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RESEARCH LETTER

Chemokine N-terminal-derived peptides differentially regulate signaling by the receptors CCR1 and CCR5

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Inflammatory chemokines are often elevated in disease settings, where the largest group of CC-chemokines are the macrophage inflammatory proteins (MIP), which are promiscuous for the receptors CCR1 and CCR5. MIP chemokines, such as CCL3 and CCL5 are processed at the N terminus, which influences signaling in a highly diverse manner. Here, we investigate the signaling capacity of peptides corresponding to truncated N termini. These 3–10-residue peptides displayed weak potency but, surprisingly, retained their signaling on CCR1. In contrast, none of the peptides generated a signal on CCR5, but a CCL3-derived tetrapeptide was a positive modulator boosting the signal of several chemokine variants on CCR5. In conclusion, chemokine N termini can be mimicked to produce small CCR1-selective agonists, as well as CCR5-selective modulators.

Keywords: allosteric modulation; chemokine; chemokine truncation; GPCR; pharmacology

Chemokines are 8–12 kDa secreted proteins that control migration of numerous cell types, immune surveillance, and inflammation by signaling through chemokine G protein-coupled receptors (GPCRs). Thereby, chemokines play a key role in inflammatory diseases, but also in cancer metastasis, although attempts to therapeutically target chemokines in this context have had limited success [1].

In humans, there are about 50 different chemokines and 23 chemokine receptors, with widely varying degrees of interaction promiscuity [2]. Chemokines are structurally defined by a flexible N terminus (typically 5–10 residues long), and a stably folded core domain made up of a 3-stranded β-sheet, an α-helix and a series of solvent-exposed loops. The chemokine core domain, also known as chemokine site 1 (CS1), interacts with the receptor's extracellular loops and N terminus, known as chemokine recognition site 1 (CRS1), and this accounts for the bulk of the interaction interface [3] (Fig. 1A). Chemokine core domain interactions are also essential for correct receptor recognition and are believed to be the first interactions that occur with

Abbreviations
ANOVA, analysis of variance; BRET, bioluminescence resonance energy transfer; cAMP, cyclic adenosine monophosphate; CCL, CC-chemokine ligand; CCR, CC-chemokine receptor; CHO, Chinese hamster ovary; CRS, chemokine recognition site; CS, chemokine site; EC_{50}, half maximal effective concentration; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PBMC, peripheral blood mononuclear cells; PTM, post-translational modification; TM, transmembrane helix.

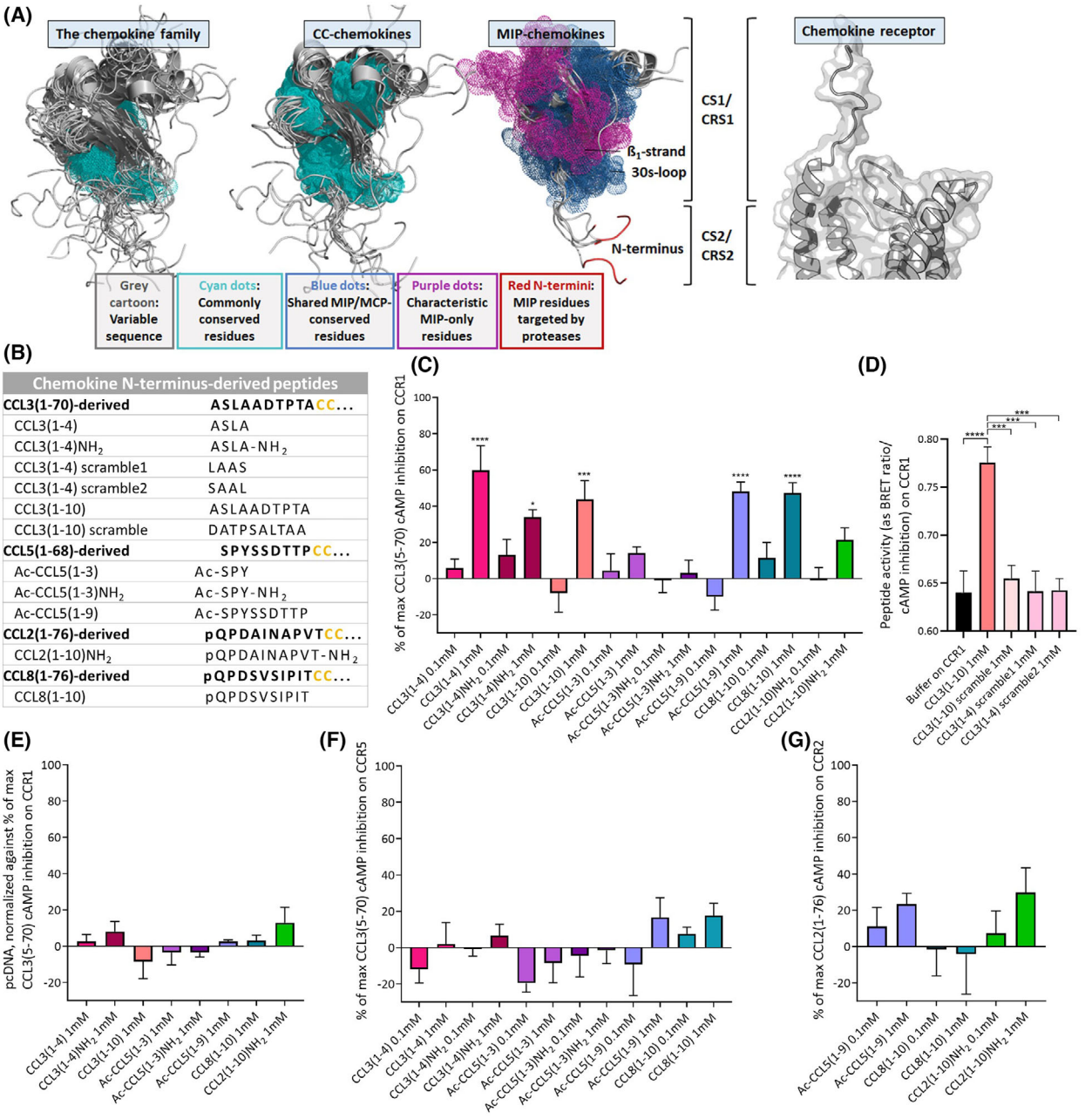

Fig. 1. Receptor-specific signaling contribution by MIP/MCP chemokine N termini. (A) Chemokines share residues within their core domains which are widely conserved (cyan), or only conserved among chemokines with similar receptor specificity (blue and purple). Most sequence conservation of MIP chemokines overlaps with the CS1/CRS1 interaction interface, whereas the chemokine N terminus sequence in the CS2/CRS2 interaction interface is highly variable. (B) Sequences of the peptides that were tested for pharmacological activity. (C) Activity of MIP- and MCP-derived peptides on CCR1 normalized to the max efficacy of CCL3(5–70), as determined by non-linear regression of a dose–response curve ranging from 1 pM to 100 nM. (D) Activity of scrambled peptide sequences of CCL3(1–4) and CCL3(1–10) on CCR1, compared to the original CCL3(1–10) sequence, not normalized to chemokine activity. (E) Activity of MIP- and MCP-derived peptides on pcDNA normalized to the max efficacy of CCL3(5–70) on CCR1. (F) Activity of MIP- and MCP-derived peptides on CCR5 normalized to the max efficacy of CCL3(5–70). (G) Activity of MIP- and MCP-derived peptides on CCR2 normalized to the max efficacy of CCL2(1–76). Data are means ± SEM of at least three experiments performed in duplicate. $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$, where *P*-values were determined using a one-way ANOVA test, and compares peptide activity at matched concentrations on receptor-transfected cells and pcDNA-transfected cells.

the receptor. The chemokine N terminus, or chemokine site 2 (CS2), interacts with the ligand binding pocket in the transmembrane space, known as chemokine recognition site 2 (CRS2), and this interaction space is often essential for receptor activation [4–7]. This two-site model is a useful framework to understand chemokine:receptor interactions, but care should be taken to not apply it habitually for all chemokine: receptor interactions, since deviations to the model are now known [8].

