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The dual function chemokine receptor CCR2 drives migration and chemokine scavenging through distinct pathways

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Abstract

C-C chemokine receptor 2 (CCR2) is a dual function receptor. Like other G protein-coupled chemo receptors, it promotes monocyte infiltration into tissues in response to the chemokine CCL2, and like atypical chemokine receptors (ACKRs), it scavenges chemokine from the extracellular environment. CCR2 therefore mediates CCL2-dependent signaling as a G protein–coupled receptor (GPCR) and also limits CCL2 signaling as scavenger receptor. We investigated the mechanisms underlying CCR2 scavenging, including the involvement of intracellular proteins typically associated with GPCR signaling and internalization. Using CRISPR knockout cell lines, we showed that CCR2 scavenged by constitutively internalizing to remove CCL2 from the extracellular space and recycling back to the cell surface for further rounds of ligand sequestration. This process occurred independently of G proteins, GPCR kinases (GRKs), β-arrestins, and clathrin, which is distinct from other “professional” chemokine scavenger receptors that couple to GRKs, β-arrestins, or both. These findings set the stage for understanding the molecular regulators that determine CCR2 scavenging and may have implications for drug development targeting this therapeutically important receptor.

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Supplementary Materials
Fig. S1–S10.

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INTRODUCTION

Chemokine receptors control the migration of many different cell types, including leukocytes, in response to specific chemokine ligands. Upon binding to chemokine, most of these receptors couple to the $G\alpha_i$ class of heterotrimeric G proteins, which in turn activate the cell motility machinery. Although most chemokine receptors are G protein-coupled receptors (GPCRs), four receptors are referred to as atypical chemokine receptors (ACKRs) because they do not couple to G proteins or directly mediate cell migration (1). Instead, some of these ACKRs (ACKR2, ACKR3, and ACKR4) scavenge chemokines to regulate extracellular ligand concentrations, which maintains the responsiveness of canonical G protein-coupled chemokine receptors that share the same ligand(s) (2). For example, proper migration and positioning of cortical interneurons not only requires CXCR4, which is directly responsible for driving neuronal migration towards CXCL12, but also ACKR3, which scavenges CXCL12. In the absence of ACKR3-mediated CXCL12 scavenging, CXCR4-mediated migration is markedly defective due to overstimulation and downregulation of CXCR4 (3). Chemokine uptake by atypical receptors also contributes to the resolution phase of inflammatory responses and the creation of chemokine gradients that promote leukocyte migration and extravasation (2, 4–8).

Scavenging is not restricted to atypical receptors, however. Early receptor knockout studies of canonical chemokine receptors (CCR2, CXCR2, CXCR3 and CX3CR1) revealed elevated plasma levels of the cognate chemokines, suggesting that these receptors mediate chemokine scavenging (9). CCR2, a GPCR and key regulator of monocyte migration, was later confirmed as a scavenger receptor (10, 11) and one study showed that CCR2 scavenges more efficiently than the atypical receptor ACKR2 (6). Thus, CCR2 is a dual function receptor that directly regulates both cell migration and scavenging, in contrast to professional scavenger receptors that cooperate with canonical GPCRs to facilitate migration.

For both atypical and canonical receptors, scavenging involves internalization and recycling of the receptor with concomitant clearance of the ligand from the extracellular space. However, knowledge of the detailed interactions and pathways that regulate scavenging is sparse and at times contradictory. In this study, we investigated the molecular mechanisms by which CCR2 scavenges, focusing on the role of intracellular proteins usually associated with GPCR signaling and internalization. Our data suggests that in addition to the G protein-dependent receptor population that controls migration, a second functional population of CCR2 scavenged CCL2 in a manner independent of G proteins, GRKs, arrestins and clathrin. This population constitutively internalized and recycled through mechanisms that have yet to be defined and accounted for the largest amount of chemokine removed from the extracellular space. We also showed that CCR1, another GPCR on monocytes that scavenges chemokine, did so in a G protein-independent but β-arrestin-dependent manner, in contrast to CCR2. This suggested that different mechanisms may operate for different receptors, possibly to avoid pathway competition in inflammatory situations and complex chemokine stimuli. CCR2 (and CCR1) have been pursued as targets for inflammatory diseases (12) and the presence of a scavenging population may affect the efficacy of antagonists directed against these receptors, warranting an understanding of the mechanisms.

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RESULTS

Internalization and chemokine scavenging by CCR2 occurs independent of Gα proteins

Like canonical chemokine receptors, CCR2 requires the activation of $G\alpha_i\beta\gamma$ heterotrimers as the first step in a cascade of events that regulate cell migration. Subsequent interactions are less well understood but are assumed to involve common mechanisms associated with GPCR signaling. This includes phosphorylation of the receptor C-terminus by G protein receptor kinases (GRKs), specifically GRK2 and GRK3, which promote CCR2 desensitization (13). β-arrestin recruitment is then triggered by CCR2 phosphorylation (14–17), and as for many GPCRs, implicated in the internalization of the receptor (18, 19). We sought to systematically investigate if and how these mediators (G proteins, GRKs and β-arrestin) contribute to internalization and scavenging by CCR2, starting with G proteins.

In contrast to CCR2-mediated cell migration, scavenging by CCR2 is $G\alpha_i$-independent by pharmacological inhibition using Pertussis Toxin (PTx) (10, 11). Additionally, in human embryonic kidney (HEK) 293 cell lines with CRISPR knockout (KO) of G proteins, scavenging is independent of $G\alpha_{q/11}$, which also couples to CCR2 (10, 11, 20). However, because $G\alpha_i$ and $G\alpha_{q/11}$ were assessed individually, which could lead to compensatory effects, and other Gα protein subtypes have not been tested, we measured CCL2 remaining in the media of CCR2-expressing cell lines with CRISPR KO of $G\alpha_i$ ($G\alpha_i$ KO) or KO of all Gα subtypes (Gα_all KO) (21). These experiments showed that the ability of CCR2 to scavenge CCL2 was only slightly perturbed in cells deficient in all Gα proteins and to a lesser extent in $G\alpha_i$ KO cells compared to the parental (WT) HEK293 cells (Fig. 1A). The slight reduction in the clearance of CCL2 from the media in the Gα_all KO cells is likely due to a slight contribution from Gαi and some other G protein (likely $G\alpha_{q/11}$) due to the canonical G protein-dependent population, which by default internalizes some chemokine along with the receptor. However, the bulk of chemokine scavenged did not depend on G proteins, consistent with prior work (10, 11).

β-arrestin1 and β-arrestin2 associate with CCR2 after stimulation with agonist in a manner that partially depends on $G\alpha_i$ (19). Because β-arrestin is often involved in receptor internalization, and receptor internalization is critical for chemokine scavenging, we examined CCL2-triggered β-arrestin recruitment to the plasma membrane (PM) in Gαi KO and Gα_all KO HEK293 cells. To monitor β-arrestin translocation, we used enhanced bystander bioluminescence resonance energy transfer (ebBRET) between RlucII-tagged β-arrestin1/2 and a fluorescently (rGFP) tagged version of the PM marker CAAX (22). The advantage of this ebBRET assay is that it allows the use of WT CCR2 without any C-terminal tags, which can potentially affect receptor trafficking and/or interactions. Recruitment of both β-arrestin1 and 2 was significantly diminished in the absence of $G\alpha_i$ and almost completely lost when all Gα proteins were absent (Fig. 1, B and C). The latter result is likely due to the loss of GRK2- and GRK3-mediated phosphorylation of the receptor C-terminus. Both kinases contain pleckstrin homology (PH) domains and because their recruitment to the PM depends on the binding of this domain to active Gβγ (23, 24), CCR2 phosphorylation is expected to be diminished in cells lacking all Gα subunits (13).

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Similar results were also observed by BRET with direct recruitment of GFP10-β-arrestin to CCR2-RlucII (fig. S1, A and B).

