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Chapter 3

Identification and profiling of CXCR3-CXCR4 chemokine receptor heteromer complexes

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Abstract

The chemokine receptors CXCR3 and CXCR4 are involved in various autoimmune diseases and cancers. Small antagonists have previously been shown to cross-inhibit chemokine binding to CCR2, CCR5, and CXCR4 heteromers. We investigated whether CXCR3 and CXCR4 can form heteromeric complexes and the binding characteristics of chemokines and small ligand compounds to these chemokine receptor heteromers.

CXCR3-CXCR4 heteromers were identified in HEK293T cells using co-immunoprecipitation, time-resolved fluorescence resonance energy transfer, saturation bioluminescence resonance energy transfer, and the GPCR-HIT approach. Equilibrium competition binding and dissociation experiments were performed to detect negative binding co-operativity.

We provide evidence that chemokine receptors CXCR3 and CXCR4 form heteromeric complexes in HEK293T cells. Chemokine binding was mutually exclusive on membranes co-expressing CXCR3 and CXCR4 as revealed by equilibrium competition binding and dissociation experiments. The small CXCR3 agonist VUF10661 impaired binding of CXCL12 to CXCR4, whereas small antagonists were unable to cross-inhibit chemokine binding to the other chemokine receptor. In contrast, negative binding co-operativity between CXCR3 and CXCR4 chemokines was not observed in intact cells. However, using the GPCR-HIT approach we have evidence for specific β-arrestin2 recruitment to CXCR3-CXCR4 heteromers in response to agonist stimulation.

This study indicates that heteromeric CXCR3-CXCR4 complexes may act as functional units in living cells, which potentially open up novel therapeutic opportunities.
**Introduction**

Chemokines are secreted chemoattractant proteins of 8-14 kDa that direct immune cell migration through interaction with G protein-coupled receptors (GPCRs). Over forty chemokines and twenty chemokine receptors have been identified in humans, forming a regulatory system in which many of these receptors promiscuously bind multiple chemokines and *vice versa*.\(^1\) In addition, chemokine receptors can also form both homo- and heteromers.\(^1\) The relative occurrence of such GPCR complexes and transiency, however, is still a matter of debate.\(^2-3\) Importantly, heteromers between the chemokine receptors CCR2, CCR5, and CXCR4 can only bind a single chemokine with high affinity.\(^4-7\) This negative binding co-operativity between the heteromerized chemokine receptors is not limited to the cognate chemokines of both receptors, but extends to synthetic allosteric antagonists of these receptors.\(^5,6\) Binding of these antagonists to one receptor allosterically inhibits chemokine binding to the other receptor within the heteromer, resulting in a cross-inhibition of intracellular signaling, and *in vitro* and *in vivo* chemotaxis.\(^5,6\) Besides inhibiting one chemokine receptor by targeting its heteromeric partner, chemokine receptor heteromers may be therapeutically targeted by heteromer-selective ligands and/or bivalent ligands that simultaneously bind both receptors in a dimer, as previously described for *e.g.* opioid receptor heteromers.\(^8-11\) Since GPCR heteromers are likely expressed on a more limited subset of cell types as compared to the individual GPCRs, heteromer-selective therapeutic agents have the potential to display improved efficacy and toxicity profiles.

CXCR3 and CXCR4 are expressed on activated T cells, natural killer cells, dendritic cells and cancer cells.\(^12-14\) CXCR4 plays an essential role in hematopoiesis, leukocyte homing/retention in secondary lymphoid tissues and recruitment to sites of inflammation, in response to local concentrations of CXCL12.\(^15\) Additionally, CXCR4 and CXCL12 are upregulated in tumors by *e.g.* hypoxia, and mediate angiogenesis, proliferation, invasion and metastasis.\(^12-14\) Expression of CXCR3 and its ligands CXCL9, CXCL10 and CXCL11 is induced under inflammatory conditions, and has been implicated in autoimmune disease, graft-versus-host disease and...
transplant rejection. Moreover, CXCR3 is also involved in cancer proliferation and metastasis.

In the present study, we show that CXCR3 and CXCR4 form heteromers at the cell surface. Negative binding co-operativity between CXCR3 and CXCR4 agonists was detected in membrane preparations of cells that co-express both receptors, but not on intact cells. However, β-arrestin2 recruitment specifically to CXCR3-CXCR4 heteromers was shown in living cells using the GPCR heteromer identification technology (GPCR-HIT) approach.

Materials and methods

**Materials.** The small CXCR3 ligands VUF10085 and VUF10661 have been previously described as AMG 487 and compound 2, respectively, and were synthesized at VU University Amsterdam. TAK-779 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. AMD3100 was obtained from Sigma-Aldrich. CXCL10 (IP-10), CXCL11 (I-TAC), and CXCL12 (SDF-1α) were from Peprotech.

