than that seen in the absence of a stimulus. Analysis of the FMI$^{\|}$ indices further clarified these findings, with glycanase treatment of neutrophils seen to ablate migration along the CXCL8 gradient, whilst migration along the fMLP gradient remained significantly greater than that seen in the absence of a stimulus (Fig 4I). From this, we conclude that effective navigation of a CXCL8 gradient requires intact GAGs on the neutrophil cell surface.

Truncation of CXCL8 by SpyCEP results in reduced binding and activation of CXCR1 and CXCR2. The inability of SpyCEP cleaved CXCL8 to provide a chemotactic gradient for neutrophils raised the possibility that CXCL8 might be unable to bind to its cognate receptors CXCR1 and CXCR2. Previous reports have detailed the effects of CXCL8 truncation by SpyCEP on neutrophil function (14), but have not determined the relative contributions of CXCR1 and CXCR2 signaling. We set out to address this using constructs encoding N-terminal HA-tagged variants of CXCR1 and CXCR2. These were transiently expressed at high levels in the mouse pre-B cell L1.2 according to published protocols (32), allowing ligand binding assays and chemotaxis assays to be performed. Full length recombinant CXCL8 (CXCL8$^{1\text{-}72}$) was subsequently incubated overnight in the presence or absence of SpyCEP using supernatants from SpyCEP-positive (H292) or isogenic SpyCEP-negative (H575) strains. The cleaved or uncleaved CXCL8 samples were then diluted in assay buffer and activity in competitive binding assays and chemotaxis assays assessed (Fig. 5). Intact CXCL8 that had been incubated with the control (H575) supernatant was readily able to displace ^{125}I-CXCL8 from both CXCR1 and CXCR2 transfectants at nanomolar concentrations (IC_{50} values of 8.1nM and 8.7nM at CXCR1 and CXCR2 respectively, Fig 5A and B). In contrast, cleaved CXCL8 that had been incubated with SpyCEP was unable to displace 50% of the ^{125}I-CXCL8 from either receptor, even when used at a 1000-fold greater concentration than the labelled ligand. We subsequently compared the ability of either cleaved or intact CXCL8 to induce migration of the CXCR1 and CXCR2 transfectants in Boyden chamber assays. Intact CXCL8 was efficacious in recruiting both CXCR1 and CXCR2 transfectants, with optimal migration seen in the 1-10 nM concentration range (Fig. 5C and D). In contrast, cleavage of CXCL8 with SpyCEP resulted in a significant reduction in efficacy compared with intact CXCL8, consistent with the reduced ability to bind either CXCR1 or CXCR2 as seen in the binding assays. Previous MS analysis has shown that the disulphide bridges within CXCL8 remain intact following SpyCEP cleavage (14), thus we conclude that it is the loss of the terminal 13 residues alone that adversely affects the binding of CXCL8 to both CXCR1 and CXCR2 and subsequent receptor activation. This is in keeping with an earlier study in which 10-fold and 30-fold reductions in chemotaxis and receptor binding were observed with a synthetic CXCL8 variant truncated at position 60 (21).

SpyCEP has broad spectrum activity for ELR+ CXC chemokines. To ascertain the specificity of SpyCEP for neutrophil-recruiting CXC chemokines, a selection of recombinant chemokines with varying degrees of homology (Figure 6A) were incubated overnight with washed wild type S. pyogenes cells (H292) (13) or the isogenic cepA mutant (H575) (15). Incubation with wild type *S. pyogenes* cells evidently resulted in the cleavage of all ELR+ CXC chemokines as deduced by SDS-PAGE analysis (Figure 6B), with a notable reduction in their molecular weight. In contrast, the non-ELR+ chemokines included as controls remained intact (CXCL4, CXCL9 and CXCL10). Incubation with control, SpyCEP-negative (H575) cells resulted in no detectable cleavage of any of the chemokines examined.

Discussion

The production of SpyCEP by *S. pyogenes* has been associated with disease severity and may be indispensible for more virulent infections, suggesting a significant contribution to pathogenesis (15, 33). Indeed, in soft tissue infections, active immunity against SpyCEP has been shown to impede bacterial dissemination and improve neutrophil infiltration to the infection site (33). SpyCEP has been shown to cleave within the first turn of the C-terminal α-helix of ELR+ chemokines (14, 15, 34). In this study, we have shown that SpyCEP cleavage of CXCL8 abolishes the ability of the chemokine to bind to heparin and greatly impairs the ability of the chemokine to bind to and elicit activation through its cognate CXCR1 and CXCR2 receptors. The observation generated the hypothesis that interaction with GAGs might be central to activation of the receptors CXCR1 and CXCR2 by CXCL8. The hypothesis was supported by the finding that C-terminal truncation severely impaired CXCL8 binding to both CXCR1 and CXCR2, reminiscent of a previous report by Hoogewerf and colleagues in which binding to CXCR1 expressing transfectants was significantly reduced following treatment with a mixture of glycanases (30). Subsequent CXCR1/2 activation by SpyCEP cleaved CXCL8 as monitored by chemo-

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taxis assays was also reduced. Further support came from our demonstration that the removal of neutrophil surface GAGs by glycanases abolished the ability of freshly isolated neutrophils to respond to CXCL8 but not to fMLP.