Chemokine receptors that respond to multiple promiscuous chemokines have proven difficult to target pharmacologically, although they are often important actors in inflammatory diseases and cancer [9,10]. Two large groups of chemokines display a very complicated pattern of promiscuity towards the receptors CCR1, CCR2, CCR3 and CCR5 – the macrophage inflammatory proteins (MIPs; CCL3, -4, -5, -14, -15, -16, -23 and five proteins encoded by CCL3 or CCL4 variant genes) and the Monocyte Chemoattractant Proteins (MCPs; CCL2, -7, -8, -11 and -13). For example, the important inflammatory MIP chemokines CCL3 (mainly produced by leukocytes) and CCL5 (produced by a broad assortment of cell types) both stimulate CCR1 and CCR5 but elicit divergent signals depending on the receptor host cell [11,12]. As these receptors are co-expressed in some differentiation stages on immune cells, most notably on monocytes and monocyte-derived macrophages and -dendritic cells, a competition – or cooperation – may occur among receptors and their cognate chemokines [13–17].

In addition to being implicated in inflammatory diseases, elevated CCR5 expression is associated with enhanced tumor survival in various cancers, while CCR1 is associated with cancer metastasis [18–20]. There is thus a great untapped potential to curb human diseases that depend on CCR1/5-based immune cell migration or inflammation, by identifying chemokine segments that give rise to distinct receptor responses, versus features that are needed for every response to a MIP-based chemokine. Presumably, therapeutics may be more successful if they are designed to be as promiscuous or specific as the chemokine:receptor interactions they should target.

While the N-terminal sequence of MCP chemokines is normally protected by a pyroglutamate, the N termini of MIP chemokines are highly variable and are naturally targeted for truncation by proteases. CCL3 is the target of cathepsin D in breast cancer, and a truncated variant, lacking the first 4 of 70 residues (denoted CCL3(5–70)) has been identified in ovarian cancer [21,22]. CCL5 is N-terminally truncated by CD26 and cathepsin G to remove the first 2 or 3 of 68 residues, respectively (denoted CCL5(3–68) and CCL5(4–68)) [23–25]. While CD26 is expressed ubiquitously in many organs, on immune cells and is present as shed active protease in plasma, cathepsin G is expressed by immune cell subsets, i.e. monocytes, macrophages, and neutrophils [26,27]. As these immune cell types dynamically regulate expression of CCR1 and CCR5, especially in response to inflammatory cues, they are able to process the CCL5 N terminus while simultaneously responding to it.

CCL3(5–70) is a potent agonist of both CCR1 and CCR5 and is a stronger agonist than the intact CCL3 (1–70) in many *in vitro* setups [28]. Truncation of full-length CCL5 to CCL5(3–68) or CCL5(4–68), on the other hand, abrogates CCR1-mediated signaling and chemotaxis, while only some signaling pathways appear to be lost on CCR5 [23,24,29]. Importantly, T cells migrate in response to CCL5(3–68), while PBMC-derived monocytes do not, presumably because the T cells used in the studies express CCR5 while the monocytes express CCR1.

Taken together, this suggests that CCR1 and CCR5 rely on the chemokine N-terminal segments in mechanistically distinct manners, which may also be observable by studying N-terminally derived peptides. In this study, we used peptides matching chemokine N-terminal sequences to assess how these segments contribute to the overall signal of CCL3 and CCL5 on CCR1 and CCR5.

Materials and methods

Mammalian cell culturing and transfection

CHO-K1 cells (American Type Culture Collection, ATCC, Manassas, VA, USA) for Gαi signaling assays were grown in RPMI1640 supplemented with 10% FBS, penicillin (180 U·mL^{-1}), streptomycin (45 µg·mL^{-1}) and L-glutamine (292 µg·mL^{-1}) and incubated at 37 °C in 5% CO_2 and 95% air humidity. Two days before the assay 25 000 cells were seeded into white 96-well culture plates. One day before the assay, Lipofectamine2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instructions to transfect the cells with the cyclic adenosine monophosphate (cAMP) Bioluminescence Resonance Energy Transfer (BRET) sensor CAMYEL and either CCR1, CCR2, CCR5 or pcDNA. The total receptor DNA used per well was 10 ng for CCR1 and CCR2, and 5 ng for CCR5, each combined with 28 ng CAMYEL DNA.

BRET cAMP assay

One day after transfection, culture medium of CHO-K1 cells was washed off and replaced with PBS with 5 µM glucose. Two timing schemes of ligand addition were used: (1,

agonism) ligands were added first (0 min), followed by addition of forskolin to a concentration of 10 μM after 5 min, coelenterazine h (Nanolight Technologies, Norman, OK, USA) addition to a total concentration of 5 μM after 10 min, and cAMP measurements were taken after 35 min as acceptor fluorescence (YFP, 525 nm) divided by donor luminescence (RLuc, 480 nm) on a 2104 Envision Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA); (2, allosteric modulation) chemokines were added first (0 min), followed by N-terminally derived peptides after 5 min, forskolin after 10 min, coelenterazine after 15 min and luminescence was measured on the plate reader after 40 min.

Sequence and structure similarity mapping to locate MIP-conserved and MIP-variable regions

PDB references of chemokine structures included in the structural alignments are: CCL1: 4OIJ chain A, CCL2: 1DOK chain A, CCL3: 2X69 chain A, CCL4: 1HUM chain A, CCL5: 5UIW chain B, CCL7: 1BO0, CCL8: 1ESR, CCL11: 1EOT, CCL13: 2RA4 chain A, CCL14: 2QR8 chain E, CCL15: 2HCC, CCL16: 5LTL chain A, CCL17: 1NR2 chain A, CCL18: 4MHE chain A, CCL19: 2MP1, CCL20: 1M8A chain A, CCL21: 2L4N state 1, CCL23: 1G91, CCL27: 2KUM state 1, CCL28: 6CWS state 1, CXCL2: 1QNK chain A state 1, CXCL4: 4R9W chain A, CXCL5: 2MGS state 1, CXCL7: 1NAP chain A, CXCL8: 5D14, CXCL10: 1LV9 state 1, CXCL11: 1RJT state 1, CXCL12: 2KEC state 1, CXCL14: 2HDL state 1, XCL1: 2N54 state 1 and CX_3CL1: 4XT3 chain B.

