Determinants of High-Affinity Binding and Receptor Activation in the N-Terminus of CCL-19 (MIP-3β)

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ABSTRACT: CC chemokine receptor 7 (CCR-7) is expressed on mature dendritic cells and T-cells. Its ligands, CCL-19 (MIP-3β) and CCL-21 (SLC), play an important role in the migration of these cells to secondary lymphoid organs where they are predominantly expressed. For most chemokines, the N-terminal domain preceding the first two conserved cysteines is involved in stabilizing the active conformation of its cognate receptors. We have chemically synthesized N-terminal analogues of CCL-19 with the aid of a native chemical ligation method to investigate structure function requirements of this ligand domain by performing ligand binding, GTP-γS binding, and chemotaxis assays. Successive truncations of the N-terminus of CCL-19 reduced the affinity of the receptor for the ligand in a size-dependent manner. Furthermore, Ala substitutions of Asn^3, Asp^4, and Asp^7 show that the side chains of these residues are important for high-affinity binding of CCL-19 to CCR-7. The effects observed were mirrored in both GTP-γS binding and chemotaxis assays, highlighting the functional importance of this ligand domain. We also describe two partial agonists of CCR-7 ([Nle^{72}]CCL-19(6−77) and Ac-[Nle^{72}]CCL-19(7−77)), and identify the first analogue of CCL-19 (Ac-[Nle^{72}]CCL-19(8−77)) that acts as a functional antagonist in vitro ($K_B \sim 350$ nM for GTP-γS binding assays). As mutations of both Glu^6 and Asp^7 to Ala did not dissociate effects on ligand binding from receptor activation, it is likely that the backbone of these two residues is crucial for agonist activity.

CC-chemokine ligand-19 (CCL-19)[1,2] and CCL-21[3] play an important role in the migration of T-cells and antigen presenting dendritic cells (DCs) to secondary lymphoid organs where they are predominantly expressed (*1*−*5*). CCL-19 and CCL-21 are members of the family of chemokines, which are small proteins that play a fundamental role as chemoattractants of leukocytes in the acute and chronic inflammatory response of the immune system (*6*, *7*), and more recently have been linked to targeting metastasis of some types of cancers (*8*, *9*). Their mechanism of action is by signaling through their cognate G-protein coupled receptors.

Two main families of chemokines have been identified: CC- and CXC-chemokines, which differ by the relative position of the first two conserved cysteines (*6*, *7*). Even though within each family most chemokines are shared by a number of receptors, there is little evidence for interactions across families. CXCR-3 agonists are the only CXC-chemokines so far that have been shown to bind to a CCR, acting as antagonists at CCR-3 (*10*, *11*). In humans, CCL-19 and CCL-21 have a single target receptor, CCR-7, which is expressed primarily on mature DCs and naïve T-cells (*12*−*14*). The combination of CCL-19 and CCL-21 expression facilitates the encounter of antigen-loaded DCs with T-cells in the lymph nodes. Furthermore, it has been shown that mice deficient in CCR-7 cannot lodge an immune response to foreign antigens because of defective T-cell and DC migration (*15*). Elucidating mechanisms underlying ligand binding and receptor activation of CCR-7 could therefore help in designing drugs for the treatment of autoimmune diseases.

As determined by NMR and X-ray crystallography, both CC- and CXC-chemokines, have very similar 3D structures: a disordered N-terminal region preceding the conserved cysteines is followed by an N-loop terminating in one turn of a 3_{10} helix, a three-stranded antiparallel β-sheet, and an α-helix that is found in close proximity to the β-sheet (*6*, *7*, *16*, *17*). The N-terminus and N-loop are also tethered to the β-sheet by the conserved disulfide bonds (*6*, *7*, *16*). At millimolar concentrations, chemokines have been shown to form dimers (*18*, *19*). However, dimerization is not necessary for high-affinity binding and receptor activation in vitro, as several mutant chemokines that are unable to form dimers have been shown to bind to and activate their cognate receptors at levels similar to wild-type chemokines (*20*−*23*). Glycosaminoglycans (GAGs) have been suggested to play a role in forming local gradients by binding chemokines

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[1] Abbreviations: CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; DC, dendtritic cells; GPCR, G-protein coupled receptor; RFU, relative fluorescence unit; GAGs, glycosaminoglycans.

[2] Also known as macrophage inflammatory protein 3β (MIP-3β), EBI1 ligand chemokine (ELC), chemokine-β 11 (CKβ-11), exodus-3, or small inducible cytokine A19 (SCYA19).

[3] Also known as secondary lymphoid organ chemokine (SLC), 6-Ckine, exodus-2, TCA-4, or small inducible cytokine A2 (SCYA2).

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(*24*−*26*). Increasing local concentrations above a certain threshold might result in chemokine dimerization. Recently, GAGs have been shown to be critical for the chemotactic properties of cells expressing chemokine receptors in vivo, suggesting that aggregation might be essential for physiological activity of chemokines (*27*).

Binding of chemokines to their receptors involves a high-affinity binding interaction, usually involving the N-loop and/or β-sheet of the chemokine and the N-terminus of the receptor. A second set of low energy interactions, usually between the N-terminus of the chemokine and the helical bundle of the receptor is thought to stabilize the active conformation of the receptor resulting in signal transduction (*28*−*30*). Most chemokine receptors have been shown to activate members of the G_i family of heterotrimeric G-proteins, and both downstream signaling and chemotaxis are pertussis toxin sensitive (*31*, *32*). It is however likely that $G_{\beta\gamma}$ and additional downstream effectors, such as MAP kinases and RGS (regulators of G-protein signaling), also play a major role in the process of signal transduction (*32*).

Here, we investigate the function of the N-terminal residues of CCL-19 through chemical synthesis of analogues with truncations and Ala-scanning substitutions. We identify residues that affect both ligand binding and the ability of the ligand to activate CCR-7, describe two analogues of CCL-19 that act as partial agonists, and identify the first CCL-19 analogue (Ac-[Nle^{72}]CCL-19(8−77)) that acts as a functional antagonist in vitro.