Placing this information in the context of what we already know about chemokine: endothelial GAG interactions (29), we propose that in the context of migration, the neutrophil glycocalyx serves several purposes. The first is the sequestration of chemokines on the neutrophil surface (both monomer and higher order species), in effect 'sampling' the gradient at the leading edge of the migrating leukocyte. This sequestration leads to a productive interaction with the chemokine receptors which can be envisaged as increasing the local concentration of chemokine in the vicinity of the receptors, or even a physical cis-presentation of chemokine to the receptors (Fig. 7). In the absence of the gradient sampling afforded by GAGs, migration along the chemokine gradient is much less efficient. As an analogy, consider an automobile taking a bend in the road after sunset. The beams from the headlights enable the driver to sample the environment and seeing the bend, steer the vehicle in the appropriate direction with negligible loss of speed. In contrast, in the absence of headlights and reduced environmental sampling, as the driver approaches the bend it is necessary to slow down to re orientate the vehicle and safely navigate the bend, leading to less fluent movement along the road.

It is interesting to note that differences in the morphology of neutrophils navigating gradients of fMLP and CXCL8 have been reported previously by Yamauchi and colleagues. Notably, neutrophils migrating along an fMLP gradient displayed a fan-like widely-spread lamellipodium at the leading edge with a compact body and short tail. In contrast, neutrophils navigating a gradient of CXCL8 showed a more focused lamellipodium with a longer cell body and tail (35).

We conclude that GAGs on the surface of the neutrophil are an essential component of effective migratory responses to CXCL8 and that SpyCEP ruthlessly exploits this to render CXCL8 inactive *in vivo* (13). This raises several interesting questions. Firstly, is sampling by GAGs required for efficient navigation of other chemokine gradients by neutrophils? This question requires additional experimentation to answer conclusively. There is perhaps a clue in the broad spectrum activity of SpyCEP for all ELR$^+$ chemokines (34) and Fig. 6B), although, it could be that in evolving activity against a principal neutrophil chemoattractant in CXCL8, SpyCEP has 'unwittingly' acquired the ability to degrade other neutrophil attractants. Secondly, are GAGs on the surface of other leukocytes critical for the navigation of chemokine gradients? Previous studies of monocytes (36) and T-cells (37) have shown that glycanase treatment results in reduced intracellular signalling in response to the chemokines CXCL4 and CCL5, although these studies were not been extended to migration. Finally, can we learn lessons from SpyCEP in terms of targeting the interactions of GAGs with chemokines for therapeutic benefits? Mutants of CXCL8 which are likely to bind to SpyCEP but have reduced activity at CXCR1 and CXCR2 have been described by others and may be a useful starting point for drug discovery (38, 39). Provided such molecules clear the typical hurdles of bioavailability and target occupancy that have beset many small molecule chemokine receptor antagonists (40), they may present themselves as candidate SpyCEP inhibitors with potential for the adjuvant management of invasive group A streptococcal infection.

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Supplementary Note 1: Supplementary Information

Supplementary Videos 1 and 2- Neutrophil migration observed via TAXIScan Migration of neutrophils along a gradient of intact CXCL8 (Supplementary Video 1), or along a gradient of CXCL8 cleaved by SpyCEP (Supplementary Video 2). Elapsed time in hours and minutes is indicated in the top left hand corner. Scale bars indicate 100 microns.