Chemokine sequences were aligned with CLUSTALW in the GENEIOUS software (Dotmatics, Boston, MA, USA) to determine conserved residues. The complete sequence alignment determined 4 residues with at least 93% conservation (C11, C34, C50, and P53, numbered according to CCL5). In the CC-chemokine alignment we determined another 6 residues with 80–100% conservation (C10, L/I19, F41, T43, V58, and L65). In the MIP chemokine alignment, another 22 conserved residues with 80–100% conservation were identified, 10 of which were uniquely conserved in the MIP subgroup (Y14, P20, D/E26, F/Y28, E29, S35, R/K44, R/K45, Q48, and S54). The additional conserved residues could also be identified in a separate MCP chemokine alignment and are thus not included as unique MIP chemokines.

Chemical synthesis and mass spectrometry of CCL5(4–68) and N-terminally derived peptides

CCL5(4–68) and short peptides were synthesized by solid-phase peptide synthesis (SPPS) on a P11 peptide synthesizer (Activotec, Cambridge, UK) using fluorenylmethoxycarbonyl (Fmoc) chemistry as previously described [30]. Briefly, peptide Fmoc deprotection was performed with 20% piperidine in *N*-methyl-2-pyrrolidone (NMP) and the next amino acid was activated with 0.45 M 2-1H-benzotriazol-1yl-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) and 0.45 M 1-hydroxybenzotriazole (HOBt) in dimethylformamide (DMF). UV absorbance was monitored to ensure adequate deprotection before each coupling step.

Purification of CCL5(4–68) and N-terminally derived peptides

After synthesis, the completed chemokine was cleaved from its resin using a solution consisting of 0.75 g crystalline phenol, 0.5 mL thioanisole, 10 mL trifluoroacetic acid (TFA), 0.25 mL 1,2-ethanedithiol and 0.25 mL ultrapure water for 90 min while shaking. The chemokine was then filtered from the resin and washed using diethyl ether. Peptides were cleaved from resins in a solution of 0.125 mL EDT, 0.125 mL ultrapure water and 4.75 mL TFA, and they were filtered and washed in tert-butyl methyl ether.

The peptides were then purified using reversed-phase high-performance liquid chromatography (RP-HPLC) with a PepMap C18 column on a Waters 600 pump and Waters 600 controller (Milford, MA, USA). The peptides were eluted by a gradient of 0 to 80% acetonitrile in 0.1% TFA, under continuous monitoring by directing 2% of the eluate to an electrospray ionization ion trap mass spectrometer. CCL3(1–4) and CCL3(1–4)NH_2 were purified using reversed-phase flash column chromatography and the peptides were eluted by a gradient on 0–5% acetonitrile. Fractions containing peptide with the correct relative molecular mass were collected for future use, and CCL5(4–68) was finally refolded in 1 M guanidium chloride, 0.3 mM reduced glutathione, 3 mM oxidized glutathione, 3 mM ethylenediaminetetraacetic acid (EDTA) and 0.01% *p*-nitrophenyl-β-D-glucuronide in 150 mM Tris (pH 8.6).

Proteins and reagents

Human CCL3(1–70), CCL5(1–68), CCL2 and CCL8 were bought from Peprotech (Neuilly-sur-Seine, France), and CCL3(5–70) was from Biotechne RnD Systems (Minneapolis, MN, USA). Chemicals for SPPS were from various suppliers: acetonitrile, NMP and TFA were from Biosolve (Valkenswaard, the Netherlands), HBTU from Activotec, tert-butyl methyl ether from Honeywell (Riedel-de-Haën, Seelze, Germany), diethyl ether, thioanisole, HOBt and DMF from Acros Organics (Geels, Belgium) and crystalline phenol and ethanedithiol from Merck KGaA (Darmstadt, Germany).

Data analysis

All signaling data are presented as means ± SEM of at least three independent experiments performed in duplicate. EC_{50} and efficacy of chemokines and peptides were determined by

non-linear regression using GRAPHPAD PRISM software version 9 (Graphpad Software, Boston, MA, USA). Comparisons were made by testing differences with one-way ANOVA with Tukey's multiple comparisons test.

Results

Chemokine N terminus-derived peptides display low-potency specificity towards CCR1

To clarify the specificity that N termini may contribute to MIP chemokines in an individual or subgroup-specific manner, we performed parallel sequence alignments and structural alignments (combinatorial extension alignment [31]) for 32 distinct chemokines with an experimentally determined structure. Sequences that are > 90% conserved were highlighted in a structural alignment, which revealed that highly conserved residues (related to the classical chemokine fold) are buried, while structural and chemical variation is found on most of the chemokine surfaces (Fig. 1A, left alignment). When only the CC-chemokines are included in the alignments, the conserved core region grows, and the variable domains shrink (shown in cyan, Fig. 1A, middle alignment). When only MIP chemokines are left in the alignments (here including CCL3, CCL4, CCL5, CCL14, and CCL15, Fig. 1A, right alignment), structural variation is minimal, and conserved residues cover most of the surfaces of the chemokines. We hypothesized that the CS2:CRS2 interaction interface between MIP chemokines and cognate receptors can define a large share of the receptor specificity of single chemokines, owing to its variability, protease targeting and key receptor binding site. We therefore synthesized a series of eight 3–10 residue peptides (Fig. 1B) directly derived from the N-terminal sequences of potent MIP (CCL3 and CCL5) and MCP (CCL2 and CCL8) chemokines, including small peptides based on the CCL3 (1–4) and CCL5(1–3) sequences, which correspond to the fragments that are removed during natural proteolytic truncation of CCL3 and CCL5. In addition, three peptides with a scrambled CCL3(1–4) or CCL3(1–10) sequence were included as controls (Fig. 1B). To improve stability and protect against degradation by exoproteases, some peptides were N-terminally acetylated (Ac) or C-terminally amidated (-NH_2) (Fig. 1B). The MCP-derived peptides were synthesized with the N-terminal pyroglutamic acid (pQ) which is typical for these chemokines.

As chemokine receptors mainly signal via Gαi activation, CCR1-expressing CHO cells were stimulated with each peptide in a cAMP BRET assay, with the most potent chemokine form, CCL3(5–70) as a reference agonist (−logEC_{50} potency of 9.5 (0.32 nM)). This revealed that CCR1 was amenable to stimulation with a wide range of chemokine N-terminal sequences administered at 100 μM or 1 mM (Fig. 1C). CCL3(1–4), CCL3(1–4)NH_2, CCL3(1–10), Ac-CCL5(1–9) and CCL8(1–10) were all able to produce a significant Gαi response at 1 mM with efficacies ranging from 33% (CCL3(1–4)NH_2) to 60% (CCL3(1–4)) on CCR1; thus, neutralizing the negatively charged C terminus of CCL3(1–4) by amidation (NH_2) did not improve agonist activity. In contrast, the scrambled variations of the CCL3(1–4) and CCL3(1–10) sequences did not produce a significant response (Fig. 1D), demonstrating that the CCR1 activation was sequence dependent. Similarly, no significant response was detected when using pcDNA-transfected cells (Fig. 1E). Gαi activity thus indicated that the chemokine core domain contributes with substantial CCR1 interactions (accounting for a million-fold potency change) but is not strictly needed as a pre-binding step before the CS2:CRS2 interaction can occur. In fact, only the very short Ac-CCL5(1–3) variants and the non-cognate CCL2(1–10)NH_2 sequences were unable to produce a Gαi response on CCR1, and thus its native chemokine specificity was still evident on N-terminal sequences. In some cases, the 10-fold lower concentration (100 μM) also appeared to elicit a trend towards activation, although not significantly. On the contrary, CCR5 was not amenable to peptide stimulation, as no N-terminal sequence could produce significant Gαi activity at 1 mM (Fig. 1F). Similarly, no response was observed in cells expressing CCR2 (Fig. 1G). Thus, certain peptide segments lacking a chemokine core domain are able to activate CCR1 autonomously while no response is detected on CCR5 or CCR2, indicating a fundamental difference in how chemokine N termini interact with MIP/MCP responsive receptors.