EXPERIMENTAL PROCEDURES

Cell Culture. All cell culture media and solutions were purchased from Cellgro (Fisher Scientific, Tustin, CA). FLP-In CHO-K cells were obtained from Invitrogen Life Technologies (Baltimore, MD). A stable FLP-In CHO-K cell line expressing CCR-7 (accession no. XM_049959) was established according to manufacturer's instructions. The stable cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/mL penicillin, 50 μg/mL streptomycin, and 200 μg/mL hygromycin (Invitrogen, Baltimore, MD). Cells were seeded in 500 cm^2 dishes and grown to 90−100% confluency prior to membrane preparations. H9 (human T-cell lymphoma) cells were obtained from ATCC (Manassas, VA) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate.

Membrane Preparations. CCR-7 expressing CHO-K cells were scraped in membrane buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, pH 7.2) and exposed to a pressure of 900 bar for 30 min in a nitrogen cavitation chamber. The homogenate was spun at 1000*g* for 10 min at 4 °C to remove nuclei and cellular debris. The supernatant was taken and spun at 45000*g* for 45 min at 4 °C to collect the membrane fraction. Membranes were resuspended in membrane buffer to a final concentration of 1 mg/mL, snap frozen in liquid nitrogen in aliquots and stored at −70 °C.

Peptide Synthesis. The N-terminal deletion analogues of CCL-19 ([Nle^{72}]CCL-19(3−77)OH, [Nle^{72}]CCL-19(4−77)-OH, [Nle^{72}]CCL-19(5−77)OH, and [Nle^{72}]CCL-19(6−77)-OH) were synthesized by solid-phase methodology (*33*) on a CS536 Peptide synthesizer (CS Bio, San Carlos, CA) with the t-Boc-Ser-4-(oxymethyl)-phenyl-acetamido-4-methylphenyl (Pam) resin, using N$^{\alpha}$-*tert*-butyloxycarbonyl (t-Boc) protection/trifluoroacetic acid (TFA) deprotection and doubling coupling of each amino acid through activation with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) chemistry as described by Clark-Lewis et al. (*34*). To eliminate complications resulting from oxidation of the methionine in the subsequent folding of the linear CCL-19 peptide in the air, the Met^{72} residue in CCL-19 was substituted with norleucine. The assembled CCL-19 peptide-resin was treated with hydrogen fluoride (HF) to cleave the completed peptide from the resin as well as to remove the side chain protecting groups. The recovered linear peptide was purified by HPLC on a Biotage KP-100 preparative HPLC system, using a gradient of acetonitrile in aqueous 0.1% TFA (Table 1). Folding of the linear peptide to the native structure was carried out by dissolving the linear peptide in 0.1 N acetic acid and then diluting the peptide solution to 0.1 M Tris-HCl buffer (pH 8.8) to a final peptide concentration of ∼0.1 mg/mL. The solution was stirred overnight at room temperature and open to the atmosphere. On the next day, the pH of the solution was adjusted to 4.5

Table 1: Peptide Yield, Calculated and Experimentally Measured Molecular Mass of CCL-19 Peptide Fragments, Ligated Linear CCL-19 Peptides, and Folded CCL-19 Peptides

	yield (mg)	calcd molec mass	measd molec mass
linear [Nle^{72}]CCL-19(3−77)OH	52.0	8624.1	8624
folded [Nle^{72}]CCL-19(3−77)OH	5.1	8620.1	8620
linear [Nle^{72}]CCL-19(4−77)OH	53.0	8510.0	8510
folded [Nle^{72}]CCL-19(4−77)OH	5.9	8506.0	8505
linear [Nle^{72}]CCL-19(5−77)OH	57.0	8393.9	8394
folded [Nle^{72}]CCL-19(5−77)OH	12.2	8389.9	8390
linear [Nle^{72}]CCL-19(6−77)OH	47.0	8324.9	8325
folded [Nle^{72}]CCL-19(6−77)OH	8.1	8320.9	8320
[Nle^{72}]CCL-19(34−77)OH	1611.0	5119.5	5120
CCL-19(1−33)MPAL	79.0	4048.0	4048
linear [Nle^{72}]CCL-19(1−77)OH	42.0	8782.6	8784
folded [Nle^{72}]CCL-19(1−77)OH	14.5	8778.2	8780
[Ala^2,Nle^{72}]CCL-19(1−33)MPAL	47.0	4017.0	4017
linear [Ala^2,Nle^{72}]CCL-19(1−77)OH	38.0	8752.2	8752
folded [Ala^2,Nle^{72}]CCL-19(1−77)OH	10.2	8748.2	8748
[Ala^3,Nle^{72}]CCL-19(1−33)MPAL	61.0	4005.0	4004
linear [Ala^3,Nle^{72}]CCL-19(1−77)OH	54.0	8739.2	8740
folded [Ala^3,Nle^{72}]CCL-19(1−77)OH	13.1	8735.2	8736
[Ala^4,Nle^{72}]CCL-19(1−33)MPAL	39.0	4003.9	4004
linear [Ala^4,Nle^{72}]CCL-19(1−77)OH	41.0	8738.2	8740
folded [Ala^4,Nle^{72}]CCL-19(1−77)OH	10.9	8734.2	8736
[Ala^6,Nle^{72}]CCL-19(1−33)MPAL	39.0	3989.9	3988
linear [Ala^6,Nle^{72}]CCL-19(1−77)OH	40.0	8724.2	8724
folded [Ala^6,Nle^{72}]CCL-19(1−77)OH	9.7	8720.2	8720
[Ala^7,Nle^{72}]CCL-19(1−33)MPAL	48.0	4004.0	4004
linear [Ala^7,Nle^{72}]CCL-19(1−77)OH	57.0	8738.2	8740
folded [Ala^7,Nle^{72}]CCL-19(1−77)OH	6.9	8734.2	8736
Ac-CCL-19(6−33)MPAL	52.0	3631.0	3632
linear Ac-[Nle^{72}]CCL-19(6−77)OH	50.0	8365.8	8368
folded Ac-[Nle^{72}]CCL-19(6−77)OH	23.0	8361.8	8364
Ac-CCL-19(7−33)MPAL	70.0	3502.5	3502
linear Ac-[Nle^{72}]CCL-19(7−77)OH	71.0	8236.7	8236
folded Ac-[Nle^{72}]CCL-19(7−77)OH	41.7	8232.7	8232
Ac-CCL-19(8−33)MPAL	62.2	3387.0	3387
linear Ac-[Nle^{72}]CCL-19(8−77)OH	57.6	8121.6	8124
folded Ac-[Nle^{72}]CCL-19(8−77)OH	28.7	8117.6	8120

with acetic acid and the folded peptide was recovered by preparative HPLC purification.

