Biased signaling at chemokine receptors

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Keywords: G protein-coupled receptor (GPCR); C-C chemokine receptor type 5 (CCR5); signaling; bioluminescence resonance energy transfer (BRET); chemokine

Abstract

The ability of GPCRs to activate selective signaling pathways according to the nature of the bound ligand is a challenging concept in the GPCR field. Signal bias has been documented for several GPCRs, including chemokine receptors. However, most of these studies examined the global signal bias between G protein- and arrestin-dependent pathways, leaving unaddressed the potential bias between particular G protein subtypes. Here, we investigated the coupling selectivity of chemokine receptors CCR2, CCR5 and CCR7 in response to various ligands with G protein subtypes by using BRET biosensors monitoring directly the activation of G proteins. We also compared data obtained with the G protein biosensors to those obtained with other functional readouts, such as β-arrestin-2 recruitment, cAMP accumulation and calcium mobilization assays. We showed that the binding of chemokines to CCR2, CCR5 and CCR7 activated the three Gαi subtypes (Gαi1, Gαi2 and Gαi3) and the two Gαo isoforms (Gαoa and Gαob) with potencies that generally correlate to their binding affinities. In addition, we showed that the binding of chemokines to CCR5 and CCR2 also activated Gα12, but not Gα13. For each receptor, we showed that the relative potency of various agonist chemokines was not identical in all assays, supporting the notion that signal bias exists at chemokine receptors.

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variants (4). Specific combinations of α, β and γ subunits in G proteins affects which downstream targets are activated, providing the means to integrate the various signals coming into a cell. Besides the canonical G protein-dependent pathways, G protein-independent signaling pathways have also been reported (5-7). Of particular interest are arrestins, which were originally considered to be solely involved in receptor desensitization and now have been identified as multifunctional scaffolds interacting with a number of signaling proteins such as MAP kinases, PI3K or PKB (8). Over the past few years, a growing number of biased ligands have been reported, which preferentially activate G protein- or arrestin-dependent pathways, and several of these biased ligands were shown to possess distinct functional properties when compared to “balanced” ligands (5,9). From a therapeutic point of view, molecules that selectively activate the arrestin pathway without affecting G protein-dependent signaling, or the inverse, could have improved efficacy or decreased side effects (6). Biased signaling therefore appears to be a novel and promising concept, which challenges our knowledge of GPCR function and raises new opportunities for the use of these receptors as therapeutic targets (10). It is therefore important to identify ligands and receptors which possess biased signaling properties, and to understand how agonist binding induces the selective activation of a signaling pathway within the cell. Chemokines are a good model system for the analysis of GPCR signaling bias. Chemokines are small basic proteins that control the homing and migration of immune cells. About forty chemokines and more than twenty chemokine receptors have been identified so far (11). Each receptor has its own specific repertoire of chemokine ligands, ranging from a one to a half a dozen. There is therefore significant redundancy in the chemokine system, but relatively little is known regarding the selectivity of pathways activated by chemokines downstream of their common receptor. Signaling selectivity has been first described for the two natural ligands of the chemokine receptor CCR7, CCL19 and CCL21. Both CCL19 and CCL21 were reported to activate G protein-dependent pathways, but only CCL19 was proposed to recruit β-arrestin and activate MAP kinases Erk1/2 (12-14). As a result, CCR7 became a prototypical example of biased signaling, particularly with respect to natural agonists. Based on these first studies, it was anticipated that other chemokine receptors might display a similar bias by preferentially activating either G protein- or arrestin-dependent pathways. Consistent with this hypothesis, Rajagopal et al. recently reported that other chemokine receptors have different tendencies to induce either G protein- or arrestin-dependent signaling (15). In addition, selectivity might exist in the activation of different G protein subtypes or isoforms. However, until recently, it was difficult to elucidate which G protein subtype was activated. In this paper, we used BRET biosensors to monitor the conformational changes in G proteins during their activation and determined which G protein subtypes were activated by various chemokines. We combined the use of these sensors with other functional assays to revisit the signaling bias previously identified for CCR7 and to analyze the signaling selectivity of various chemokine ligands through activation of the closely related receptors CCR2 and CCR5.

Experimental procedures
Reagents, plasmids and cell lines. Chemokines and Maraviroc were purchased from R&D Systems. TAK-779 was purchased from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID. Plasmids encoding G protein and arrestin constructs were described previously (16). The plasmid encoding the cAMP sensor YFP-Epac-RLuc (CAMYEL) was purchased from ATCC. Human embryonic kidney cells (HEK293T) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (GIBCO), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). CHO-K1 cells expressing apoaequorin were cultured in Ham’s F12 medium supplemented with 10% fetal bovine serum (GIBCO), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Cells stably expressing apoaequorin and chemokine receptors were cultured in presence of 10 µg/ml zeocin and G418 (Invitrogen).

Binding assays. Binding experiments were performed as previously described (16). CHO-K1 cells were incubated for 45 min at 25°C in the assay buffer (50 mM Hepes pH 7.4, 1 mM CaCl2, 5 mM MgCl2, 250 mM sucrose, 0.5% BSA) with 0.1 nM [125I]-CCL2, -CCL4 or -CCL19 as tracers and
variable concentrations of unlabeled competitors. Tubes were incubated for one hour at room temperature and bound tracer was separated by filtration through GF/B filters presoaked in 1% polyethyleneimine (PEI). Filters were counted in a γ-scintillation counter. Binding parameters were determined with the PRISM software (GraphPad Softwares) using nonlinear regression applied to a single site model. Ki values were calculated from IC50 values based on the Cheng-Prusoff equation.

**G-protein BRET assay.** G protein activation was assayed by BRET as previously described (17,18). Briefly, plasmids encoding G protein biosensors and receptors of interest were cotransfected into HEK293T cells by using the calcium phosphate method. Forty-eight hours after transfection, cells were washed twice with PBS, detached and resuspended in PBS plus 0.1% (w/v) glucose at room temperature. Cells were then distributed (80 µg of proteins per well) in a 96-well microplate (Optiplate, PerkinElmer, Lifescience). BRET between RLuc8 and GFP10 was measured 1 min after addition of coelenterazine 400a/Deep blue C (5 µM, Gentaur). BRET readings were collected using an Infinite F200 reader (Tecan Group Ltd). The BRET signal was calculated as the ratio of emission of GFP10 (510–540 nm) to RLuc8 (370–450 nm).