Allosteric modulation of CCR5 by chemokine-derived peptides

It is well known that N-terminal truncations of CCL3 and CCL5 have a major impact on chemokine signals and immune cell response and that these occur as PTMs in nature [32]. Available experimental structures of CCL:CCR complexes reveal that the distal N termini of CCL3 and CCL5 follow different paths and interact differently within CRS2 of the receptor (Fig. 2A). Our comparison of full-length and truncated CCL3(1–70 and 5–70) and CCL5(1–68 and 4–68) on CCR5 in the BRET-based Gαi cAMP assay supports this notion (Fig. 2B,C). MIP:CCR5 signaling is strongly influenced by the N-terminal 3–4 amino acids of the chemokine, where CCL3 truncation is

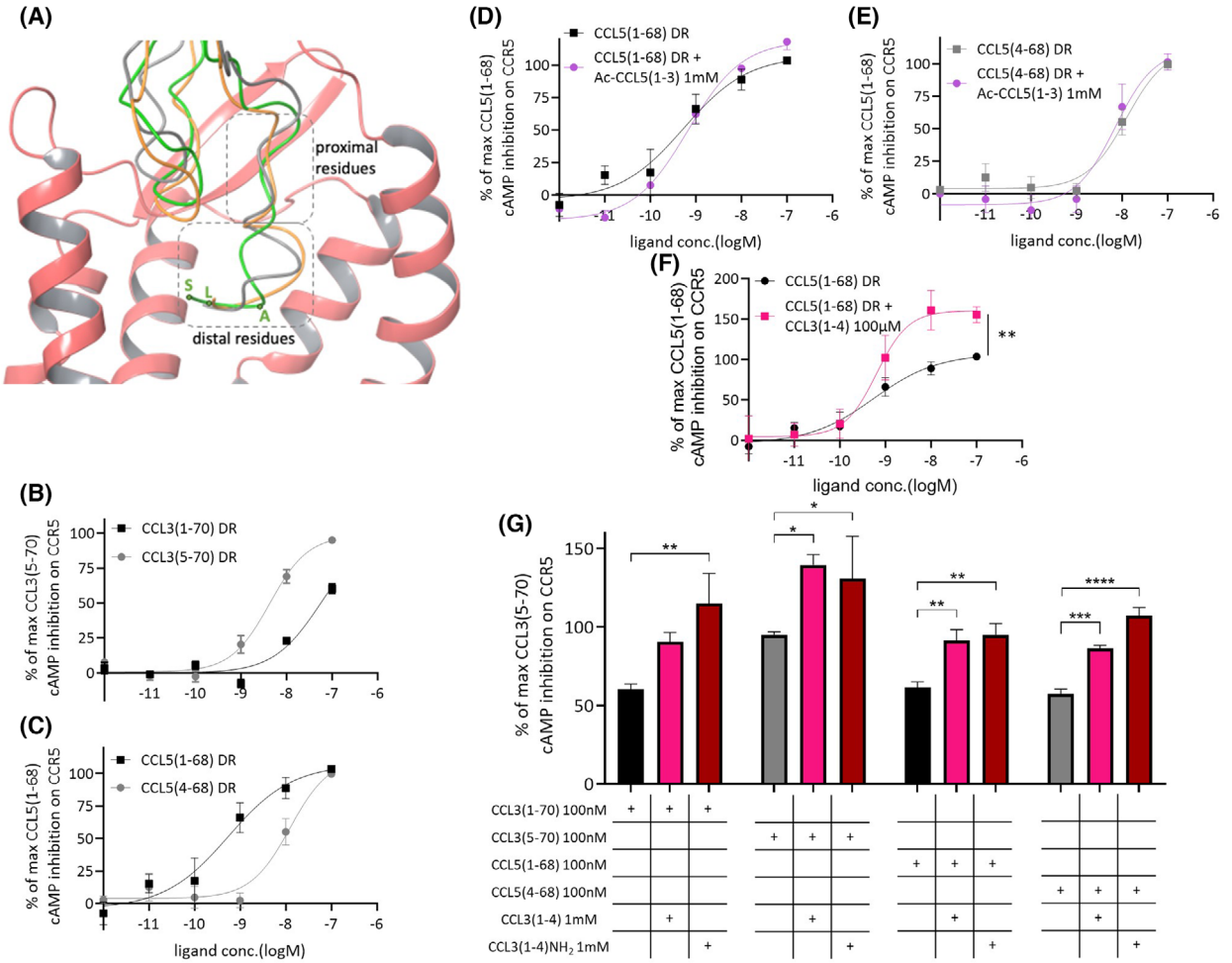

Fig. 2. Peptides as allosteric modulators of CCR5 signaling. (A) Orientation of the N termini of CCL3 (green), CCL5 (orange), and CCL2 (gray) in the context of a CCR (salmon) showing good overlap of the proximal segments (residues 6–9), but different paths for the distal ends (residues 1–5) in CRS2. The distal S-L-A residues of CCL3 are highlighted with green dots/letters. The figure was created by superimposing the receptor component of PDB structures 7F1Q (CCL3:CCR5 complex), 7F1R (CCL5:CCR5 complex), 7XA3 (CCL2:CCR2 complex), and 7VL9 (CCL15:CCR1 complex), and displaying the underlined components; TM6 and TM7 are removed for clarity. Note that the orientation of a specific CCL N terminus could vary for different CCRs. (B) $G\alpha_i$ cAMP dose–response curve (DR) of full-length CCL5(1–68) and truncated CCL5(4–68) on CCR5. (C) Dose–response curve of full-length CCL3(1–70) and truncated CCL3(5–70) on CCR5. (D) Dose–response curve of CCL5(1–68) alone or in the presence of Ac-CCL5(1–3) at 1 mM. (E) Dose–response curve of CCL5(4–68) alone or in the presence of Ac-CCL5(1–3) at 1 mM. (F) Dose–response curve of CCL5(1–68) alone or in the presence of CCL3(1–4) at 100 μM. (G) Efficacy of 100 nM chemokine (CCL3(1–70), CCL3(5–70), CCL5(1–68) or CCL5(4–68)) alone or in the presence of CCL3(1–4) or CCL3(1–4)-NH_2 at 1 mM. Data are means ± SEM of at least three experiments performed in duplicate. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, where *P*-values were determined using a one-way ANOVA test.

