JPET Fast Forward. Published on March 17, 2004 as DOI: 10.1124/jpet.103.063131

JPET #63131

Cloning and pharmacological characterization of CXCR1 and CXCR2 from Macaca fascicularis

R. William Hipkin, Gregory Deno, Jay Fine, Yongliang Sun, Brian Wilburn,

Xuedong Fan, Waldemar Gonsiorek and Maria T. Wiekowski

Department of Immunology, Schering-Plough Research Institute

2015 Galloping Hill Road, Kenilworth, NJ 07033

JPET #63131

Macaca fascicularis CXCR1 and CXCR2 cloning and phamacology

Dr. Maria Wiekowski

Department of Immunology

Schering-Plough Research Institute

2015 Galloping Hill Rd

Kenilworth, NJ 07033

phone: 908-740-3082

fax: 908-740-3084

e-mail: maria.wiekowski@spcorp.com

Number of text pages: 30

Number of tables: 1

Number of figures: 7

Number of references: 37

Number of words in Abstract: 213

Number of words in Introduction: 453

Number of words in Discussion: 1312

Inflammation & Immunopharmacology

JPET Fast Forward. Published on March 17, 2004 as DOI: 10.1124/jpet.103.063131
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Two genes with high sequence homology to human CXCR1 (hCXCR1) and CXCR2 (hCXCR2) were cloned from blood of cynomolgus monkey (Macaca fascicularis). Comparison of the expression pattern of these receptors in different species demonstrated that, like in humans, cynomolgus CXCR1 (cCXCR1) and CXCR2 (cCXCR2) are highly expressed in blood. Membranes from transfected BaF3 cells expressing cCXCR1 bind IL-8 with an affinity similar to hCXCR1 (K_d: 170±87 pM and 103±37 pM, respectively), and show low binding affinity to Gro-α. Cynomolgus CXCR2 also binds hIL-8 but with somewhat higher affinity than the hCXCR2 (46±28 pM and 220±14 pM, respectively). Surprisingly, cCXCR2 has a reduced binding affinity to hGro-α (3.7±2.2 nM), a specific ligand of hCXCR2 (540±140 pM). Furthermore, the CXCR2 specific antagonist SB 225002 is 10-fold more potent in inhibiting IL-8 binding to hCXCR2 than to cCXCR2, suggesting that some of the observed differences in the amino acid sequences of the human and monkey receptor affect ligand binding sites or the conformation of the receptor. Both cynomolgus receptors were functionally active in inducing GTPγS exchange on membranes in response to IL-8 and Gro-α and in mediating chemotactic activity of recombinant BA/F3 cells in response to IL-8 and Gro-α. These results identify the products of the novel cynomolgus genes as functional homologs of hCXCR1 and hCXCR2.

JPET Fast Forward. Published on March 17, 2004 as DOI: 10.1124/jpet.103.063131
This article has not been copyedited and formatted. The final version may differ from this version.

An immune response is characterized by the activation of leukocytes and migration of hematopoietic cells into secondary lymphoid organs or to the site of inflammation. Under inflammatory conditions the expression of specific chemokines (Yoshie et al., 2001), a class of small proteins with potent chemotactic activity, is upregulated. For instance, the expression of the chemokines IL-8 (CXCL8), Gro-α (CXCL1) and ENA78 (CXCL5) is induced in inflammatory human diseases like rheumatoid arthritis, psoriasis, inflammatory bowel disease, acute respiratory distress syndrome, and COPD (Gillitzer et al., 1996; Woods et al., 2000; Kurdowska et al., 2002; Banks et al., 2003; Beeh et al., 2003). IL-8, Gro-α and ENA78 are potent activators of neutrophil chemotaxis (Patel et al., 2001; Feniger-Barish et al., 2003) and are ligands for the G-protein coupled seven-transmembrane receptor CXCR2 (IL-8Rb). In addition, IL-8 is also a high affinity ligand for CXCR1 (IL-8Ra). Additional ligands for CXCR2 are GCP-2 (granulocyte chemotactic protein-2; CXCL6) and NAP-2 (neutrophil-activating peptide-2; CXCL7) (Ahuja et al., 1996).

The role of CXCR1 and CXCR2 in the pathogenesis of inflammatory responses has been demonstrated in a number of animal models. For instance, neutralization of IL-8 by a monoclonal antibody resulted in the suppression of a delayed type hypersensitivity reaction, lung inflammation or tissue injury in rabbits (Larsen et al., 1995; Matsumoto et al., 1997; Yamamoto et al., 1998). Similarly, a small, nonpeptide CXCR2 antagonist (SB225002; (White et al., 1998) reduces significantly the number of infiltrating leukocytes into the inflamed joint and the expression of proinflammaotry mediators in a rabbit model of arthritis (Podolin et al., 2002).

JPET Fast Forward. Published on March 17, 2004 as DOI: 10.1124/jpet.103.063131
This article has not been copyedited and formatted. The final version may differ from this version.

Human neutrophils express both receptors, CXCR1 and CXCR2, and chemotaxis of human neutrophils can be induced by IL-8, Gro-α or ENA78 (Patel et al., 2001; Feniger-Barish et al., 2003). Interestingly, while in rodents neutrophil chemotaxis is mediated by a unique homolog of hCXCR2 (Bozic et al., 1994; Harada et al., 1994; Shibata et al., 2000), rabbit neutrophils express both homologs (Beckmann et al., 1991; Thomas et al., 1991; Prado et al., 1994). Although homologs for CXCR1 and CXCR2 have been described for a variety on non-human species (Figs. 1 and 2), we wanted to model CXCR1 and CXCR2 mediated responses in cynomolgus monkey, a species where diseases with a neutrophil component like COPD, acute lung injury and rheumatoid arthritis can be studied (Rose et al., 1992; Mihara et al., 2001; Billah et al., 2002). Thus, we cloned the genes encoding CXCR1 and CXCR2 from cynomolgus monkey, analyzed the pharmacological characteristics of these receptors and identified them as functional homologs of hCXCR1 and hCXCR2. In addition, comparison of the binding affinities of human chemokines to cynomolgus and human receptors with their specific amino acid sequences allowed the confirmation of formerly established sites critical for ligand binding.

JPET Fast Forward. Published on March 17, 2004 as DOI: 10.1124/jpet.103.063131
This article has not been copyedited and formatted. The final version may differ from this version.

Methods

Cloning and expression of monkey CXCR1 and CXCR2. Cynomolgus CXCR1 was cloned from genomic DNA using PCR primers derived from the human sequence for CXCR1 (genebank acc# L19591). For cloning and expression purposes the 5' primer (CAAGAATTC*GCCGCCACC*ATGTCAAATATTACAGATCCACA) contained am EcoR1 restriction site (underlined) and a kozak sequence (*italic*) and the 3 primer (CAACGCGGCCGC*TCA*GAGGTTGGAAGAGACATTGA) a Not1 restriction site (underlined) and a stop codon (*italic*). The resulting PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced. To obtain the 5' and 3' prime ends of the cCXCR1 gene, primers (5' primer GGCATAGGTGACAATCACAACATAC and 3' primer CCAGGCTTATCATCCAAACAACGCCACTCC) were designed for the rapid amplification of cDNA ends (RACE) based on the existing cynomolgus sequence. RACE amplification (Clonetech; Palo Alto, CA) was performed on RNA purified from cynomolgus blood using the PAXgene blood RNA kit by PreAnalytiX (Hombrechtikon, CH). Similarly, cCXCR2 was cloned from genomic cynomolgus DNA using PCR primers based on the hCXCR2 sequence (genebank accession # NM_001557): 5' primer CACTGATGTCTACCTGCTGAACCT starting a position +230f and 3' primer CCGGTGCTTCTGCCCCATGT starting at +729r (ATG = 1). The PCR product was cloned and sequenced, and based on the sequence of this CXCR2 fragment, RACE primers were designed to obtain the 5' and 3' segments of the cynomolgus gene: 5' RACE primer TGTCCTCATAGCAGACTGGGCTAATATA; 3'RACE primer TCTTACTTTTCCGAAGGACTGTCTACCT. The RACE reaction was performed on

RNA from cynomolgus blood. After sequencing of the RACE products, PCR primers were designed based on the sequence of the 5' and 3' end of cCXCR2 to amplify the entire CXCR2 cDNA from blood RNA. For cloning and expression purposes the 5' primer contained am EcoR1 restriction site and a kozak sequence (CAAGAATTC*GCCGCCACC*ATGCAGAGTTTCAACTTTGAAGAT) and the 3' primer a Not I restriction site (underlined) and a stop signal (italic) (CAACGCGGCCGC*TCA*GAGAGTAGTGGAAGTGTGCCC). Cynomolgus CXCR1 and CXCR2 were expressed in BaF3 cells; positive transfectants were selected by G418 resistance. BaF3 cells were maintained in RPMI containing 10% FCS, 1% Penicillin-Streptomycin & Glutamine, 1 ng/ml murine IL-3 (R&D systems, Minneapolis, MI). All assays using these recombinant BaF3 cells were performed using pools of cells without further selection for high expressors.