**cAMP BRET assay.** cAMP inhibition was assayed by BRET as previously described (19). Briefly, plasmids encoding YFP-Epac-RLuc biosensor and receptors of interest were cotransfected into HEK293T cells by using the calcium phosphate method. Twenty-four hours post-transfection, cells were collected and seeded in 96-well microplates (165306, Nunc) and cultured for an additional 24 h. Cells were then rinsed once with PBS and incubated in PBS plus 0.1% (w/v) glucose at 25°C to slow down kinetics of arrestin recruitment and improve temporal resolution. BRET between RLuc and YFP was measured after the addition of coelenterazine h (5 µM, Promega). Chemokines were added 5 min after coelenterazine h and BRET readings were collected using a Mithras LB940 Multilabel Reader (Berthold Technologies). The BRET signal was calculated as the ratio of emission of YFP (520–570 nm) to RLuc (370–480 nm).

**β-arrestin BRET assay.** β-arrestin recruitment was measured by a BRET proximity assay as previously described (17). Briefly, plasmids encoding Rluc-β-arrestin 2 and receptors fused to Venus were cotransfected into HEK293T cells by using the calcium phosphate method. Twenty-four hours post-transfection, cells were collected and seeded in 96-well microplates (165306, Nunc) and cultured for an additional 24 h. Cells were then rinsed once with PBS and incubated in PBS plus 0.1% (w/v) glucose at 25°C to slow down kinetics of arrestin recruitment and improve temporal resolution. BRET between RLuc8 and YFP was measured after the addition of coelenterazine h (5 µM, Promega). Chemokines were added 5 min after coelenterazine h and BRET readings were collected using a Mithras LB940 Multilabel Reader (Berthold Technologies). The BRET signal was calculated as the ratio of emission of YFP (520–570 nm) to RLuc (370–480 nm).

**Intracellular calcium mobilization assay.** Calcium mobilization was measured in HEK293 cells expressing CCR2 or CCR7 with an aequorin-based assay as previously described (16). As signals induced by chemokines were barely detectable in HEK293 cells expressing CCR5, calcium mobilization was performed in CHO-K1 cells stably expressing CCR5. Briefly, cells expressing apoaequorin and the receptor of interest were incubated for 4 h in the dark in the presence of 5 µM coelenterazine h (Promega), then diluted before use to reach the appropriate cell density. The cell suspension (25,000 cells/well) was added to wells containing various concentrations of chemokines and luminescence was recorded for 30 sec in an EG&G Berthold luminometer (PerkinElmer Life Sciences).

**MAP kinases assays.** HEK293 cells expressing CCR7 were starved for 12–18 h in serum-free medium prior to stimulation. After stimulation, cells were collected by centrifugation and heated to 100°C for 5 min in 2X Laemmli sample buffer. Whole cell lysates were resolved on 10% Tris/Tricine polyacrylamide gels and transferred to nitrocellulose membranes. Phosphorylated ERK1/2, total ERK1/2, and β-arrestins were detected by using rabbit polyclonal anti-phospho-ERK1/2 (Cell Signaling #4370, 1:1,000) and anti-
ERK1/2 (Cell Signaling #4695S, 1:2,000) antibodies. Chemiluminescence detection was performed using ECL-Plus reagent (Perkin Elmer).

**Bias analysis.** The bias index (β) was estimated by using the following equation where RA denotes the relative efficacy (RA) of a ligand (Lig) through pathway a and pathway b relative to a reference agonist (ref) chosen arbitrary (15).

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\text{Bias index} = \log \left( \frac{R_{\text{A}}^{\text{ref}}}{R_{\text{A}}^{\text{b}}} \right) = \log \left( \frac{E_{\text{Cmax,b}} \cdot E_{\text{Cmax,ref}} / \text{Lig}}{E_{\text{Cmax,a}} \cdot E_{\text{Cmax,ref}} / \text{ref}} \right)
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Statistical analyses were performed where appropriate using one-way ANOVA with Dunnett’s post-test, and statistical significance related to the reference balanced ligand was taken as p < 0.05

**Results**

1. **Signaling selectivity at chemokine receptor CCR7**

   In a competition binding assay, we first confirmed that CCL19 and CCL21 compete with radiolabelled CCL19 for CCR7 binding with nearly equivalent pIC\textsubscript{50} values (Figure 1A, Table 1). We next investigated the panel of G proteins activated by CCR7 upon binding of CCL19 or CCL21 by using BRET-based biosensors directly monitoring the activation of G proteins. This technology relies on the large interdomain movement that occurs within heterotrimeric G proteins upon GDP/GTP exchange, resulting in a decrease of the BRET signal between probes inserted at specific locations within G\textalpha and G\textgamma subunits (17,18). This technology detects early signaling events that occur shortly after receptor stimulation, enabling us to identify the G protein subtypes activated by a receptor. CCR7 was transiently co-expressed in HEK-293T cells with ten different biosensors belonging to the four major classes of G proteins (G\textalpha12, G\textalpha13, G\textalphaq11 and G\textalpha12/13), and stimulated with either CCL19 or CCL21. Among the ten G protein isoforms tested, neither chemokine significantly activated the G\textalpha1, G\textalpha11, G\textalpha5, G\textalpha12 or G\textalpha13 proteins at 100 nM (data not shown). CCL19 binding triggered a significant activation of the three G\textalpha subtypes (G\textalpha1, G\textalpha2, G\textalpha3) and the two G\textalpha isoforms (G\textalpha6, G\textalpha16) (Figure 1B). In contrast, CCL21 binding led to less efficient activation of the G\textalpha10 proteins. A dose response curve indicated that CCL21 activates G\textalpha1 and G\textalphao at least ten times less potently than CCL19 (Figure 1 C-E and Table 1). We are however uncertain of the maximal efficacy of G\textalpha1 activation by CCL21 as we did not saturate the BRET signal at 100 nM. We next measured the inhibition of cAMP production as a representative readout of G\textalpha1 proteins activation and showed that CCL21 inhibited cAMP production triggered by forskolin at lower potency but similar efficacy compare to CCL19, in complete agreement with data generated with the G protein biosensors (Figure 1F). We also investigated the ability of CCR7 to recruit β-arrestin by a BRET proximity assay, which measures the energy transfer between β-arrestin-2-Rluc and CCR7 fused to the yellow fluorescent protein Venus. Due to the rapid recruitment of arrestin at 37°C, our assays were performed at 25°C to decrease the reaction kinetics and gain temporal resolution. CCL21 (at 100 nM) induced the recruitment of β-arrestin-2 slower than CCL19 (Tau values of 4.6 min and 2.4 min respectively), in perfect agreement with previously published data (12,13) (Figure 1G). It should be noted that the maximal BRET value (BRET\textsuperscript{MAX}) reached with CCL21 stimulation is also 30% lower than the BRET\textsuperscript{MAX} with CCL19 stimulation. This might reflect distinct conformations of receptor-arrestin complexes (12), but is most likely the consequence of decreased arrestin recruitment at the tested concentrations. Indeed, we showed that CCL21 activates β-arrestin-2 ten times less potently than CCL19 (Figure 1H, Table 1). Finally, we showed that CCL21 also triggered calcium mobilization and ERK1/2 phosphorylation less potently than CCL19 (Figure 1 I-J). Collectively, our data indicate that CCL21 binds CCR7 as efficiently as CCL19 but is less potent in activating all the assays including G proteins and β-arrestin-2, challenging thus the notion of signaling bias as previously claimed between these two pathways.

