Fluorescence Resonance Energy Transfer Imaging Reveals that Chemokine-Binding Modulates Heterodimers of CXCR4 and CCR5 Receptors

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Abstract

Background: Dimerization has emerged as an important feature of chemokine G-protein-coupled receptors. CXCR4 and CCR5 regulate leukocyte chemotaxis and also serve as a co-receptor for HIV entry. Both receptors are recruited to the immunological synapse during T-cell activation. However, it is not clear whether they form heterodimers and whether ligand binding modulates the dimer formation.

Methodology/Principal Findings: Using a sensitive Fluorescence Resonance Energy Transfer (FRET) imaging method, we investigated the formation of CCR5 and CXCR4 heterodimers on the plasma membrane of live cells. We found that CCR5 and CXCR4 exist as constitutive heterodimers and ligands of CCR5 and CXCR4 promote different conformational changes within these preexisting heterodimers. Ligands of CCR5, in contrast to a ligand of CXCR4, induced a clear increase in FRET efficiency, indicating that selective ligands promote and stabilize a distinct conformation of the heterodimers. We also found that mutations at C-terminus of CCR5 reduced its ability to form heterodimers with CXCR4. In addition, ligands induce different conformational transitions of heterodimers of CXCR4 and CCR5 or CCR5STA and CCR5

Conclusions/Significance: Taken together, our data suggest a model in which CXCR4 and CCR5 spontaneously form heterodimers and ligand-binding to CXCR4 or CCR5 causes different conformational changes affecting heterodimerization, indicating the complexity of regulation of dimerization/function of these chemokine receptors by ligand binding.

Introduction

Chemokine receptors are members of the superfamily of G-protein-coupled receptors (GPCRs), which posses seven transmembrane domains that are interconnected by multiple extracellular and intracellular loops, and an intracellular C-terminal tal [1]. Chemokines are a large family of small proteins that mediate recruitment of leukocytes to sites of inflammation and coordinate their trafficking throughout the human body [2,3]. Gradients of chemokines that are detected by their receptors control cell traffic in homeostasis and inflammation in vivo [3]. Chemokines regulate leukocyte function by binding to specific chemokine receptors expressed on their surface, typically leading to the activation of receptor-associated Janus tyrosine kinases (JAKs) and the heterotrimeric G-protein Gs [4–7]. One basic question is how different chemokine receptors receive and transduce signals from the surface of a cell on which multiple GPCRs are expressed. Initially, GPCRs were believed to signal as simple monomers [8,9]. However, mounting evidence now indicates that many GPCRs, including several chemokine receptors, function as dimers or higher-order oligomers [8,9].

CXCR4 and CCR5 receptors regulate leukocyte chemotaxis in inflammation and also serve in conjunction with CD4 as co-receptors for HIV entry [1,3]. CXCR4 normally functions as the receptor for the chemokine CXCL12/SDF-1, whereas CCR5 mediates responses to several chemokines, including CCL3/MIP1-α, CCL4/MIP1-β and CCL5/RANTES [2]. CXCR4 and CCR5 are co-expressed in several leukocyte populations including lymphocyte and monocytes [3,10]. In addition to their roles in regulating leukocyte chemotaxis, CXCR4 and CCR5 serve as the entry co-receptors for T-tropic or M-tropic strains of HIV virus, respectively. Upon the binding of envelope protein gp120, CD4 receptor physically associates with either CXCR4 or CCR5 receptors to initiate the formation of the HIV entry complex [11,12]. CXCR4 or CCR5 can also form heterodimers with other GPCR receptors for initiation or alteration of signaling by these involved receptors. For example, CXCR4 and the δ-opioid receptor (DOR), both of which are expressed on the surface of monocytes and other immune cells, form heterodimers the presence of ligands for each receptor. The formation of the CXCR4:DOR heterodimer prevents each of them from signaling [6]. CCR5 and CCR2 can form heterodimers on the surface of the
cells when they are stimulated with both CCL5 and CCL2 (ligands of CCR5 and CCR2, respectively) [5]. The CCR5:CCR2 heterodimers activate heterotrimERIC-G-protein Gq/11, instead of Gq, which is activated by CCR5 or CCR2 alone [3]. It appears that heterodimerization in response to chemokine binding is required for the termination or alteration of signaling by an increasing number of chemokine receptors [13].