Comparison of receptor sequences from different species. The amino acid sequences were aligned using the Vector NTI software program. The transmembrane regions of the receptors were predicted using TMpred on ISREC server.

RNA Extraction and cDNA synthesis. RNA was prepared from blood from different species using the PAXgene blood RNA kit (PreAnalytix, Hombrechtikon, CH) according to manufactures protocol. RNA was isolated from other tissues using RNA STAT-60 (Tel-Test, Friendsword, TX) according to manufactures protocol. All samples were DNase treated using DNase 1 (Roche Diagnostics Corp. Indianapolis, IN) prior to cDNA synthesis. cDNA was generated using SuperScript First Strand Synthesis System for RT-

PCR (Invitrogen). Both Oligo dT (Invitrogen) and random hexomers (Promega) were used to prime first strand synthesis.

Quantitative PCR analysis. Quantitative PCR analysis was performed on an ABI 9700 sequence-detection instrument (TaqMan) following manufacturer's instructions. SYBR green TaqMan analysis was performed using primer sequences specific for mouse, rat, rabbit, cynomolgus and human CXCR1/CXCR2 (Table 1). Ribosomal RNA primers (PE Applied Biosystems, Foster City, CA) were used to normalize cDNA levels to correct for sample to sample variation. TaqMan PCR conditions were as follows: 48^0C for 30 min; 95^0C for 10 min; 40 cycles of 95^0C for 15 s, 60^0C for 1 min. Data was analyzed using Sequence Detection Systems software version 1.7.

Flowcytometry. Cynomolgus neutrophils were isolated from blood and stained with 20 µL of the PE-conjugated anti-human CXCR1 antibody clone #42705 (R&D Systems) or the PE-conjugated anti-human CXCR2 antibody clone #48311 (R&D Systems) in PBS, 1% BSA and 0.1% sodium azide. Events were acquired on a BD Biosciences FACScan and analyzed using the CellQuest software.

Cell membrane preparation. Ba/F3-monkeyCXCR1 and monkeyCXCR2 cell membranes were prepared as previously described (Hipkin et al., 1997). Cells were pelleted by centrifugation, incubated in homogenization buffer (10 mM Tris-HCl, 5 mM EDTA, 3 mM EGTA, pH 7.6) and 1 mM PMSF for 30 min. on ice. The cells were then lysed with a Dounce homogenizer using a stirrer type RZR3 polytron homogenizer

JPET Fast Forward. Published on March 17, 2004 as DOI: 10.1124/jpet.103.063131
This article has not been copyedited and formatted. The final version may differ from this version.

(Caframo, Wiarton, Ont.) with 12 strokes at 900 RPM. The intact cells and nuclei were removed by centrifugation at 500xg for 5 min. The cell membranes in the supernatant were then pelleted by centrifugation at 100,000xg for 30 min. The membranes were resuspended in glygly buffer (20 mM glycylglycine, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.2), aliquoted, quick frozen and stored at -80^{0}C. Protein concentration in membrane preparations was determined using the method of Bradford (Bradford, 1976).

[^{35}S]GTPγS binding assay. The exchange of guanosine 5'-[γ–^{35}S]triphospate ([^{35}S]GTPγS, triethylammonium salt; specific activity = 1250 Ci/mmol; NEN Boston, MA) was measured using a scintillation proximity assay (SPA) as previously described (Gonsiorek et al., 2003). For each assay point, 2 µg of membrane was preincubated for 15 min at room temperature with 200 µg wheat germ agglutinin-coated SPA beads (WGA-SPA; Amersham, Arlington Heights, IL) in SPA binding buffer (50 mM TRIS-HCL, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 50 mM NaCl, 0.002% NaN_3, 0.1 % BSA, 10 µg/ml saponin, pH=7.6). The beads and membranes were transferred to a 96-well Isoplate (Wallac, Gaithersburg, MD) and pre-incubated for 60 min with 10 µM guanosine 5'-diphosphate (GDP) in the presence of increasing concentrations of recombinant hIL-8, Gro-α or ENA-70 (R&D Systems). The GTPγS exchange reaction was initiated by the addition of 0.1 nM [^{35}S]GTPγS and was carried out for 60 min at room temperature. Membrane-bound [^{35}S]GTPγS was measured using a 1450 Microbeta Trilux counter (Wallac, Gaithersburg, MD).

JPET Fast Forward. Published on March 17, 2004 as DOI: 10.1124/jpet.103.063131
This article has not been copyedited and formatted. The final version may differ from this version.

Radioligand binding assay. ^{125}I-IL-8 (specific activity = 2200 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Radioligand competition and saturation binding assays were done using SPA technology (Cox et al., 2001). Membranes (2 µg per assay point) in SPA binding buffer were preincubated for 30 min at room temperature with 200 µg WGA-SPA, transferred to a 96-well Isoplate and further incubated at room temperature with the radioliganḍand and the indicated concentrations of chemokines for 6h. Ligand affinities (Ki) from competition binding experiments were calculated from binding IC_{50} using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Chemotaxis assay. The 96-well 5-micron pore size disposable ChemoTx # 101-5 were purchased from Neuro Probe Inc. (Gaithersburg, MD). Lower chambers were filled with 30 µl of various concentrations of IL-8 (R&D Systems, Minneapolis, MN) in PBS containing 0.2% BSA. Wells were then covered with a filter. Onto each filter spot $5x10^4$ cells in 25 µl volume were loaded and incubated at 37^0C for 90 min. The migrated cells in the lower chamber were transferred into a Microfluor 2 White plate (ThermoLabsystems, Franklin, MA) by spinning through a Funnel plate (Neuro Probe Inc. Gaithersburg, MD). 25 µl of PBS was added to each well and then filled with 50 µl of CelTiter-Glo luminescent solution (Promega Madison, MI). After 10 min at room temperature, the plate was read on a Luminoskan (ThermoLabsystems, Franklin, MA) using Acsent Software.

Results

Sequence analysis of the cynomolgus monkey CXCR1 and CXCR2 receptors. To clone the CXCR1 and CXCR2 receptor from cynomolgus monkey (Macaca fascularis), primers based on the 5' and 3' sequences of the respective human receptor were used to amplify gene fragments from monkey blood or spleen. Gene fragments of the expected size were sequenced. Sequence analysis confirmed that these gene fragments indeed resembled primate CXCR1 and CXCR2 sequences. Subsequent RACE reactions then allowed us to obtain the entire cynomolgus genes including the 5' and 3 'ends.

Comparison of the amino acid sequences of primate CXCR1 receptors revealed that the cCXCR1 receptor is most closely related to CXCR1 of rhesus monkey (Macaca mulatto; 98% homology), and has about 94% homology to human, chimpanzee or gorilla CXCR1 (Figure 1). In contrast to the amino acid sequences of other primate CXCR1, both macaque CXCR1 receptors have an additional tyrosine inserted at position 14 in their N-terminus. More surprisingly, however, we observed differences in the cynomolgus sequence at amino acid positions that otherwise were highly conserved in primates and non-primate sequences, i.e. a tyrosine to histidine change at the end of the transmembrane domain 1 (TM1) at position 74, a serine to alanine change at position 190, and an arginine to histidine change at the c-terminal border of TM5 (Fig. 1). Since amino acids at these positions are highly conserved among species, the sequence for cCXCR1 was confirmed by sequencing this gene from at least five individual monkeys.

In contrast to CXCR1, cCXCR2 was only 93% homologus to rhesus CXCR2, and had even less homology to human, chimpanzee or gorilla CXCR1 (~ 92%). The cCXCR2 receptor sequence showed the highest variance within the first twelve N-

terminal amino-acids when compared to rhesus and primate CXCR2 (Figure 2). Additional differences in the amino acid sequence were noted in regions that otherwise were highly conserved among primate and non-primate sequences (rabbit, rat and mouse), i.e. a tyrosine to histidine exchange at position 75 similar to CXCR1, a valine to isoleucine change at position 82, a serine to alanine change at position 107, a glutamic acid to glutamine change at position 287, and a stretch of four non-conserved amino acids at position 189 to 192 (Fig. 2).