Because a substantial decrease in β-arrestin recruitment in the absence of G proteins would be expected to impair receptor internalization, we also investigated the ability of CCR2 to internalize in the Gαi and Gα_all KO cells with CCR2-RlucII and rGFP-CAAX BRET pairs. As compared to WT HEK293 cells, cells deficient in all Gα subunits showed only a minor loss of CCL2-induced receptor internalization (Fig. 1D), which was surprising given the lack of β-arrestin recruitment. Similar results were also observed when using CCR2-RlucII association with the early endosome marker rGFP-FYVE as an orthogonal measure of receptor internalization (fig. S2, A through E). However, we previously showed that BRET-based methods cannot detect the constitutive internalization of CCR2 in the absence of chemokine (11), because processes that occur continuously and effectively at equilibrium give rise to a baseline BRET state. It is only after a perturbation, such as ligand addition, that a BRET signal corresponding to a change in receptor internalization, can be detected above the baseline (fig. S3A). Therefore, to monitor constitutive receptor internalization, another method is required. Accordingly, we developed a "pre-label" flow cytometry assay (11) in which cell surface receptors are labeled at 4°C, a temperature that does not permit internalization and therefore enables quantification of the initial level of surface receptor. Upon warming the cells to 37°C (without ligand), constitutive internalization and endocytic trafficking of the receptor resume, and the amount of labeled receptor at the cell surface decreases. The amount of original (or pre-labeled) receptor remaining at the surface can then be detected with a fluorescent secondary antibody (fig. S3B), and loss of receptor due to constitutive internalization can be quantified by the decrease in fluorescence signal compared to that at 4°C. Using this new method, we showed that constitutive internalization of CCR2 was not reduced by the loss of $G\alpha_i$ and in fact was slightly increased by the loss of all Gα proteins (Fig. 1E). Confirmation of G protein-independent receptor internalization was visualized by confocal fluorescence microscopy using surface-labeled SNAP-CCR2 (Fig. 1F). Together, these data suggest that CCR2 constitutively internalizes in a ligand-dependent and -independent manner and scavenges chemokine independently of G proteins, similar to atypical chemokine receptors (1).

GRKs and receptor C-terminal phosphorylation are not required for CCR2 internalization and scavenging

Phosphorylation of the C-terminus of agonist stimulated GPCRs is a crucial step in the recruitment of β-arrestins, and often results in arrestin-mediated receptor internalization (25–27). Phosphorylation of CCR2 contributes to receptor internalization (28, 29), but which GRKs play a role and whether phosphorylation contributes to constitutive as well as agonist-induced internalization is unclear. To address these questions, we conducted receptor internalization and chemokine scavenging experiments in CCR2-expressing HEK293A CRISPR KO cells lacking either GRK2/3, GRK5/6, or GRK2/3/5/6. ELISA-based quantification of CCR2 scavenging revealed that only the GRK2/3/5/6 KO cell lines showed a significant reduction in extracellular CCL2 (Fig. 2A). However, even in these cells, the difference compared to CCR2 in WT cells was minor. Assessment of β-arrestin recruitment by ebBRET showed a substantial loss of CCL2-mediated β-arrestin

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recruitment in both GRK2/3 and GRK2/3/5/6 KO cells (Fig. 2, B and C), consistent with a contribution of GRK2 to the phosphorylation of CCR2 (28). The further decrease in β-arrestin2 recruitment in GRK2/3/5/6 KO cells indicated that GRK5 and/or 6 had only a minor effect. Assessment of CCR2 internalization by BRET and flow cytometry analysis revealed that CCL2-dependent internalization was partially decreased in GRK2/3 KO cells and almost completely lost in the GRK2/3/5/6 KO cells (Fig. 2D), whereas constitutive receptor internalization was unaffected (Fig. 2E). Fluorescence microscopy imaging also confirmed that constitutive internalization of CCR2 was independent of GRKs (Fig. 2F).

Although GRKs contribute to the phosphorylation of CCR2, we sought to determine whether any C-terminal phosphorylation was required for chemokine scavenging using a mutant of CCR2 (CCR2-ST/9A) with nine C-terminal serine/threonine residues mutated to alanine. Ser^{356} at the extreme C-terminus of CCR2 was left unchanged because it is part of a putative class II PDZ-binding motif, which may be required for receptor trafficking or recycling (30, 31). Moreover, CCR2-ST/9A with an additional S356A mutation (CCR2-ST/10A) behaved similarly as the CCR2-ST/9A mutant (fig. S4, A through C). Consistent with the GRK KO cells, cells expressing the CCR2-ST/9A mutant showed only a minor loss of CCL2 scavenging (Fig. 3A), but a significant loss of β-arrestin recruitment (Fig. 3, B and C) and CCL2-dependent internalization (Fig. 3D). However, as with the loss of Gα proteins or GRKs, constitutive internalization of CCR2 was unaffected (Fig. 3, E and F). Fluorescence microscopy confirmed that CCL2-mCherry was efficiently scavenged in the absence of GRK2/3/5/6 and C-terminal serine/threonine residues (fig. S5, A and B) and that the loss of extracellular CCL2 was due to chemokine uptake into cells and not just binding at the surface. These results suggest that CCR2 phosphorylation is important for β-arrestin recruitment and canonical ligand-induced internalization but not for constitutive internalization and chemokine scavenging. The data also further support the role of constitutive CCR2 internalization in chemokine scavenging.

β-arrestins play a minor role in chemokine scavenging by CCR2

Upon activation and phosphorylation by GRKs, many GPCRs interact with β-arrestins, which mediate internalization by engaging endocytic machinery components such as AP2 and clathrin (32, 33). The above data showing the sensitivity of β-arrestin recruitment to the loss of Gα proteins, GRKs and C-terminal phosphorylation suggested a canonical role for β-arrestins in CCR2 function, similar to many GPCRs. Indeed, β-arrestin is recruited to CCR2 following chemokine stimulation (19) and is involved in receptor desensitization in primary monocytes, such that disruption of GRK2-mediated receptor phosphorylation and β-arrestin recruitment leads to increased monocyte migration (13, 34, 35). Through their effects on internalization, β-arrestins also contribute to chemokine scavenging by the atypical receptors ACKR2 (36), ACKR4 (37), and possibly ACKR3, although there are conflicting reports for this receptor (38–40). By contrast, the reduction of CCL2-stimulated β-arrestin recruitment but not CCL2 scavenging in the G protein or GRK KO cells or cells expressing the CCR2 phosphorylation mutants suggested that β-arrestins are not necessary for CCR2-mediated scavenging. However, these experiments do not exclude a role for a constitutive interaction between CCR2 and β-arrestin, which is not detectable in the β-arrestin recruitment assay. To more directly address the role of β-arrestin, we investigated

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CCR2-mediated scavenging using HEK293 cells with CRISPR KO of both β-arrestin1 and 2, and observed only a small (~19%) but measurable loss of CCL2 scavenging (Figure 4A). Moreover, CCL2-mCherry was efficiently scavenged in the absence of β-arrestin1/2 (fig. S6). On the other hand, knockout of β-arrestin1/2 led to a complete loss of CCL2-induced receptor internalization (Fig. 4B), similar to the effects of the GRK2/3/5/6 deletion, but it did not affect constitutive receptor internalization as determined by flow cytometry (Fig. 4C) and confocal microscopy (Fig. 4D). We confirmed these observations in CCR2-expressing THP-1 monocytic cell line with CRISPR KO of β-arrestin1 and β-arrestin2 (fig. S7, A and B). As for the CCR2-expressing HEK293 cells, only a small (~19%) reduction in scavenging (Fig. 4E) and an even smaller (~8%) loss of constitutive internalization (Fig. 4F) was observed.