**DNA constructs.** Human CXCR3 and CXCR4 were tagged at the N-terminus with FLAG (DYKDDDDK) or HA (YPYDVPDYA) epitope using polymerase chain reaction based methods and subcloned in the pCD4 expression plasmid (a gift from Dr. Langer, Robert Wood Johnson Medical School, Piscataway, NJ). The receptor fusion proteins CXCR3-Renilla luciferase (Rluc), CXCR3-enhanced yellow fluorescent protein (EYFP), CXCR4-Rluc, and CXCR4-EYFP have been previously described. CXCR3-Rluc8 was constructed by substitution of Rluc with the optimized Rluc8 variant as previously described. CXCR4-Rluc8 and β-arrestin2-Venus constructs have been described previously. For sensitized emission fluorescence resonance energy transfer (FRET) studies, CXCR3, CXCR4, and GABA

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**Cell culture and transfection.** HEK293T cells were cultured in Dulbecco’s modified eagle’s medium containing 10% fetal calf serum, 100 Units/mL penicillin and 100 µg/mL streptomycin (PAA Laboratories GmbH, Cölbe, Germany) were maintained at 37°C in a humidified atmosphere containing 5% CO2. For saturation bioluminescence resonance energy transfer (BRET), time-resolved FRET (trFRET), immunoprecipitation (IP) and radioligand binding studies, HEK293T cells were transfected with indicated amounts of plasmid DNA using 25-kDa linear polyethyleneimine (Polysciences, Eppelheim, Germany) as described previously.24 Transfected DNA was held constant by adjusting the total amount of DNA with the empty vector pcDEF3. For GPCR-HIT assays, HEK293T cells were maintained at 37°C in 5% CO2 and complete media (Dulbecco’s modified Eagle’s medium (DMEM) containing 0.3 mg/mL glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco)) supplemented with 10% foetal calf serum (FCS) and 400 µg/mL Geneticin (Gibco). Transient transfections were carried out 24 h after seeding about 550,000 cells/well of a 6-well plate. Genejuice (Novagen) transfection reagent was used according to the manufacturer’s instructions. Cells were harvested with 0.05% Trypsin-EDTA (Gibco).

**Saturation BRET.** HEK293T cells were cultured and transfected in 96 well plates. GPCR-EYFP and GPCR-Rluc expression and saturation BRET between these receptors were measured as described previously.24

**Time-resolved FRET.** HEK293T cells were transfected with plasmid DNA encoding N-terminally tagged CXCR3 and/or CXCR4. trFRET was measured on a Novostar plate reader (BMG Lab Technologies, Offenburg, Germany) 48 h post-transfection, as described previously (van Rijn et al., 2006) with minor modifications. Briefly, following incubation with both 0.8 nM Eu³⁺-labeled anti-HA and 13 nM XL665-labeled anti-Flag antibodies (CisBio Bioassays, 30204 Bagnols/Cesse Cedex, France), the cells were washed and resuspended in phosphate-buffered saline (107 cells/mL). Next, 50 µl of each sample was dispensed in triplicate in a white-walled 384 well microtiter plate.

**Sensitized emission FRET.** Imaging of HEK293T cells expressing receptor CFP or Venus fusion proteins was performed on a Leica AOBS_TCS SP2 confocal laser scanning microscope using a 63X NA 1.4 oil-immersion objective (Leica Microsystems, Rijswijk, The Netherlands) and the 458 nm line of an Ar/Kr laser. FRET was imaged by detecting sensitized emission.27 FRET efficiency was determined by background subtraction, bleed-through correction, and correction of intensity.28 Values were measured by scaling all samples to the same level of CXCR3-CFP/CXCR3-Venus followed by detecting the intensity at different regions of interest on the cell membrane.

**Co-immunoprecipitation and immunoblotting.** HEK293T cells were transfected with plasmid DNA encoding N-terminally tagged receptors. 24 h after transfection, cell lysates were prepared and IP with anti-HA-agarose antibody (clone HA-7, Sigma, St Louis, MO) was performed according to
manufacturer’s instructions. Immunoprecipitated protein was eluted from anti-HA-agarose antibody by incubation with 6x sample buffer (0.35 M Tris.HCl, pH 6.8, 10.3 % sodium dodecyl sulfate, 30% glycerol, 0.6 M dithiothreitol, 180 µM bromophenol blue; all chemicals were obtained from Sigma-Aldrich) at room temperature for 5 min. Protein samples were resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (AppliChem, Darmstadt, Germany). Blots were probed with the primary antibodies rat anti-HA (Roche, Indianapolis, IN) or mouse anti-FLAG (Sigma-Aldrich, St-Louis, MO) followed by horseradish peroxidase-conjugated secondary antibodies (Thermo Scientific, Rockford, IL, and Bio-Rad, Richmond, CA) and visualized with an enhanced chemiluminescent reagent (Thermo Scientific, Rockford, IL).

**Radioligand binding.** Preparation of HEK293T cell membrane fractions and [125I]-chemokine competition binding were performed as described previously. Cell membrane fractions were prepared 48 h post-transfection. Dissociation of [125I]-chemokine was determined using infinite dilution. To this end, membranes were incubated in binding buffer with approximately 100 pM of radioligand at room temperature for 60 min, followed by centrifugation. The pellet was then resuspended in 3% of the supernatant volume and 3 µL was dispensed per well of a 96 well plate. At the indicated time points, 300 µL of binding buffer or 100 nM unlabeled chemokine solution was added to the membrane suspension. Whole cell binding experiments were performed essentially as previously described. Briefly, 24 h after transfections, cells were collected and seeded into poly-L-lysine coated 48 well plates. The next day, culture medium was replaced with ice-cold binding buffer containing 50 pM [125I]-CXCL10 or [125I]-CXCL12 in the absence or presence of 100 nM unlabeled chemokines. After 4 h the cells were washed, solubilized, and collected to measure bound radioligand in a Wallac Compugamma counter (Perkin Elmer Life Sciences).