Because the double-coupling method is rather time-consuming and gave relatively low yield, a native chemical ligation method (*35*) was introduced to make the other CCL-19 peptides. The native chemical ligation method takes advantage of a Xaa−Cys bond in the peptide sequence, where Xaa is any amino acid except proline, and a Xaa−Cys bond is conveniently located at the Gly^{33}−Cys^{34} position of CCL-19. Thus, two peptide fragments of CCL-19, one corresponding to N-terminal CCL-19(8−33) and the other to the C-terminal CCL-19(34−77), were synthesized by solid-phase peptide synthesis methodology. The C-terminal [Nle^{72}]CCL-19(34−77)OH fragment was synthesized with the t-Boc-Ser-Pam resin using the t-Boc protection/TFA deprotection and single coupling of each amino acid through activation with 1,3-di-isopropyl-carbodiimide chemistry as described (*36*). The protected N-terminal CCL-19(8−33) fragment was synthesized through the use of a trityl-associated mercaptopropionic acid-leucine (TAMPAL) resin as described by Hackeng et al. (*37*). The N-terminal protected CCL-19(8−33)-mercaptopropionic acid-leucine (MPAL)-resin intermediate was then divided into aliquots for subsequent synthesis of the CCL-19(1−33)-MPAL as well as the Ala-substituted and deletion plus acetylation fragments of CCL-19. The assembled peptide fragments were deprotected and cleaved from the resin anchor with HF and the recovered peptide purified by preparative HPLC. The purified C-terminal [Nle^{72}]CCL-19(34−77)OH fragment was ligated with an equimolar amount of the appropriate N-terminal CCL-19(1−33)-MPAL fragment at ~2 mM peptide concentration in 0.1 M phosphate buffer (pH 7.5) containing 6 M guanidine and 2% (v/v) benzyl-mercaptan and 2% (v/v) thiophenol at 37 °C overnight as described (*37*). The ligation product was again purified by preparative HPLC to isolate the ligated linear peptide (Table 1). Folding of the linear peptide to the native structure was carried out as above and the folded peptide recovered by preparative HPLC purification. The structure of each of the N-terminal and C-terminal [Nle^{72}]CCL-19 peptide fragments, each of the ligated linear [Nle^{72}]CCL-19 peptides and each of the folded [Nle^{72}]CCL-19 peptides were verified by mass spectrometric analysis using a PE SCIEX-API mass spectrometer equipped with an electrospray ion source (Table 1).

GTP-γS Binding Assays. CCL-19 and CCL-21 were obtained from ID Labs (London, ON). Five micrograms of membranes were incubated at 37 °C for 30 min in assay buffer (50 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% BSA, pH 7.2) in the presence of 10 μM GDP, 0.5 nM ^{35}S-GTP-γS (Perkin-Elmer Life Sciences, Atlanta, GA) and varying concentrations of agonist (total volume 100 μL in a 96-well plate). For Schild analysis, membranes were preincubated with the various amounts of Ac-[Nle^{72}]CCL-19(8−77) for 10 min before the addition of agonist. Membranes were filtered onto a 96-well GF/C filterplate (Packard Bioscience, Hartford CT) and washed with 500 mL wash buffer (50 mM Tris-HCl, 5 mM $MgCl_2$) using a Filtermate 196 Harvester (Packard Instruments, Downers Grove, IL). The filter plates were dried under a heat lamp before addition of 50 μL of scintillation fluid to each well (Scintisafe, 30% cocktail, Fisher Scientific, Tustin, CA) and counted on a Topcount NXT (Packard, Downers Grove, IL).

Radioligand Binding Assays. One microgram of membranes were incubated with 80 000 cpm (0.1 μCi) ^{125}I-CCL-19 (Perkin-Elmer Life Sciences, Atlanta, GA) in binding buffer (50 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA, pH 7.2) in the presence/absence of unlabeled ligand at room temperature for 1 h (100 μL in a 96-well plate). Membranes were filtered onto a 96-well GF/C plate (Packard Bioscience, Hartford, CT) that had been presoaked for a minimum of 1 h in 0.25% polyethylenimine/0.5% BSA/0.5× PBS and washed with 500 mL of wash buffer (50 mM HEPES, 0.5 M NaCl, 0.1%BSA) using a Filtermate 196 Harvester (Packard Instruments, Downers Grove, IL). The filter plates were dried under a heat lamp before addition of 50 μL of scintillation fluid (Scintisafe, 30% cocktail, Fisher Scientific, Tustin, CA) to each well, and counted on a Topcount NXT (Packard, Downers Grove, IL). Nonspecific binding was determined by performing the assay on membranes from untransfected FLP-In CHO-K cells and was found to be not significantly different from binding in the presence of high amounts of cold ligand (data not shown).

Chemotaxis. Chemotaxis assays were performed in MBA96 chemotaxis chambers (Neuroprobe, Gaithersburg, MD) using 8-micron polycarbonate membranes coated with poly-(vinylpyrrolidone) (PVP). Cells were serum starved for 2 h prior to the experiment in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 10 mM HEPES, and 2 mM L-glutamine. Chemotactic chambers were assembled according to manufacturer's instructions. Briefly, chemoattractant was added at various concentrations (final volume of 355 μL) to a 96-well U-bottom plate (E&K, Los Gatos, CA), which was placed into the chemotactic chamber. 5×10^5 H9 cells were added to the upper wells and incubated for 6 h at 37 °C. For antagonist assays, 100 nM [Nle^{72}]CCL-19-(1−77) was added to the bottom chamber in the presence of varying concentrations of antagonist. H9 cells were pre-incubated with various amounts of antagonist for 5 min before addition to the top chamber. To stop the assay, the top wells were washed twice with phosphate buffered saline (PBS). Cells sticking to the top of the filter were detached with trypsin-EDTA and swiping with a filter wiper before the filter was removed from the bottom chamber. Cells that migrated to the bottom chamber were collected by centrifugation at 400*g* for 10 min. The number of cells that migrated to the bottom chamber was detected using a Cyquant cell proliferation kit (Molecular Probes, Eugene, OR). Briefly, cell pellets were lysed in cell lysis buffer containing the Cyquant dye. After a 10-min incubation at room temperature, lysates were transferred to a black flat-bottom 96-well polystyrene plate (Costar, Fisher Scientific, Tustin, CA), which was read in an LJL Analyst (LJL Biosystems, Sunnyvale, CA) using a fluorescence intensity program (480 nm/530 nm emission/excitation wavelengths). To obtain a standard curve, known numbers of H9 cells were included to determine relative fluorescence units (RFUs). The standard curve was plotted in Prism (Version 3.03, GraphPad, San Diego, CA) using the "one-phase exponential association" analysis. Samples with RFUs outside the linear range of the standard curve were diluted in lysis buffer. Each experiment was performed in triplicate, and data are shown as mean ± SEM from at least three independent experiments. 100 and