Thus, in comparison to known primate IL-8 receptors, the CXCR1 and CXCR2 receptors of cynomolgus monkey have a significant number of differences in their amino acid sequence. Most noteworthy are a considerable number of changes in domains that are highly conserved in primate and non-primate species.

Expression analysis of CXCR1 and CXCR2. We compared the pattern of cCXCR1 and cCXCR2 expression in selected monkey tissues. Highest expression of both receptors was found in blood (Fig. 3 A and B). The expression level of both receptors was about 100-fold lower in spleen, and more than 1000-fold lower in brain and spinal cord when compared to blood. The predominant expression of CXCR1 and CXCR2 in cynomolgus blood is similar to the expression pattern seen in human, where high expression of both receptors was found in blood and spleen (Fig. 3C and D). In a non-primate species like rabbit, CXCR1 and CXCR2 are again predominantly expressed in blood (Fig. E and F), while in contrast rats express only CXCR2 in blood and spleen. Rat CXCR1, in contrast to the other species investigated, is expressed at only very low levels in the analyzed tissues and blood (Fig. 3F).

JPET Fast Forward. Published on March 17, 2004 as DOI: 10.1124/jpet.103.063131
This article has not been copyedited and formatted. The final version may differ from this version.

Thus, cCXCR1 and cCXCR2 are expressed, like in human, primarily in blood indicating that the novel cynomolgus genes are likely homologs of the human genes. Like human neutrophils, cynomolgus neutrophils express CXCR1 and CXCR2 as was demonstrated by flowcytometry using antibodies to human CXCR1 or CXCR2 (Fig. 3I).

Pharmacological characterization of cynomolgus CXCR1 and CXCR2. Because the cloned cynomolgus receptors CXCR1 and CXCR2 showed differences in otherwise conserved amino acid residues, we tested whether cCXCR1 and cCXCR2 had ligand binding characteristics similar to their human homologs. To this end we expressed recombinant human and monkey receptors in the pro-B cell line BaF3. To determine the affinity of the receptors to IL-8 and Gro-α, membranes of recombinant BA/F3 cells were incubated in the presence of ^{125}I-IL-8 and increasing concentrations of unlabelled human chemokines. The amount of radiolabeled ligand bound to the receptor was determined by Scintillation Proximity Assay technology.

As shown in Fig. 4, cCXCR1 binds human IL-8 (B_{max} = 1.96 pmol/mg; data not shown) with an affinity similar to that of hCXCR1 (Kd = 170±84 pM and 103±37 pM, respectively). Similar to hCXCR1, cCXCR1 has very low binding activity to the CXCR2 specific ligand Gro-α (250±84 nM and 1.4±1.1 μM, respectively). Comparison of the chemokine binding affinities to CXCR2 of both species revealed that hCXCR2 binds hIL-8 with an affinity of 210±14 pM, while IL-8 binds to cCXCR2 receptor with higher affinity at 46±28 pM (B_{max} = 0.75 pmol/mg; data not shown). However, while hCXCR2 binds Gro-α with an affinity of 540±140 pM, Gro-α binding to cCXCR2 was 12-fold lower at about 3.7±2.2 nM. Binding of radiolabeled ligand to membranes of parental

BA/F3 cells could not be detected (data not shown). Thus, the cynomolgus receptors CXCR1 and CXCR2 have a ligand binding profile similar to that of their human homologs; both receptors bind IL-8 with comparable affinity, while Gro-α binding occurs preferentially to CXCR2. However, Gro-α binds with significantly lower affinity to cCXCR2 than to hCXCR2.

To test whether chemokine binding by cynomolgus receptors translated in receptor activation, ligand dependent [^{35}S]GTPγS exchange was tested on membranes prepared from BaF3 cells expressing cynomolgus CXCR1 or CXCR2. Cynomolgus CXCR1 dependent GTPγS exchange was half-maximal stimulated by IL-8 concentrations between 84 and 240 pM, while 1000-fold higher concentrations of Gro-α were required (EC_{50} = 260±160 nM; Fig. 5A). Similarly, a half-maximal response of cCXCR2 to IL-8 was reached at concentrations between 1.8 and 10 pM, while Gro-α induced CXCR2 activation occurred at only 1000-fold higher concentrations (EC_{50} = 27±14 nM; Fig. 5B). In contrast, while ENA-70 was less potent than IL-8 on CXCR1 mediated GTP-γ-S exchange, it was more potent than IL-8 and Gro-α in activating the CXCR2 receptor.

Cynomolgus receptors CXCR1 and CXCR2 confer IL-8 dependent chemotactic activity to recombinant BaF3 cells. Chemokine binding and activation of hCXCR1 and hCXCR2 results in chemotaxis of cells (Feniger-Barish et al., 2003). To test whether the newly identified cynomolgus receptors CXCR1 and CXCR2 can induce chemotaxis, we tested the migration of recombinant BaF3 cells in response to increasing concentrations of hIL-8. As shown in Figure 6, recombinant BaF3 cells expressing either cCXCR1 or cCXCR2 migrate towards hIL-8 in a dose dependent manner (EC_{50} = 190 pM and 930

pM, respectively). Parental BaF3 cells did not chemotax to hIL-8, nor did recombinant cCXCR1 or cCXCR2 BaF3 chemotax to hMCP-1, a ligand for CCR2 (data not shown). This indicated that the newly identified cynomolgus receptors CXCR1 and CXCR2 are functional homologs of hCXCR1 and hCXCR2 in their abilities to bind chemokines, activate signal transduction and confer chemotactic activity to cells.

Ligand binding sites on the hCXCR1 and hCXCR2 receptors have been mapped by analysis of ligand binding to receptor chimeras, to receptors with point mutations, or by inhibition of ligand binding in the presence of an antagonist like SB225002 (Catusse et al., 2003). Since the cynomolgus receptors CXCR1 and CXCR2 show differences in the amino acid sequences at sites that are usually highly conserved among species, we determined the effect of SB225002 on the ligand binding activities of hCXCR1 and hCXCR2 to that of cCXCR1 and cCXCR2. SB225002 only poorly inhibited IL-8 binding to CXCR1 of either species (IC_{50} in low μM range; Fig. 7) confirming it as a CXCR2 selective antagonist (White et al., 1998). However, the binding affinity of SB225002 was about 10-fold more potent in inhibiting IL-8 binding to hCXCR2 (IC_{50} = 4.8 ± 1.6 nM) than to cCXCR2 (IC_{50} = 34 ± 11 nM; Fig. 7). This data was consistent with the IC_{50} of 9.9 nM reported by Catusse et al. for the effect of SB225002 on IL-8 binding to hCXCR2 (Catusse et al., 2003).

JPET Fast Forward. Published on March 17, 2004 as DOI: 10.1124/jpet.103.063131
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

We identified the novel genes encoding for cynomolgus monkey CXCR1 and CXCR2 and successfully expressed them in recombinant cells. Like for hCXCR1, cCXCR1 binds preferentially IL-8 with high affinity, while cCXCR2 binds IL-8 and Gro-α, albeit the binding affinity of human Gro-α to cCXCR2 occurred with a 10-fold lower affinity than to the human receptor. Ligand binding to cCXCR1 or cCXCR2 resulted in receptor activation as determined by GTPγS exchange with an EC_{50} that correlated with their binding activity, and IL-8 induced a chemotactic response of recombinant cells expressing either cynomolgus receptor. This identifies the cynomolgus receptors as functional homologs of hCXCR1 and hCXCR2.

Alignment of primate and non-primate receptor sequences reveals a number of unique amino acid differences in the cynomolgus receptors when compared to homologus genes of other primates (Figs 1 and 2). Outside the N-terminal domain, the majority of amino acid changes are located in transmembrane regions, thus they are not likely to affect ligand binding. Indeed this is supported by the fact that binding of IL-8 to both cynomolgus receptors occurs with an affinity similar to that of IL-8 binding to the human receptors.