2. **Activation of G protein subtypes by CCR5**

   To further analyse the signaling selectivity of chemokine receptors, we also investigated the activation of G proteins through CCR5, a receptor which binds multiple chemokines. We first measured the binding affinities of some of these chemokines (CCL-3, -4, -5, -8 and -13) and showed that they compete with radiolabelled CCL4 for CCR5 binding with calculated pIC\textsubscript{50} values consistent with previous estimates (Figure 2 and...
Table 2) (23). We next compared the ability of these chemokines to trigger G protein activation by using BRET-based G protein biosensors. CCR5 was coexpressed with the agonist chemokines CCL3, CCL4, CCL5, CCL8 and CCL13. As controls, cells were exposed to the antagonist chemokine CCL7 and to TAK779 and Maraviroc, two small molecules displaying inverse agonist properties. Stimulation of CCR5 by CCL3, CCL4, CCL5, CCL8 and CCL13 at 100 nM significantly activated all Gαi and Gαo isoforms and also Gα12, with CCL13 activating each G protein the least (Figure 3). Stimulation of cells expressing the biosensors without CCR5 was used as negative control, and generated signals of much weaker magnitude (Figure 3). No significant BRET signal was detected for the Gαq, Gα11, Gαi or Gα13 proteins (Figure 3 and data not shown). The chemokine antagonist CCL7 had no significant effect, while TAK779 and Maraviroc displayed a tendency to increase the BRET signal for some biosensors (Gα2, Gα3, Gα12) in presence of CCR5, in agreement with their inverse agonist properties. Dose response curves performed with some representative G protein biosensors also indicated that the calculated pEC50 values are similar for the different Gα proteins. CCL5 was the most potent agonist in these assays, despite having a similar CCR5 binding affinity than CCL3 and CCL4. CCL13 was the least potent agonist, in agreement with its lower affinity for CCR2 (Figure 4, Table 2). However, the calculated Emax values were more variable for specific chemokines and Gα proteins, with CCL13 being the agonist with the lowest efficacy. Collectively, our data did not reveal major activation bias amongst chemokines and between the Gαi, Gαo and Gα12 protein subtypes, although CCL5 displayed higher potency in most assays and CCL8 tended to be less effective in activating Gαo proteins. We next used cAMP accumulation as a representative readout of Gαo protein activation. As expected, all chemokine agonists inhibited the cAMP production triggered by forskolin with relative potencies compatible with our binding data. The calculated pEC50 values are lower than those estimated with the G protein biosensors likely as a consequence of the higher sensitivity of this assay due to signal amplification along the cascade (Figure 5A, Table 2). CCL13 inhibited cAMP accumulation with a lower potency, but with a similar efficiency compared to the other agonist chemokines. Finally, we showed that agonist chemokines activated calcium mobilization with similar EC50 and Emax values, with the exception of CCL13, which was again less potent in this assay (Figure 5B and Table 2). In contrast to the stimulation of CCR7, our data showed that stimulation of CCR5 triggered the activation of G12, indicating that chemokine receptors can activate distinct G proteins. Moreover, our results also showed that CCR5 activated Gα12 but not Gα13, although they belong to the same family (Figure 3).

3 Activation of G protein subtypes by CCR2

As CCL7, CCL8 and CCL13 also bind to the closely related receptor CCR2, we next tested which G protein subtypes were activated by these chemokines when bound to CCR2. We showed that these chemokines competed with radiolabelled CCL2 for CCR2 binding with calculated pIC50 values compatible with previous estimates (Figure 2 and Table 3) (24). CCL7, CCL8 and CCL13 (at 100 nM) activated Gαi and Gαo proteins at similar potencies to CCL2, which was used as a positive control (Figures 6, 7 and Table 3). However, CCL8 and CCL13 were less efficient agonists than CCL2 and CCL7 in most assays. In contrast to its effect on CCR5, CCL7 efficiently activated Gα40 proteins through CCR2, in agreement with its known role as a CCR2 agonist. Interestingly, CCL2 and CCL7 also activated Gα12 with similar potencies as those of Gαi and Gαo. In contrast, CCL13 poorly activated Gα12 in comparison to Gαi and Gαo, while CCL8 was almost silent in this assay. The relative efficacy of these four CCR2 agonists therefore differs depending on the specific G protein and the activation readout. Furthermore, since our results suggest that CCL8 and CCL13 bind to CCR2 less efficiently than CCL2 and CCL7, the relative potency of various chemokines in the various G protein assays does not strictly follow their binding affinity for CCR2. This is therefore suggestive of signaling bias. Similar to CCR5, none of the Gαq, Gα11, Gαi or Gα13 proteins was activated upon chemokine binding to CCR2 (Figure 6 and data not shown). Finally, we showed that all chemokines inhibited cAMP accumulation with relative potencies compatible with binding data (Figure 8A). In contrast, CCL2 induced significantly more calcium influx than CCL7, despite their similar binding affinities. Likewise, CCL13 induced significantly higher calcium influx than CCL8,
despite a lower binding affinity. The calculated calcium mobilization efficacies were similar for the various chemokines, with the exception of CCL8, for which no values could be determined (Figure 8B and Table 3).