CCR4 and CCR5 are expressed on the surface of T lymphocytes and, during T cell activation, are both recruited to the immunological synapse (IS). This recruitment requires chemokine secretion by antigen-presenting cells (APCs) [14]. Therefore, it has been proposed that APC-derived chemokines promote formation of CXCR4:CCR5 heterodimers, resulting in accumulation of these receptors at the IS. Despite the important roles of CXCR4 and CCR5 in chemotaxis, HIV entry, and T cell activation, it is still not clear whether CXCR4 and CCR5 form heterodimers on the surface of live cells.

In this report, we investigated the formation of heterodimers between CXCR4 and CCR5 on the surface of live cells using FRET imaging coupled with quantitative microscopic analyses. CXCR4 was tagged with CFP (FRET donor), and CCR5 and two CCR5 mutants with altered C-termini, CCR5STA and CCR5AA, were fused with YFP (FRET acceptor). We observed that CXCR4CFP, CCR5YFP, CCR5STA-YFP and CCR5AA-YFP could be expressed on the surface of live cells. When co-expressed, CXCR4CFP and CCR5YFP displayed a high level of FRET signal, suggesting that mutations in the C-terminus of CCR5 caused a decrease in CCR5’s ability to form dimers with CXCR4. Furthermore, CCR5 chemokines, CCL5/MIP1α or CCL5/RANTES, induced a clear increase, while CXCR4 ligand, CXC12/SDF-1, triggered a decrease in FRET between CXCR4CFP and CCR5YFP, suggesting that the binding of these chemokines differentially modulates the stability or conformation of CXCR4:CCR5 heterodimers in the plasma membrane.

Materials and Methods

Chemicals and reagents

pEYFP-N1 and pECFP-N1 were purchased from Clontech (Palo Alto, CA). Lipofectamine 2000 was purchased from Invitrogen (Grand Island, NY). CCL3/MIP1α, CCL5/RANTES and CXCL12/SDF1α were purchased from BioSource (Camarillo, CA). Fluor-4-AM was from Molecular Probes (Eugene, OR). Anti-GFP monoclonal antibody (JL-8) was from BD Biosciences Clontech (Palo Alto, CA). All of the other reagents were of reagent grade and were obtained from standard suppliers.

Plasmid, cell line, and transfection

Human CXCR4 and CCR5 gene were generated by PCR. Plasmids carrying mutant receptors CCR5STA and CCR5AA were generous gifts from Dr. Murphy’s group at NIAID, NIH. The plasmids encoding CCR5YFP, CCR5STA-YFP and CCR5AA-YFP were constructed by inserting the PCR product of CCR5, CCR5STA and CCR5AA into the pEYFP-N1 vectors in multicloning sites. The plasmid encoding CXCR4CFP was constructed by inserting the PCR product of CXCR4 into the pECFP-N1 vector. HEK293T cells were cultured in Dulbecco modified Eagle medium supplemented with fetal calf serum (10%), penicillin (5 μg/ml), and streptomycin (5 μg/ml) and were grown in 5% CO2 at 37°C. HEK293T cells were transfected or co-transfected with the plasmids encoding CCR5YFP, CCR5STA-YFP, CCR5AA-YFP and/or CXCR4CFP mediated by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions [16].

Calcium assay

HEK293T cells were seeded in four-well chambers at 10^4/ml, 24–36 hrs before the experiments. After 3 hours of starvation, the cells were labeled by incubation with Fluor-4-AM in Hanks balanced salt solution for half an hour, washed twice, and incubated for half an hour before being taken images under the microscope. Upon the addition of SDF-1 (50 nM) or RANTES (50 nM) to the cell chamber, time-lapse images were collected with multi-track mode (Zeiss 510), and CFP and Fluor-4 images were digitally separated. Changes in Ca2⁺ concentration were represented as the changes in the intensity of Fluor-4 (I/I0, where I is the intensity at time t, and I0 is the intensity at time 0) as previously described [7].