Because loss of GRK2-mediated receptor phosphorylation and β-arrestin recruitment was previously reported to result in increased monocyte migration (13, 34, 35), we tested the effect of β-arrestin1/2 KD in THP-1 cells and also observed a significant increase in migration, but only at CCL2 concentrations of 100 nM and above (Fig. 4G). This is consistent with the role of β-arrestin in receptor desensitization; however, the lack of increased migration in the β-arrestin1/2 KD cells at lower, more physiological levels of CCL2 indicates that CCR2 remains responsive even when β-arrestin is present. Together with minor contributions of G proteins and GRKs to scavenging (described above) but the significant loss of β-arrestin recruitment in the absence of Gα subunits or GRKs, these data suggest that β-arrestins only play a small role in CCR2-mediated scavenging, which is associated with canonical ligand-stimulated receptor internalization dependent on G protein, GRK, and phosphorylation. The data also suggest that constitutive internalization, which appears to play a major role in CCR2-mediated scavenging, is β-arrestin independent.

Chemokine scavenging occurs independently of clathrin-mediated endocytosis

The above data implicate constitutive internalization as a consistent feature of CCR2 scavenging. However, the pathways by which CCR2 (and other chemokine receptors) constitutively internalize are poorly understood. Although clathrin is often involved in canonical ligand-dependent internalization of many GPCRs (41), some GPCRs, such as protease-activated receptor-1 (PAR1) and thromboxane-A2β receptor, constitutively internalize through clathrin-coated pits independently of phosphorylation and β-arrestin (42–44). We therefore evaluated the ability of CCR2 to internalize and scavenge using a dominant-negative form of dynamin-2 (DNM2-K44A) that inhibits clathrin-mediated endocytosis (CME) (45). DNM2-K44A had no effect on the ability of CCR2 to scavenge chemokine (Figure 5A) and only a small effect on constitutive internalization (Fig. 5, B and C). By contrast, CCL2-dependent internalization was significantly inhibited by the dominant-negative dynamin mutant (Fig. 5D), consistent with a canonical mechanism of internalization. Consistent with this finding, confocal microscopy showed that the dynamin inhibitor Dyngo-4a (46) did not affect constitutive internalization (fig. S8).

CCR2 undergoes rapid recycling and is resistant to degradation

The above data suggests that CCR2-mediated ligand scavenging is robust and largely unaffected by perturbations of mediators frequently associated with GPCR function. To

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further probe CCR2 scavenging mechanisms, we investigated its endosomal trafficking patterns in comparison to the scavenging receptor ACKR3 (47, 48) and the non-scavenging receptor CXCR4 (11). BRET experiments were conducted using C-terminally RlucII-tagged receptors in combination with rGFP-tagged Rab4, Rab11 or Rab7 endosomal markers that represent fast recycling, slow recycling and late endosomal association, respectively (49). CCR2 rapidly sorted into fast recycling Rab4-positive endosomes and Rab7-positive late endosomes but did not associate with Rab11-positive slow recycling endosomes (Figure 6, A to C). Sorting of CCR2 into Rab4-positive fast recycling endosomes was more extensive and sustained compared to the atypical scavenging receptor ACKR3, which sorted to a greater extent into slow recycling endosomes. Additionally, although Rab7 is generally regarded to be associated with lysosomal degradation, Rab7 also recycles through the trans-Golgi network (TGN) (50–52), and due to the lack of observed degradation (shown below) we believe CCR2 is trafficking through this TGN recycling pathway. In contrast to CCR2 and ACKR3, the chemokine-activated GPCR CXCR4 showed minimal association with recycling endosomes, consistent with the fact that it does not scavenge chemokine. These results highlight the general differences between endosomal trafficking patterns observed in chemokine receptor endocytosis.

Using a BRET-based method (22), we also demonstrated that CCR2 internalized following chemokine stimulation but robustly recycled back to the plasma membrane upon chemokine washout (Fig. 6, D to F) or after addition of the CCR2 inhibitor BMS681 (fig. S9). Compared to ACKR3, which also constitutively internalizes and recycles, CCR2 recycled back to the plasma membrane more efficiently. As an orthogonal strategy, we used a surface receptor labeling method (39), which also revealed that cell surface CCR2 decreased upon chemokine stimulation but was quickly replenished following CCL2 removal (Fig. 6G). Finally, although CCR2 associated with Rab7-positive late endosomes, Western blot analysis revealed that the expression level of CCR2 was maintained following CCL2 stimulation, in contrast to the control GPCR PAR1 (Fig. 6, H and I) which was degraded following agonist addition. These data suggest that CCR2 has distinct trafficking mechanisms compared to other chemokine and non-chemokine receptors, which is not surprising because it functions as both a scavenging receptor and a canonical GPCR. The ability to rapidly recycle and avoid depletion may also contribute to its ability to efficiently scavenge chemokine.

CCR2 and CCR1 have distinct mechanisms of scavenging

CCR1 is another chemokine receptor expressed on monocytes that regulates migration (53, 54). We and others previously showed that despite being a G protein-coupled chemokine receptor like CCR2, CCR1 scavenges chemokine (55, 56). Furthermore, we determined that CCR1 is constitutively phosphorylated, constitutively interacts with β-arrestin2 and constitutively internalizes in a β-arrestin2-dependent manner (55). Given that scavenging relies on constitutive internalization, we hypothesized that chemokine scavenging by CCR1 may be β-arrestin-dependent. Indeed, β-arrestin KO cells showed a major reduction (~54%) in CCL14 scavenging by CCR1 (Figure 7A), which contrasts with the minor effect of β-arrestin KO on chemokine sequestration by CCR2 (19%).

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Given the different β-arrestin dependencies of CCR1 and CCR2, as well as the general role of β-arrestins in receptor desensitization, we also evaluated the impact of the loss of β-arrestin on $G\alpha_i$ activation using $G\alpha_i$-Nluc and Gβγ-cpVenus in a BRET-based Gαβγ dissociation assay (37). In the β-arrestin KO cells, both CCR1 and CCR2 triggered similar, sustained $G\alpha_i$:Gβγ dissociation upon stimulation with chemokine, as expected (Fig. 7, B and C). However, in WT cells, CCR1 quickly activated $G\alpha_i$, but $G\alpha_i$ and Gβγ rapidly reassociated, likely because the receptor returned to a state in which it was constitutively phosphorylated and associated with β-arrestin (Fig. 7D). By contrast, CCR2 had a similar activation profile in the presence or absence of β-arrestin (Fig. 7E), indicating that β-arrestin mediated desensitization of CCR2 does not occur to the same extent as for CCR1. The prolonged activation of G protein as observed in this assay may reflect the ability of CCR2 to avoid desensitization and/or downregulation (Fig. 6H), allowing cells to migrate, even when exposed to high concentrations of chemokine (10). Consistent with these findings, activation by the formyl peptide receptor-1 (FPR1) results in heterologous desensitization of CCR1 but not CCR2 (57). Comparisons to endosomal trafficking of CCR1 were attempted but proved unsuccessful due to the lack of cell surface expression of C-terminally RlucII-tagged CCR1 (CCR1-RlucII) (fig. S10, A and B). Together, the results suggest that β-arrestin regulates scavenging and signaling of CCR1 to a greater extent than CCR2. Moreover, the greater susceptibility of CCR1 to desensitization may reflect a functional hierarchy of these two receptors in monocytes.

DISCUSSION

The physiological importance of clearing circulating chemokines as well as chemokines from tissue microenvironments has been documented most extensively for ACKRs. These receptors work in concert with canonical G protein-coupled chemokine receptors to regulate extracellular chemokine availability during normal immunological responses and inflammatory conditions. However, several canonical chemokine receptors including CCR1, CCR2 and CCR5, also act as scavenger receptors (2, 9–11, 55, 56). In this study we sought to define the mechanisms that regulate CCR2 scavenging as they are poorly understood.