4. Arrestin recruitment by CCR5 and CCR2

We finally investigated differences in β-arrestin-2 recruitment to CCR2 and CCR5 in response to the various chemokines. Stimulation by chemokines induced a progressive increase in the energy transfer between β-arrestin-2-Rluc and CCR5-Venus, indicating recruitment of the arrestin to CCR5 (Figure 9). CCL5 and CCL8 recruited β-arrestin-2 with the fastest kinetics of the chemokines tested (Tau values CCL3: 5.0 min; CCL4: 6.5 min; CCL5: 3.5 min; and CCL8: 3.5 min). The BRET$_{\text{MAX}}$ values reached for the various chemokines were reflective of the respective pEC$_{50}$ values combined with the lack of saturation for some chemokines at 100 nM. Very low β-arrestin-2 recruitment to CCR5 was detected upon CCL13 stimulation, consistent with the weak activation detected in other readouts for this chemokine. Stimulating CCR5 with increasing concentrations of chemokines showed that all tested chemokines activated β-arrestin-2 recruitment with potencies comparable to those estimated for G protein activation (Figure 9 and Table 2). No differences in arrestin recruitment were observed for CCL5 and CCL3/CCL4 in this assay, suggesting the absence of strong signaling bias between G protein- and arrestin-dependent pathways for CCR5 agonists. We also investigated the recruitment of β-arrestin-2 by CCR2 and showed that the various chemokines triggered the recruitment of β-arrestin-2-Rluc to CCR2-Venus, but with variable kinetics and BRET$_{\text{MAX}}$ values (Figure 9 and Table 3). In this assay, CCL2 was more potent than CCL7 despite similar affinities, and CCL13 efficacy was substantially higher than that of CCL8, which is similar to our results for calcium mobilization. These data, in agreement with a previous report (24) and support a model with bias in the activation of signaling pathways by CCR2 agonists.

5. Assessment of chemokines bias

We performed a quantitative analysis of chemokines bias based on functional parameters from Tables 2 and 3 as previously described for other chemokine receptors (15). Bias factors between pathways were determined for most chemokines with the exception of the cases where signaling parameters could not be determined. Most of the calculated bias values ranged between zero and 1, suggesting no or weak bias, and none of these biases was statistically significant (Figure 10, 11 and Table 4 and 5). Only few bias factors raised above 1 with statistical significance. At CCR5, CCL5 and CCL8 showed some level of bias for Goi1 and Gœob relative to arrestin, the bias of CCL5 between Gœob and arrestin being the only one to reach statistical significance. Similarly, CCL8 showed at CCR2 a statistically significant bias for Gœob activation relative to arrestin. These results suggest the existence of some bias between G protein and arrestin activation. At CCR5, significant bias could also be determined for some chemokines (CCL4, CCL8) between calcium and arrestin or cAMP. However, we have to keep in mind that calcium mobilization with CCR5 was performed in another cell type, which may influence the estimation of bias. It is also of note that CCL8 showed at CCR5 significant bias for Goi1 and Gœob relative to cAMP. This result may appear somehow paradoxical as inhibition of cAMP constitutes a representative readout of Ga$\alpha_{i/o}$ proteins activation. Nevertheless, it may not be relevant to compare cAMP inhibition and a single G protein or to calculate bias between assays displaying different levels of amplification.

Discussion

Over the past decade, biased signaling has emerged as a novel and challenging concept in the GPCR field. This concept relies on the hypothesis that receptors can oscillate among multiple conformational states, each of which is able to activate its own set of signaling pathways (7,25). Selective activation of signaling pathways by specific GPCR ligands has been demonstrated for several receptors, including angiotensin AT$R$, dopamine D$\tau$R, serotonin 5-HT$\tau$C$R$ and the pituitary adenylate cyclase-activating peptide (PACAP) receptors (26-28). Differential effects of chemokines on their common receptor was reported in terms of signaling cascade activation, receptor phosphorylation or internalization (15,29-33). So far, one of the prototypical examples of signaling bias in GPCRs is the chemokine receptor CCR7. It was originally shown that both natural ligands of CCR7, CCL19 and CCL21, equally activate G
protein-dependent signaling, while only CCL19 is able to promote efficient β-arrestin recruitment and MAPK phosphorylation (12-14). The precise molecular mechanisms underlying this signaling bias is not known, though the involvement of CCR7 phosphorylation by selective GRKs has been suggested (12). Although discrepancies regarding the extent of arrestin activation and MAPK phosphorylation exist between studies, they all supported that CCL19 and CCL21 lead to similar G protein-dependent signaling (12,13). In the present study, we confirmed that CCL21 binding induces β-arrestin recruitment with a lower potency than CCL19, but in contrast to previous reports, we also showed that CCL21 is less potent at activating G proteins, as measured by BRET biosensors directly monitoring the conformational changes associated to Gαi or Gαo activation. We also confirmed that the lower Gαo activation following stimulation by CCL21 is associated with a reduced cAMP accumulation, in perfect agreement with the role of Gαi/o proteins in inhibiting cAMP generation. Similarly, CCL21 activates less efficiently calcium mobilization and ERK1/2 phosphorylation, indicating that several signals downstream of G proteins and arrestin behave similarly. Collectively, our data suggest that CCL21 interacts with CCR7 as efficiently as CCL19 but is less potent in activating G proteins and recruiting β-arrestin, challenging thus the notion of bias claimed between these two pathways. The exact reason for the discrepancy between our results and those of Kohout et al. (13) is not known for sure but may be linked to the various expression systems used in the original study, while we performed all our assays in HEK293T cells. In the line with this hypothesis, we found that calcium mobilization and Erk1/2 phosphorylation can be activated by CCL21 as efficiently as CCL19 in CHO cells expressing CCR7 (not shown). It should thus be kept in mind that the nature of the cells used to monitor receptor signaling can impact some assays. BRET biosensors could thus constitute an interesting alternative tool to investigate early signaling events at the level of G protein activation as they measure conformational changes of the ligand/receptor/G protein complex.