potentiating (indicating that the CCL3(1–4) segment suppresses the CCL3 signal) and CCL5 truncation is inhibitory (indicating that the CCL5(1–3) sequence bolsters the CCL5 signal) (Fig. 2B,C). Furthermore, we previously demonstrated that CCL3 truncation creates space for allosteric modulation by small metal ion chelators [33,34]. We therefore tested the N-terminal peptides for allosteric activity. The CCL5-derived peptide Ac-CCL5(1–3), which corresponds to a CCL5 sequence that positively affects chemokine potency (Fig. 2C), did not affect the potency or efficacy of full-length CCL5 (Fig. 2D). Furthermore, Ac-CCL5(1–3) did not rescue or otherwise alter the signal of truncated CCL5(4–68) on CCR5, although we expected that the peptide and truncated chemokine might be able to bind to CCR5 simultaneously (Fig. 2E). However, when we combined full-length CCL5 with the CCL3(1–4) peptide (100 μM), the Gαi signaling efficacy

was enhanced to 160% of the chemokine alone (*P*-value of 0.0023, Fig. 2F). At this concentration, CCL3 (1–4) did not elicit signaling when applied alone on any of the receptors tested (CCR1, CCR2 or CCR5, Fig. 1C–G). In the presence of short peptides, the baselines of the chemokine dose–response curves were not affected, in line with the lack of intrinsic peptide agonism on CCR5 (Fig. 1F). We went on to test the specificity of CCL3(1–4) (with or without C-terminal amidation, to ensure that the introduction of a negative charge at the CCL3(1–4) C terminus was not responsible for the outcome) as an allosteric modulator of full-length CCL5, and found that this peptide was in fact able to enhance the signaling of both CCL3 and CCL5 in both their full-length and truncated forms, albeit 1 mM was required (Fig. 2G). Notably, the CCL3(1–4) peptide corresponds to a CCL3 segment that contributes by suppressing signaling, since the full-length form of CCL3 is less potent and efficacious than the truncated form. These findings indicate that MIP chemokines on CCR5 are particularly receptive to positive allosteric modulation of maximal efficacy without affecting chemokine potency.

The CCL3(1–4) peptide can produce an additive effect with chemokines on CCR1

As several different N-terminal chemokine-derived peptides could activate CCR1 at low potency (Fig. 1C), we hypothesized that they also might be able to interact with CCR1 simultaneously with a chemokine, depending on the space available in the receptor. The N-terminally truncated CCL5(4–68) displayed a particularly low potency on CCR1 compared to full-length CCL5 (Fig. 3A) and compared to its potency on CCR5 (Fig. 2C). The corresponding peptide fragment, Ac-CCL5(1–3), appeared to boost the baseline of CCR1 signaling when used as an allosteric modulator of full-length CCL5, although we did not observe a significant difference (Fig. 3B). When combining CCL3(1–4) (100 μM) and full-length CCL5, we found that the peptide was not able to positively modulate the chemokine signal on CCR1 (Fig. 3C), as was the case on CCR5 (Fig. 2F). Instead, at 1 mM, the peptide lifted the baseline of the dose–response curve (Fig. 3C), owing to its intrinsic activity at this concentration (Fig. 1C). On CCR1, the CCL3 chemokine signal is both more potent and efficacious when N-terminally truncated, i.e. without the CCL3(1–4) sequence (Fig. 3D). In the presence of 1 mM CCL3(1–4) and CCL3(1–4)NH_2, we found that dose–response curve baselines of full-length and truncated CCL3 were shifted upwards, while the chemokine potency remained unchanged (Fig. 3E,F). When compared to the truncated CCL3(5–70), the full-length CCL3(1–70) and CCL5(1–68) chemokines exhibited sub-maximal efficacy at 100 nM (70% and 72%, respectively), but 100% efficacy could be reached by an additive effect between CCL3(1–4) and the full-length chemokines (Fig. 3G). The efficacy of truncated chemokines, on the other hand, could not be enhanced in the presence of CCL3 (1–4), which could perhaps indicate that CCL3(1–4) cooperates with the N terminus of chemokines directly, or that only the full-length chemokines induce a CCR1 conformation that can simultaneously accommodate CCL3(1–4). As such, CCL3(1–4) appears to be involved in distinct mechanisms on CCR1 and CCR5, cooperatively signaling through CCR1 while only producing a CCR5 effect in the presence of chemokines, which could be an opportune characteristic if applied to cells expressing both receptors simultaneously.

Discussion

With this study, we show that CCR1 and CCR5 can be distinguished by the role they assign to peptides derived from chemokine N termini. We suggest that CCR1 relies on the chemokine core domains for binding affinity and chemokine N termini to regulate activation, while CCR5 instead responds to the chemokine as a whole for binding and activation. Furthermore, we propose that this is proof-of-concept that drugs can be optimized to mimic the CCL3(1–4) sequence, which would allow them to enhance MIP chemokine signaling in a manner dependent on local tissue expression of CCR1 and CCR5, as well as MIP chemokine truncations.

Many inflammatory chemokines, such as CCL3 and CCL5, interact promiscuously with the receptors CCR1 and CCR5, and they are therefore difficult to control pharmacologically. It is currently a topic of discussion if the chemokine ensemble recognizing each receptor serves as "backups" for each other (i.e. are redundant), or if differences in signaling bias, post-translational modifications or local tissue concentrations allow receptor-expressing cells to distinguish and respond differently to each chemokine [35,36].

In particular, the tissue expression and specificity of chemokines must be better understood to improve success in attempts to block pathological chemokine signals. Most of the chemokines activating CCR1 and CCR5 are expressed on the MIP gene cluster (CCL3, -4, -5, -14, -15, -16 and -23) and are widely reported to exhibit signaling bias, as well as differential tissue diffusion due to specific affinities within the extracellular matrix (ECM) [37–39]. Since CCR1 and CCR5 are