Like most chemokine receptors, CCR2 is a GPCR that activates G proteins, is phosphorylated by GRKs, recruits β-arrestin and is internalized upon agonist stimulation (13, 19, 29). However, we showed that in contrast to its function in controlling cell migration, CCR2 robustly scavenged chemokine in a manner that was largely independent of these classical GPCR signaling pathways. We hypothesize that the minor contributions to scavenging from G proteins, GRKs and β-arrestins are attributable to chemokine uptake that occurs by default in canonical GPCR agonist-stimulated pathways. However, the bulk of chemokine scavenging arises from constitutive “passive” internalization of CCR2 through mediators that have yet to be identified. Thus, CCR2 appears to have two mechanistically distinct functional populations, one that regulates migration and one involved in scavenging (Fig. 8). Receptor molecules in these populations likely interchange, with constitutively internalized and recycling CCR2 acting as a feeder pathway that provides non-desensitized receptor for persistent CCL2-stimulated cell migration by the signaling pathway. The importance of rapid receptor recycling for continuous CCR2 signaling at the leading edge of monocytes was suggested by Volpe and coworkers to explain why receptor internalization

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does not reduce the responsiveness of the cells as they migrate towards CCL2 (10). The existence of a large population of internalized/recycling CCR2 molecules that do not need to be resensitized by dephosphorylation may contribute to the efficiency by which signaling-ready receptor molecules can be replenished at the leading edge of migrating cells from the scavenger pathway.

We originally hypothesized that β-arrestin might be an essential component of CCR2 scavenging because of its common role in receptor internalization. Additionally, we previously identified CCR1 as a scavenger that relies on β-arrestin to constitutively internalize (55), a key feature of scavenging, and we confirmed here that β-arrestin played a major role in chemokine sequestration by CCR1. However, unlike CCR1, we found that CCR2 constitutively internalizes in the absence of β-arrestin and that knockout of β-arrestin has only a small effect on scavenging (possible due to the agonist-stimulated canonical pathway). Consistent with these contrasting dependencies on β-arrestin, CCR2 scavenges largely independently of GRKs and phosphorylation of its C-terminus whereas CCR1 is basally phosphorylated (55). Thus, it appears that different receptors use different mechanisms for scavenging. Because CCR1 and CCR2 are both chemoattractant receptors on monocytes that predominantly scavenge distinct ligands, the use of different scavenging mechanisms might be important for avoiding competition for pathway regulators, which could impair chemokine clearance. CCR2 and CCR1 also differ with respect to the duration of G protein activation, with CCR1-activated G proteins returning more rapidly to an inactive state when β-arrestin is present. The prolonged activation of G proteins following stimulation by CCR2 both in the presence and absence of β-arrestin may reflect its relative functional independence from arrestin.

Whereas CCR2 scavenges chemokine independently of the most common agonist-triggered GPCR internalization mechanisms, constitutive internalization and recycling is crucial. In fact, we previously showed that the entire surface population of CCR2 in monocytes constitutively turns over and is replaced in under 30 minutes (11). Accordingly, we investigated the role of clathrin. However, perturbing CME did not inhibit CCR2-mediated chemokine scavenging (Fig. 5A). β-arrestin often acts as an adapter along with AP-2 to bridge clathrin (32, 33, 59), and these results are consistent with a lack of a role for β-arrestin in scavenging. Therefore, future endeavors will be focused on identifying mediators of clathrin-independent endocytosis (CIE). Possibilities include Ddc42 and Arf in the Rho family of GTPases, which regulate both clathrin- and dynamin-independent internalization pathways and recycling of membrane proteins, including GPCRs. For example, Arf3 is required for maintaining the integrity of the recycling endosome (60), whereas other members are involved in CIE (61–63). Arf6 is also involved in actin cytoskeleton remodeling, phagocytosis and migration, as well as regulating the recycling of GPCRs, such as the β_2-adrenergic receptor (64–66). Cdc42 is involved in macropinocytosis and phagocytosis in macrophages and dendritic cells, as well as transmigration of monocytes, all of which express CCR2, and could contribute to its passive scavenging (67–69). Pathways involved in the natural turnover of the cell membrane and the constitutive endocytic processes may also play a role (70). For example, GPCRs and other membrane proteins can sense and sort according to membrane curvature (71). The specific niches in which CCR2 resides within the membrane and its capacity to recycle back to the surface following

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constitutive endocytosis could influence its ability to scavenge. These pathways are less understood but may involve micro- and macropinocytosis mentioned above, as well as the CLIC-GEEC pathway, and other CME and CIE mechanisms (72–74).

In addition to understanding the molecular controls of scavenging, a key question relates to the functional relevance of scavenging by canonical receptors. As suggested above and by Volpe and coworkers (10), scavenging may allow cells to continuously migrate by remaining responsive to chemoattractant, which may be particularly important for monocytes during pro-inflammatory immune responses. For CCR2, sustained G protein activation following agonist stimulation (Fig. 7, E) may also contribute to persistent migration, thereby synergizing with scavenging and rapid recycling. On the other hand, migration without desensitizing may be inappropriate for certain cell types in some contexts, which could be why some canonical receptors, like CXCR4, do not scavenge but instead rely on atypical receptors when scavenging is needed. Scavenging by canonical chemokine receptors may also enable migrating cells to create their own chemokine gradients and undergo self-directed migration or contribute to dampening or terminating the inflammatory response when such a response is no longer needed (5, 75–80). Finally, because some ligands of CCR2 are shared with other chemokine receptors (such as chemokine CCL7 with receptor CCR1), scavenging by CCR2 may impact other chemokine receptors and affect the migration of cells that express both receptors in trans (on different cells), or in cis, such as monocytes that express both CCR1 and CCR2. Along these lines, desensitization and downregulation of CCR1 occurs in PBMCs from CCR2 KO mice which fail to scavenge CCR2 ligands (9).

A final question concerns what mechanisms regulate whether CCR2 scavenges chemokine or is directed into G protein-dependent pathways to promote cell migration, and the factors that determine the balance of scavenging versus migration. In monocytes and dendritic cells exposed to treatments mimicking inflammation (LPS or IFNγ plus IL-10), CCR1, CCR2 and CCR5 switch purely to scavenging (56). They simultaneously become incapable of promoting cell migration as if uncoupled from G proteins, despite continued cell surface expression of the receptors. This is likely due to inhibition of some component of the cell motility machinery rather than a receptor-specific phenomenon, but whether there are also receptor-specific switches that determine relative amounts of scavenging versus migration remains to be determined. Changes in trafficking patterns could change the balance; for example, the β_2 adrenergic and M3 muscarinic receptors are reported to constitutively internalize via by CIE and shift to clathrin-dependent endocytosis after agonist stimulation (81).

The scavenging function of chemokine receptors needs to be considered when evaluating the safety and therapeutic efficacy of blocking receptor-ligand binding, especially considering the above potential roles of scavenging. CCR2 is being pursued as a target for several diseases (12, 82–89) but inhibition with small molecule antagonists leads to inhibition of scavenging and elevated plasma levels of CCL2 (90–93), the consequences of which are unknown (11). The presence of elevated levels of chemokine may also compete with receptor antagonists, thereby decreasing the efficacy of therapeutic drugs aimed at blocking CCR2-mediated cell migration (90, 91). To fully understand the role of scavenging in

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normal physiology and potential consequences of blocking it, a better understanding of the regulatory mechanisms will be required. This study provides insight in this direction by demonstrating that CCR2 has two functional populations, one that controls migration through canonical G protein mechanisms and one that controls scavenging through G protein-independent mechanisms. The results also provide a framework for understanding a body of prior studies directed at defining the network of intracellular proteins involved in CCR2 pharmacology.