**GPCR-Heteromer Identification Technology (GPCR-HIT).** As described previously, HEK293T cells were transiently transfected with cDNA encoding a GPCR fused to Rluc8 (GPRC-Rluc8) and β-arrestin2 fused to Venus (β-arrestin2-Venus), along with a second GPCR that was untagged with respect to BRET signaling, or empty vector. DNA amounts of 0.1, 0.3 and 0.1 µg/well of a 6-well plate were used for each construct respectively. 48 h post-transfection, cells were incubated at 37 °C, 5% CO2 for 2 h with 30 µM EnduRen (Promega) in Freestyle293 medium with 25 mM HEPES (Gibco) to ensure substrate equilibrium was reached. BRET measurements were taken at 37 °C using the VICTOR Light plate reader with Wallac 1420 software (PerkinElmer). Filtered light emissions were sequentially measured at 400–475 and 520–540 nm. The BRET signal was calculated by subtracting the ratio of 520–540 nm emission over 400–475 nm emission for a vehicle-treated cell sample from the same ratio for a second aliquot of the same cells treated with agonist, as described previously. In this calculation, the vehicle-treated cell sample represents the background, eliminating the requirement for measuring a donor-only control sample. For these BRET kinetic assays, the final pre-treatment reading is presented at the zero time point (time of ligand/vehicle addition). The situation where addition of ligand specific for the untagged
GPCR results in a ligand-induced BRET signal indicates β-arrestin binding specifically to a heteromer complex.19

**Data analysis.** Statistical analysis as well as nonlinear regression analysis of saturation BRET and radioligand binding data was performed using Prism 4.03 (GraphPad Software Inc, San Diego, CA).

**Results**

**Identification of CXCR3-CXCR4 heteromers.** Physical interactions between CXCR3 and CXCR4 were assessed by co-immunoprecipitation of differentially N-terminal epitope-tagged receptors from HEK293T cells co-expressing HA-CXCR3 and FLAG-CXCR4. To this end, cell lysates were subjected to IP with anti-HA beads. Both lysates and immunoprecipitates were resolved by SDS-PAGE and probed with anti-HA or anti-FLAG antibodies. Detection of a FLAG-immunoreactive band at 39 kDa demonstrated co-immunoprecipitation of co-expressed (*i.e.* co) FLAG-CXCR4 with HA-CXCR3 (Fig. 1A lower panel), indicating that CXCR3 and CXCR4 are physically associated in a ligand-independent manner. Mixing of cells expressing either HA-CXCR3 or FLAG-CXCR4 (*i.e.* mix) prior to solubilization and anti-HA IP did not result in FLAG immunoreactivity following SDS-PAGE immunoblotting, ruling out the formation of non-specific HA-CXCR3/FLAG-CXCR4 aggregates during the solubilization process (Fig. 1A lower panel). FLAG-CXCR4 was undetectable in direct immunoblots of lysates produced from cells expressing FLAG-CXCR4 in the absence (mix) or presence of HA-CXCR3 (co), which may be the consequence of low FLAG-CXCR4 expression levels (data not shown). In contrast, HA-CXCR3 was detected in lysate as well as anti-HA IP immunoblots prepared from both mixed and co-expressed cell samples (Fig. 1A upper panel). To demonstrate the presence of HA-CXCR3/FLAG-CXCR4 heteromers on the cell surface, trFRET was measured on intact cells in parallel with the co-immunoprecipitation experiments. Intact cells co-expressing HA-CXCR3 and FLAG-CXCR4 (*i.e.* co), and mixed cells expressing either HA-CXCR3 or FLAG-CXCR4 (*i.e.* mix), were labeled with Eu³⁺-conjugated anti-HA and XL665-conjugated anti-FLAG antibodies.
HEK293T cells were transfected with HA-CXCR3 and/or FLAG-CXCR4 (500 ng/10^6 cells). Cells expressing HA-CXCR3 were collected and mixed (1:1) with cells expressing FLAG-CXCR4 (*i.e.* mix), whereas cells co-expressing HA-CXCR3 and FLAG-CXCR4 were mixed (1:1) with cells transfected with the empty vector pcDEF3 (*i.e.* co). **(A)** For co-immunoprecipitation experiments, cells were solubilized and lysates were immunoprecipitated with anti-HA beads, and both lysates and immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-HA (top) or anti-FLAG antibodies (bottom). Immunoblots shown are from a representative experiment performed three times. **(B)** For trFRET analysis, cell surface expressed HA-CXCR3 and FLAG-CXCR4 were labeled with Eu^{3+}-conjugated anti-HA and XL665-conjugated anti-Flag antibodies, respectively, and trFRET was determined by measuring emission at 665 nm 100 µs after excitation of Eu^{3+} at 337 nm. Specific trFRET between GPCR heteromers is given by the trFRETco/trFRET mix ratio. Pooled data from five independent experiments are shown. ***Co-transfected cells emitted a significantly higher FRET signal in comparison to the mix control (P<0.0001).

A higher trFRET signal was observed for cells co-expressing both receptors in comparison to cells expressing either HA-CXCR3 or FLAG-CXCR4, which were mixed prior to antibody incubation. This indicates that CXCR3 and CXCR4 indeed exist in close proximity (<10 nm) on the surface of living cells that co-express both receptors (Fig. 1B). Due to poor expression of the FLAG-CXCR3, as determined by ELISA (data not shown), the FLAG-CXCR3/HA-CXCR4 combination could not be investigated in either co-immunoprecipitation or trFRET experiments.
Figure 2. CXCR3-CXCR4 heteromers are present on the cell surface. (A) HEK293T cells were co-transfected with CXCR3-CFP and either CXCR3-Venus (left panels), CXCR4-Venus (middle panels), or GABA<sub>B2</sub>-Venus (right panels). CXCR3-CFP fluorescence images are shown in the upper panels, receptor-Venus fluorescence images are shown in the middle panels, whereas sensitized emission FRET is shown in the bottom panels. (B) Quantification of FRET efficiency from the sensitized emission FRET images.