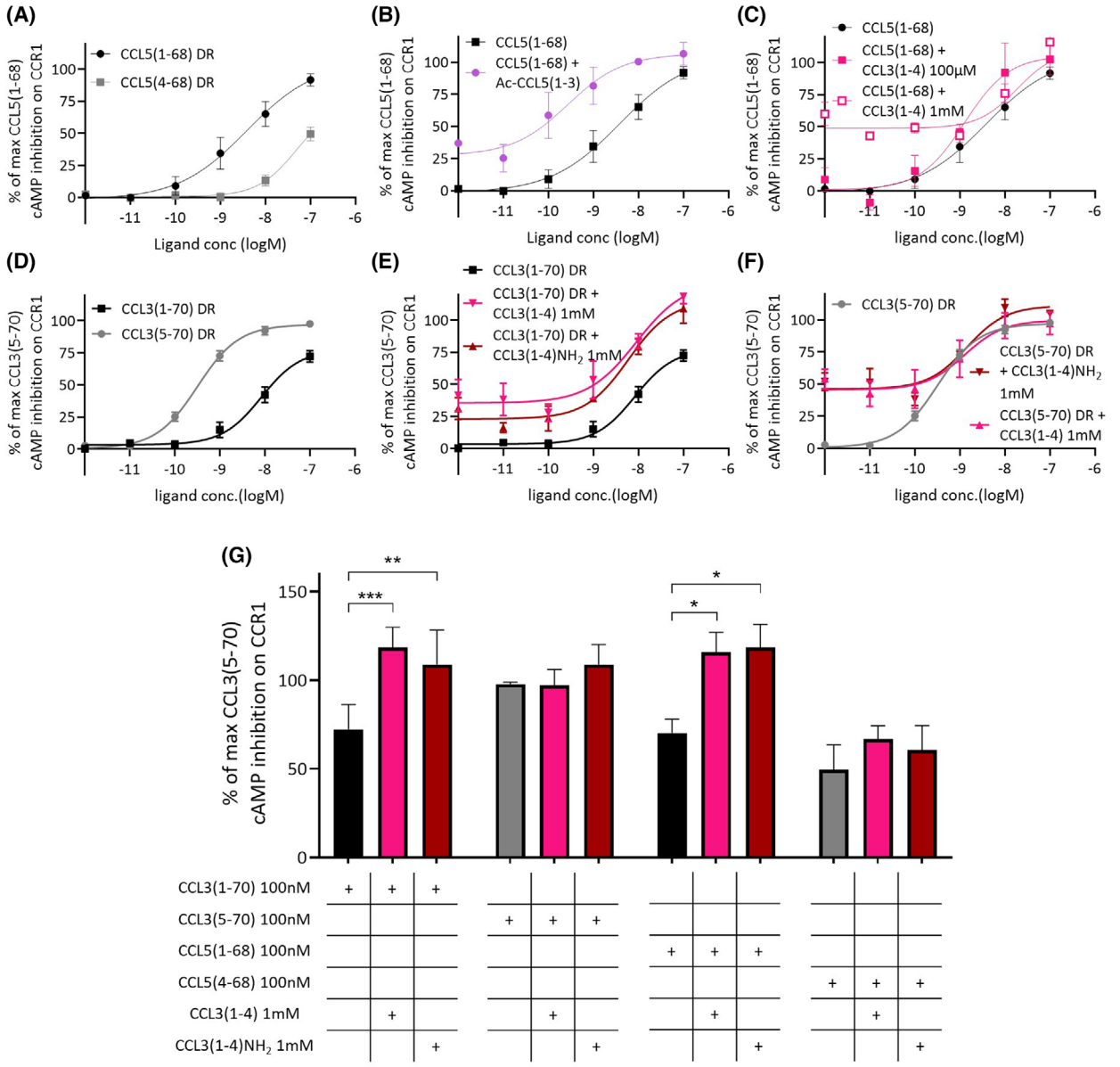

Fig. 3. Peptides with an additive effect on MIP:CCR1 signaling. (A) $G\alpha_i$ cAMP dose–response curve (DR) of full-length CCL5(1–68) and truncated CCL5(4–68) on CCR1. (B) Dose–response curve of CCL5(1–68) alone or in the presence of Ac-CCL5(1–3) at 1 mM. (C) Dose–response curve of CCL5(1–68) alone or in the presence of CCL3(1–4) at 100 µM or 1 mM. (D) Dose–response curve of full-length CCL3(1–70) and truncated CCL3(5–70) on CCR1. (E) Dose–response curve of CCL3(1–70) alone or in the presence of CCL3(1–4) at 100 µM or 1 mM. (F) Dose–response curve of CCL3(5–70) alone or in the presence of CCL3(1–4) at 100 µM or 1 mM. (G) Efficacy of 100 nM chemokine (CCL3(1–70), CCL3(5–70), CCL5(1–68) or CCL5(4–68)) alone or in the presence of CCL3 (1–4) or CCL3(1–4)-NH_2 at 1 mM. Data are means ± SEM of at least 3 experiments performed in duplicate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, where *P*-values were determined using a one-way ANOVA test.

co-expressed at several stages of differentiation and polarization on human monocytes, macrophages, eosinophils, and neutrophils [13,14,40], it is evident that they can both compete and cooperate for immune cell responses. CCL3 and CCL5 represent two of the most potent chemokines for CCR1 and CCR5, and expression is upregulated by many peripheral tissues and mature hematopoietic cells upon stimulation with pro-inflammatory cytokines, lipopolysaccharide (LPS) and virus protein [41].

We have previously shown that MIP chemokines share structure and sequence segments at the 30s-loop

that are essential for the interaction with an aromatic residue cluster on CCR1 and CCR5, which connects TM4, TM5, and ECL2 [28]. This interaction represents a pharmacologically accessible, shared signaling domain on CCR1 and CCR5 needed for recognition by the core domains of MIP chemokines such as CCL3 and CCL5. Importantly, other CCR1 and CCR5 cognate chemokines from the Monocyte Chemoattractant Protein (MCP) group, such as CCL7 and CCL8, do not depend on the aromatic cluster on CCR1 and CCR5, and thus pathologies involving MIP chemokines can conceivably be targeted without interfering with the normal functions of the MCP chemokines.

In contrast, the receptor aromatic cluster was sensitive to chemokine N-terminal truncations, but the influence of the chemokine N terminus is defined by each specific chemokine:receptor match [28]. This agreed with the notion that the receptor transmembrane pocket is sensitive to ligand stimulation, but also implied that this interaction interface can be very specific to each chemokine:receptor pair. In this study, we used combinatorial extension alignment of > 30 chemokine structures combined with multiple sequence alignments to illustrate that the N terminus is perhaps the most important distinction between MIP chemokines (see Fig. 1A). Consequently, targeting the chemokine N-terminal interaction site may also be the most promising opportunity to interfere with pathological signals arising from only a single MIP chemokine.

In this study, we moreover show that there is not only a distinction between the MIP chemokine N termini that interact with the receptor transmembrane pocket, but also a systematic difference in how CCR1 and CCR5 read these N termini. CCR1 could be activated by sequences from all cognate chemokine N termini, with the exception of the short Ac-CCL5(1–3) peptides, indicating that transmembrane pocket interactions dominate CCR1 activation by chemokines (see Fig. 1C). This finding is corroborated by the reported conversion of some chemokines into CCR1 antagonists by N-terminal truncation [23]. In fact, even a chimeric chemokine that combined the CCL3 N terminus and the CCL5 core domain was unable to activate CCR1 [42].

CCR5 appears to recognize chemokine N termini in the transmembrane pocket differently from CCR1. As we previously described, an important interplay exists between the MIP chemokine core domain and the N terminus via the CCR5 aromatic cluster [28]. CCL3 cannot signal through CCR5 if mutations have been made in the aromatic cluster, but will still bind to the receptor, as long as the chemokine N terminus remains intact. Since truncated MIP chemokines can still activate CCR5, the chemokine N terminus may rather serve to "regulate" signals originating from other parts of the chemokine [29]. This may be why one short peptide, CCL3(1–4), was able to act as an agonist of CCR1 and allosteric modulator of CCR5 at the same time and could inspire development of drugs that retain these properties.

The natural processing of CCL3 and CCL5 by CD26 and cathepsins is a mechanism that regulates the balance between CCR1 and CCR5 signaling, which affects the immune cell composition that is attracted to sites of inflammation. We have previously reported that it is possible to specifically target truncated CCL3 variants for allosteric modulation in CCR1 and CCR5, using divalent metal ion chelators that bind deep in the transmembrane receptor pocket as agonists while simultaneously enhancing specific binding of CCL3(5–70) by 3–5-fold to both receptors [33,34,43]. By increasing the size of the chelator from a bipyridine to a terpyridine, the chelator compound becomes specific to enhance only truncated chemokine binding to CCR5, and further enlargements of the compounds completely precluded allosteric action, whereas the intrinsic agonistic activity was retained. In contrast, allosteric modulation by the CCL3(1–4) peptide did not require truncation of CCL3 or CCL5, and furthermore did not act as an intrinsic agonist on CCR5, meaning that it would theoretically be able to boost only endogenous CCR5 signaling in immune settings.