Imaging and FRET assay

Cells were washed twice with 1×HBSS and then starved in 1×HBSS+1%BSA for 3 hrs. Before imaging, the cells were treated with chemokines for 20 min. Zeiss Plan-apochromat 40× oil immersion objective was used for image acquisition. Images were collected with multi-track mode (Zeiss 510). In Track I, there were two channels, cells were excited with 458 nm, CFP emission signals were collected through Channel I (475–525 nm) and FRET emission signals were collected through Channel II (>530 nm). In Track II, there was only one YFP channel, YFP emission signals were collected with this channel (>530 nm). FRET efficiency between CFP and YFP was analyzed using Zeiss LSM Software.

Intermolecular FRET efficiency was shown as N-FRET using macro of Zeiss LSM Software. Briefly, Sensitized Emission bleed-through (or crosstalk) coefficients were determined using control cells that expressed only CFP or YFP and expressed as correction factors as follows. Donor coefficients Fd/Dd: the amount of crosstalk of donor signal into the FRET channel. Acceptor coefficients: Fa/Aa: the amount of crosstalk of the acceptor signal into the acceptor channel. Acceptor coefficients: Fa/Aa: the amount of crosstalk of the acceptor signal into the FRET channel. Da/Fd: the amount of crosstalk of donor signal into the FRET channel. Da/Fd: the amount of crosstalk of the acceptor signal into the donor channel. Da/Fa: the amount of crosstalk of the donor signal into the donor channel. Da/Fa: the amount of crosstalk of the FRET channel or the donor channel. Display N-FRET image with intensities that is converted from the FRET index is calculated for each pixel by LSM FRET tool for Carl Zeiss AIM software using the method Xia and Liu [17]. For quantification of N-FRET, regions of interest (ROIs) covering the plasma membrane from the acquired images were chosen, processed as above, and calculated automatically using the FRET macro of LSM imaging software as previously described [16]. Means and SD are shown. Statistical significance was determined with Student’s t-test.

Results

Expression of CXCR4, CCR5, and mutant CCR5 receptors tagged with fluorescent proteins

To investigate the distributions of CXCR4 and CCR5 in the plasma membrane of live cells, we fused CFP to the C-terminus of CXCR4, and YFP to the C-terminus of CCR5. It is well known that upon activation of CCR5 the C-terminal tail interacts with GPCR kinase(s) and arrestin to carry out receptor functions [9]. In this study, we selected two CCR5 mutants, CCR5STA and CCR5AA, and fused YFP to their C-termini. CCR5STA is a CCR5 mutant in which all serines and threonines in the C-terminal tail were replaced by alanines; while CCR5AA is a mutant

Dimerization of CXCR4 and CCR5
in which the last 46 amino acids (307–352 a.a.) of the C-terminus were removed [15]. HEK293 cells were transfected with the CXCR4CFP, CCR5YFP, CCR554YFP or CCR55A4YFP constructs and expression of appropriately sized fusion proteins was verified by western blotting with anti-GFP antibodies (Figure 1A). Fluorescence microscopy revealed that the majority of CXCR4CFP, CCR5YFP, CCR55A4YFP was expressed uniformly on the plasma membrane (Figure 1B). The surface expression of CCR55A4YFP, on the other hand, was less efficient compared to the other tagged receptors. Consistent with a previous report [15], CCR55A4YFP was frequently localized to the interior of transfected cells (data not shown). However, it was expressed on the cell surface in a small fraction of the cells under our experimental condition, which allowed us to carry out the measurement of CCR55A4YFP on the surface of live cells (Figure 1B).