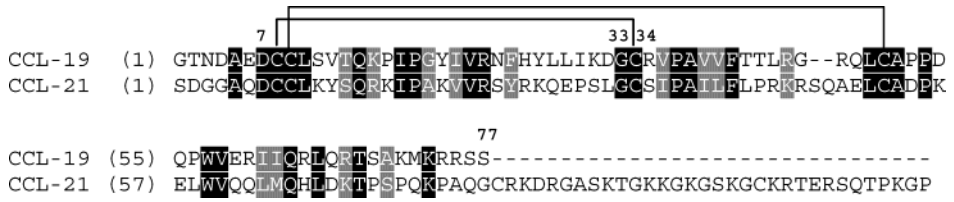

FIGURE 1: Alignments of CCL-19 and CCL-21. Conserved residues are highlighted in black, while conservatively substituted residues are highlighted in gray. The disulfide links described for chemokines are indicated by lines above the sequence.

Table 2: Binding Affinity (pIC_{50}) and Potency (pEC_{50}) of N-Terminal Ala-Scan Analogues of CCL-19[a]

ligand	pIC_{50}	pEC_{50}
CCL-19	8.71 ± 0.16 (1.9 nM)	8.93 ± 0.18 (1.2 nM)
[Nle^{72}]CCL-19(1−77)	8.64 ± 0.13 (2.3 nM)	8.83 ± 0.10 (1.5 nM)
[Ala^{2}, Nle^{72}]CCL-19(1−77)	8.37 ± 0.03 (4.3 nM)	8.37 ± 0.21 (4.3 nM)
[Ala^{3}, Nle^{72}]CCL-19(1−77)	7.74 ± 0.01 (18 nM)	7.37 ± 0.12 (43 nM)
[Ala^{4}, Nle^{72}]CCL-19(1−77)	8.02 ± 0.08 (9.5 nM)	8.84 ± 0.16 (14 nM)
[Ala^{6}, Nle^{72}]CCL-19(1−77)	8.36 ± 0.05 (4.4 nM)	8.36 ± 0.20 (4.4 nM)
[Ala^{7}, Nle^{72}]CCL-19(1−77)	6.88 ± 0.11 (132 nM)	6.53 ± 0.21 (295 nM)

[a] pIC_{50} and pEC_{50} values of truncated CCL-19 analogues were determined as described in the methods section. Data presented are the mean ± SEM of at least three independent experiments; mean IC_{50} and EC_{50} values are shown in brackets.

0% migration were defined as number of cells that migrated in response to 0.3 μM (maximum) and no (background) [Nle^{72}]CCL-19(1−77).

Data Analysis. GTP-γS and radioligand binding experiments were carried out in duplicate. Data are presented as mean ± SEM of pooled or representative data of at least three independent experiments as indicated in legends. Data were plotted, and IC_{50} and EC_{50} values were calculated using the "one site competition" formula of Prism (Version 3.03, GraphPad, San Diego, CA) for the IC_{50} values and the "Sigmoidal dose response" formula of Prism (Version 3.03, GraphPad, San Diego, CA) for the EC_{50} values. The K_B of Ac-[Nle^{72}]CCL-19(8−77) was determined by calculating the mean -pK_B values from three independent Schild plots.

RESULTS

Comparison of [Nle^{72}]CCL-19(1−77) and Commercial CCL-19. To eliminate complications resulting from oxidating Met^{72} during the chemical synthesis of CCL-19, this residue was substituted by norleucine. The resultant [Nle^{72}]CCL-19(1−77) was shown to behave similar to commercially available CCL-19 in both GTP-γS and radioligand binding assays (Table 2)

Comparison of CCL-19 and CCL-21 N-Termini. For a number of chemokines, it has been reported that the N-terminal domain preceding the conserved cysteines plays a major role in high-affinity ligand binding and/or receptor activation (*38*−*41*). As CCR-7's exclusive ligands are CCL-19 and CCL-21, it comes as a surprise that these ligands share very little sequence identity in this region (Figure 1). Only two out of seven residues preceding the conserved cysteines are conserved: Ala^{5} and Asp^{7}. A second Asp is found in position 2 of CCL-21, while an Asp is found in position 4 of CCL-19 (Figure 1). Furthermore, CCL-19 carries a third negative charge in position 6 of this domain (Glu^{6}), which is substituted by a Gln in CCL-21 (Figure 1). Another substantial difference between CCL-19 and CCL-21 is an Asn in position 3 of CCL-19, while Gly is found in the equivalent position of CCL-21 (Figure 1). To investigate the role of these N-terminal residues, we mutated and successively truncated the N-terminus of [Nle^{72}]CCL-19(1−77) and tested the resultant analogues for their ability to activate CCR-7 with the aid of a GTP-γS binding assay. We also performed ligand binding assays to investigate the effect of N-terminal mutations on high-affinity ligand binding to CCR-7. Finally, we tested the CCL-19 analogues in chemotactic assays to show physiological relevance of our findings.