Extensive analysis of the hCXCR1 receptor has identified two domains as important ligand binding sites, the N-terminal domain(LaRosa et al., 1992) and the third extracellular loop (EL3) (Hebert et al., 1993) suggesting that, in its three-dimensional conformation, these domains are in close contact. Cynomolgus CXCR1 has 98% homology to hCXCR1, the highest number of differences in the amino acid sequence occur in the N-terminal domain, the fourth transmembrane domain, and the third

extracellular loop. Despite these differences in proposed ligand binding sites, cynomolgus and human CXCR1 bind human IL-8 with similar affinity. This suggests that the conformation of the N-terminal domain is flexible to the extent that the addition of an amino acid residue like tyrosine at position 14 in cCXCR1 does not influence IL-8 binding. The role of EL3 in ligand binding to CXCR1 has been demonstrated by binding studies on CXCR1 molecules with mutated amino acid residues. Substitution of amino acids in EL3 at positions 271, 274, 275or 278 to 280 by alanine abolished IL-8 binding (Hebert et al., 1993; Leong et al., 1994). While in cCXCR1 the amino acid residues at positions 275 and 278 to 280 are conserved, glutamine is changed to histidine or lysine at position 271 and 274, respectively. These amino acid changes do not affect the affinity of hIL-8 to cCXCR1, suggesting that substitution of polar amino acids by positively charged residues in EL3 does not alter IL-8 binding sites or the conformation of the receptor.

Cynomolgus CXCR2 binds human IL-8 with an affinity comparable to its human homolog, but the binding affinity of Gro-α is approximately 10-fold lower. Alignment of human and cynomolgus receptors identify major differences in the amino acid sequence of the N-terminal domain and the second and third extracellular loop. These regions were implicated as important chemokine binding sites by analysis of ligand binding to human CXCR1-CXCR2 chimeras (Ahuja et al., 1996) and prediction from molecular modeling of CXCR2 (Luo et al., 1997). Within the N-terminal domain point mutational analysis identified glutamic acid at position seven (E7Q) and aspartic acid at position nine (D9F) of hCXCR2 as sites for interaction with IL-8 and Gro-α (Katancik et al., 2000). Comparison with the cynomolgus N-terminan domain showed differences in the

amino acid sequence at these position (E7Q) and (D9F) and other changes (K16E, G17N, T28D). In addition, a stretch of amino acids in EL2 of cCXCR2 differs from the human sequence (LTYI compared to SSNV starting at position 188). While previous studies have identified these regions as critical IL-8 binding sites of hCXCR2 (Luo et al., 1997; Catusse et al., 2003), binding of IL-8 to cCXCR2 occurs with an affinity similar to hCXCR2. This suggests that despite differences in the amino acid sequence the three-dimensional conformation and net charge of IL-8 binding sites on cCXCR2 is similar to hCXCR2. However, while these amino acid differences between cynomolgus and human CXCR2 do not influence IL-8 binding, they appear to affect Gro-α binding. An important Gro-α binding site is located in the third extracellular loop of hCXCR2. When within this loop the sequence HID at position 291 was changed to NXG in the human receptor, binding of Gro-α occurred with a 10-fold lower affinity (Catusse et al., 2003). Interestingly, the cynomolgus amino acid sequence differs from the human sequence at this site (human HIDR to cynomolgus NIDQ) which could explain the 10-fold lower binding affinity of human Gro-α to the monkey receptor. Interestingly, the human ligand ENA-70, a truncated version of the hCXCR2 ligand ENA-78, is highly efficacious on cCXCR2 (Fig. 5), suggesting that binding restrictions caused by species specific differences in the receptor structure do not apply to all ligands.

The influence of HID291 on chemokine binding is further supported by the fact that this sequence has been identified as one of the binding sites for the CXCR2 selective antagonist SB225002 (Catusse et al., 2003). SB225002 inhibits binding of Gro-α and IL-8 to hCXCR2, but has no effect on binding of IL-8 to hCXCR1 (Catusse et al., 2003). Compared to hCXCR2, 10-fold higher concentrations of SB225002 are required to inhibit

IL-8 binding to cCXCR2 than to hCXCR2, suggesting that in cCXCR2 at least one binding site for SB225002 is altered. In summary, correlation of the amino acid sequence of human and non-human receptors with their ligand binding characteristics substantiates the proposed position of ligand binding sites on these receptors. Additional studies with receptor antagonists support these results, suggesting that comparison of sequences and function of receptors from different species can identify potential ligand binding sites and may guide rational drug design.

The products of the newly identified cynomolgus genes were shown by sequence analysis and functional characterization to be true homologs of hCXCR1 and hCXCR2. These receptors are, like their human and rabbit homologs, highly expressed in blood at similar levels. Similarly, rat CXCR2 is highly expressed in blood, but only low levels of CXCR1 expression could be detected in selected rat tissues and blood. This low expression of rat CXCR1 might occur in macrophages, since expression of CXCR1 on rat neutrophils has not been detected (Dunstan et al., 1996). Interestingly, a gene with 86% homology to rat CXCR1, but only 76% homology to hCXCR1 has recently been identified in mice (genebank accession #BC051677) but the function of the proposed rodent CXCR1 receptors remains to be identified. Thus, evaluation of the function of CXCR1 and CXCR2 on neutrophil migration in vivo and their effect on the pathogenesis of inflammatory diseases can only be evaluated in species like rabbit or monkey that show a pattern of CXCR1 and CXCR2 expression similar to that in humans while neutrophil function in inflammatory models in rodents is most likely only dependent on the CXCR2 receptor only.

So far the cynomolgus ligands for CXCR1 and CXCR2 have not been identified, but the sequence for rhesus macaque IL-8 and Gro-α have been reported (Minnerly et al., 1995; Basu et al., 2002). The amino acid sequence of rhesus IL-8 and Gro-a are 94% and 90% identical to the sequence of their human homologs. Based on the low binding affinity of human Gro-α to cCXCR2 and the observed differences in the cCXCR2 amino acid sequence compared to the human and rhesus receptors, we would predict that the sequence for at least cynomolgus Gro-α is significantly different from that of other primates.

In conclusion, this study demonstrates that the pharmacology of homologus gene products can differ substantially even between closely related species. This is emphasized by our observation that the CXCR2 antagonist SB 225002 is at least 10-fold less effective in inhibiting ligand binding to cCXCR2 than to hCXCR2. This emphasizes the importance of information on the pharmacology of receptors targeted for the interpretation of animal models and testing the effects of antagonists in vivo.

References

Ahuja SK, Lee JC and Murphy PM (1996) CXC chemokines bind to unique sets of selectivity determinants that can function independently and are broadly distributed on multiple domains of human interleukin-8 receptor B. Determinants of high affinity binding and receptor activation are distinct. *J Biol Chem* **271**:225-232.

Banks C, Bateman A, Payne R, Johnson P and Sheron N (2003) Chemokine expression in IBD. Mucosal chemokine expression is unselectively increased in both ulcerative colitis and Crohn's disease. *J Pathol* **199**:28-35.

Basu S, Schaefer TM, Ghosh M, Fuller CL and Reinhart TA (2002) Molecular cloning and sequencing of 25 different rhesus macaque chemokine cDNAs reveals evolutionary conservation among C, CC, CXC, AND CX3C families of chemokines. *Cytokine* **18**:140-148.

Beckmann MP, Munger WE, Kozlosky C, VandenBos T, Price V, Lyman S, Gerard NP, Gerard C and Cerretti DP (1991) Molecular characterization of the interleukin-8 receptor. *Biochem Biophys Res Commun* **179**:784-789.

Beeh KM, Kornmann O, Buhl R, Culpitt SV, Giembycz MA and Barnes PJ (2003) Neutrophil chemotactic activity of sputum from patients with COPD: role of interleukin 8 and leukotriene B4. *Chest* **123**:1240-1247.

Billah MM, Cooper N, Minnicozzi M, Warneck J, Wang P, Hey JA, Kreutner W, Rizzo CA, Smith SR, Young S, Chapman RW, Dyke H, Shih NY, Piwinski JJ, Cuss FM, Montana J, Ganguly AK and Egan RW (2002) Pharmacology of N-(3,5-dichloro-1-oxido-4-pyridinyl)-8-methoxy-2-(trifluoromethyl)-5-quino line carboxamide (SCH

351591), a novel, orally active phosphodiesterase 4 inhibitor. *J Pharmacol Exp Ther* **302**:127-137.

Bozic CR, Gerard NP, von Uexkull-Guldenband C, Kolakowski LF, Jr., Conklyn MJ, Breslow R, Showell HJ and Gerard C (1994) The murine interleukin 8 type B receptor homologue and its ligands. Expression and biological characterization. *J Biol Chem* **269**:29355-29358.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248-254.

Catusse J, Liotard A, Loillier B, Pruneau D and Paquet JL (2003) Characterization of the molecular interactions of interleukin-8 (CXCL8), growth related oncogen alpha (CXCL1) and a non-peptide antagonist (SB 225002) with the human CXCR2. *Biochem Pharmacol* **65**:813-821.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.