Functional characterization of the tagged receptors

To test the functionality of CXCR4CFP, we examined ligand-induced Ca2+ responses in the cells that expressed CXCR4CFP on the cell surface (Figure 2A). We imaged fluorescence intensity change of Fluo-4, a calcium indicator, triggered by the CXCR4 ligand SDF-1. Using a confocal fluorescence microscope (Zeiss 510 META), fluorescence images of CXCR4CFP and Fluo-4 were simultaneously recorded in a time-lapse experiment (Figure 2A). CXCR4CFP primarily localized to the cell surface, while Fluo-4 was distributed throughout the cytosol. Upon addition of SDF-1 to the cell chamber, the Fluo-4 fluorescence signal transiently increased in the cytosol of the CXCR4CFP cells but not that of the parental HEK293 cells, indicating that the CXCL12/SDF-1-elicited Ca2+ response was specifically mediated by the expressed CXCR4CFP (Figure 2A and 2B). Our previous study demonstrated that the CCR5 receptor fused with CFP at its C-terminus is also functional in the ligand-induced Ca2+ response [16]. We also investigated ligand-induced Ca2+ response in cells expressing CCR55A4YFP and CCR55A4YFP under the same live cell imaging conditions (Figure 2C). Upon addition of MIP1α, a ligand for CCR5, to the cell chamber, Fluo-4 fluorescence signal increased in the cells expressing CCR5-YFP, CCR55A4YFP and CCR55A4YFP, indicating that these tagged receptors also retained their ligand-binding and signaling functions (Figure 2C). However, CCR55A4YFP and CCR55A4YFP triggered the Ca2+ responses with different kinetics compared with that induced by CCR5YFP, indicating that mutations of the C-terminal tail of CCR5 affected its ability in signaling (Figure 2C). Taken together, cells expressing CXCR4CFP, CCR5YFP, CCR55A4YFP and CCR55A4YFP provide a system for probing extracellular ligand-induced changes in

Figure 1. Expression of CXCR4 or CCR5 and CCR5 mutant receptors tagged with fluorescent proteins. A. The indicated CFP- or YFP-tagged receptors, transiently expressed in HEK293 cells, were detected in whole cell lysates by western blotting with an anti-GFP antibody. Untransfected HEK293 cells were included as a negative control. B. Schematic diagram of CXCR4CFP, CCR5YFP, CCR55A4YFP and CCR55A4YFP on the cell membrane. Confocal images of living cells expressing membrane-localized CXCR4CFP (cyan), CCR5YFP (red), CCR55A4YFP (red) and CCR55A4YFP (red); top panels, fluorescence images. Bottom panels, the merged images of the fluorescence and differential-interference-contrast (DIC) images. Scale bar, 10 μm.

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Co-localization of CXCR4 and CCR5 or CCR5 mutant receptors

We investigated membrane distribution of CXCR4 and CCR5 or CCR5 mutants in HEK293 cells that co-expressed CXCR4CFP and either CCR5YFP, CCR5STAYFP or CCR5D4YFP. Using multitrack and line-scanning mode of a laser-scanning confocal microscope, cells were simultaneously recorded in the CFP and YFP detection channel. We observed that CXCR4CFP co-localized on the cell surface with each of the CCR5 variants (Figure 3). However, this co-localization does not prove that the receptors are physically associated given that the spatial resolution of light microscopy is more than 200 nm. Therefore, we used the FRET imaging method to determine if CXCR4CFP was in close proximity to CCR5YFP, CCR5STAYFP and CCR5D4YFP on the cell surface.