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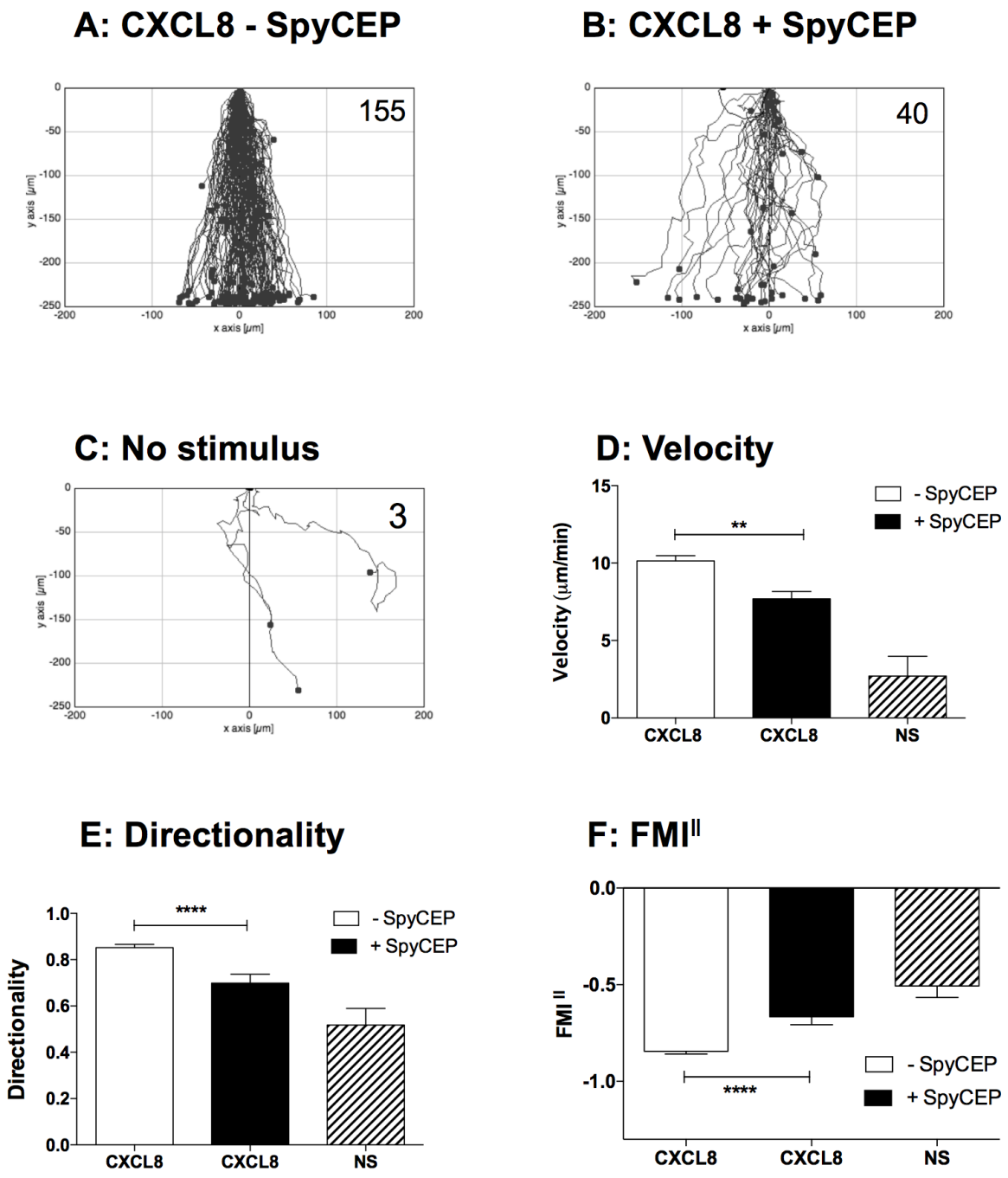

Fig. 1. SpyCEP cleavage of CXCL8 results in significantly impaired migration of neutrophils as visualized in realtime by TAXIScan. Panels A-C show the collated tracks of individual migrating neutrophils (duplicate conditions) pooled from three independent experiments using different donors. Gradients of H575 or H292 treated CXCL8 were established in panels A and B respectively, while panel C shows the lack of neutrophil migration in the absence of chemokine. The total number of tracked cells from all three experiments is shown in the top right hand corner. Panels D-F show significant differences in velocity, directionality and forward migration index parallel to the gradient (FMI||) of the data in panels A-C following tracking analysis. Error bars represent the SEM. Statistical significances between CXCL8 treatments were determined by one-way ANOVA with Tukey's' post-test.