Cox MA, Jenh CH, Gonsiorek W, Fine J, Narula SK, Zavodny PJ and Hipkin RW (2001) Human interferon-inducible 10-kDa protein and human interferon-inducible T cell alpha chemoattractant are allotopic ligands for human CXCR3: differential binding to receptor states. *Mol Pharmacol* **59**:707-715.

Dunstan CA, Salafranca MN, Adhikari S, Xia Y, Feng L and Harrison JK (1996) Identification of two rat genes orthologous to the human interleukin-8 receptors. *J Biol Chem* **271**:32770-32776.

Feniger-Barish R, Yron I, Meshel T, Matityahu E and Ben-Baruch A (2003) IL-8-induced migratory responses through CXCR1 and CXCR2: association with phosphorylation and cellular redistribution of focal adhesion kinase. *Biochemistry* **42**:2874-2886.

Gillitzer R, Ritter U, Spandau U, Goebeler M and Brocker EB (1996) Differential expression of GRO-alpha and IL-8 mRNA in psoriasis: a model for neutrophil migration and accumulation in vivo. *J Invest Dermatol* **107**:778-782.

Gonsiorek W, Zavodny P and Hipkin RW (2003) The study of CXCR3 and CCR7 pharmacology using [35S]GTPgammaS exchange assays in cell membranes and permeabilized peripheral blood lymphocytes. *J Immunol Methods* **273**:15-27.

Harada A, Kuno K, Nomura H, Mukaida N, Murakami S and Matsushima K (1994) Cloning of a cDNA encoding a mouse homolog of the interleukin-8 receptor. *Gene* **142**:297-300.

Hebert CA, Chuntharapai A, Smith M, Colby T, Kim J and Horuk R (1993) Partial functional mapping of the human interleukin-8 type A receptor. Identification of a major ligand binding domain. *J Biol Chem* **268**:18549-18553.

Hipkin RW, Friedman J, Clark RB, Eppler CM and Schonbrunn A (1997) Agonist-induced desensitization, internalization, and phosphorylation of the sst2A somatostatin receptor. *J Biol Chem* **272**:13869-13876.

Katancik JA, Sharma A and de Nardin E (2000) Interleukin 8, neutrophil-activating peptide-2 and GRO-alpha bind to and elicit cell activation via specific and different amino acid residues of CXCR2. *Cytokine* **12**:1480-1488.

Kurdowska A, Noble JM, Grant IS, Robertson CR, Haslett C and Donnelly SC (2002) Anti-interleukin-8 autoantibodies in patients at risk for acute respiratory distress syndrome. *Crit Care Med* **30**:2335-2337.

LaRosa GJ, Thomas KM, Kaufmann ME, Mark R, White M, Taylor L, Gray G, Witt D and Navarro J (1992) Amino terminus of the interleukin-8 receptor is a major determinant of receptor subtype specificity. *J Biol Chem* **267**:25402-25406.

Larsen CG, Thomsen MK, Gesser B, Thomsen PD, Deleuran BW, Nowak J, Skodt V, Thomsen HK, Deleuran M, Thestrup-Pedersen K and et al. (1995) The delayed-type hypersensitivity reaction is dependent on IL-8. Inhibition of a tuberculin skin reaction by an anti-IL-8 monoclonal antibody. *J Immunol* **155**:2151-2157.

Leong SR, Kabakoff RC and Hebert CA (1994) Complete mutagenesis of the extracellular domain of interleukin-8 (IL-8) type A receptor identifies charged residues mediating IL-8 binding and signal transduction. *J Biol Chem* **269**:19343-19348.

Luo Z, Butcher DJ and Huang Z (1997) Molecular modeling of interleukin-8 receptor beta and analysis of the receptor-ligand interaction. *Protein Eng* **10**:1039-1045.

Matsumoto T, Ikeda K, Mukaida N, Harada A, Matsumoto Y, Yamashita J and Matsushima K (1997) Prevention of cerebral edema and infarct in cerebral reperfusion injury by an antibody to interleukin-8. *Lab Invest* **77**:119-125.

Mihara M, Kotoh M, Nishimoto N, Oda Y, Kumagai E, Takagi N, Tsunemi K, Ohsugi Y, Kishimoto T, Yoshizaki K and Takeda Y (2001) Humanized antibody to human interleukin-6 receptor inhibits the development of collagen arthritis in cynomolgus monkeys. *Clin Immunol* **98**:319-326.

Minnerly JC, Baganoff MP, Deppeler CL, Keller BT, Rapp SR, Widomski DL, Fretland DJ and Bolanowski MA (1995) Identification and characterization of rhesus macaque interleukin-8. *Inflammation* **19**:313-331.

Patel L, Charlton SJ, Chambers JK and Macphee CH (2001) Expression and functional analysis of chemokine receptors in human peripheral blood leukocyte populations. *Cytokine* **14**:27-36.

Podolin PL, Bolognese BJ, Foley JJ, Schmidt DB, Buckley PT, Widdowson KL, Jin Q, White JR, Lee JM, Goodman RB, Hagen TR, Kajikawa O, Marshall LA, Hay DW and Sarau HM (2002) A potent and selective nonpeptide antagonist of CXCR2 inhibits acute and chronic models of arthritis in the rabbit. *J Immunol* **169**:6435-6444.

Prado GN, Thomas KM, Suzuki H, LaRosa GJ, Wilkinson N, Folco E and Navarro J (1994) Molecular characterization of a novel rabbit interleukin-8 receptor isotype. *J Biol Chem* **269**:12391-12394.

Rose RM, Kobzik L, Dushay K, Wolfthal S, Hondalus M, Metzger M, Stoudemire J, Brain JD, Garnick M, O'Donnell C and et al. (1992) The effect of aerosolized recombinant human granulocyte macrophage colony-stimulating factor on lung leukocytes in nonhuman primates. *Am Rev Respir Dis* **146**:1279-1286.

Shibata F, Konishi K and Nakagawa H (2000) Identification of a common receptor for three types of rat cytokine-induced neutrophil chemoattractants (CINCs). *Cytokine* **12**:1368-1373.

Thomas KM, Taylor L and Navarro J (1991) The interleukin-8 receptor is encoded by a neutrophil-specific cDNA clone, F3R. *J Biol Chem* **266**:14839-14841.

White JR, Lee JM, Young PR, Hertzberg RP, Jurewicz AJ, Chaikin MA, Widdowson K, Foley JJ, Martin LD, Griswold DE and Sarau HM (1998) Identification of a potent, selective non-peptide CXCR2 antagonist that inhibits interleukin-8-induced neutrophil migration. *J Biol Chem* **273**:10095-10098.

Woods JM, Katschke KJ, Jr., Tokuhira M, Kurata H, Arai KI, Campbell PL and Koch AE (2000) Reduction of inflammatory cytokines and prostaglandin E2 by IL-13 gene therapy in rheumatoid arthritis synovium. *J Immunol* **165**:2755-2763.

Yamamoto T, Kajikawa O, Martin TR, Sharar SR, Harlan JM and Winn RK (1998) The role of leukocyte emigration and IL-8 on the development of lipopolysaccharide-induced lung injury in rabbits. *J Immunol* **161**:5704-5709.

Yoshie O, Imai T and Nomiyama H (2001) Chemokines in immunity. *Adv Immunol* **78**:57-110.

Figure Legends

Fig. 1. Comparison of the CXCR1 aminoacid sequence between different species. The amino acid sequence of hCXCR1 (genebank acc# L19591) and the CXCR1 sequence of several non-human primates like chimpanzee (genebank acc# X91109), gorilla (genebank acc# X91110) , rhesus (genebank acc# X91112), were compared to the sequence of cCXCR1. The sequences of rabbit CXCR1 (genebank acc# M58021), rat CXCR1 (genebank acc# X77797) are also included. Boxed sequences indicate predicted transmembrane domains (TM); extracellular loops (EL) are indicated. Amino acid residues mentioned in the text are depicted in bold and are underlined.

Fig. 2. Comparison of the CXCR2 aminoacid sequence between different species. The aminoacid sequence of hCXCR2 (genebank acc# NM_001557) and the CXCR2 sequence of several non-human primates like chimpanzee (genebank acc# X91113), gorilla (genebank acc# X91114), rhesus (genebank acc# X91116), were compared toe the sequence of cCXCR2. The sequences of rabbit CXCR2 (genebank acc# L24445), rat CXCR 2 (genebank acc# NM_017183) and mouse CXCR2 (genebank acc# NM_009909), are also included. Boxed sequences indicate predicted transmembrane domains (TM); extracellular loops (EL) are indicated. Amino acid residues mentioned in the text are depicted in bold and are underlined.