CXCR4 and CCR5 exist as preformed dimers on the cell surface

To examine interactions between CXCR4 and CCR5, we measured FRET between CFP and YFP in cells co-expressing CXCR4CFP and CCR5YFP. For this we used confocal microscopy and the sensitized emission method to calculate FRET efficiency between the CFP and YFP moieties. Using a multitrack and line-scanning mode of a laser-scanning confocal microscope, cells were simultaneously recorded in the CFP and YFP, and FRET detection channels. Fluorescence was simultaneously collected pixel-by-pixel from three detection channels: CFP (458 nm, CFP excitation, 475–525 nm, CFP emission); FRET (458 nm, CFP excitation; long pass filter 530 nm, YFP emission); and YFP (514 nm, YFP excitation, long pass filter 530 nm, YFP emission) (Figure 4A). We first obtained bleed-through (cross talk) co-efficient by analyzing images from cells expressing only CXCR4CFP or CCR5YFP, which were imaged with the identical configuration and scanning setup as the controls (Figure 4B). We then obtained normalized FRET (N-FRET) efficiency in the plasma membrane of cells expressing both CXCR4CFP and CCR5YFP. Using the FRET analysis tool, the FRET macro for the Ziess LSM 510 META microscope, the normalized FRET (N-FRET) image with intensities was converted from the FRET index calculated from each pixel as previously described [16,17] (details in Materials and Methods).

An advantage of using confocal microscopy to evaluate FRET is that individual region of interests (ROIs) within a cell can be selectively examined for FRET efficiency [18]. In contrast, fluorometric [19] or flow cytometric [20,21] approaches can only
measure total cellular FRET, which often includes concentrations of fluorophores in intracellular compartments. Because we were interested in CXCR4 and CCR5 interactions at the cell surface, only the plasma membrane region of the cell was selected as the ROIs (Figure 3C). FRET efficiency is usually sensitive to the relative amounts of donors and acceptors in the selected regions. However, we took the following steps to minimize the possibility that our measurements were strongly influenced by high levels of receptor expression. First, all the FRET images were recorded using identical parameters, including excitation laser intensity, detector gains and magnification. Therefore, the levels of CXCR4CFP and CCR5YFP, CCR5STAYFP or CCR5D4YFP were clearly monitored pixel-by-pixel by CFP and YFP intensities.

Second, in our analyses, we selected regions having mean CFP and YFP intensities between 600 and 2800 to ensure the proper receptor levels. Finally, FRET values were normalized pixel-by-pixel by dividing by the square root of the donor and acceptor concentrations to yield N-FRET values. We have previously shown that such N-FRET values are relatively independent of the CFP or YFP concentrations and, therefore, likely reflect receptor interactions [16,22].

We used tagged receptors for IL17 and TNF (IL17RA-YFP and TNFR-CFP), which both localize to the cell membrane and do not associate as a receptor complex, as negative controls for determining the baseline for FRET efficiency [20,22]. Strikingly, cells expressing CXCR4CFP and CCR5YFP showed a marked enhancement of FRET (Figure 4B and 4D). In clear contrast, cells that expressed membrane-localized CXCR4CFP and CCR5D4YFP showed very low FRET intensity, while cells expressing CXCR4CFP and CCR5STAYFP displayed moderate FRET signal (Figure 4B and 4D). Quantitative analyses of FRET efficiency indicated that normalized FRET (N-FRET) between CXCR4CFP:CCR5YFP (n = 198) was significantly greater than in the case of CXCR4CFP:CCR5D4YFP (n = 148, p < 0.01) or the negative control (n = 58, p < 0.01) (Figure 4D). In addition, the intermediate N-FRET value obtained for CXCR4CFP:CCR5STAYFP (n = 156) was significantly lower than that of CXCR4CFP:CCR5YFP (p < 0.01) but still higher than those of CXCR4CFP:CCR5D4YFP (p < 0.01) and the negative control (p < 0.01). On the other hand, N-FRET of CXCR4CFP:CCR5STAYFP did not significantly differ from that of the control (p > 0.01). Together, these data suggest that CCR5 dimerizes with CXCR4 on the plasma membrane in the