The chelator compounds coordinate Zn(II) binding to the important activation cascade residue $Glu^{7.39}$ found in both CCR1 and CCR5 and depend on this residue for intrinsic signaling and enhancement of chemokine binding. The recent cryo-EM structures of CCR5 reveal that CCL3 and CCL5 also interact with $Glu^{7.39}$, but only CCL5 significantly depends on this residue for signaling, which may explain why the CCL3(1–4) peptide acts as a neutral allosteric modulator on CCR5 [43,44].

Pharmaceuticals that target CCR1 and CCR5 have been in development for decades and have yielded useful compounds that mainly target the receptor transmembrane pockets [45,46]. Synthetic compounds have chiefly displayed antagonism by direct blocking of larger ligands, or agonism by coupling to activity constraining or signal transmission residues found in the receptor pockets. The main alternative group of artificial ligands are N-terminally modified CCL5 variants, displaying high affinity/potency combined with an extensive range of properties, such as aminooxypentane (AOP)-RANTES (CCR5-specific cell surface down-regulation), [4P6]RANTES (an agonist with modified signaling profile) and [5P7]RANTES (a

potent CCR5 antagonist) [47–50]. With this study, we suggest that if small peptides are developed for greater potency, half-life and CCR5-selectivity, they could be useful as "silent" allosteric drugs that only produce an effect when the endogenous chemokine is naturally present. Indeed, N-terminal chemokine-derived peptides are unlikely to display significant GAG binding properties, are not intrinsic CCR5 agonists, and would thus not elicit systemic immune effects *in vivo* unless a chemokine gradient is already present. This, obviously, hinges on developing such peptides further, so that CCR1, being more sensitive to chemokine N-terminal activation, is not activated inadvertently.

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Author contributions

OL, PP, and MMR designed the study. OL, SS, AW, SL, IAL, KQ, PP, and MMR planned or carried out experiments. OL, JV, SS, PP and MMR analyzed data and OL wrote the first manuscript draft.

Conflict of interest statement

The authors declare no conflict of interests.

Peer review

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Data accessibility

The data that support the findings of this study are available in Figs 1–3.

References

1 Shi C and Pamer EG (2011) Monocyte recruitment during infection and inflammation. *Nat Rev Immunol* **11**, 762–774.
2 Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ, Horuk R, Sparre-Ulrich AH, Locati M, Luster AD *et al.* (2014) International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol Rev* **66**, 1–79.
3 Scholten DJ, Canals M, Maussang D, Roumen L, Smit MJ, Wijtmans M, de Graaf C, Vischer HF and Leurs R (2012) Pharmacological modulation of chemokine receptor function. *Br J Pharmacol* **165**, 1617–1643.
4 Pease JE, Wang J, Ponath PD and Murphy PM (1998) The N-terminal extracellular segments of the chemokine receptors CCR1 and CCR3 are determinants for MIP-1alpha and eotaxin binding, respectively, but a second domain is essential for efficient receptor activation. *J Biol Chem* **273**, 19972–19976.
5 Zoffmann S, Chollet A and Galzi JL (2002) Identification of the extracellular loop 2 as the point of interaction between the N terminus of the chemokine MIP-1alpha and its CCR1 receptor. *Mol Pharmacol* **62**, 729–736.
6 Wu L, Ruffing N, Shi X, Newman W, Soler D, Mackay CR and Qin S (1996) Discrete steps in binding and signaling of interleukin-8 with its receptor. *J Biol Chem* **271**, 31202–31209.
7 Blanpain C, Doranz BJ, Bondue A, Govaerts C, De Leener A, Vassart G, Doms RW, Proudfoot A and Parmentier M (2003) The core domain of chemokines binds CCR5 extracellular domains while their amino terminus interacts with the transmembrane helix bundle. *J Biol Chem* **278**, 5179–5187.
8 Kleist AB, Getschman AE, Ziarek JJ, Nevins AM, Gauthier PA, Chevigne A, Szpakowska M and Volkman BF (2016) New paradigms in chemokine receptor signal transduction: moving beyond the two-site model. *Biochem Pharmacol* **114**, 53–68.
9 Solari R, Pease JE and Begg M (2015) Chemokine receptors as therapeutic targets: why aren't there more drugs? *Eur J Pharmacol* **746**, 363–367.
10 Steen A, Larsen O, Thiele S and Rosenkilde MM (2014) Biased and g protein-independent signaling of chemokine receptors. *Front Immunol* **5**, 277.
11 Corbisier J, Gales C, Huszagh A, Parmentier M and Springael JY (2015) Biased signaling at chemokine receptors. *J Biol Chem* **290**, 9542–9554.
12 Metzemaekers M, Van Damme J, Mortier A and Proost P (2016) Regulation of chemokine activity – a focus on the role of dipeptidyl peptidase IV/CD26. *Front Immunol* **7**, 483.
13 Medina-Ruiz L, Bartolini R, Wilson GJ, Dyer DP, Vidler F, Hughes CE, Schuette F, Love S, Pingen M, Hayes AJ *et al.* (2022) Analysis of combinatorial chemokine receptor expression dynamics using multi-receptor reporter mice. *Elife* **11**, e72418.
14 Sandblad KG, Jones P, Kostalla MJ, Linton L, Glise H and Winqvist O (2015) Chemokine receptor expression