Next, sensitized emission FRET using confocal laser scanning microscopy was used to visualize CXCR3-CXCR4 heteromers. CXCR3 and CXCR4 are colocalized at the cell surface and exist as heteromers as revealed by FRET between ECFP and Venus fluorophores that are fused to these receptors (Fig. 2A). In contrast, CXCR3 and GABA<sub>B2</sub> do not form heteromers as revealed by significantly lower FRET levels (Fig. 2B), even though both receptors are colocalized at the cell surface (Fig. 2A).

To determine relative propensities of CXCR3 and CXCR4 to form homo- and heteromers, cells were co-transfected with a constant amount of BRET donor constructs CXCR3-Rluc or CXCR4-Rluc, and increasing amounts of BRET acceptor constructs CXCR3-EYFP or CXCR4-EYFP. Hyperbolic BRET signals were observed between CXCR4-Rluc and CXCR4-EYFP (Fig. 3A), CXCR4-Rluc and CXCR3-EYFP (Fig. 3B), CXCR3-Rluc and CXCR4-EYFP (Fig. 3C), and CXCR3-Rluc and CXCR3-EYFP (Fig. 3D).
Figure 3. Hetero- and homomerization of CXCR3 and CXCR4. HEK293T cells were transiently co-transfected with a constant amount (150 ng/10^6 cells) of CXCR4-Rluc (A and B) or CXCR3-Rluc (C and D) DNA and increasing amounts of CXCR3-EYFP (B and D) or CXCR4-EYFP (A and C) (0-2200 ng/10^6 cells). Saturation curves were obtained by measuring BRET ratio as function of acceptor/donor ratio (i.e. EYFP/Rluc). Data were obtained from at least three independent experiments each performed in triplicate. Curves were fitted using nonlinear regression, assuming a single binding site.

Saturation of the BRET signal with increasing EYFP/Rluc ratios indicates that close proximity (<10 nm) between Rluc and EYFP is due to specific interactions between receptor-Rluc and receptor-EYFP fusion constructs, and not the consequence of random collisions between BRET partners. Saturated BRET (BRET_{max}) was higher between CXCR4-Rluc and CXCR4-EYFP in comparison to the other combinations. BRET_{max} was comparable between CXCR4-Rluc and CXCR3-EYFP, and CXCR3-Rluc...
and CXCR3-EYFP, whereas the CXCR3-Rluc and CXCR4-EYFP combination yielded the lowest \( \text{BRET}_{\text{max}} \) value. The \( \text{BRET}_{50} \) value of a BRET saturation curve is the EYFP/Rluc ratio resulting in half-maximal BRET, and is considered to be a measure of the relative affinity of the interacting proteins for each other. Comparable \( \text{BRET}_{50} \) values were obtained for all CXCR3 and CXCR4 homo- and heteromer combinations, indicating that CXCR3 and CXCR4 have comparable propensities to form homo- and heteromeric complexes (Table 1).

### Table 1. Saturation Bioluminescence Resonance Energy Transfer between CXCR3 and CXCR4.
HEK293T cells were transfected with a constant amount of CXCR3-Rluc or CXCR4-Rluc DNA (150 ng/10^6 cells) and increasing amounts of CXCR3-EYFP or CXCR4-EYFP DNA (10-2200 ng/10^6 cells). \( \text{BRET}_{50} \) and \( \text{BRET}_{\text{max}} \) values were determined by fitting pooled data from three or more independent experiments to a single binding site isotherm.

<table>
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<th>Combination</th>
<th>( \text{BRET}_{50} )</th>
<th>( \text{BRET}_{\text{max}} )</th>
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<tr>
<td>CXCR4-Rluc / CXCR4-EYFP</td>
<td>2.73</td>
<td>1.06</td>
</tr>
<tr>
<td>CXCR4-Rluc / CXCR3-EYFP</td>
<td>3.58</td>
<td>0.35</td>
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<td>CXCR3-Rluc / CXCR4-EYFP</td>
<td>4.11</td>
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</tr>
<tr>
<td>CXCR3-Rluc / CXCR3-EYFP</td>
<td>1.97</td>
<td>0.33</td>
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</table>

The recruitment of β-arrestin2 to CXCR3 (Fig. 4A) and CXCR4 (Fig. 4B) was assessed using BRET with Rluc8-tagged receptors co-expressed with β-arrestin2-Venus. CXCL11 resulted in clear β-arrestin2-Venus recruitment to CXCR3-Rluc8 and no CXCL12-induced signal was observed (Fig. 4A). CXCL11 was used in preference to CXCL10 in these experiments as the CXCL10-induced β-arrestin2-Venus recruitment to CXCR3-Rluc8 was very weak compared with that induced by CXCL11 (data not shown), as shown previously.22 In contrast, CXCL12 resulted in clear β-arrestin2-Venus recruitment to CXCR4-Rluc8, with no ligand-induced signal observed with CXCL11 (Fig. 4B). Combined treatment with both CXCL11 and CXCL12 in this case resulted in a similar BRET profile to that observed with CXCL12 alone.
Figure 4. Evidence for the CXCR3–CXCR4 heteromer recruiting β-arrestin2 using the GPCR-HIT assay. Extended BRET (eBRET) kinetic profiles were generated with live HEK293FT cells co-expressing CXCR3-Rluc8 and β-arrestin2-Venus (A), CXCR4-Rluc8 and β-arrestin2-Venus (B) or CXCR4-Rluc8, β-arrestin2-Venus and CXCR3 (C). These cells were treated with 100 nM CXCL11, CXCL12 or both. Data are mean ± SEM of three independent experiments.