Ala Substitutions of Residues in the N-Terminus of CCL-19. To characterize the function of the side chains of each N-terminal residue, we made analogues of CCL-19 with Ala substitutions in positions 2, 3, 4, 6, and 7. Thr^{2}Ala and Glu^{6}-Ala substitutions affected neither ligand binding, nor receptor activation (Figure 2, Table 2). Asn^{3}Ala, Asp^{4}Ala, and Asp^{7}-Ala substitutions, however, shifted GTP-γS binding 29-, 9-, and 197-fold, respectively (Figure 2a, Table 2). At the same time these analogues showed about 8-, 4-, and 57-fold reduced binding affinities (Figure 2b, Table 2), showing only slight (∼2−4-fold) separation between the loss in functional potency and the loss in binding affinity. The rank order of potency of the CCL-19 analogues to induce chemotaxis of H9 cells, a cell line derived from a T-cell lymphoma, was found to be very similar to their rank order of potency to activate CCR-7 as measured by GTP-γS binding assays, except for the Ala^{3} and Ala^{7} analogues, which were able to induce chemotaxis, albeit with reduced efficacies (Figure 2c).

N-Terminal Truncation of CCL-19. Truncation of the first two N-terminal residues resulted in an analogue that exhibited a 7-fold reduced potency to activate CCR-7 as measured by GTP-γS binding assays, with maximum levels of activation being retained (Figure 3a, Table 3). Furthermore, deletion of three ([Nle^{72}]CCL-19(4−77)), four ([Nle^{72}]CCL-19(5−77)), and five ([Nle^{72}]CCL-19(6−77)) N-terminal residues reduced the potency of the analogues to activate CCR-7 by 36-, 55-, and 530-fold, respectively, with [Nle^{72}]CCL-19-(6−77) also showing a reduced maximal level of activation (Figure 3a, Table 3), suggesting it is acting as a partial agonist. The loss in potency was found to be correlated to the loss in binding affinity (Figure 3b, Table 3). This series of structure activity relationships was preserved in the ability of the peptides to stimulate chemotaxis of H9 cells with the exception of [Nle^{72}]CCL-19(6−77), which showed almost no ability to induce chemotaxis (Figure 3c).

Acetylation of N-Terminal Truncations of CCL-19. One possible explanation for the loss in activity for [Nle^{72}]CCL-

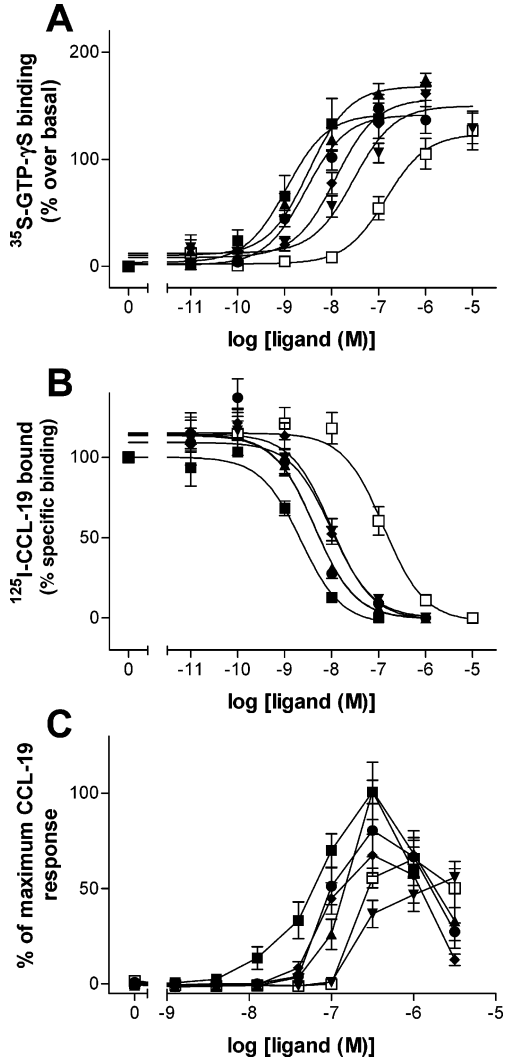

FIGURE 2: Effects of Ala substitutions in the N-terminus of [Nle^{72}]-CCL-19 on receptor activation (A), ligand binding (B), and chemotaxis (C). GTP-γS and ligand binding assays were performed in the presence of increasing concentrations of [Nle^{72}]CCL-19(1−77) (■), [Ala^{2}, Nle^{72}]CCL-19 (▲), [Ala^{3}, Nle^{72}]CCL-19 (▼), [Ala^{4}, Nle^{72}]CCL-19 (◆), [Ala^{6}, Nle^{72}]CCL-19 (●), and [Ala^{7}, Nle^{72}]CCL-19 (□) as described in the methods section. Data shown are mean ± SEM from at least three independent experiments.

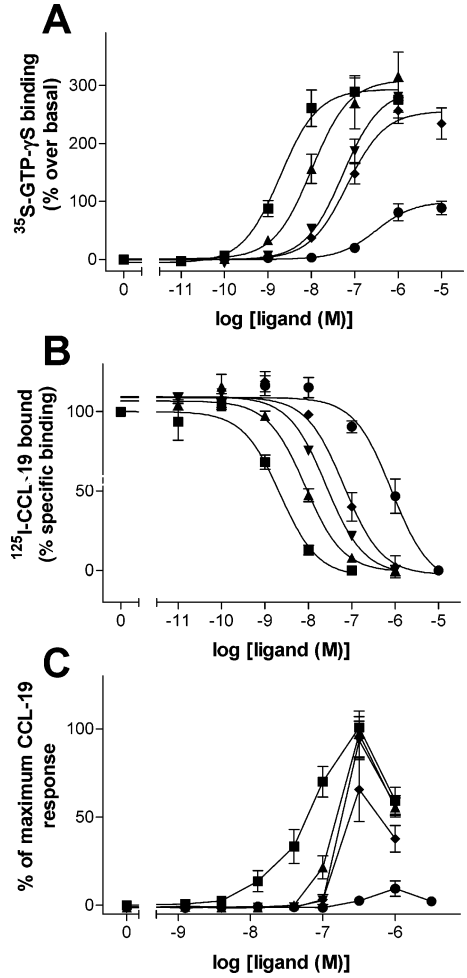

FIGURE 3: Effects of truncation of the N-terminus of [Nle^{72}]CCL-19 on receptor activation (A), ligand binding (B), and chemotaxis (C). GTP-γS and ligand binding assays were performed in the presence of increasing concentrations of [Nle^{72}]CCL-19(1−77) (■), [Nle^{72}]CCL-19(3−77) (▲), [Nle^{72}]CCL-19(4−77) (▼), [Nle^{72}]CCL-19(5−77) (◆), and [Nle^{72}]CCL-19(6−77) (●) as described in the methods section. Data shown are mean ± SEM from at least three independent experiments.