Fig. 3. Analysis of CXCR1 and CXCR2 expression. Expression of CXCR1 (panels A, C, E and G) or CXCR2 (panels B, D, F and H) in tissues and whole blood of selected species as determined by real-time PCR. (I) Expression of CXCR1 and CXCR2 on

cynomolgus neutrophils detected by flowcytometry using anti-human CXCR1 or CXCR2 antibodies.

Fig. 4. Competition binding of chemokines to cynmolgus and human CXCR1 and CXCR2. Membranes of BA/F3 cells expressing either cynomolgus (A) or human (B) CXCR1 or CXCR2 receptor were incubated with increasing concentrations of hIL-8 or Gro-α in the presence of radiolabeled ^{125}I-hIL-8. Radioligand binding to the membranes was measured by WGA-SPA scintillation. Ligand affinities from competition bindings were calculated from binding IC_{50} using the Cheng-Prusoff equation.

Fig. 5. Chemokines induce GTP-gS exchange on BA/F3 cell membranes expressing cCXCR1 or cCXCR2. Membranes of recombinant BA/F3 cells expressing cCXCR1 (A) or cCXCR2 (B) receptor were incubated in the presence of GTP-γS, GDP and increasing concentrations of chemokines. Membrane associated radioactivity was measured by scintillation proximity. Data from a representative experiment in a series of three experiments is presented.

Fig. 6. IL-8 dependent chemotaxis of cells expressing cynomolgus CXCR1 or CXCR2. Recombinant BA/F3 cells expressing either cCXCR1 (open circle) or cCXCR2 (open square) were tested for their migratory potential in response to increasing concentrations of IL-8 (A) or Gro-α (B) in a 96-well chemotaxis apparatus. Data is expressed as the percentage of total cells migrating towards the respective chemokine.

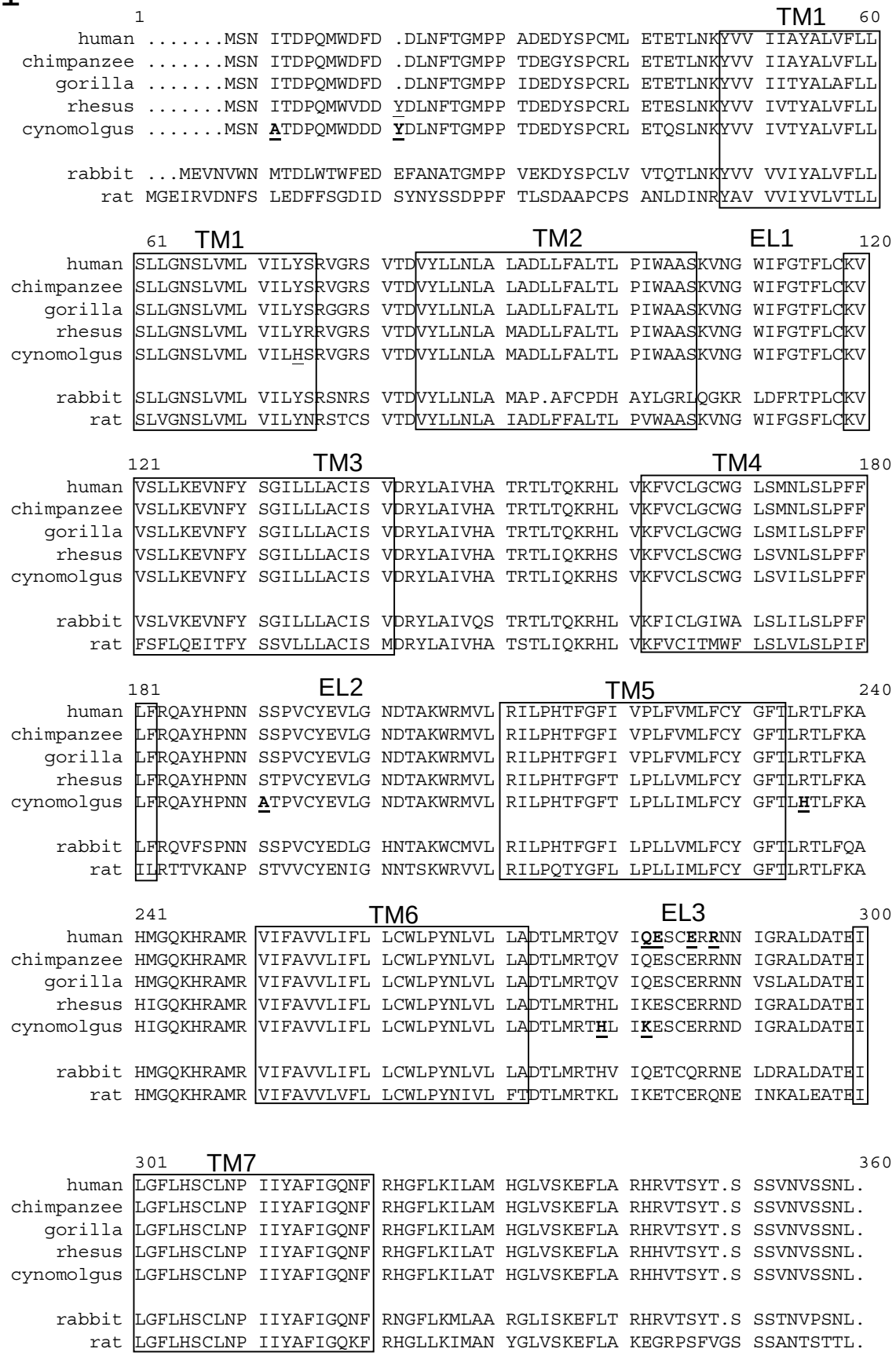
Fig. 7. IL-8 competition binding in the presence of SB225002. BA/F3 membranes from cells expressing either the human CXCR1 (panel A) or CXCR2 (panel B) or cynomolgus CXCR1 (panel C) or CXCR2 (panel D) receptors were incubated in the presence of cold and radiolabeled IL-8 and increasing concentrations of SB225002. Radioligand binding to the membranes was measured by WGA-SPA scintillation. Ligand affinities from competition bindings were calculated from binding IC_{50} using the Cheng-Prusoff equation.

TABLE 1. Primer sequences for CXCR1/CXCR2 of different species.

receptor	species	forward primer	reverse primer
CXCR1	human	TGCCACCTGCAGATGAAGATTA	AGGCGATGATCACAACATACTTGT
CXCR2	human	TCCGAAGGACCGTCTACTCATC	CCGCCAGTTTGCTGTATTGTT
CXCR1	cyno	GGGATGATGACTATGATCTAAATTTCACT	GGCATAGGTGACAATCACAACATAC
CXCR2	cyno	CTGCCCCCTTCTCTACCAGAT	AATACTTGTTGATTTCCAGGGATTCT
CXCR1	rat	AATGCTGCCCACTGGAGAGT	CAGCCTGCCTGTTGGTCAT
CXCR2	rat	GGGTGGATAATTTCAGCCTTGA	AGGGCGGGTCAGAACTGTAA
CXCR1	rabbit	TGGAAGTAAACGTATGGAATATGACTGA	CAGGAGGCATACCAGTAGCATTT
CXCR2	rabbit	TTCGGCGATTTCAGCAATTAC	GCTGTTGGTTTCCAGAGATTCTG