![Figure 4](image-url). An analysis of CXCR4 and CCR5 association on the surface of live cells by FRET imaging. A. Schematic diagram of FRET measurement between CFP-tagged CXCR4 and YFP-tagged CCR5. When cells were excited at 458 nm, emissions are simultaneously recorded in CFP channel and FRET channel, and when cells were excited at 514 nm, emissions were recorded in YFP channels. We used multi-channel and line-scanning mode so that images of three channels were recorded simultaneously. Cells expressing only CXCR4CFP or CCR5YFP were used as controls for calculating real FRET efficiency that is expressed as normalized FRET (N-FRET). B. Images of cells expressing only CXCR4CFP or CCR5YFP; CXCR4CFP with CCR5YFP, and CCR5STAYFP or CCR5D4YFP. N-FRET images show FRET intensities. C. FRET efficiency, N-FRET, in the plasma membrane was measured in selected regions of interest (ROIs). D. Quantitative analysis of N-FRET on cells expressing CXCR4CFP and CCR5YFP (n = 198), CCR5STAYFP (n = 156) or CCR5D4YFP (n = 148) is shown as means ± S.D. Cells expressing IL17RA-YFP and TNFR-CFP (n = 58) were used as negative control. Statistical significance was assessed using a t-test. doi:10.1371/journal.pone.0003424.g004
absence of ligands and, moreover, that the C-terminal tail mutations of CCR5^ST4 or CCR5^M4 reduce or abolish the receptor’s ability to form pre-existing dimers with CXCR4.

**Ligand binding to CCR5 or CXCR4 differentially affects heterodimers**

To examine the effects of CCR5 and CXCR4 ligands on the pre-existing dimers, we measured the FRET efficiency between CXCR4:CFP and CXCR4:YFP upon stimulation with CCL3/MIP1α and CCL5/RANTES (ligands of CCR5) and CXCL12/SDF-1 (a ligand of CXCR4). Relative to the unstimulated control, both of the CCR5 ligands induced N-FRET increases between CXCR4:CFP and CXCR4:YFP of roughly 30% (p<0.01 for both), while CXCL12/SDF-1 (n = 119) triggered a slight decrease in the N-FRET signal (p<0.01) (Figure 5A and 5B). These results suggest that ligands binding to each receptor comprising the heterodimer distinctly alter its conformation. In contrast to the results obtained with CCR5:YFP, addition of CCL3/MIP1α (n = 148) in the absence of ligands, addition of CCL3/MIP1α (n = 85) and CXCL12/SDF-1 (n = 96) caused clear increases in N-FRET between these receptors (p<0.01 for both), suggesting that binding of either CXCL12/SDF-1 to CXCR4 or CCL3/MIP1α (but not CCL5/RANTES n = 138, p>0.05) to CCR5^SA4 promotes the formation of CXCR4:CXL5^AA heterodimers. Taken together, our results suggest that the formation of heterodimers between CXCR4 and CCR5 is a dynamic process and that ligand binding may cause conformational changes in pre-existing CXCR4:CCR5 dimers, destabilize CXCR4:CCR5^ST4 heterodimers or promote CXCR4:CCR5^AA heterodimer formation.

**Discussion**

In recent years, a number of GPCRs have been shown to exist as dimers in the plasma membrane, raising a number of interesting questions regarding the molecular dynamics and functional significance of receptor dimer formation [9]. One of the highly debated questions is whether dimerization in the cell membrane occurs spontaneously or is induced by ligand binding. The chemokine receptors CXCR4 and CCR5 regulate leukocyte chemotaxis and are also instrumental in the entry of HIV into immune cells [12,23]. However, their dimerization properties have not been clearly defined. In this study using a fluorescence resonance energy transfer imaging, we provide the first evidence that CXCR4 and CCR5 form heterodimers on the plasma membrane in the absence of chemokines and that their chemokine ligands induced different conformational changes that either promote or destabilize the formation of heterodimers.