19(6−77) might be that truncating the N-terminal domain of CCL-19 exposes the positively charged amino-terminus to regions of the receptor where counterions for the negatively charged residues in the CCL-19 amino-terminus are usually found, resulting in an unfavorable electrostatic interaction. To eliminate the possibility of these charge repulsions, [Nle^{72}]CCL-19(6−77) was acetylated. In GTP-γS binding assays, Ac-[Nle^{72}]CCL-19(6−77) showed a 17-fold increase in potency compared with the nonacetylated analogue, while maximum levels of both GTP-γS binding and chemotactic activity were restored (Figure 4a, Table 3). This translates to a 30-fold loss in potency compared with CCL-19. Similarly, binding affinity of Ac-[Nle^{72}]CCL-19-(6−77) increased 20-fold compared with the unacetylated analogue (10-fold drop compared with CCL-19) (Figure 4b, Table 3). To eliminate the possibility of further charge repulsions, [Nle^{72}]CCL-19(7−77) and [Nle^{72}]CCL-19(8−77) were synthesized acetylated. Ac-[Nle^{72}]CCL-19(7−77) showed a 472-fold drop in potency for G-protein activation compared with CCL-19, with the maximal activity being reduced to similar levels as the [Nle^{72}]CCL-19(6−77) analogue (Figure 4a, Table 3), suggesting it is a partial agonist. The binding affinity of this analogue was found to be 120-fold lower than the binding affinity of CCL-19 (Figure 4b, Table 3). As seen with [Nle^{72}]CCL-19(6−77), Ac-[Nle^{72}]CCL-19(7−77) showed almost no ability to induce chemotaxis (Figure 4c).

Correlation of G-Protein Activation with Ligand Binding in Vitro. For all analogues described above, loss in potency for G-protein activation was found to be proportional to the loss in binding affinity. This finding was substantiated when we plotted -pEC$_{50}$ values against -pIC$_{50}$ values for all analogues described above (Figure 5). When the data were analyzed by linear regression, a line with a slope of 1.1 and $r^2 = 0.97$ was obtained, showing a remarkably tight fit for all analogues on the graph.

Ac-[Nle^{72}]CCL-19(8−77) Behaves as an Antagonist at CCR-7. Ac-[Nle^{72}]CCL-19(8−77) showed no detectable receptor activation, even at 10 μM concentrations (Figure 4a, Table 3). Furthermore, it was unable to stimulate chemotaxis of H9 cells at 3 μM concentrations (Figure 4c). As Ac-[Nle^{72}]CCL-19(8−77) showed an affinity of ~700

Table 3: Binding Affinity (pIC_{50}) and Potency (pEC_{50}) of Truncated CCL-19 Analogues[a]

ligand	binding pIC_{50}	GTP-γS pEC_{50}
[Nle^{72}]CCL-19(1−77)	8.64 ± 0.13 (2.3 nM)	8.83 ± 0.10 (1.5 nM)
[Nle^{72}]CCL-19(3−77)	8.05 ± 0.08 (8.9 nM)	7.98 ± 0.04 (10 nM)
[Nle^{72}]CCL-19(4−77)	7.59 ± 0.03 (26 nM)	7.27 ± 0.03 (54 nM)
[Nle^{72}]CCL-19(5−77)	7.16 ± 0.08 (69 nM)	7.08 ± 0.03 (83 nM)
[Nle^{72}]CCL-19(6−77)	6.22 ± 0.08 (603 nM)	6.10 ± 0.18[b] (794 nM)
Ac-[Nle^{72}]CCL-19(6−77)	7.56 ± 0.15 (28 nM)	7.32 ± 0.02 (48 nM)
Ac-[Nle^{72}]CCL-19(7−77)	6.56 ± 0.09 (275 nM)	6.15 ± 0.06[b] (708 nM)
Ac-[Nle^{72}]CCL-19(8−77)	6.16 ± 0.08 (692 nM)	n.m.[c]

[a] pIC_{50} and pEC_{50} values of truncated CCL-19 analogues were determined as described in the methods section. Data presented are the mean ± SEM of at least three independent experiments; mean IC_{50} and EC_{50} values are shown in brackets. [b] Partial agonists. [c] n.m.= not measurable.

nM in ligand binding experiments (Figure 4b, Table 3), it appeared likely that this analogue acts as an antagonist at CCR-7. To investigate this, we performed a Schild analysis on the [Nle^{72}]CCL-19(1−77) response. Indeed, Ac-[Nle^{72}]-CCL-19(8−77) exhibited antagonist activity with a K_B of ~350 nM (Figure 6a). Furthermore, Ac-[Nle^{72}]CCL-19(8−77) was able to block CCL-19-mediated chemotaxis of H9 cells (Figure 6b).

DISCUSSION

Despite the low sequence homology of chemokines, NMR and crystallography studies indicate that the overall 3D structure of chemokines is very similar (*16*, *17*, *19*, *42*−*44*). As well as the overall configuration, the mechanism of receptor activation of chemokines appears to be conserved as well. It is thought that in addition to interactions resulting in high-affinity binding, a second set of interactions leads to the receptor being stabilized in the active conformation (*28*−*30*).