In the GPCR-HIT configuration of CXCR4-Rluc8, β-arrestin2-Venus and CXCR3 (Fig. 4C), the CXCL12-induced BRET signal for β-arrestin2-Venus recruitment to CXCR4-Rluc8 was smaller and more transient than that observed in the absence of co-expressed non-BRET-tagged CXCR3. Such a change in profile was observed...
previously when comparing β-arrestin2-Venus recruitment to CXCR4-Rluc8 with and without untagged CCR2.\textsuperscript{19} A very weak signal was now observed with CXCL11, but most interesting was the BRET signal observed with both CXCL11 and CXCL12 with this configuration, which was substantially stronger than that observed with CXCL12 alone (Fig. 4C). As published previously for other chemokine receptor combinations, this is indicative of either β-arrestin2 recruitment being facilitated by both types of receptor in the complex being in active conformations, or that the proximity of the donor and acceptor in these complexes is sufficiently close to enable detection of changes in donor–acceptor distance and/or relative orientation resulting from both receptors being stabilized in active conformations instead of just one, both scenarios being consistent with β-arrestin2 recruitment specifically to the CXCR3-CXCR4 heteromer.\textsuperscript{19}

\textbf{CXCR3 and CXCR4 agonists display negative binding co-operativity on membranes co-expressing CXCR3 and CXCR4.} The CXCR3 chemokine CXCL10 and small CXCR3 agonist VUF10661 inhibited binding of [\textsuperscript{125}I]-CXCL10 to CXCR3-expressing membranes in a concentration-dependent manner under equilibrium conditions (Fig. 5A), which is not affected by the co-expression of CXCR4 (Fig. 5B and Table 2).\textsuperscript{21,22} As expected, the CXCR4 chemokine CXCL12 was unable to displace [\textsuperscript{125}I]-CXCL10 from membranes expressing only CXCR3. However, CXCL12 competed with [\textsuperscript{125}I]-CXCL10 for binding to membranes co-expressing CXCR3 with CXCR4 (Fig. 5B). This heterologous trans-inhibition of [\textsuperscript{125}I]-CXCL10 binding to CXCR3/CXCR4 co-expressing membranes by CXCL12 was only partial in comparison to homologous inhibition by CXCL10 (\textit{i.e.} 70 ± 8% and 100% displacement, respectively), which corresponded to the anticipated number of CXCR3 and CXCR4 being associated as homo- and heteromers. Conversely, in membranes expressing CXCR4 alone (Fig. 5C), [\textsuperscript{125}I]-CXCL12 was displaced by unlabeled CXCL12, but not by the CXCR3 agonists CXCL10 or VUF10661. However, on co-expression of CXCR3 with CXCR4, both CXCL10 and VUF10661 inhibited [\textsuperscript{125}I]-CXCL12 binding to CXCR4 (Fig. 5D).
Figure 5. CXCR3 and CXCR4 heteromers display negative ligand binding co-operativity for endogenous and low molecular weight agonists. Membranes for [\textsuperscript{125}I]uCXCL10 and [\textsuperscript{125}I]uCXCL12 binding experiments were prepared from HEK293T cells transfected with 500 ng/\(10^6\) cells CXCR3 DNA (A), 125 ng/\(10^6\) cells CXCR4 DNA (C) or co-transfected with CXCR3 and CXCR4 DNA (B and D). Competition binding experiments were performed with approximately 50 pM of [\textsuperscript{125}I]uCXCL10 (A and B) and [\textsuperscript{125}I]uCXCL12 (C and D) and increasing concentrations of the CXCR3 chemokine CXCL10, small CXCR3 agonist VUF10661 and the CXCR4 chemokine CXCL12. Graphs shown are representative of three or more independent experiments performed in triplicate.

In agreement with the expected presence of both CXCR3/CXCR4 homo- and heteromers, CXCL10 and VUF10661 could only partially displace [\textsuperscript{125}I]uCXCL12 (i.e. 53 \(\pm\) 6% and 75 \(\pm\) 3% displacement, respectively).
Figure 6. Low molecular weight antagonists of CXCR3 and CXCR4 do not have negative binding co-operativity with endogenous agonists. Membranes for [\(^{125}\)I]-CXCL10 and [\(^{125}\)I]-CXCL12 binding experiments were prepared from HEK293T cells transfected with 500 ng/10^6 cells CXCR3 DNA (A), 125 ng/10^6 cells CXCR4 DNA (C) or co-transfected with CXCR3 and CXCR4 DNA (B and D). Competition binding experiments were performed with approximately 50 pM of [\(^{125}\)I]-CXCL10 (A and B) and [\(^{125}\)I]-CXCL12 (C and D) and increasing concentrations of the CXCR3 chemokine antagonists VUF10085 and TAK-779 and the CXCR4 antagonist AMD3100. Graphs shown are representative of three or more independent experiments performed in triplicate.