MATERIALS AND METHODS

Cell lines

KO and parental (WT) control HEK293 cells were a kind gift of Dr. Asuka Inoue (Tohoku University, Japan). HEK293 cells lacking functional $G\alpha_i$ (Gi KO), or a combination of $G\alpha_i$, $G\alpha_o$, $G\alpha_q$, $G\alpha_z$, $G\alpha_{olf}$, $G\alpha_{11}$, $G\alpha_s$, $G\alpha_{12}$ and $G\alpha_{13}$ knockouts (Gs/i/o/olf/q/z/11/12/13 = G_all KO) were generated by CRISPR/Cas9 as previously reported (21, 94). A dual β-arrestin1 and β-arrestin2 knockout (β-arrestin1/2 KO) was prepared by CRISPR/Cas9 targeting of ARRB1 and ARRB2 as described previously (95). Similarly, CRISPR/Cas9 was used to generate GRK2/3 KO, GRK5/6 KO and GRK2/3/5/6 KO in the HEK293A cell line (96). THP-1 β-arrestin1/2 KD and corresponding THP-1 negative gRNA control cells were prepared using lentiCRISPRv2 (97), a gift from Dr. Feng Zhang (Broad Institute, MA, USA) and gRNAs generated using CHOPCHOPv3 (98). THP-1 β-arrestin1/2 KD was confirmed by Western blot (fig. S6, A) using anti-β-arrestin1 (#D8O3J, Cell Signaling Technology, MA, USA), and anti-β-arrestin2 (#C16D9, Cell Signaling Technology, MA, USA) antibodies. Stable chemokine receptor-expressing cells (CCR1 or CCR2) were generated in the HEK293 WT and all KO cell lines by lentiviral transduction using pLenti-CMV-Hygro expression vector, a gift from Eric Campeau & Paul Kaufman (University of Massachusetts Medical School, MA, USA) (99) and receptor surface expression confirmed by flow cytometry (Guava EasyCyte™ 8HT, Luminex) with an anti-hCCR2-PE (phycoerythrin) antibody (FAB151P, R&D Systems). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with GlutaMax (Gibco) and 10% fetal bovine serum (FBS) and grown at 37°C with 5% CO_2.

DNA plasmids and cloning

The pcDNA3.1(+) constructs of β-arrestin1-RlucII, β-arrestin2-RlucII, rGFP-CAAX, rGFP-Rab4 and rGFP-Rab11 (22) were kindly gifted by Dr. Michel Bouvier (Université de Montréal, Canada). The FLAG-PAR1 construct was kindly gifted by Dr. JoAnn Trejo (UC San Diego, La Jolla, CA, USA). The rGFP-Rab7 construct was generated by PCR amplification of the Rab7 coding sequence of EGFP-Rab7A, a gift from Qing Zhong (UC Berkeley, CA, USA) (100) and in-frame insertion into the XbaI and PmeI sites of rGFP-Rab4 by DNA ligation. Receptor-RlucII (CCR2, CXCR4, ACKR3) constructs were created by PCR amplification of the coding region of CCR2, CXCR4 and ACKR3; products were subcloned in-frame at the N-terminus of the RlucII sequence into the pcDNA3.1 RlucII vector. The CCR2-ST/9A (S316A, T324A, T338A, T343A, S344A, T345A, T347A, S349A and T350A) phosphorylation-deficient mutant was created using site-directed mutagenesis with the QuikChange mutagenesis kit (Agilent Technologies, La Jolla, CA, USA). To

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obtain FLAG-SNAP-CCR2, the coding sequence of pcDNA3.1(+) CCR2 vectors were PCR amplified and then inserted into the pRK5–FLAG-ST–CXCR4 plasmid (101), containing a mGlu5 receptor signal peptide (102) that promotes proper receptor trafficking to the cell surface (kind gift of Dr. Angélique Levoye, University of Paris, France). Introduction of two gRNAs into the lentiCRISPRv2 plasmid for β-arrestin1/2 KD was performed using the Multiplex CRISPR/Cas9 Assembly System, a gift from Dr. Takashi Yamamoto (Hiroshima University, Japan) (103), following the Golden Gate assembly method as previously described previously (104).

Chemokine scavenging ELISA assay

WT non-receptor-expressing or stable receptor-expressing (CCR1 or CCR2) HEK293 cells and corresponding KO cells were seeded in triplicate in 96-well dishes at 50,000 cells/well and allowed to adhere for ~8 h. Subsequently, media was replaced with DMEM/10% FBS media containing 5 nM CCL2 or CCL14 and incubated for ~16 h. Remaining chemokine levels in the supernatants of cultured cells were measured in triplicate using commercially available CCL2 and CCL14 Invitrogen™ ELISA kits according to the manufacturer's instructions (Fisher Scientific, Hampton, NH, USA) and read with a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA). Remaining levels of exogenous chemokine are reported as the percentage of levels in supernatants of the corresponding non-receptor expressing cells.

BRET assays

HEK293 cells were seeded directly into 96-well plates (20,000 cells per well) and transfected the next day using Mirus TransIT-Lt1 transfection reagent at ~70% confluency. Cells were transfected with a BRET donor (1.2 ng of Receptor-RlucII or 0.72 ng of β-arrestin1- or β-arrestin2-RlucII per well) along with 4.8 ng of BRET acceptor per well (for example, rGFP-CAAX, rGFP-Rab4, rGFP-Rab11 or rGFP-Rab7). All assays were performed ~30 h after transfection, modified from previously described methods (22). Cells were washed once with pre-warmed Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.5 mM $MgCl_2$, 0.37 mM NaH_2PO_4, 25 mM HEPES, pH 7.4), then cell-permeable RlucII substrate, Prolume Purple (NanoLight Technologies) at a final concentration of 5 μM was added, ~3 to 6 min before BRET measurements. Three baseline BRET measurements were performed approximately 1 min apart, and were followed by the addition of the indicated concentrations of chemokine. Subsequent BRET measurements were taken approximately 1 min apart for 50 min or the indicated times.

For evaluation of receptor recycling to the plasma membrane, cells were seeded onto poly-D-lysine coated 96-well plates and transfected as described above. After 13 min of chemokine stimulation, cells were washed three times with pre-warmed Tyrode's buffer and subsequent BRET measurements were taken approximately 1 min apart for an additional 25 min. Values are presented as the percentage of mock treated (no ligand) [($BRET_{ligand}$/ $BRET_{basal}$) × 100]. All BRET measurements were read using a VictorX Light luminescence reader (Perkin Elmer, Waltham, MA, USA) or Spark microplate reader (Tecan, Männedorf, Switzerland).

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“Pre-label” assay to measure receptor constitutive internalization

CCR2 receptor surface expression was determined by a pre-label flow cytometry-based internalization assay (11, 55), which measures the number of labeled CCR2 molecules remaining on the cell surface after 45 min of incubation at 37°C. HEK293 cells stably expressing CCR2 were labeled with mouse anti-hCCR2 antibody (Clone# 48607, R&D Systems) or its isotype-matched control for 30 min on ice and protected from light. Unbound antibody was washed away with wash buffer (PBS, 0.5% bovine serum albumin (BSA)). Cells were then resuspended in Assay Buffer (DMEM, 0.5% BSA) and either held at 4°C (which prevents receptor trafficking and internalization) or transferred to 37°C (which allows for normal receptor trafficking) and incubated for 45 min. After incubation, cells were transferred to wet ice and the remaining surface receptor was labeled with anti-mouse antibody conjugated to PE (Clone# 344701, R&D Systems) for 40 min on ice and protected from light. CCR2 expression was assessed by flow cytometry using a Guava Easycyte™ 8HT flow cytometer (Luminex) and analyzed with FlowJo software (FlowJo, Ashland, OR, USA). The geometric mean fluorescent intensity of analyzed cells was used to quantify surface expression of CCR2 and compared to non-internalized control to determine relative percent of receptor remaining at the surface.