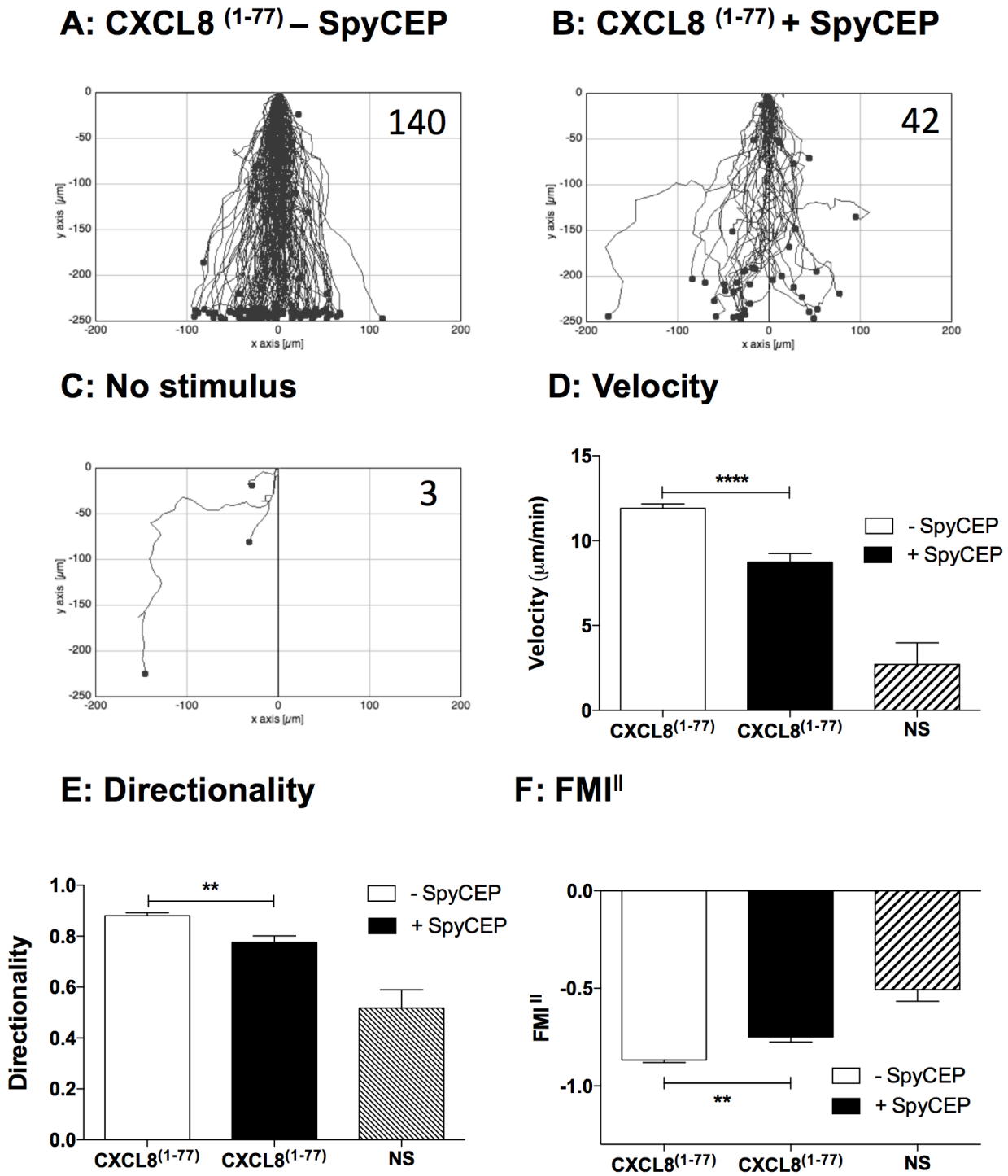

Fig. 2. SpyCEP cleavage of the extended form of CXCL8 ($CXCL8^{1-77}$) results in significantly impaired migration of neutrophils. Panels A-C show the collated tracks of individual migrating neutrophils (duplicate conditions) pooled from three independent experiments using different donors. Gradients of H575 or H292 treated $CXCL8^{1-77}$ were established in panels A and B respectively, while panel C shows the lack of neutrophil migration in the absence of chemokine. The total number of tracked cells from all three experiments is shown in the top right hand corner. Panels D-F show significant differences in velocity, directionality and FMI|| of the data in panels A-C following tracking analysis. Error bars represent the SEM. Statistical significances between $CXCL8^{1-77}$ treatments were determined by one-way ANOVA with Tukey's' post-test.