We also investigated the putative signaling selectivity at two other chemokine receptors, CCR2 and CCR5, and compared the data generated using the G protein biosensors with those obtained with β-arrestin recruitment, cAMP accumulation and calcium mobilization assays. We first identified the set of G protein subtypes activated by CCR2 and CCR5, and showed that both receptors activate proteins of the Gαi, Gαo, and Gα12 subfamilies. Not surprisingly, an antagonist chemokine was silent in such assays, while small molecule inverse agonists produced changes in some BRET signals compatible with an inhibition of the constitutive activity of these receptors. These results confirm that G protein biosensors constitute valuable tools to discriminate properties of receptor ligands. All agonist chemokines activated G proteins with potencies that generally correlate with the potencies calculated for β-arrestin recruitment and their binding affinities for the receptors. Gα1 activation and arrestin recruitment have previously been reported to be correlated for CCR2 (24). There were however some changes in the relative efficiency of the chemokines according to the assay used. CCL5 was, for example, more potent on CCR5 than CCL3 and CCL4 in some G protein assays while these three chemokines displayed similar binding parameters and did not differ significantly in other assays. CCL8 also displayed a lower relative potency on CCR5 in the β-arrestin-2 recruitment assay. Quantitative analysis revealed that CCL5 and CCL8 display some selectivity for G proteins activation over β-arrestin-2 recruitment, even if only one bias value reached statistical significance. Likewise, CCL7 and CCL8 displayed lower relative potencies on CCR2 in the calcium mobilization and β-arrestin recruitment assays, while they behaved similarly to CCL2 and CCL13 in other assays. CCL8 also activated Gαi1 and Gαo12 much more efficiently than Gαi12 through CCR2. Quantitative analysis showed that CCL8 displayed significant bias for Gαob activation relative to arrestin but no other significant bias could be determined. One explanation could be that most of our concentration-response data showed moderate variations, yielding bias values ranging between zero and 1. Mathematical calculation of bias factors is therefore useful to quantify to some extent the selectivity of a ligand for one pathway relative to another. However, it remains to be determined precisely whether those values reflect distinct physiological responses.
Collectively, these data suggest that different chemokines acting on CCR5 or CCR2 may trigger overlapping but distinct sets of G protein subtypes, providing some selectivity in downstream signaling cascades. Finally, our data also revealed that CCR5 and CCR2 activate $G\alpha_{12}$, whereas CCR7 does not at all. Our results also revealed that CCR5/2 activate $G\alpha_{12}$ but not $G\alpha_{13}$, which belongs to the same family. This activation profile is clearly distinct from other receptors that activate both $G\alpha_{12}$ and $G\alpha_{13}$, such as the thromboxane receptor (TPaR) (18), illustrating the utility of biosensors in discriminating the activation of G protein subtypes that cannot be otherwise measured by "classical" readouts.

In summary, we showed here that G protein BRET-biosensors enable the pragmatic analysis of G protein subtype activation by chemokine receptors upon interaction with their various ligands. We also showed that the chemokines tested in this study activated G proteins with potencies that generally match those detected for $\beta$-arrestin recruitment and other assays, but with subtle changes in the rank order according to assay. Thus, the behavior of chemokines contrast to the situation encountered in other studies with some synthetic small molecules that show agonism in one assay but antagonism in another (38, 39). Collectively, our results suggest the existence of a moderate signaling bias between chemokines acting on the same receptor. Although variations in G proteins or arrestins activation were detected, the structural basis underlying biased signaling remains to be identified precisely. It also appears that the bias remains relatively subtle for natural ligands such as chemokines, while more overt bias has been described for synthetic small molecule agonists.
REFERENCES


**Authors contribution** Conceived and designed the experiments: J-Y S.; Performed the experiments: J.C., A.H. and J-Y S. Analyzed the data: J.C., C.G., M.P and J-Y S.; Wrote the paper: M. P. and J-Y S.

**FOOTNOTES**

J.C. holds a studentship from the Belgian Fonds pour la formation à la Recherche dans l’Industrie et l’Agriculture (FRIA). This work was supported by the Fonds de la Recherche Scientifique Médicale of Belgium, the Actions de Recherche Concertées, and the Interuniversity Attraction Poles Programme (P7–14), Belgian State, Belgian Science Policy.
Figure legends

Figure 1. A. Competition binding assays performed on CHO-K1 cells expressing CCR7. Cells were incubated with 0.1 nM $^{125}$I-CCL19 as tracer and unlabelled CCL19 (●) or CCL21 (○) as competitors. The data were normalized for nonspecific binding (0%) in the presence of 300 nM of competitor (CCL19), and specific binding in the absence of competitor (100%). All points were run in triplicates (error bars indicate S.E.M.). B-E. G protein activation by CCR7. Real-time measurement of BRET signal in HEK293T cells coexpressing G protein biosensors and CCR7, and stimulated for 1 minute with 50 nM CCL19 (black bars and circles) or CCL21 (open bars and circles). Results are expressed as the difference in BRET signals measured in the presence and absence of stimulation. Data represent the mean ± S.E.M. of three to six independent experiments. Statistical significance between stimulated and unstimulated cells was assessed using Tukey’s test (**P < 0.001). F. Inhibition of cAMP by CCR7. Measurement of BRET signal in HEK293T cells coexpressing cAMP biosensor and CCR7, and stimulated sequentially by CCL19 (●) or CCL21 (○) and forskolin. The results were normalized for the basal signal in absence of stimulation (0%) and the maximal response obtained with forskolin only (100%). Data represent the mean ± S.E.M. of three independent experiments. G-H. Recruitment of β-arrestin 2 by CCR7. Real-time measurement of BRET signal in HEK293T cells expressing β-arrestine2-RLuc8 and CCR7-Venus and stimulated with CCL19 (back circles) or CCL21 (open circles). Cells were stimulated by 100 nM chemokines in E, and for 30 min in F. Results are expressed as the difference in BRET signals measured in the presence and absence of stimulation. Data represent the mean ± S.E.M. of three independent experiments. I. Calcium mobilization by CCR7. Calcium mobilization was measured in HEK293 cells using the aequorin-based functional assay. Cells expressing CCR7 were stimulated with increasing concentrations of CCL19 (●) or CCL21 (○) and luminescence was recorded for 30 s. The results were normalized for the basal luminescence in absence of agonist (0%) and the maximal response obtained with 50 µM acetylcholine (100%). Data represent the mean ± S.E.M. of three independent experiments J. Phosphorylation of ERK1/2 by CCR7. HEK293 cells stably expressing CCR7 were stimulated with 100 nM CCL19 or CCL21 for two minutes. Results are expressed as the ratio between the amount of phospho-ERK1/2 and total ERK1/2 following quantification on Western blots. Data represent the mean ± S.E.M. of three independent experiments.