The main function of the N-terminus of chemokines is thought to be stabilizing the active form of the receptor (*38*−*41*). These interactions are normally not measurable in radioligand binding assays. It has been shown that various truncations of the N-termini of CCL-2 (MCP-1) (*39*, *45*, *46*), CCL-4 (MIP-1β) (*23*), CCL-5 (*39*, *47*, *48*), CCL-7 (MCP-3) (*39*), and CXCL-12 (SDF-1) (*30*) resulted in analogues that were not able to activate but had little change in their ability to bind to their cognate receptors. Furthermore, the addition of Met to CCL-5 (*49*) and CCL-2 (*50*) as well as the addition of aminooxypentane to the amino-terminus of the CCL-5 (*51*) and CCL-3 (MIP-1α) (*52*) resulted in functional antagonists, indicating that the length of the N-terminus is crucial for activity for most chemokines, without having major effects on ligand binding. However, the change in activity is not necessarily size dependent, as seen with CXCL-5 (ENA-78) (*53*). Furthermore, it has been shown that the N-terminus of CXCL-12 contains some residues involved in high-affinity binding between residues 3 and 9 (*30*), while the N-terminal domain alone of CXCL-11 (I-TAC) and CXCL-12 is able to activate CXCR-4 (*54*−*56*), implying that the N-terminus of at least some chemokines might contribute to high-affinity binding to the receptor.

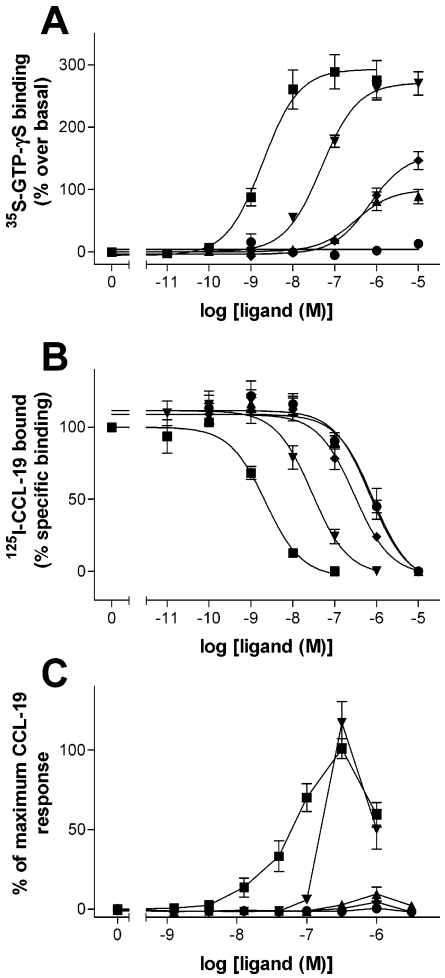

FIGURE 4: Acetylated truncation analogues of CCL-19 activate CCR-7 (A), bind to CCR-7 (B), and induce chemotaxis of H9 cells. GTP-γS and ligand binding assays were performed in the presence of increasing concentrations of [Nle^{72}]CCL-19(1−77) (■), [Nle^{72}]-CCL-19(6−77) (▲), Ac-[Nle^{72}]CCL-19(6−77) (▼), Ac-[Nle^{72}]CCL-19(7−77) (◆), and Ac-[Nle^{72}]CCL-19(8−77) (●) as described in the methods section. Data shown are mean ± SEM from at least three independent experiments.

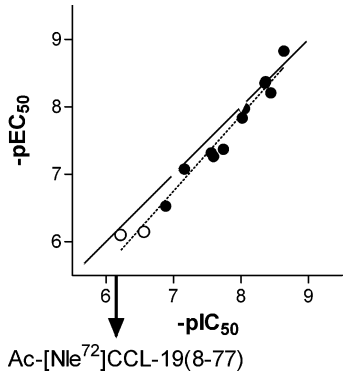

FIGURE 5: Correlation between -pEC_{50} and -pIC_{50} values to show the dependence of receptor activation on high-affinity binding for all analogues of CCL-19 investigated in this paper. The dashed line indicates a line with a slope of 1, while the solid line is linear regression. Solid dots (●) indicate agonists, while open dots (○) indicate partial agonists.

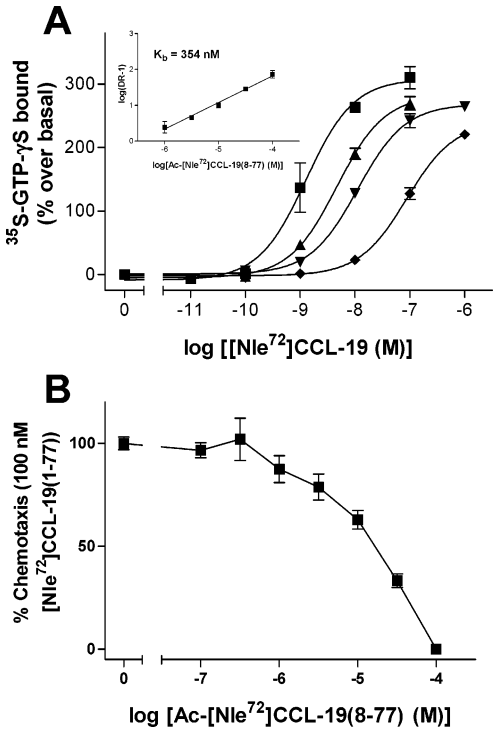

FIGURE 6: Antagonist activity of Ac-[Nle^{72}]CCL-19(8−77). CHO-K-CCR7 membranes were incubated with ^{35}S-GTP-γS in the presence of increasing amounts of [Nle^{72}]CCL-19(1−77) after being preincubated for 10 min with assay buffer (■), 1 μM (▲), 10 μM (▼), and 100 μM (◆) Ac-[Nle^{72}]CCL-19(8−77) (A). H9 cells were preincubated with varying concentrations of Ac-[Nle^{72}]CCL-19-(8−77) for 5 min before chemotaxis was measured in response to 100 nM [Nle^{72}]CCL-19(1−77) (B). Data shown are representative (A) and mean (B) ± SEM from at least three independent experiments.

Here, Ala substitutions in the N-terminus of [Nle^{72}]CCL-19(1−77) showed the importance of Asn^3, Asp^4, and Asp^7 in high-affinity binding to CCR-7 with the most dramatic loss in binding affinities stemming from the Asp^{7}Ala mutation. The role of the side chains of these residues in receptor activation is minimal as the loss in functional potency of these mutant analogues is directly proportional to the loss in binding affinity and no significant differences in maximal stimulation were observed.