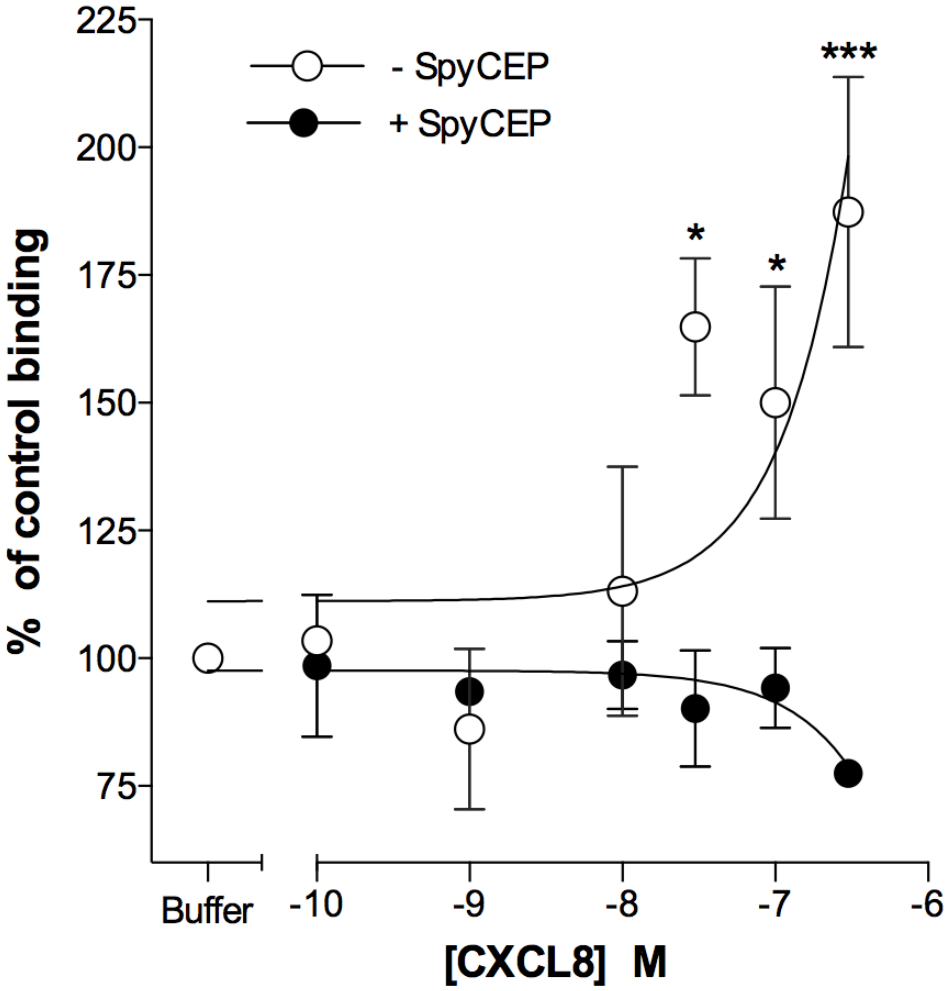

Fig. 3. SpyCEP cleavage of CXCL8 ablates binding to heparin.Graph shows the relative abilities of H292 and H575-treated CXCL8 in binding to heparin sepharose beads. Error bars represent the mean ± SEM (n=6). Statistical significance between CXCL8 treatments was determined by two-way ANOVA with Sidak's post-test.