Figure 2. A. Competition binding assays performed on cells expressing CCR5. CHO-K1 cells expressing CCR5 were incubated with 0.1 nM $^{125}$I-CCL4 as tracer and unlabelled CCL3, CCL4, CCL5, CCL8 and CCL13 as competitors. The data were normalized for nonspecific binding (0%) in the presence of 300 nM CCL4 and specific binding in the absence of competitor (100%). All points were run in triplicates (error bars indicate S.E.M.). B. Competition binding assays performed on cells expressing CCR2. CHO-K1 cells expressing CCR2 were incubated with 0.05 nM $^{125}$I-CCL2 as
tracer and unlabelled CCL5, CCL7, CCL8 and CCL13 as competitors. The data were normalized for nonspecific binding (0%) in the presence of 300 nM CCL2 and specific binding in the absence of competitor (100%). All points were run in triplicates (error bars indicate S.E.M.).

**Figure 3. Panel of G proteins activated by CCR5.** Real-time measurement of BRET signal in HEK293T cells coexpressing CCR5 and G protein biosensors (black bars) or G protein biosensors only (open bars) and stimulated for one minute with 100 nM of the chemokines CCL3, CCL4, CCL5, CCL7, CCL8, CCL13 or 1 µM of the small molecules TAK779 (T) and Maraviroc (M). Results are expressed as the difference in BRET signal measured in the presence and absence of stimulation. Data represent the mean ± S.E.M. of at least six independent experiments. Statistical significance between cells expressing or not CCR5 (** P<0.01, * P<0.1) and between chemokines (### P<0.001, ## P<0.01, # P<0.1) was assessed using Tukey's test.

**Figure 4. Activation of G_{i1}, G_{ob} and G_{12} by CCR5.** Real-time measurement of BRET signal in HEK293T cells coexpressing CCR5 and G protein biosensors and stimulated with increasing concentration of chemokines. Results are expressed as the difference in BRET signal measured in the presence and absence of chemokines. Data represent the mean ± S.E.M. of three independent experiments.

**Figure 5. Inhibition of cAMP and calcium mobilization triggered by CCR5**

**Panel A** Measurement of BRET signal in HEK293T cells coexpressing the cAMP biosensor and CCR5, and stimulated sequentially by chemokines and forskolin. The results were normalized for the basal signal in absence of stimulation (0%) and the maximal response obtained with forskolin only (100%). Data represent the mean ± S.E.M. of three independent experiments. **Panel B** Calcium mobilization was measured in CHO-K1 cells using the aequorin-based functional assay. Cells expressing CCR5 were stimulated with increasing concentrations of chemokines and luminescence was recorded for 30 s. The results were normalized for the basal luminescence in absence of agonist (0%) and the maximal response obtained with 25 µM ATP (100%). Data represent the mean ± S.E.M. of three independent experiments.

**Figure 6. Panel of G proteins activated by CCR2.** Real-time measurement of BRET signal in HEK293T cells coexpressing CCR2 and G protein biosensors (black bars) or G protein biosensors only (open bars) and stimulated for 1 minute with 100 nM of the chemokines CCL2, CCL7, CCL8, CCL13, or 1 µM of the small molecules TAK779 (T) and Maraviroc (M). Results are expressed as the difference in BRET signal measured in the presence and absence of stimulation. Data represent the mean ± S.E.M. of at least six independent experiments. Statistical significance between cells expressing or not CCR2 (** P<0.001, * P<0.1) and between chemokines (** P<0.01, * P<0.1) was assessed using Tukey's test.
Figure 7. Activation of $G_{11}$, $G_{ob}$ and $G_{12}$ by CCR2. Real-time measurement of BRET signal in HEK293T cells coexpressing CCR2 and G protein biosensors and stimulated with increasing concentrations of chemokines. Results are expressed as the difference in BRET signal measured in the presence and absence of chemokines. Data represent the mean ± S.E.M. of three independent experiments.

Figure 8. Inhibition of cAMP and calcium mobilization triggered by CCR2. A. Measurement of BRET signal in HEK293T cells coexpressing the cAMP biosensor and CCR2, and stimulated sequentially by chemokines and forskolin. The results were normalized for the basal signal in absence of stimulation (0%) and the maximal response obtained with forskolin only (100%). Data represent the mean ± S.E.M. of three independent experiments. B. Calcium mobilization was measured in HEK293 cells using the aequorin-based functional assay. Cells expressing CCR2 were stimulated with increasing concentrations of chemokines and luminescence was recorded for 30 s. The results were normalized for the basal luminescence in absence of agonist (0%) and the maximal response obtained with 50 µM acetylcholine (100%). Data represent the mean ± S.E.M. of three independent experiments.

Figure 9. Recruitment of $\beta$-arrestin 2 by CCR5 and CCR2. Real-time measurement of BRET signal in HEK293T cells expressing either $\beta$-arrestin2-RLuc8 and CCR5-Venus (A and B) or $\beta$-arrestin2-RLuc8 and CCR2-Venus (C and D) and stimulated with chemokines. For kinetics, BRET signals were measured after addition of 100 nM chemokines. For dose response curves, BRET was recorded 30 minutes after stimulation with various concentrations of chemokines. Results are expressed as net BRET, corresponding to the difference in BRET signal between cells expressing arrestin plus receptor and cells expressing arrestin only. Data represent the mean ± S.E.M. of three independent experiments.

Figure 10 Chemokine bias factors at CCR5. Bias factors between different pathways were calculated for each chemokine, using CCL3 as the reference chemokine. Bias factors that are significantly different (Tukey’s test) from the reference chemokine CCL3 are in bold and colored according to the preferred pathway. Data represent the mean ± S.E.M. from Table 4. ND, not determined.