The small molecule CXCR3 antagonists VUF10085\(^{20}\) and TAK-779\(^{21}\) inhibited [\(^{125}\)I]-CXCL10 binding to membranes expressing CXCR3 alone (Fig. 6A) or together with CXCR4 (Fig. 6B). Calculated IC\(_{50}\) values of both CXCR3 antagonists were not affected by the co-expression of CXCR4 (Table 2). The CXCR4 antagonist AMD3100
did not affect $^{125\text{I}}$-CXCL10 binding to CXCR3-expressing membranes (Fig. 6A). In contrast to the negative binding co-operativity between CXCL10 and CXCL12 at the CXCR3-CXCR4 heteromer, AMD3100 did not trans-inhibit $^{125\text{I}}$-CXCL10 binding to membranes co-expressing CXCR3 and CXCR4 (Fig. 6B). Similarly, $^{125\text{I}}$-CXCL12 binding to CXCR4 in membranes expressing this receptor alone (Fig. 6C) or in combination with CXCR3 (Fig. 6D) was inhibited by AMD3100 with similar potencies (Table 2), whereas VUF10085 and TAK-779 could not inhibit $^{125\text{I}}$-CXCL12 binding to either membrane preparation.

**Table 2. Binding parameters of ligands on membranes expressing CXCR3 and/or CXCR4.**

Binding parameters of the CXCR3 ligands CXCL10 (chemokine), VUF10661 (small agonist), TAK and VUF10085 (antagonists) and the CXCR4 ligands CXCR4 (chemokine) and AMD3100 (antagonist) for their cognate receptors were determined in the absence and presence of CXCR4 and CXCR3, respectively. pIC$_{50}$ values were determined using displacement of $^{125\text{I}}$-CXCL10 and $^{125\text{I}}$-CXCL12 from membrane preparations of HEK293T cells transfected with CXCR3, CXCR4 or both receptors. pIC$_{50}$ values are given as averages ± SEM of two or more independent experiments performed in triplicate.

<table>
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<th>Radioligand</th>
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<th>CXCR3</th>
<th>CXCR4</th>
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<td>$^{125\text{I}}$-CXCL10</td>
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<td>9.6 ± 0.1</td>
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<td>TAK-779</td>
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<td></td>
<td>AMD3100</td>
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<td></td>
<td>$B_{max}$ (fmol/µg)</td>
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<td>0.26 ± 0.04</td>
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<td>ND</td>
<td>ND</td>
<td>8.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>CXCL12</td>
<td>ND</td>
<td>9.5 ± 0.1</td>
<td>9.5 ± 0.2</td>
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<tr>
<td></td>
<td>VUF10661</td>
<td>ND</td>
<td>ND</td>
<td>6.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>VUF10085</td>
<td>ND</td>
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<td>TAK-779</td>
<td>ND</td>
<td>ND</td>
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<td>AMD3100</td>
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<td>6.8 ± 0.2</td>
<td>7.3 ± 0.1</td>
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<td></td>
<td>$B_{max}$ (fmol/µg)</td>
<td>ND</td>
<td>1.58 ± 0.15</td>
<td>1.08 ± 0.14</td>
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CXCR3-CXCR4 heteromerization alters ligand-binding kinetics. Trans-inhibition of CXCL12 equilibrium binding to CXCR4 by both CXCL10 and VUF10661 suggests an allosteric mode of action between agonist-occupied CXCR3 and CXCR4, rather than steric hindrance between these agonists to bind receptor heteromers. To confirm allosteric interactions between the CXCR3 and CXCR4 ligand binding sites, [\(^{125}\)I]-CXCL12 dissociation rates from CXCR4 and CXCR3-CXCR4 heteromers were determined in the absence or presence of CXCR3 agonists, using an infinite radioligand dilution approach. To this end, membranes were pre-equilibrated with [\(^{125}\)I]-CXCL12, and free [\(^{125}\)I]-CXCL12 was removed by centrifugation. Dissociation kinetics of bound [\(^{125}\)I]-CXCL12 was measured upon 100-fold dilution in binding buffer in the absence or presence of unlabeled chemokines.

**Figure 7. Heteromerization of CXCR3 and CXCR4 increases the dissociation rate of CXCL12.** [\(^{125}\)I]-CXCL12 dissociation half-life was determined in HEK293T membranes expressing CXCR4 (A) alone or (B) in combination with CXCR3, in the absence (asterisk with dotted line) and presence of the CXCR3 endogenous agonist CXCL10 (open circles), the small CXCR3 agonist VUF10661 (open squares), and the CXCR4 chemokine CXCL12 (closed circles). Representative graphs of three or more independent experiments performed in triplicate are shown.

Dissociation of [\(^{125}\)I]-CXCL12 from membranes expressing CXCR4 alone was significantly accelerated in the presence of 100 nM unlabelled CXCL12 as compared to the basal dissociation rate in binding buffer (Fig. 7A and Table 3), suggesting negative binding co-operativity within CXCR4 homomers. In agreement with
equilibrium binding (Fig. 5C), both CXCR3 agonists did not affect CXCL12 dissociation in the absence of CXCR3 (Fig. 7A). Interestingly, CXCR3 co-expression accelerated basal CXCL12 dissociation from CXCR4, which was further accelerated by 100 nM CXCL10 or 10µM VUF10661 (Fig. 7B and Table 3). The very rapid dissociation of [\(^{125}\)I]uCXCL10 from CXCR3 in the absence or presence of CXCR4 did not allow the measurement of accelerated effects of unlabeled ligands within this short time frame (data not shown).

Table 3. [\(^{125}\)I]CXCL12 dissociation half-life from CXCR4 is decreased in the presence of CXCR3. Results shown are average ± SEM of three or more independent experiments performed in triplicate.