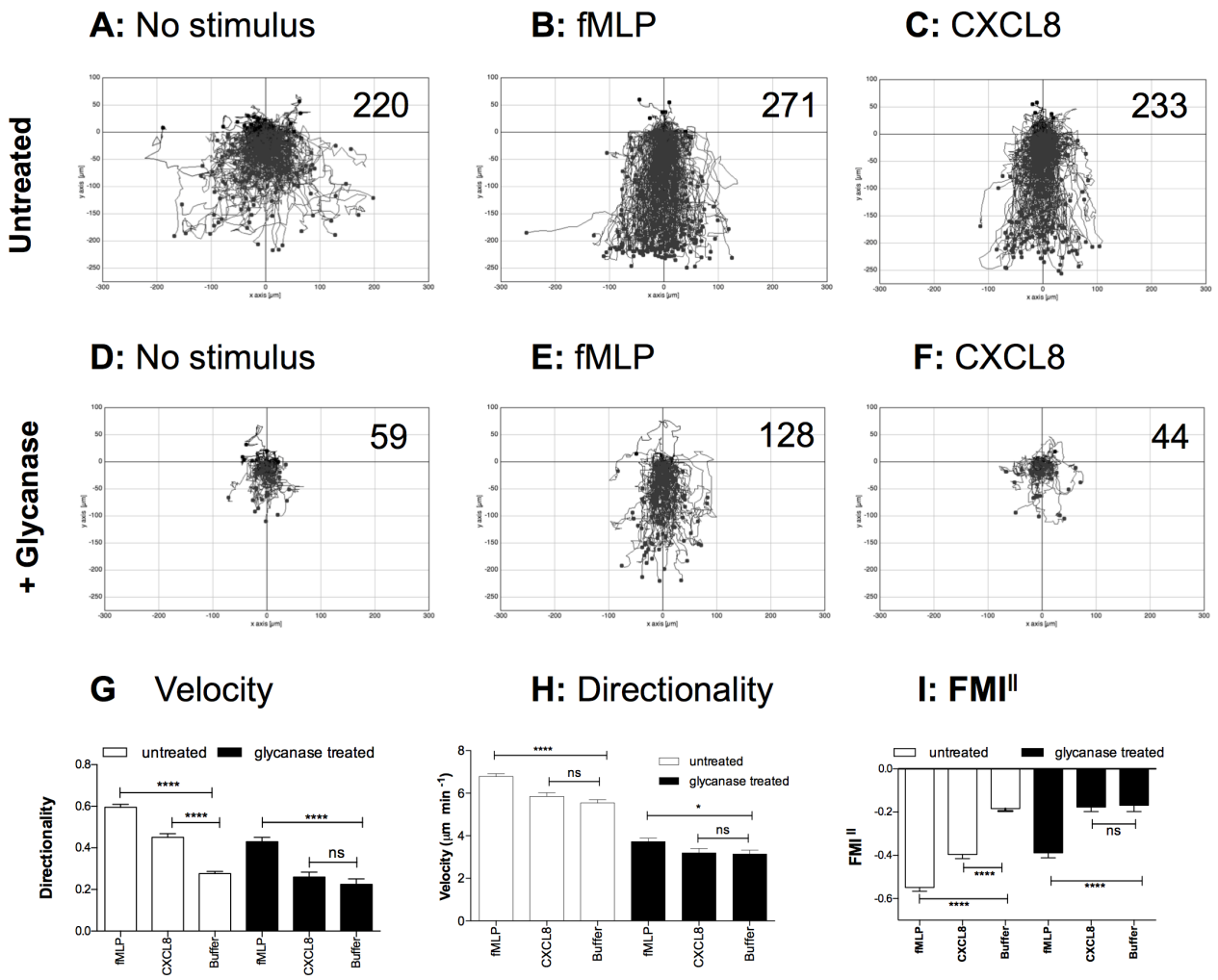

Fig. 4. Removal of neutrophil GAGs specifically ablates migration along a CXCL8 gradient.Panels A-F show the collated tracks of individual migrating neutrophils (duplicate conditions) pooled from three independent experiments using different donors. In panels A-C, neutrophils were incubated in buffer alone prior to migration, while in panels D-F, cells were treated with a glycanase cocktail. Gradients of fMLP, CXCL8 were established where noted, with the total number of tracked cells shown in the top right hand corner. Panels G-I show significant differences in velocity, directionality and FMI‖ of the data in panels A-C following tracking analysis. Error bars represent the SEM. Statistical significances between conditions were determined by one-way ANOVA with Tukey's' post-test.