Receptor constitutive internalization microscopy

Constitutive internalization of CCR2 was qualitatively assessed based on a previously described method (102). HEK293 cells or the corresponding KO cell lines expressing FLAG-SNAP-CCR2 or FLAG-SNAP-CCR2-ST/9A were seeded onto fibronectin-coated 10 mm glass-bottom dishes (FluoroDish FD3510, WPI). For cells expressing dynamin-2 dominant negative mutant DNM2-K44A, cells were transfected with DNM2-K44A-EGFP, a kind gift of Dr. Jin Zhang (UC San Diego, La Jolla, CA, USA) and re-seeded 24 h after transfection. The next day, cells were stained with 5 μM cell impermeable SNAP-Surface Alexa Fluor 488 or 647 (New England Biolabs) in complete media (DMEM + 10% FBS) at 4°C in the dark for 45 min. Excess SNAP substrate was removed by washing with ice-cold complete media. Cells were imaged in phenol-red free DMEM media containing 2% FBS at 4°C (a temperature that prevents receptor trafficking and internalization) or transferred to 37°C for 45 min (a temperature that allows for normal receptor trafficking) and then imaged on an Eclipse Ti2-E (Nikon) equipped with a CSU-X1 (Yokogawa) spinning disk field scanning confocal system and stage top incubator (Tokai Hit).

SNAP-receptor recycling assay

The ability of CCR2 to recover to the cell surface following internalization was assessed based on a modified method as described (39). HEK293 cells stably expressing SNAP-tagged CCR2 were preincubated for 2 h with 5 mg/mL cycloheximide. Cells were then left untreated or stimulated with 100 nM CCL2 at 37°C for 45 min. Subsequently, cells were washed and remaining non-internalized SNAP-CCR2 at the cell surface was blocked at 4°C with SNAP-Surface block (New England Biolabs) for 45 min. The cells were then washed and shifted to 37°C for 15, 45 or 75 min. Following the specified timepoints, cells were transferred to wet ice and receptors were labeled with SNAP-Surface Alexa Fluor 649 (New

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England Biolabs) at 4°C. Data are displayed as a percentage of receptor compared to surface receptor measured at the start of the protocol.

Chemotaxis assay

Chemotaxis assays were carried out by using Transwell chemotaxis chambers (Corning Inc., Corning, New York, USA). Cells were harvested by centrifugation and resuspended in complete media, Roswell Park Memorial Institute 1640 (RPMI) + 10% FBS, at a density of 6.6×10^5 cells/ml. Various concentrations of CCL2 was added to the bottom of the chambers and covered with a 5-μm pore-sized polycarbonate membrane filter while 5×10^4 cells were added to the top of the filter. After 2 h incubation at 37°C, media containing migrated cells was removed from the bottom chamber and cells were counted by flow cytometry using a Guava Easycyte™ 8HT flow cytometer (Luminex) and analyzed with FlowJo software (FlowJo, Ashland, OR, USA). Data are presented as percentage of migrated cells compared to initial number of cells added before migration.

Receptor degradation assay

CCR2 degradation was assessed as described previously described (105). HEK293 cells were seeded in 12-well dishes (1.5×10^5 cells/well) and transfected the next day with either FLAG-CCR2 or FLAG-PAR1 using Mirus TransIT-Lt1 transfection reagent. After 48 h, cells were treated with 10 μg/mL cycloheximide for 90 min at 37°C. Cells were then incubated in the same media with or without 100 nM CCL2 or 100 μM TFLLRN (PAR1-specific agonist) for 2 h at 37°C. Cells were placed on ice, washed with PBS, and lysed in RIPA lysis buffer (Thermo Fisher Scientific) containing cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche). Cell lysates were collected and rotated end-over-end for 1 h at 4°C, and protein concentrations were determined by Pierce™ Rapid Gold bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Equivalent amounts of lysates were used for immunoprecipitation (IP) using M2 anti-FLAG affinity resin (Millipore Sigma) according to the manufacturer's instructions. Equivalent volumes of IP elution and equal amounts of lysates were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% nonfat dry milk (Bio-Rad) diluted in wash buffer (50 mm Tris-HCl, pH 7.4, 15 mm NaCl, 0.1% Tween 20) and subsequently washed. IP elution Western blots were incubated overnight at 4°C with anti-FLAG antibody (F4725, Millipore Sigma) diluted in TBS containing 5% BSA and lysate Western blots were incubated overnight at 4°C with anti-α-Tubulin (T6074, Millipore Sigma). Membranes were washed and probed with corresponding secondary IRDye® 800CW antibody (LI-COR Biosciences) diluted in TBS containing 5% BSA at room temperature for 1 h. Membranes were washed and imaged on an Odyssey Infrared Imaging System (LI-COR Biosciences). Densitometry was performed using LI-COR Image Studio software.

G protein dissociation assay

HEK293 cells and corresponding KO cells stably expressing CCR1 or CCR2 were seeded directly into 96-well plates (20,000 cells/well) and transfected the next day with pIRES Gα-Nluc Gβγ-cpVenus, a kind gift from Dr. Daniel Legler (University of Konstanz, Germany) using Mirus TransIT-Lt1 transfection reagent with cells at ~70% confluency (37). After ~30 h, cells were washed once with pre-warmed Tyrode's buffer followed by

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addition of 5 μM luciferase substrate coelenterazine-H (Biotium, CA, USA). Three baseline BRET measurements were performed approximately 1 min apart, followed by addition of indicated concentrations of chemokine ligand and subsequent BRET measurements were taken approximately 1 min apart for 25 min or indicated times.

Statistics

Data points are the mean ± SEM of at least three independent experiments. Data were analyzed using GraphPad Prism with statistically significant differences ($P < 0.05$) using one-way ANOVAs with Dunnett's multiple comparison post hoc test or unpaired t test with Welch's correction post hoc test. For comparison of BRET internalization and β-arrestin recruitment, the area under the curve (AUC) was determined using GraphPad Prism and statistical significance was determined using the appropriate tests mentioned above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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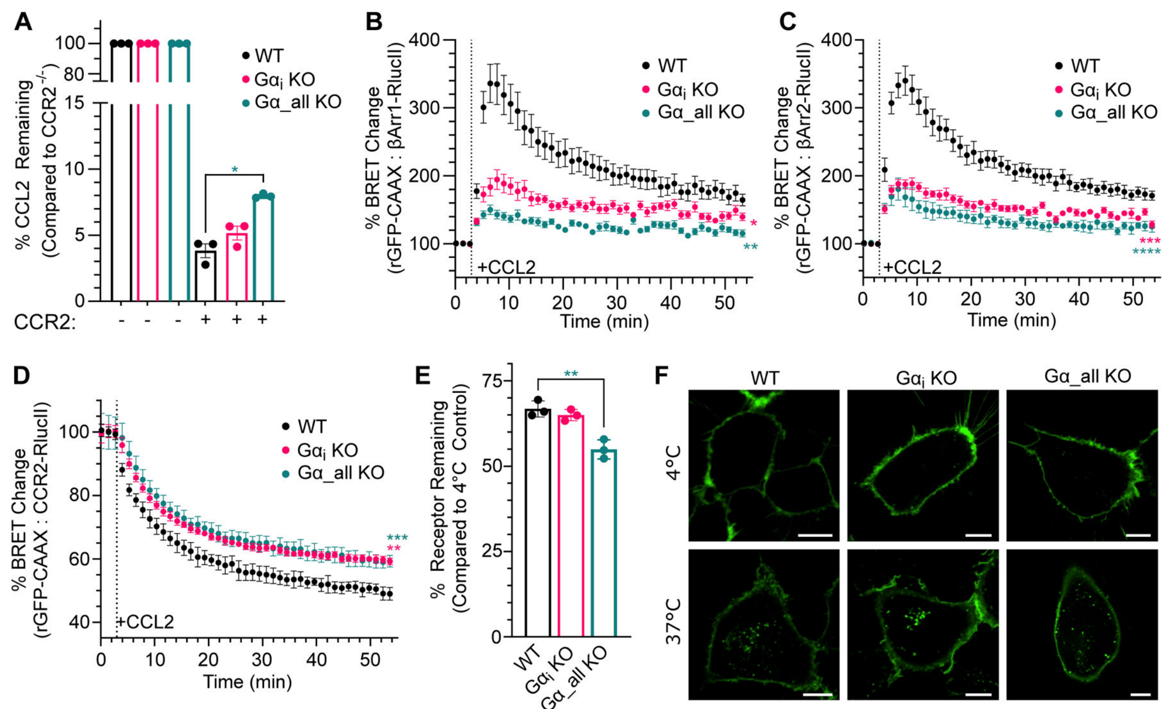

Fig. 1. Scavenging of CCR2 is G protein-independent.