Figure 11 Chemokine bias factors at CCR2. Bias factor between different pathways were calculated for each chemokine, using CCL2 as the reference chemokine. Bias factors that are significantly different (Tukey’s test) from the reference are in bold and colored according to the preferred pathway. Data represent the mean ± S.E.M. from Table 4. ND, not determined.
Table 1. Binding and signaling properties of CCR7

<table>
<thead>
<tr>
<th>Ligands</th>
<th>[¹²⁵I]-CCL19</th>
<th>Gαi2</th>
<th>Gαi3</th>
<th>Gαoa</th>
<th>β-Arr2</th>
<th>cAMP</th>
<th>Ca++</th>
<th>MAPK</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL19</td>
<td>-8.5 ± 0.1</td>
<td>-8.8 ± 0.1</td>
<td>-8.1 ± 0.2</td>
<td>-0.11 ± 0.01</td>
<td>-8.3 ± 0.1</td>
<td>-0.19 ± 0.01</td>
<td>-8.2 ± 0.1</td>
<td>-0.11 ± 0.01</td>
</tr>
<tr>
<td>CCL21</td>
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<td>ND</td>
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<td>-0.15 ± 0.03</td>
<td>-7.6 ± 0.1</td>
<td>-0.07 ± 0.01</td>
</tr>
</tbody>
</table>

Binding and signaling parameters were measured on HEK293 or CHO-K1 cells expressing CCR7. The plC₅₀, pEC₅₀ and E_MAX values were obtained from experiments displayed in Fig. 1. Values represent the mean ± S.E.M. of at least three independent experiments. ND, not determined.
### Table 2. Binding and signaling parameters of CCR5

<table>
<thead>
<tr>
<th>Ligands</th>
<th>[125I]-CCL4 pIC50</th>
<th>pKi</th>
<th>pEC50</th>
<th>Emax</th>
<th>pEC50</th>
<th>Emax</th>
<th>pEC50</th>
<th>Emax</th>
<th>pEC50</th>
<th>Emax</th>
<th>pEC50</th>
<th>Emax</th>
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<th>Emax</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>CCL3</td>
<td>−8.7 ± 0.1</td>
<td>−8.9 ± 0.1</td>
<td>−8.1 ± 0.4</td>
<td>−0.09 ± 0.02</td>
<td>−8.1 ± 0.4</td>
<td>−0.08 ± 0.02</td>
<td>−8.6 ± 0.3</td>
<td>−0.017 ± 0.002</td>
<td>−8.6 ± 0.2</td>
<td>0.08 ± 0.01</td>
<td>−10.6 ± 0.2</td>
<td>43 ± 3</td>
<td>−7.2 ± 0.1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>CCL4</td>
<td>−9.2 ± 0.1</td>
<td>−9.4 ± 0.1</td>
<td>−8.5 ± 0.2</td>
<td>−0.07 ± 0.02</td>
<td>−8.3 ± 0.2</td>
<td>−0.10 ± 0.01</td>
<td>−8.1 ± 0.2</td>
<td>−0.016 ± 0.002</td>
<td>−8.1 ± 0.1</td>
<td>0.10 ± 0.01</td>
<td>−9.1 ± 0.1</td>
<td>44 ± 3</td>
<td>−8.2 ± 0.2</td>
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<td>−9.2 ± 0.1</td>
<td>−0.10 ± 0.01</td>
<td>−9.0 ± 0.3</td>
<td>−0.020 ± 0.001</td>
<td>−8.1 ± 0.2</td>
<td>0.10 ± 0.01</td>
<td>−10.3 ± 0.3</td>
<td>34 ± 2</td>
<td>−8.0 ± 0.2</td>
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<tr>
<td>CCL8</td>
<td>−8.2 ± 0.1</td>
<td>−8.4 ± 0.1</td>
<td>−8.2 ± 0.1</td>
<td>−0.09 ± 0.01</td>
<td>−8.1 ± 0.3</td>
<td>−0.06 ± 0.01</td>
<td>−8.1 ± 0.4</td>
<td>−0.015 ± 0.003</td>
<td>−7.0 ± 0.2</td>
<td>0.08 ± 0.01</td>
<td>−9.2 ± 0.2</td>
<td>39 ± 3</td>
<td>−7.4 ± 0.1</td>
<td>89 ± 3</td>
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<tr>
<td>CCL13</td>
<td>−7.2 ± 0.1</td>
<td>−7.4 ± 0.1</td>
<td>ND</td>
<td>ND</td>
<td>−7.6 ± 0.1</td>
<td>−0.05 ± 0.01</td>
<td>ND</td>
<td>ND</td>
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</table>

Binding and functional parameters were measured on HEK293 or CHO-K1 cells expressing CCR5. The pIC<sub>50</sub> and pEC<sub>50</sub> and Emax values were obtained from experiments as displayed in Fig. 2, 4, 5, 9. Values represent the mean ± S.E.M. of at least three independent experiments. ND, not determined.
Table 3. Binding and signaling parameters of CCR2

<table>
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<tr>
<th>Ligands</th>
<th>[125I]-CCL2</th>
<th>Gq11</th>
<th>Gαob</th>
<th>Gα12</th>
<th>β-Arr2</th>
<th>cAMP</th>
<th>Ca++</th>
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<tbody>
<tr>
<td></td>
<td>pIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>pKi</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>E&lt;sub&gt;MAX&lt;/sub&gt;</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>E&lt;sub&gt;MAX&lt;/sub&gt;</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>CCL2</td>
<td>-9.6 ± 0.1</td>
<td>-10.0 ± 0.1</td>
<td>-8.7 ± 0.2</td>
<td>-0.13 ± 0.01</td>
<td>-8.0 ± 0.1</td>
<td>-0.15 ± 0.01</td>
<td>-8.4 ± 0.4</td>
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<tr>
<td>CCL7</td>
<td>-9.2 ± 0.1</td>
<td>-9.6 ± 0.1</td>
<td>-8.4 ± 0.2</td>
<td>-0.10 ± 0.01</td>
<td>-8.2 ± 0.1</td>
<td>-0.12 ± 0.01</td>
<td>-8.2 ± 0.2</td>
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<tr>
<td>CCL8</td>
<td>-8.5 ± 0.1</td>
<td>-8.9 ± 0.1</td>
<td>-8.5 ± 0.2</td>
<td>-0.06 ± 0.01</td>
<td>-8.8 ± 0.4</td>
<td>-0.05 ± 0.01</td>
<td>ND</td>
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<tr>
<td>CCL13</td>
<td>-7.9 ± 0.1</td>
<td>-8.3 ± 0.1</td>
<td>-8.5 ± 0.4</td>
<td>-0.06 ± 0.01</td>
<td>-7.9 ± 0.2</td>
<td>-0.08 ± 0.01</td>
<td>ND</td>
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</table>