on monocytes from healthy individuals. *Clin Immunol* **161**, 348–353.
15 Gouwy M, Struyf S, Leutenez L, Portner N, Sozzani S and Van Damme J (2014) Chemokines and other GPCR ligands synergize in receptor-mediated migration of monocyte-derived immature and mature dendritic cells. *Immunobiology* **219**, 218–229.
16 Dyer DP, Medina-Ruiz L, Bartolini R, Schuette F, Hughes CE, Pallas K, Vidler F, Macleod MKL, Kelly CJ, Lee KM *et al.* (2019) Chemokine receptor redundancy and specificity are context dependent. *Immunity* **50**, 378–389.e5.
17 Bartolini R, Medina-Ruiz L, Hayes AJ, Kelly CJ, Halawa HA and Graham GJ (2022) Inflammatory chemokine receptors support inflammatory macrophage and dendritic cell maturation. *Immunohorizons* **6**, 743–759.
18 Hemmatazad H and Berger MD (2021) CCR5 is a potential therapeutic target for cancer. *Expert Opin Ther Targets* **25**, 311–327.
19 Karash A, Mazzoni MR and Gilchrist A (2014) Pharmacological intervention at CCR1 and CCR5 as an approach for cancer: help or hindrance. *Curr Top Med Chem* **14**, 1553–1573.
20 White GE, Iqbal AJ and Greaves DR (2013) CC chemokine receptors and chronic inflammation – therapeutic opportunities and pharmacological challenges. *Pharmacol Rev* **65**, 47–89.
21 Wolf M, Clark-Lewis I, Buri C, Langen H, Lis M and Mazzucchelli L (2003) Cathepsin D specifically cleaves the chemokines macrophage inflammatory protein-1 alpha, macrophage inflammatory protein-1 beta, and SLC that are expressed in human breast cancer. *Am J Pathol* **162**, 1183–1190.
22 Schutyser E, Struyf S, Proost P, Opdenakker G, Laureys G, Verhasselt B, Peperstraete L, Van de Putte I, Saccani A, Allavena P *et al.* (2002) Identification of biologically active chemokine isoforms from ascitic fluid and elevated levels of CCL18/pulmonary and activation-regulated chemokine in ovarian carcinoma. *J Biol Chem* **277**, 24584–24593.
23 Struyf S, De Meester I, Scharpe S, Lenaerts JP, Menten P, Wang JM, Proost P and Van Damme J (1998) Natural truncation of RANTES abolishes signaling through the CC chemokine receptors CCR1 and CCR3, impairs its chemotactic potency and generates a CC chemokine inhibitor. *Eur J Immunol* **28**, 1262–1271.
24 Lim JK, Burns JM, Lu W and DeVico AL (2005) Multiple pathways of amino terminal processing produce two truncated variants of RANTES/CCL5. *J Leukoc Biol* **78**, 442–452.
25 Lim JK, Lu W, Hartley O and DeVico AL (2006) N-terminal proteolytic processing by cathepsin G converts RANTES/CCL5 and related analogs into a truncated 4-68 variant. *J Leukoc Biol* **80**, 1395–1404.
26 Delgado MB, Clark-Lewis I, Loetscher P, Langen H, Thelen M, Baggiolini M and Wolf M (2001) Rapid inactivation of stromal cell-derived factor-1 by cathepsin G associated with lymphocytes. *Eur J Immunol* **31**, 699–707.
27 Owen CA, Campbell MA, Sannes PL, Boukedes SS and Campbell EJ (1995) Cell surface-bound elastase and cathepsin G on human neutrophils: a novel, non-oxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases. *J Cell Biol* **131**, 775–789.
28 Larsen O, van der Velden WJC, Mavri M, Schuermans S, Rummel PC, Karlshoj S, Gustavsson M, Proost P, Vabeno J and Rosenkilde MM (2022) Identification of a conserved chemokine receptor motif that enables ligand discrimination. *Sci Signal* **15**, eabg7042.
29 Oravecz T, Pall M, Roderiquez G, Gorrell MD, Ditto M, Nguyen NY, Boykins R, Unsworth E and Norcross MA (1997) Regulation of the receptor specificity and function of the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) by dipeptidyl peptidase IV (CD26)-mediated cleavage. *J Exp Med* **186**, 1865–1872.
30 Loos T, Mortier A and Proost P (2009) Chapter 1. Isolation, identification, and production of posttranslationally modified chemokines. *Methods Enzymol* **461**, 3–29.
31 Shindyalov IN and Bourne PE (1998) Protein structure alignment by incremental combinatorial extension (CE) of the optimal path. *Protein Eng* **11**, 739–747.
32 Proost P, Struyf S, Van Damme J, Fiten P, Ugarte-Berzal E and Opdenakker G (2017) Chemokine isoforms and processing in inflammation and immunity. *J Autoimmun* **85**, 45–57.
33 Thiele S, Malmgaard-Clausen M, Engel-Andreasen J, Steen A, Rummel PC, Nielsen MC, Gloriam DE, Frimurer TM, Ulven T and Rosenkilde MM (2012) Modulation in selectivity and allosteric properties of small-molecule ligands for CC-chemokine receptors. *J Med Chem* **55**, 8164–8177.
34 Larsen O, Lückmann M, van der Velden WJ, Oliva-Santiago M, Brvar M, Ulven T, Frimurer TM, Karlshøj S and Rosenkilde MM (2019) Selective allosteric modulation of N-terminally cleaved, but not full length CCL3 in CCR1. *ACS Pharmacol Transl Sci* **2**, 429–441.
35 Mantovani A (2018) Redundancy and robustness versus division of labour and specialization in innate immunity. *Semin Immunol* **36**, 28–30.
36 Dyer DP (2020) Understanding the mechanisms that facilitate specificity, not redundancy, of chemokine mediated leukocyte recruitment. *Immunology* **160**, 336–344.
37 Zlotnik A, Yoshie O and Nomiyama H (2006) The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biol* **7**, 243.

38 Rajagopal S, Bassoni DL, Campbell JJ, Gerard NP, Gerard C and Wehrman TS (2013) Biased agonism as a mechanism for differential signaling by chemokine receptors. *J Biol Chem* **288**, 35039–35048.
39 Allen SJ, Crown SE and Handel TM (2007) Chemokine: receptor structure, interactions, and antagonism. *Annu Rev Immunol* **25**, 787–820.
40 Repeke CE, Ferreira SB Jr, Claudino M, Silveira EM, de Assis GF, Avila-Campos MJ, Silva JS and Garlet GP (2010) Evidences of the cooperative role of the chemokines CCL3, CCL4 and CCL5 and its receptors CCR1+ and CCR5+ in RANKL+ cell migration throughout experimental periodontitis in mice. *Bone* **46**, 1122–1130.
41 Melchjorsen J, Sorensen LN and Paludan SR (2003) Expression and function of chemokines during viral infections: from molecular mechanisms to in vivo function. *J Leukoc Biol* **74**, 331–343.
42 Blanpain C, Buser R, Power CA, Edgerton M, Buchanan C, Mack M, Simmons G, Clapham PR, Parmentier M and Proudfoot AE (2001) A chimeric MIP-1alpha/RANTES protein demonstrates the use of different regions of the RANTES protein to bind and activate its receptors. *J Leukoc Biol* **69**, 977–985.
43 Karlshoj S, Amarandi RM, Larsen O, Daugvilaite V, Steen A, Brvar M, Pui A, Frimurer TM, Ulven T and Rosenkilde MM (2016) Molecular mechanism of action for allosteric modulators and agonists in CC-chemokine receptor 5 (CCR5). *J Biol Chem* **291**, 26860–26874.
44 Zhang H, Chen K, Tan Q, Shao Q, Han S, Zhang C, Yi C, Chu X, Zhu Y, Xu Y *et al.* (2021) Structural basis for chemokine recognition and receptor activation of chemokine receptor CCR5. *Nat Commun* **12**, 4151.
45 Gladue RP, Brown MF and Zwillich SH (2010) CCR1 antagonists: what have we learned from clinical trials. *Curr Top Med Chem* **10**, 1268–1277.
46 Gilliam BL, Riedel DJ and Redfield RR (2011) Clinical use of CCR5 inhibitors in HIV and beyond. *J Transl Med* **9** (Suppl 1), S9.
47 Simmons G, Clapham PR, Picard L, Offord RE, Rosenkilde MM, Schwartz TW, Buser R, Wells TN and Proudfoot AE (1997) Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science* **276**, 276–279.
48 Elsner J, Mack M, Bruhl H, Dulkys Y, Kimmig D, Simmons G, Clapham PR, Schlondorff D, Kapp A, Wells TN *et al.* (2000) Differential activation of CC chemokine receptors by AOP-RANTES. *J Biol Chem* **275**, 7787–7794.
49 Choi WT, Nedellec R, Coetzer M, Colin P, Lagane B, Offord RE, Hartley O and Mosier DE (2012) CCR5 mutations distinguish N-terminal modifications of RANTES (CCL5) with agonist versus antagonist activity. *J Virol* **86**, 10218–10220.
50 Gaertner H, Cerini F, Escola JM, Kuenzi G, Melotti A, Offord R, Rossitto-Borlat I, Nedellec R, Salkowitz J, Gorochov G *et al.* (2008) Highly potent, fully recombinant anti-HIV chemokines: reengineering a low-cost microbicide. *Proc Natl Acad Sci USA* **105**, 17706–17711.