<table>
<thead>
<tr>
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<th>t(_{1/2}) ± SEM (min)</th>
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<tr>
<td>Buffer</td>
<td>93 ± 15</td>
</tr>
<tr>
<td>CXCL10</td>
<td>81 ± 9</td>
</tr>
<tr>
<td>CXCL12</td>
<td>12 ± 1(^{*})</td>
</tr>
<tr>
<td>VUF10661</td>
<td>83 ± 25</td>
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</table>

\(^{*}\) t\(_{1/2}\) differs significantly from t\(_{1/2}\) in presence of vehicle (p < 0.05)

\(^{\#}\) t\(_{1/2}\) differs significantly from t\(_{1/2}\) in membranes expressing CXCR4 alone (p < 0.05)

Negative binding co-operativity between CXCR3 and CXCR4 chemokines is not apparent in intact cells. High affinity agonist binding to chemokine receptors requires the coupling of G proteins, as revealed by decreased membrane binding of CXCL12 to CXCR4 and CXCL10 to CXCR3 upon G protein uncoupling using GTP\(_\gamma\)S and pertussis toxin, respectively.\(^7,26,33,34\) Hence, negative co-operativity observed in equilibrium binding experiments on isolated membrane fractions between two agonists that interact with different receptors, may be the consequence of G protein scavenging, if these receptors couple to the same and limited pool of G proteins.\(^35,36\) In contrast, to our observations on isolated membranes, CXCL10 and CXCL12 do not affect each other’s equilibrium binding on intact cells co-expressing CXCR3 and CXCR4 at comparable levels (Fig. 8).
Figure 8. Chemokines display no negative binding co-operativity on intact cells. Binding of approximately 50 pM $[^{125}\text{I}]$uCXCL10 (A) or $[^{125}\text{I}]$uCXCL12 (B) was measured on intact HEK293T cells transiently transfected with 500 ng CXCR3 DNA/10$^6$ cells (open bars), 125 ng CXCR4 DNA/10$^6$ cells (hatched bars), or co-transfected with CXCR3 and CXCR4 DNA (closed bars). Graphs show mean ± SEM of two or more independent experiments performed in triplicate.