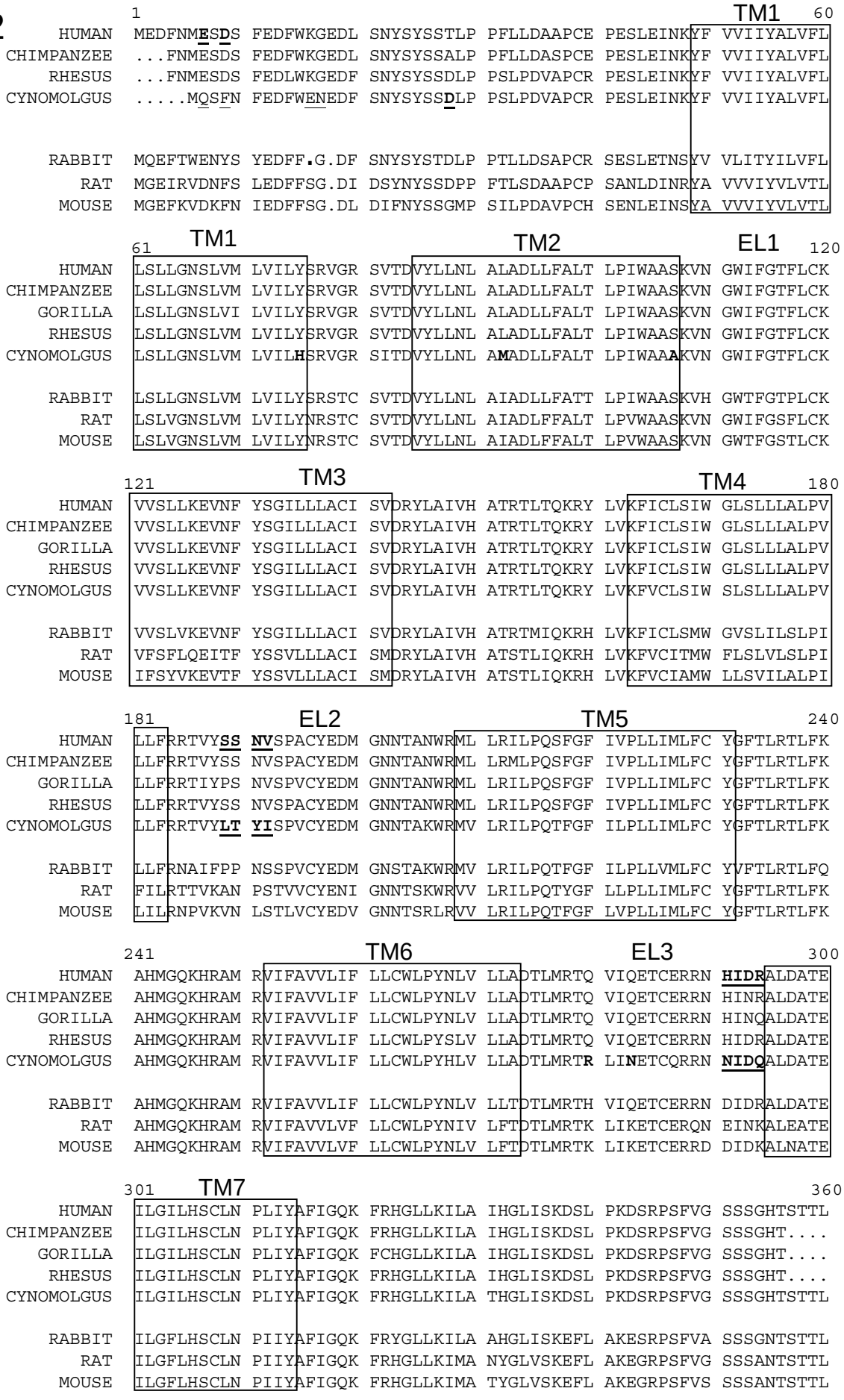
JPET Fast Forward. Published on March 17, 2004 as DOI: 10.1124/jpet.103.063131
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 1



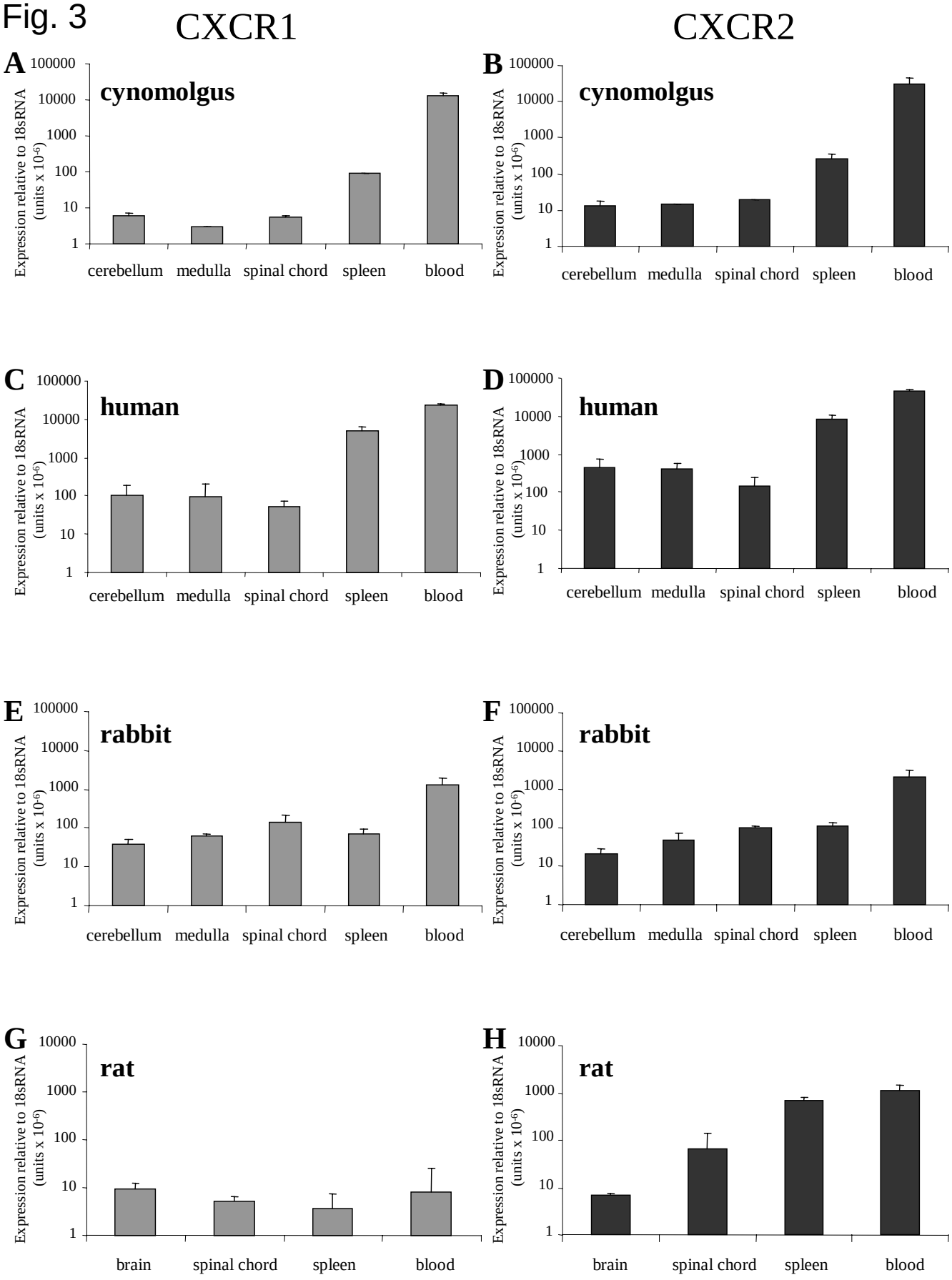
JPET Fast Forward. Published on March 17, 2004 as DOI: 10.1124/jpet.103.063131
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 2



JPET Fast Forward. Published on March 17, 2004 as DOI: 10.1124/jpet.103.063131
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 3



JPET Fast Forward. Published on March 17, 2004 as DOI: 10.1124/jpet.103.063131
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 3 part I