Truncation of the N-terminal domain of [Nle^{72}]CCL-19-(1−77) decreased the potency of the ligand to activate CCR-7 in a size-dependent manner, similar to effects seen for other chemokines. This loss in potency was proportional to the loss in high-affinity binding up to and including the truncation of residue 4. When the first five residues were deleted, the resultant analogue was found to be a partial agonist in GTP-γS binding assays and was unable to induce chemotaxis. As [Nle^{72}]CCL-19(5−77) is still a full agonist, the removal of Ala^5 with its short hydrophobic side chain would appear unlikely to be the cause for this dramatic effect. The N-terminus of CCR-7 ligands carries a number of negatively charged residues (Asp^4, Glu^6, and Asp^7 for CCL-19 and Asp^2 and Asp^7 for CCL-21). Some of the loss in binding affinity and activity as seen when the N-terminus of CCL-19 is truncated might therefore be attributed to the introduction of the positively charged N-terminal amine in positions where a negative charge is usually found. We therefore acetylated [Nle^{72}]CCL-19(6−77) and found that removal of the positive charge from the N-terminus improved the ligand binding affinity and restored the ability of the ligand to activate the receptor in GTP-γS assays and the ability to induce chemotaxis to levels similar to those observed for [Nle^{72}]CCL-19-(5−77). This observation suggests that the positively charged N-terminal amine of truncated CCL-19 analogues potentially interferes with positive charges in the binding sites that are usually in close proximity or interact with negative charges that are found on the N-terminus of the ligands. For the design of novel peptide ligands, it is therefore important to take these charge requirements into consideration. We therefore acetylated subsequent truncation analogues of CCL-19.

The deletion of residues 1−6 converted the ligand into a partial agonist in GTP-γS binding assays. Ac-[Nle^{72}]CCL-19(7−77) was also unable to induce chemotaxis, indicating that Glu^6 might be essential for stabilizing the active conformation of the receptor. Because this analogue is acetylated, masking the positively charged amino group, this reduction in activity is not due to unfavorable charge interactions, as seen with [Nle^{72}]CCL-19(6−77). As mutating the side chain of Glu^6 to Ala had very little effect on ligand binding, GTP-γS binding and chemotaxis assays, it is unlikely that the side chain of Glu^6 is required for receptor activation. It is therefore possible that there is a steric requirement or an interaction of the backbone NH or Cβ with the receptor that is needed to stabilize the active conformation of CCR-7.

Further removal of Asp^8 resulted in a ligand that acted as a functional antagonist in both GTP-γS binding and chemotaxis assays. As Ac-[Nle^{72}]CCL-19(7−77) is an agonist, albeit with reduced activity, it is likely that Asp^7 of CCL-19 is crucial for agonist activity. This finding would be consistent with CXC ligands that contain a Asp−Leu−Arg sequence preceding the conserved cysteines, where the positively charged Arg is crucial for agonist activity (*57*, *58*). Although mutating Asp^7 to Ala had a dramatic effect on GTP-γS binding assays, the loss in functional potency was proportional to the loss in binding affinity. The mechanism underlying receptor activation can therefore not be explained by a simple charge−charge interaction. One explanation might be that the side chain of Asp^7 is crucial for high-affinity binding, while there is a second interaction with, or a steric requirement of the backbone NH or Cβ of Asp^7, that is crucial for receptor activation similar to the observations from the removal of Glu^6. In the absence of the correct side chain in position 7, the backbone interaction might still be able to form, but in a less efficient manner, as the Asp^7 side chain interaction with the receptor may be needed for the appropriate positioning of the backbone. Ac-[Nle^{72}]CCL-19(8−77) is the first reported peptide analogue of CCL-19 that acts as an antagonist. Previously, a CCL-21 antagonist had been described, although its potency is not yet known (*59*). Because of the low potency of our CCL-19 antagonist, improvements need to be made before this analogue can be used in models investigating the physiologic role of CCR-7 in immune system homeostasis and the development of immune responses.

From these data, it appears that the main function of the five N-terminal residues is related to high-affinity binding

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to CCR-7. It is, however, not possible to assess from our experiments whether any of these residues are directly involved in receptor activation independent of high-affinity binding. In other chemokine/receptor interactions, the N-terminus of the chemokine is usually involved in receptor activation but not high-affinity binding. However, residues 3−9 of CXCR-12 have also been shown to be important for high-affinity binding, although removal of residues 1 and 2 resulted in a functional antagonist (*30*). Our data suggest that the backbone of the two residues immediately preceding the conserved cysteines are likely to be involved in stabilizing the active conformation of CCR-7, which correlates with findings from CXCL-8, where the three residues immediately preceding the conserved cysteines have been shown to be important for activity (*57*, *60*). Plotting -pEC_{50} values against -pIC_{50} values showed the linear relationship between potency for receptor activation and binding affinity. As even the partial agonists can be found very close to the theoretical line, it is likely that interactions stabilizing the active conformation are independent of high-affinity binding and partially disrupted in these two analogues. This hypothesis is strengthened by mutations of CCR-7 that decrease ligand mediated receptor activation but not ligand binding (unpublished observations[4]), which indicates that CCL-19 and CCL-21 activate CCR-7 in the "two-step" mechanism which has been proposed for other chemokines and some non-chemokine GPCRs as well (*61*). However, other residues important for receptor activation might be found outside the amino-terminus on CCL-19 as seen with CCL-11 (Eotaxin-1), where Phe^{11} (the residue immediately following the two conserved Cys), Asn^{15}, and Lys^{17} were shown to be involved in receptor activation (*62*).

In summary, we showed that the N-terminus of CCL-19 is crucial for both high-affinity ligand binding and receptor activation. Major determinants for high-affinity binding are the side chains of Asn^3, Asp^4, and Asp^7. The backbones of Glu^6 and Asp^7 appear to be the major contributors to agonist activity. In [Nle^{72}]CCL-19(6−77) and Ac-[Nle^{72}]CCL-19-(7−77), we describe two partial agonists of GTP-γS binding assays that are unable to induce chemotaxis of H9 cells. We furthermore describe the first peptide analogue of CCL-19 (Ac-[Nle^{72}]CCL-19(8−77)) that acts as an antagonist at CCR-7 in vitro. As the results shown are pertinent in both in membrane assays as well as chemotaxis, these findings may form the basis for designing analogues that could be used in models investigating autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.

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