Binding and functional parameters were measured on HEK293 or CHO-K1 cells expressing CCR2. The pIC<sub>50</sub>, pEC<sub>50</sub> and E<sub>MAX</sub> values were obtained from experiments as displayed in Fig. 2, 7, 8, 9. Values represent the mean ± S.E.M. of at least three independent experiments. ND, not determined.
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<tbody>
<tr>
<td>CCL3</td>
<td>0.00 ± 0.76</td>
<td>0.00 ± 0.67</td>
<td>0.00 ± 0.60</td>
<td>0.00 ± 0.58</td>
<td>0.00 ± 0.62</td>
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<td>0.00 ± 0.58</td>
<td>0.00 ± 0.50</td>
<td>0.00 ± 0.65</td>
<td>0.00 ± 0.45</td>
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<td>0.00 ± 0.34</td>
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<tr>
<td>CCL4</td>
<td>-0.06 ± 0.61</td>
<td>0.80 ± 0.57</td>
<td>0.59 ± 0.50</td>
<td>0.93 ± 0.50</td>
<td>-0.54 ± 0.48</td>
<td>0.86 ± 0.55</td>
<td>0.65 ± 0.48</td>
<td>0.99 ± 0.47</td>
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<td>-0.21 ± 0.43</td>
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<td>-1.34 ± 0.41</td>
<td>0.34 ± 0.32</td>
<td>-1.13 ± 0.30</td>
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<tr>
<td>CCL5</td>
<td>-0.33 ± 0.74</td>
<td>0.47 ± 0.70</td>
<td>1.58 ± 0.68</td>
<td>1.01 ± 0.66</td>
<td>0.34 ± 0.63</td>
<td>0.80 ± 0.53</td>
<td>1.91 ± 0.50*</td>
<td>1.35 ± 0.48</td>
<td>0.67 ± 0.44</td>
<td>1.11 ± 0.43</td>
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<tr>
<td>CCL8</td>
<td>0.13 ± 0.64</td>
<td>0.72 ± 0.65</td>
<td>1.72 ± 0.50</td>
<td>2.59 ± 0.49*</td>
<td>0.03 ± 0.44</td>
<td>0.59 ± 0.71</td>
<td>1.59 ± 0.58</td>
<td>2.46 ± 0.56*</td>
<td>-0.10 ± 0.53</td>
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<td>1.87 ± 0.58</td>
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<td>0.88 ± 0.41</td>
<td>-1.69 ± 0.36*</td>
</tr>
<tr>
<td>CCL13</td>
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</tbody>
</table>

The bias factors were calculated as described in "Experimental Procedures". The agonist CCL3 was arbitrarily defined as the reference with a bias factor of zero. Bold values indicate bias factors with statistically significant difference with the CCL3 reference (* p< 0.05). Values represent the mean ± S.E.M. of at least three independent experiments. ND, not determined.
### Table 5 Bias factors at CCR2

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<tr>
<td>CCL2</td>
<td>0.00 ± 0.46</td>
<td>0.00 ± 0.49</td>
<td>0.00 ± 0.43</td>
<td>0.00 ± 0.44</td>
<td>0.00 ± 0.32</td>
<td>0.00 ± 0.52</td>
<td>0.00 ± 0.49</td>
<td>0.00 ± 0.24</td>
<td>0.00 ± 0.27</td>
<td>0.00 ± 0.52</td>
<td>0.00 ± 0.30</td>
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<td>0.00 ± 0.17</td>
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<td>CCL7</td>
<td>−0.72 ± 0.58</td>
<td>−0.26 ± 0.61</td>
<td>0.27 ± 0.56</td>
<td>−0.83 ± 0.68</td>
<td>ND</td>
<td>0.46 ± 0.32</td>
<td>0.98 ± 0.48</td>
<td>−0.11 ± 0.44</td>
<td>0.72 ± 0.26</td>
<td>0.52 ± 0.29</td>
<td>−0.8 ± 0.48</td>
<td>0.26 ± 0.33</td>
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<td>0.83 ± 0.56</td>
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<td>0.80 ± 0.73</td>
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<td>ND</td>
<td>1.89 ± 0.96*</td>
<td>1.02 ± 0.53</td>
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<td>−0.87 ± 0.37</td>
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<td>CCL13</td>
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<td>ND</td>
<td>0.26 ± 0.54</td>
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<td>1.12 ± 0.57</td>
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</table>

The bias factors were calculated as described in "Experimental Procedures". The agonist CCL2 was arbitrarily defined as the reference with a bias factor of zero. Bold values indicate bias factors with statistically significant differences with the CCL2 reference (* p < 0.05). Values represent the mean ± S.E.M. of at least three independent experiments. ND, not determined.
Corbisier Figure 1
Corbisier Figure 2
Corbisier Figure 3
Corbisier Figure 4
Corbisier Figure 5

A  B  

110 100 100 90 90 80 80 70 70 60 60 50 50 40 40 30 30 125 125 110 110 100 100 90 90 80 80 70 70 60 60 50 50 40 40 30 30

Calcium mobilization (% of ATP response)  
cAMP production (% of Forskolin response)  

Log[chemokine] M  

Log[chemokine] M  

CCR5  

CCL3  CCL4  CCL5  CCL8  CCL13
Corbisier Figure 7
Corbisier Figure 8
Corbisier Figure 9
Corbisier Figure 10
Corbisier Figure 11
Biased signaling at chemokine receptors
Jenny Corbisier, Céline Galès, Alexandre Huszagh, Marc Parmentier and Jean-Yves Springael

J. Biol. Chem. published online January 22, 2015

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