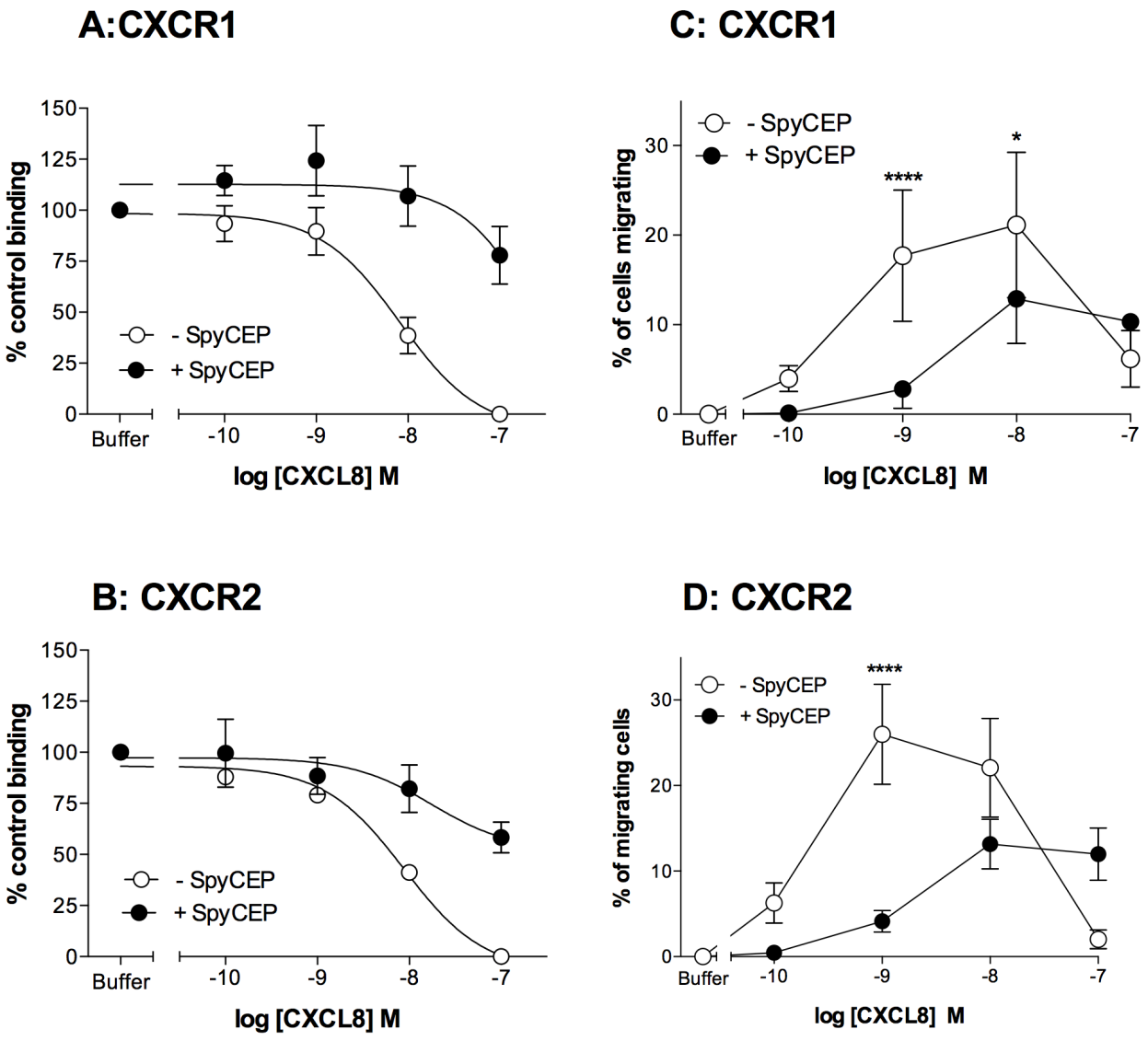

Fig. 5. SpyCEP cleavage of CXCL8 inhibits binding and activation of CXCR1 and CXCR2. Panels A and B show the ability of CXCL8 to compete for the binding of 125I-CXCL8 to CXCR1 or CXCR2 transfectants following incubation with supernatants from either the H292 or H575 GAS strains (n=6). Panels C and D show the activities of identically treated CXCL8 in inducing the migration of CXCR1 or CXCR2 transfectants (n=6). Error bars represent the mean ± SEM.

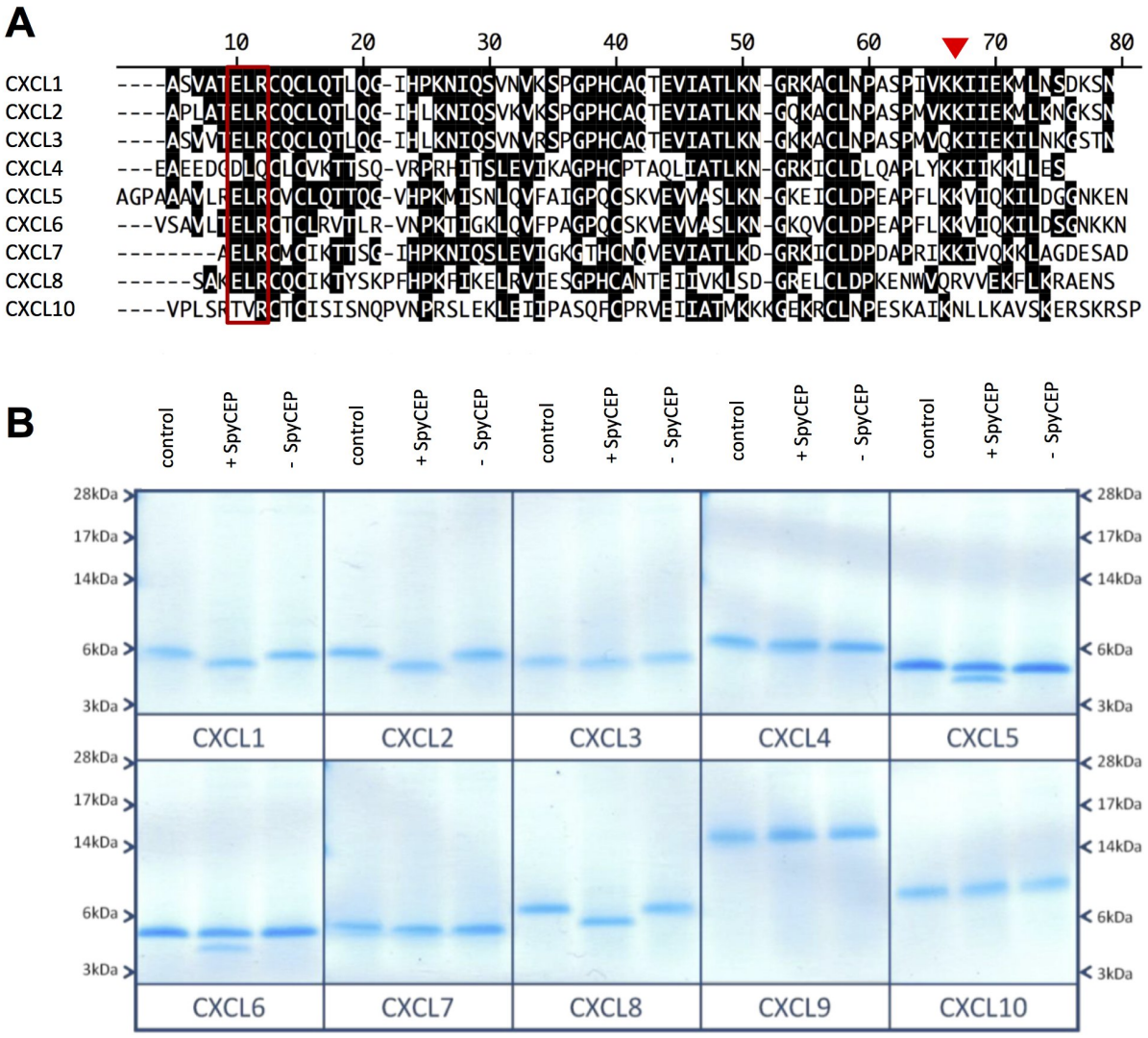

Fig. 6. SpyCEP has broad spectrum activity for ELR^{+} CXC chemokines. Panel A shows an alignment of a selection of mature human CXC chemokine sequences. The ELR motif shared by several of the chemokines is boxed and the previously determined site of cleavage of CXCL8 by SpyCEP denoted by a red triangle (15). Panel B shows the effects of SpyCEP cleavage on chemokine. 500ng of chemokine was incubated with $2\,\mu l$ of washed wild type GAS cells (H292) or an isogenic cepA mutant (H575). Cleavage was allowed to proceed for 18hr at $37\,^{\circ}C$ after which the proteins were separated by SLS-PAGE and stained with colloidal blue (Panel B).