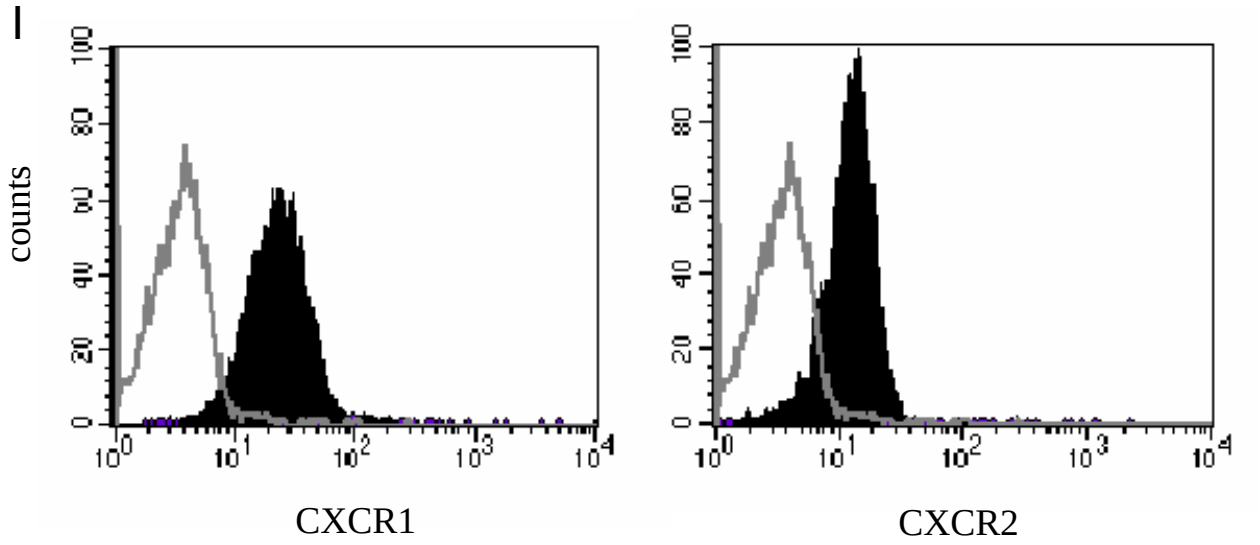


Fig. 4

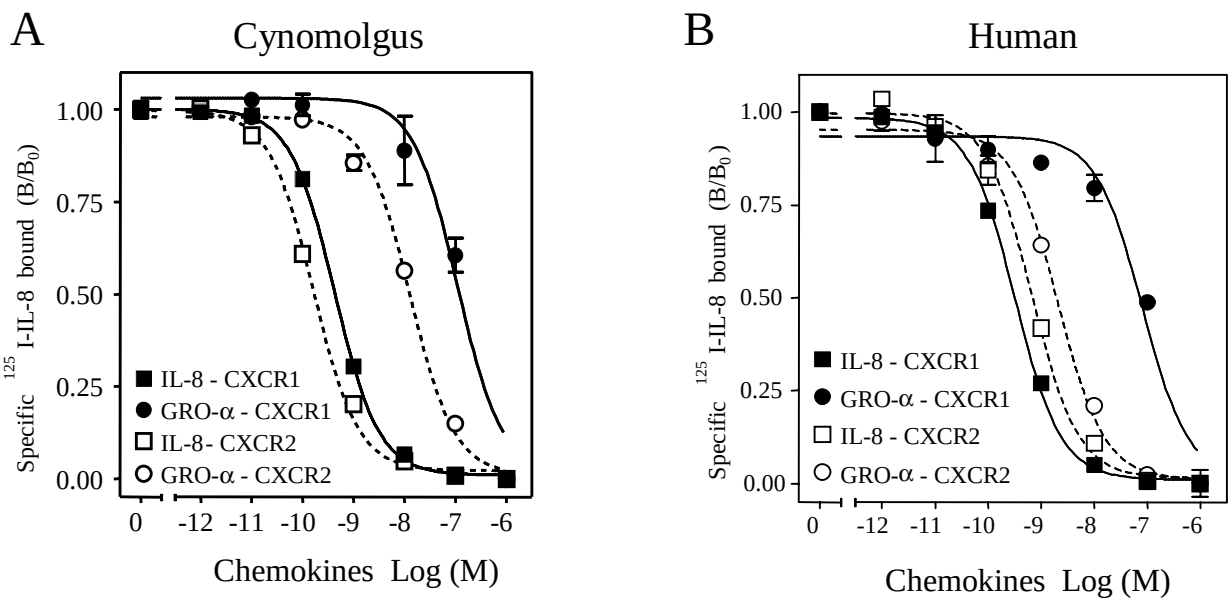


Fig. 5

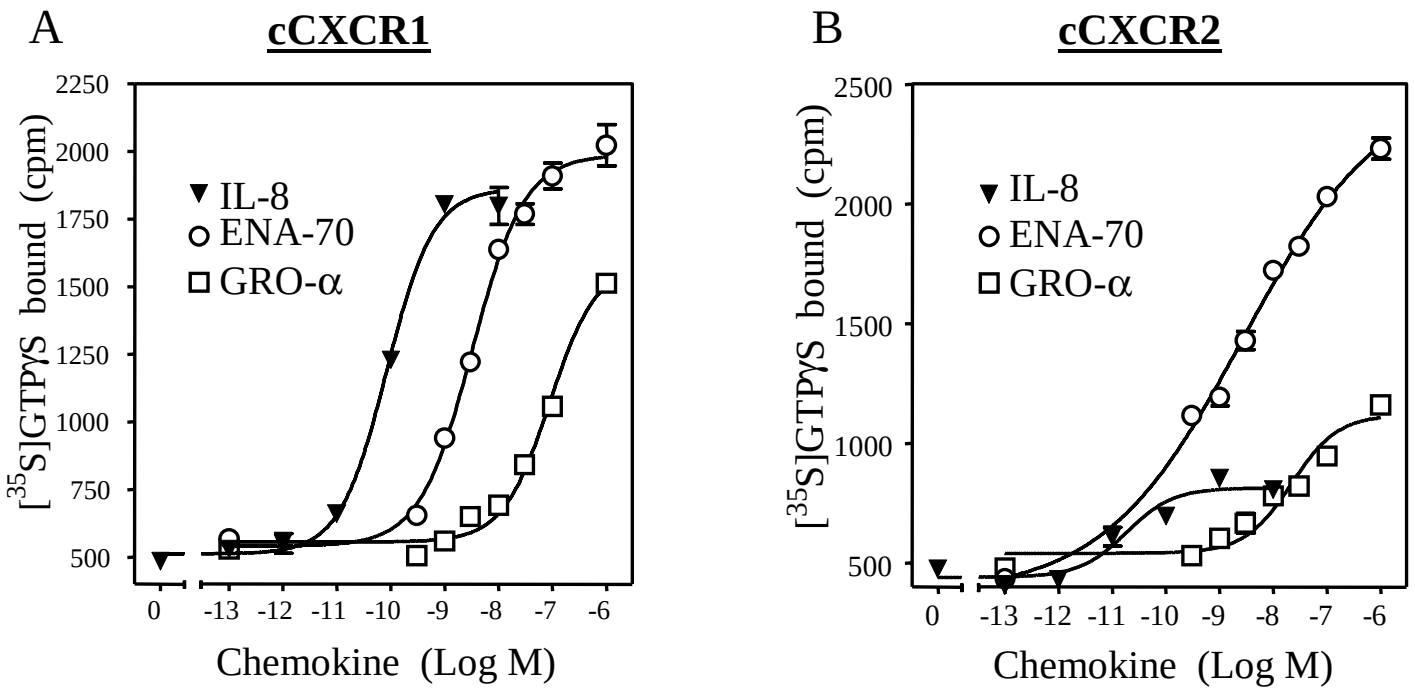


Fig. 6

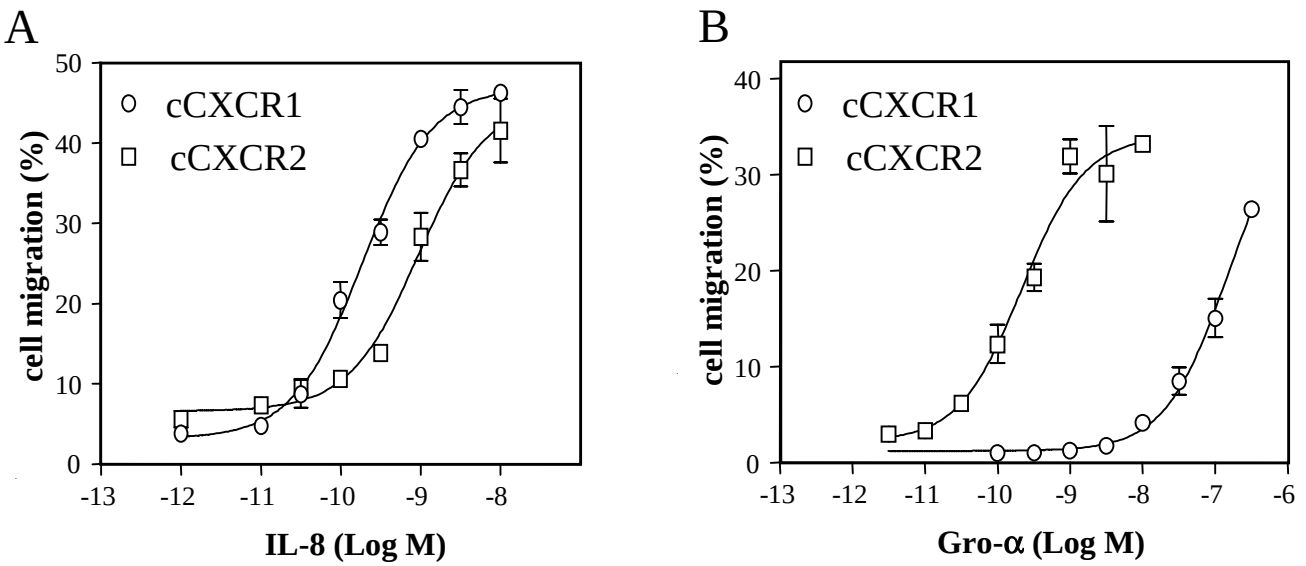


Fig. 7