Discussion and conclusions

The chemokine receptors CXCR3 and CXCR4 are implicated in the pathogenesis of autoimmune disease and various cancers, and are gaining recognition as attractive targets for therapeutical intervention.\textsuperscript{13,14,37} Interestingly, chemokines and small antagonists that specifically interact with either CCR2 or CCR5 inhibited CXCL12-induced CXCR4 activity, and \textit{vice versa}, provided that these chemokine receptors are co-expressed.\textsuperscript{5,6} This cross-inhibition is observed in both recombinant and native cells, and is the consequence of negative binding co-operativity between chemokine receptors that are situated in heteromeric complexes. Since CXCR3 and CXCR4 are co-expressed on various immune and cancer cell types, we investigated whether these chemokine receptors heteromerize and display negative ligand-binding co-operativity. In the present study, we demonstrated that CXCR3 and CXCR4 form heteromers using the following five different technologies: co-immunoprecipitation, trFRET, FRET, saturation BRET, and/or GPCR-HIT. The
detection of CXCR4 homomers is in agreement with previous studies.\textsuperscript{5,6,38-42} On the other hand, the detection of CXCR3 homomers and CXCR3/CXCR4 heteromers is in conflict with the conclusions of a recent study in which homo- and heteromerization of CXCR3 and CXCR4 was evaluated using BRET analyses.\textsuperscript{39} In that study, various combinations of chemokine receptors fused to either Rluc or green fluorescent protein (GFP) were co-expressed in cells at equal luminescence and fluorescence levels, respectively. High BRET was observed between CXCR4-Rluc and CXCR4-GFP, whereas the significantly lower BRET levels between CXCR3/CXCR3, CXCR3/CXCR4, CXCR2/CXCR2, CXCR2/CXCR4 and CCR5/CXCR4 were considered to be marginal. On the basis of these results, Hamatake \textit{et al.} concluded that only CXCR4 is situated in higher order complexes.\textsuperscript{39} Indeed, we and others observed higher BRET levels for CXCR4 homomers as compared to CXCR4 heteromers and homomers between CCR2, CCR5 and CXCR3 in saturation BRET experiments.\textsuperscript{6,42} However, BRET levels per se cannot be used as a quantitative measure of the relative number of complexes being formed between different receptor combinations, as BRET is not only determined by numbers of complexes but also heavily depends on the close proximity and relative orientation between the BRET donor (\textit{i.e.} Rluc) and acceptor (\textit{i.e.} GFP or EYFP) proteins within each of the different receptor complexes. On the other hand, the relative propensity of receptors to form complexes can be extracted from saturation BRET analysis as the BRET\textsubscript{50} value, which corresponds to the BRET acceptor/donor ratio of receptor fusion proteins resulting in 50\% of the saturated BRET signal.\textsuperscript{32} The BRET\textsubscript{50} values were comparable for CXCR3-CXCR3, CXCR3-CXCR4, and CXCR4-CXCR4 complexes in our experiments, suggesting that CXCR3 and CXCR4 have comparable probability to form homo- and heteromers. Likewise, comparable BRET\textsubscript{50} values have been observed for homo- and heteromers between chemokine receptors CCR2 and CCR5\textsuperscript{4,7}, CCR2 and CXCR4\textsuperscript{5,42}, CCR5 and CXCR4\textsuperscript{6,43}, CXCR1 and CXCR2\textsuperscript{44}, and CXCR4 and CXCR7\textsuperscript{45}. Importantly, chemokine receptor homo- and heteromerization were confirmed in these studies by other biophysical,
biochemical, and/or pharmacological evidence. Negative binding co-operativity has been described within heteromers of CCR2, CCR5, or CXCR4.4-7 In this study, we showed that negative binding co-operativity also occurs within CXCR3-CXCR4 heteromers, as binding of the chemokines CXCL10 and CXCL12 is mutually exclusive on membranes co-expressing these receptors. Moreover, inhibition of CXCL12 binding to CXCR4 by the small CXCR3 agonist VUF10661 suggests that the observed negative binding co-operativity is not due to steric hindrance, but rather results from agonist-induced conformational changes transmitted from one receptor to the other within CXCR3-CXCR4 heteromers. Such transmission of conformational changes across receptor pairs has been directly shown for the norepinephrine-occupied "α2A"-adrenergic receptor upon morphine binding to the associated µ-opioid receptor within the heteromer using intramolecular FRET analysis.46 Hence, agonist binding to CXCR3 constrains CXCR4 to a conformation with lower affinity for CXCL12 and vice versa. Negative binding co-operativity within CXCR3/CXCR4 heteromers results in partial inhibition of radiolabeled chemokine binding in equilibrium binding experiments, which reflects the proportion of CXCR3/CXCR4 heteromers next to homomers of both receptor types. This is in agreement with the comparable propensities of CXCR3 and CXCR4 to form homo- and heteromers as observed in our saturation BRET analyses. Interestingly, negative binding co-operativity within CXCR3/CXCR4 heteromers is limited to agonists, which contrasts with previous observations on CCR2/CXCR4 and CCR5/CXCR4 heteromers, in which small antagonists cross-inhibited both chemokine binding and chemokine-induced in vitro and in vivo activity.5,6 This discrepancy may be related to distinct interfaces between various chemokine receptor pairs, resulting in different efficiencies with which a conformational change in one protomer is conveyed to the other. Hence, our experimental data suggest that CXCR3 or CXCR4 antagonists do not therapeutically cross-inhibit chemokine-induced activation of the other receptor within the heteromer. The validity of negative binding co-operativity detected in membrane preparations expressing two GPCRs has recently been questioned.36 Based on a catalytic model,
Chabre et al. argued that G protein coupling to an agonist-occupied receptor is almost irreversible in equilibrium binding assays on membrane preparations, as free GTP is not available to occupy the empty nucleotide binding pocket in the G protein upon GDP release in this experimental setup. Since the pool of shared G proteins might be smaller than the total number of cognate receptors, this agonist-induced or constitutive G protein scavenging by one of the receptor subtypes results in a permanent depletion of G proteins from other receptors. As a consequence, the latter GPCRs often display low agonist binding affinity, which is observed as apparent negative binding co-operativity between two agonists of co-expressed GPCRs but in fact does not necessarily require receptor heteromerization. Sohy et al. confirmed negative binding co-operativity between CCR2, CCR5, and CXCR4 chemokine receptor heteromers in both recombinant and native intact cells in which GTP is readily available in the cytoplasm. In contrast, CXCL10 and CXCL12 did not affect each other’s binding to intact HEK293T cells that co-express CXCR3 and CXCR4. Furthermore it could be argued that the results of the GPCR-HIT studies, again in live cells, are also more consistent with a lack of negative co-operativity as we observe greater BRET signals with dual agonist treatment, although we have previously suggested that these findings could be reconciled because the significant conformational changes believed to result in negative binding co-operativity may also influence the ability of the heteromer to recruit β-arrestin2. The contradiction between intact cells and isolated membranes indeed suggests G protein scavenging in the latter format as proposed by Chabre et al. Yet, this “G protein scavenging” model cannot explain the increased dissociation rate of CXCL12 from membranes co-expressing CXCR3 and CXCR4 in the presence of CXCR3 agonists. This decreased affinity of CXCR4 for bound CXCL12 can only be explained by allosteric interactions between CXCR4 and CXCR3 upon binding of agonists to the latter, as initial binding of radiolabeled CXCL12 takes place in the absence of these CXCR3 agonists, and is consequently not hampered by G protein availability. To distinguish direct allosteric interactions between these receptors from downstream crosstalk events such as G protein
scavenging, intramolecular FRET approaches might be applied on CXCR3 and CXCR4 in the future.\textsuperscript{46,47} At present, however, we cannot explain the observed discrepancy in CXCR3/CXCR4 binding co-operativity.

In summary, we show that CXCR3 and CXCR4 form heteromers at the cell surface using multiple experimental approaches. CXCR3 co-expression increased the dissociation of CXCL12 from CXCR4 membranes, which is further accelerated by CXCR3 agonists. Although specific CXCR3 and CXCR4 agonists inhibit each other’s equilibrium binding on isolated membranes as well, this apparent negative binding co-operativity was not observed on intact cells.

\section*{Acknowledgements}

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\section*{Conflicts of interest}

In addition to being Head of the Laboratory for Molecular Endocrinology–GPCRs, Western Australian Institute for Medical Research and Centre for Medical Research, University of Western Australia, K.D.G.P. is Chief Scientific Officer of Dimerix Bioscience Pty Ltd, a spin-out company of the University of Western Australia that has been assigned the rights to the “GPCR-HIT” technology. K.D.G.P. has a minor shareholding in Dimerix.

\section*{References}