Our results indicate that CXCR4 and CCR5 heterodimers exist on the surface of cells in the absence of ligand stimulation and that point mutations within or deletion of the C-terminus of CCR5 reduce the ability of the receptor to form heterodimers with CXCR4 (Fig. 3). CCR5 has been previously shown to form homodimers and heterodimers with CCR2 chemokine receptor [5]. Previous studies indicated that transmembrane regions TM1, TM2 and TM4 of CCR5 are involved in the homodimer formation. Several amino acids, such as Ile52 in TM1 and Val150 in TM4, have been shown to be crucial for CCR5 function and homodimerization [24]. The role of the C-terminus of CCR5 has been examined in receptor surface expression, receptor signaling and HIV entry. The C-terminal deletion mutants reduced the surface expression [15,25,26]. We found the CCR5^ST4 mutant was expressed on the cell surface, and its chemokine-induced Ca^2+ response prolonged, which is consistent with the defect of the receptor in desensitization that is required for phosphorylation at C-terminus [15]. We also found that the deletion mutant CCR5^M4 was aberrantly expressed in the cytosol in a majority of transfected cells, but surface expression of CCR5^M4 was detected in a small number of cells. Using live single cell imaging, we were able to use these mutants to analyze functions of the C-terminus in chemokine-induced Ca^2+ responses and heterodimer formation. Our data suggested that the C-terminal tail of CCR5 may be involved in the formation of CXCR4 and CCR5 heterodimers.

Our data revealed that ligand binding to CCR5 or CXCR4 could either promote or destabilize the formation of heterodimers. Previous studies indicated that ligand binding could promote the formation of homo- or heterodimers. For example, the CXCR4 and δ-opioid receptor (DOR) form both homo- and heterodimers and extracellular ligands could alter the complexes of these dimers [6]. DPDPE, a ligand of DOR, induces DOR homodimers; CXCL12/SDF-1, a ligand of CXCR4, triggers the formation of CXCR4 homodimers; and DPDPE and SDF-1 together promote assembly of heterodimeric CXCR4:DOR complexes. It has been proposed that the formation of homo- or heterodimers on the cell membrane, where various receptors co-exist, is a dynamic process,
and various ligands bind to their respective receptors and alter the complexes of receptors. Our study indicated a new level of complexity regarding to the effect of ligand binding on the formation of receptor heterodimers. While binding of CCL3/MIP1α and CCL5/RANTES to CCR5 promoted the formation of CCR5:CXCR4 heterodimers, CXCL12/SD1-1 binding to CXCR4 reduced the level of preexisting CCR5:CXCR4 heterodimers. Mutations in the C-terminal tail of CCR5 not only reduced or abolished its ability to form heterodimers with CXCR4 but also altered effects of ligand binding on the formation of heterodimers with CXCR4. CCL3/MIP1α binding to CCR5STA mutant receptor reduced its ability to form heterodimer with CXCR4, while the binding of CCL5/RANTES to CCR5 or CXCL12/SD1-1 to CXCR4 had no apparent effect. Furthermore, CCL3/MIP1α and CXCL12/SD1-1 binding to either CCR5STA mutant or CXCR4 promoted formation of heterodimers. Our results suggest that ligand binding to a chemokine receptor causes a conformational change in the receptor that can either promote or destabilize the formation of receptor dimers. Mutations of the C-terminal domain of a receptor affect ligand-induced conformational changes, thereby altering its potential to form dimers.

Our results have clear implication for the in vivo physiology of chemokine and their receptors. It has been suggested that chemokines secreted by antigen-presenting cells recruit both CXCR4 and CCR5 to the immunological synapse (IS) during T cell activation [14]. Our data suggest that this accumulation of both CXCR4 and CCR5 could be mediated by chemokine-promoted formation of CXCR4:CCR5 heterodimers. Our results also provide information on the functions of the C-terminal tail of chemokine receptors in dimerization and thus define additional targets for potential drugs in chemokine-related diseases. It should be noted that our study was performed using HEK293 cells expressing CFP- or YFP-tagged receptors. Whether or not the CXCR4:CCR5 dimerization and ligand-induced changes we observed occur in actual leukocytes in vivo remains to be determined. New techniques are to be developed in order to apply this imaging approach in vivo [27].

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Author Contributions
Conceived and designed the experiments: NI TJ. Performed the experiments: NI. Analyzed the data: NI TJ. Contributed reagents/materials/analysis tools: NI TJ. Wrote the paper: DH TJ.

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