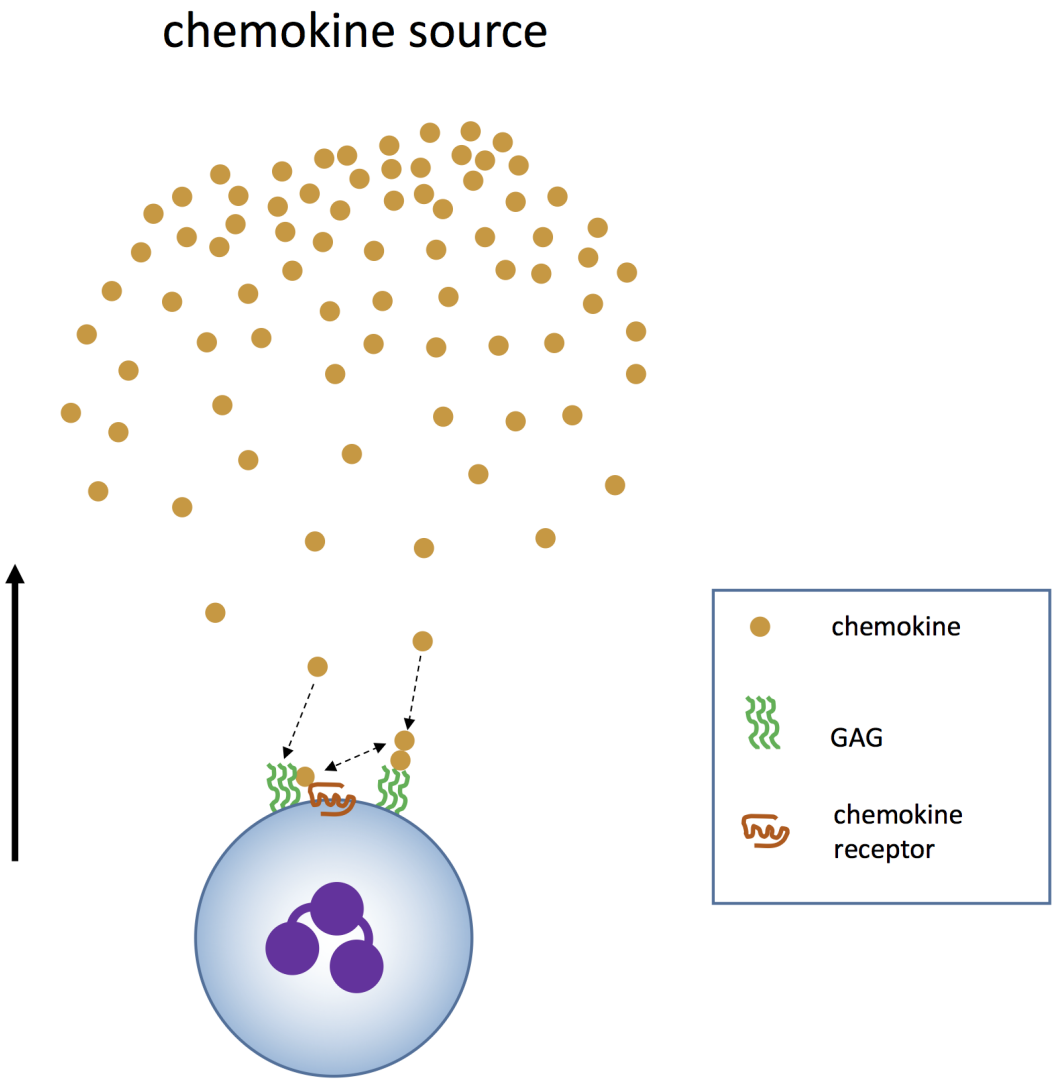

Fig. 7. Model of enhanced gradient navigation by neutrophils due to the cooperation of chemokine receptors and GAGs. The cartoon shows a neutrophil migrating along a gradient of chemokine towards the source (direction indicated by the bold arrow). Cell surface GAGs encounter chemokine and bind it, enabling presentation to the chemokine receptor (dashed arrow, a) or increasing the local concentration of chemokine surrounding the neutrophil by assisting in chemokine oligomerization (dashed arrow, b). Chemokine monomer can dissociate from the oligomer and activate the chemokine receptor (double headed dashed arrow, c).