(**A**) WT, $G\alpha_i$ KO and Gα_all KO HEK293 cells stably expressing CCR2 and respective non-expressing cells were cultured in media containing 5 nM CCL2 for 16 h. The remaining levels of CCL2 were measured by ELISA and interpolated from CCL2 standards, and are presented as percentages of remaining CCL2 relative to non-CCR2 expressing cells. (**B** and **C**) Cells transfected with β-arrestin1- or β-arrestin2-RlucII and rGFP-CAAX were stimulated with 100 nM CCL2 or left untreated. β-arrestin recruitment was assessed by ebBRET. Data are presented as percentages of BRET values for untreated controls. (**D**) CCR2 internalization assessed by BRET. Cells transfected with CCR2-RlucII and rGFP-CAAX were stimulated with 100 nM CCL2 or left untreated. The BRET ratio changes upon agonist treatment are expressed as percentages of the BRET ratio observed in untreated controls. (**E**) Constitutive internalization of WT, $G\alpha_i$ KO and Gα_all KO HEK293 cells stably expressing CCR2 were assessed by pre-label flow cytometry. Data are presented as the percentage of surface receptor remaining compared to non-internalized control. (**F**) Constitutive internalization was visualized by fluorescence confocal microscopy in WT, $G\alpha_i$ KO and Gα_all KO HEK293 cells expressing SNAP-CCR2 that was labeled with cell impermeable SNAP-Surface Alexa Fluor 488 at 4°C for 1 h. The cells were held at 4°C for 45 min (top panel) or transferred to 37°C for 45 min (bottom panel) before being imaged. Images are representative of three independent experiments. Scale bars, 10 μm. Data are expressed as the means ± SEM of n 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared to controls by one-way analysis of variance (ANOVA) with Dunnet’s multiple comparison test.

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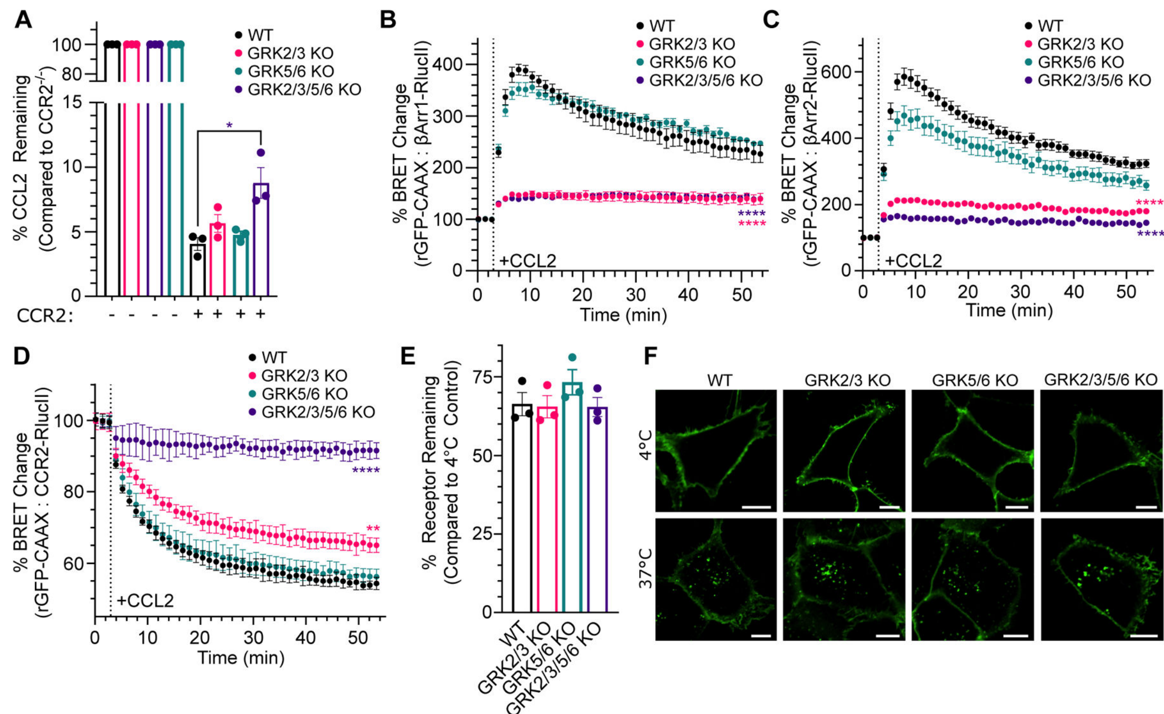

Fig. 2. CCR2 scavenging of CCL2 is not dependent on GRKs.

(**A**) WT HEK293A, HEK293A GRK2/3 KO, HEK293A GRK5/6 KO and HEK293A GRK2/3/5/6 KO cells stably expressing CCR2 and the corresponding CCR2 non-expressing cells were cultured in media containing 5 nM CCL2 for 16 h. The remaining levels of CCL2 were interpolated from CCL2 standards and are presented as percentages of remaining CCL2 relative to CCR2 non-expressing cells. (**B** and **C**) Cells transfected with β-arrestin1- or β-arrestin2-RlucII and rGFP-CAAX were stimulated with 100 nM CCL2 or left untreated. β-arrestin recruitment was assessed by ebBRET. Data are presented as percentages of BRET values for untreated controls. (**D**) CCR2 internalization assessed by BRET. Cells transfected with CCR2-RlucII and rGFP-CAAX were stimulated with 100 nM CCL2 or left untreated. The BRET ratio changes upon agonist treatment are expressed as percentages of the BRET ratio observed in the untreated controls. (**E**) Constitutive internalization of WT HEK293A, HEK293A GRK2/3 KO, HEK293A GRK5/6 KO and HEK293A GRK2/3/5/6 KO stably expressing CCR2 was assessed by pre-label flow cytometry. Data are presented as percentages of surface receptor remaining as compared to non-internalized control. (**F**) Constitutive internalization was visualized by fluorescence confocal microscopy in WT and corresponding GRK KO HEK293A cells expressing SNAP-CCR2 that was labeled with cell impermeable SNAP-Surface Alexa Fluor 488 at 4°C for 1 h. The cells were then held at 4°C for 45 min (top panel) or transferred to 37°C for 45 min (bottom panel) before being imaged. Images are representative of three independent experiments. Scale bars, 10 μm. Data are expressed as the means ± SEM of n 3 independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ compared to controls by one-way analysis of variance (ANOVA) with Dunnet's multiple comparison test.

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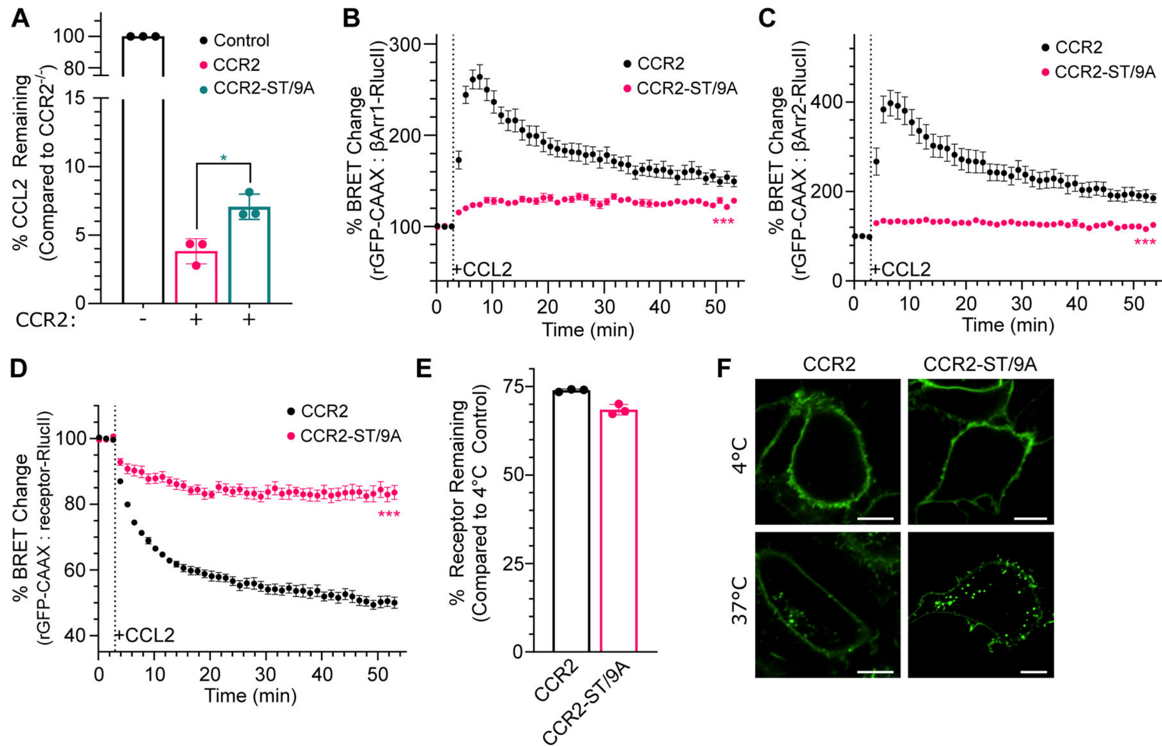

Fig. 3. CCR2 scavenging of CCL2 occurs independently of receptor phosphorylation.

(**A**) HEK293 cells stably expressing CCR2 or CCR2 with nine C-terminal serine/threonine residues mutated to alanine (CCR2-ST/9A) and non-expressing cells were cultured in media containing 5 nM CCL2 for 16 h. The remaining levels of CCL2 were interpolated from CCL2 standards and are presented as percentages of the respective non-CCR2 expressing cells. (**B** and **C**) Cells expressing CCR2 or CCR2-ST9/A transfected with β-arrestin1- or β-arrestin2-RlucII and rGFP-CAAX were stimulated with 100 nM CCL2 or left untreated. β-arrestin recruitment was assessed by ebBRET. (**D**) Cells transfected with CCR2-RlucII or CCR2-ST/9A-RlucII and rGFP-CAAX were stimulated with 100 nM CCL2 or left untreated. CCR2 internalization was assessed by BRET. The BRET ratio changes upon agonist treatment are expressed as percentages of the BRET ratio for untreated controls. (**E**) Constitutive internalization of WT CCR2 and CCR2-ST/9A in HEK293 cells was assessed by pre-label flow cytometry. (**F**) Constitutive internalization was visualized by fluorescence confocal microscopy in HEK293 cells expressing SNAP-CCR2 or SNAP-CCR2-ST/9A labeled with cell impermeable SNAP-Surface Alexa Fluor 488 at 4°C for 1 h. Cells were then either held at 4°C for 45 min (top panel) or transferred to 37°C for 45 min (bottom panel) before being imaged. Images are representative of three independent experiments. Scale bars, 10 μm. Data are expressed as the means ± SEM of n 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared to controls by unpaired t test.

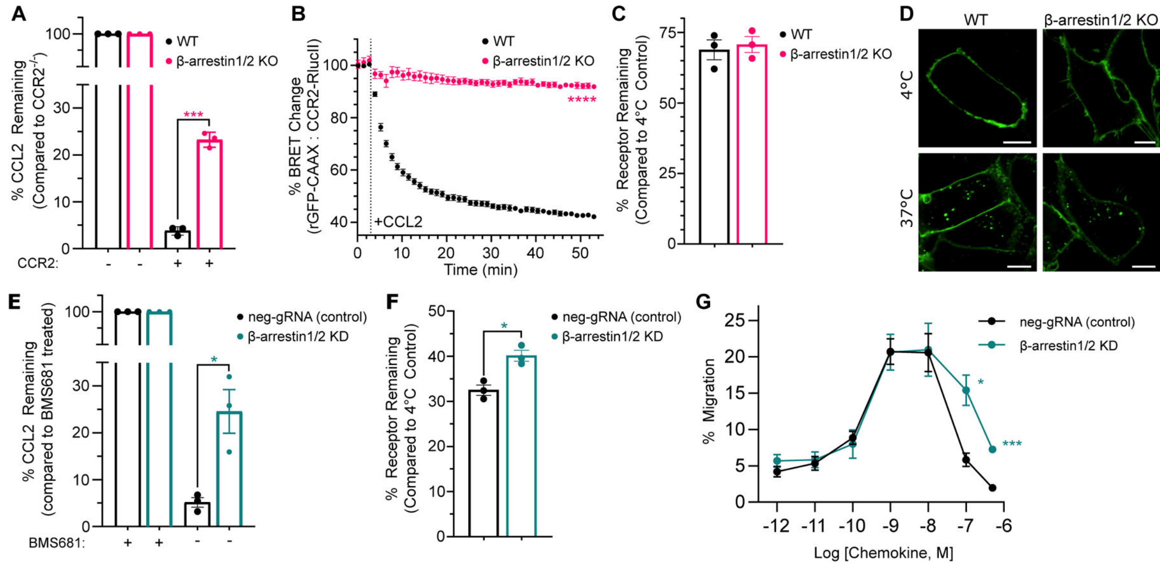

Fig. 4. β-arrestins are dispensable for constitutive internalization and chemokine scavenging by CCR2.

(**A**) WT HEK293 and β-arrestin1/2 KO HEK293 cells stably expressing CCR2 and respective non-expressing cells were cultured in media containing 5 nM CCL2 for 16 h. The remaining levels of CCL2 were interpolated from CCL2 standards and are presented as percentages of respective non-CCR2 expressing cells. (**B**) Cells transfected with CCR2-RlucII and rGFP-CAAX were stimulated with 100 nM CCL2 or left untreated. CCR2 internalization was assessed by BRET. The BRET ratio changes upon agonist treatment are expressed as percentages of the BRET ratio observed in untreated controls. (**C**) Constitutive internalization of WT HEK293 and β-arrestin1/2 KO HEK293 cells stably expressing CCR2 was assessed by pre-label flow cytometry. Data are presented as percentages of surface receptor remaining as compared to non-internalized control. (**D**) Constitutive internalization was visualized by fluorescence confocal microscopy in WT and β-arrestin1/2 KO HEK293 cells expressing SNAP-CCR2 that was labeled with cell impermeable SNAP-Surface Alexa Fluor 488 at 4°C for 1 h. The cells were then held at 4°C for 45 min (top panel) or transferred to 37°C for 45 min (bottom panel) before being imaged. Images are representative of three independent experiments. Scale bars, 10 μm. (**E**) THP-1 cells transduced with non-targeting gRNA (neg-gRNA) and THP-1 β-arrestin1/2 KD cells were treated with vehicle or the CCR2 inhibitor BMS681 and cultured in media containing 5 nM CCL2 for 16 h. The remaining levels of CCL2 were interpolated from CCL2 standards and are presented as percentages of non-scavenging control BMS681 treated cells. (**F**) Constitutive internalization of CCR2 in THP-1 neg-gRNA and β-arrestin1/2 KD THP-1 cells was assessed by pre-label flow cytometry. Data are presented as percentages of surface receptor remaining as compared to non-internalized control. (**G**) Transwell migration of THP-1 neg-gRNA and β-arrestin1/2 KD THP-1 cells at various concentrations of chemokine. Data are presented as percentages of migrated cells as compared to initial number of cells added to each well. Data are expressed as the means ± SEM of n 3 independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ compared to controls by unpaired t test.

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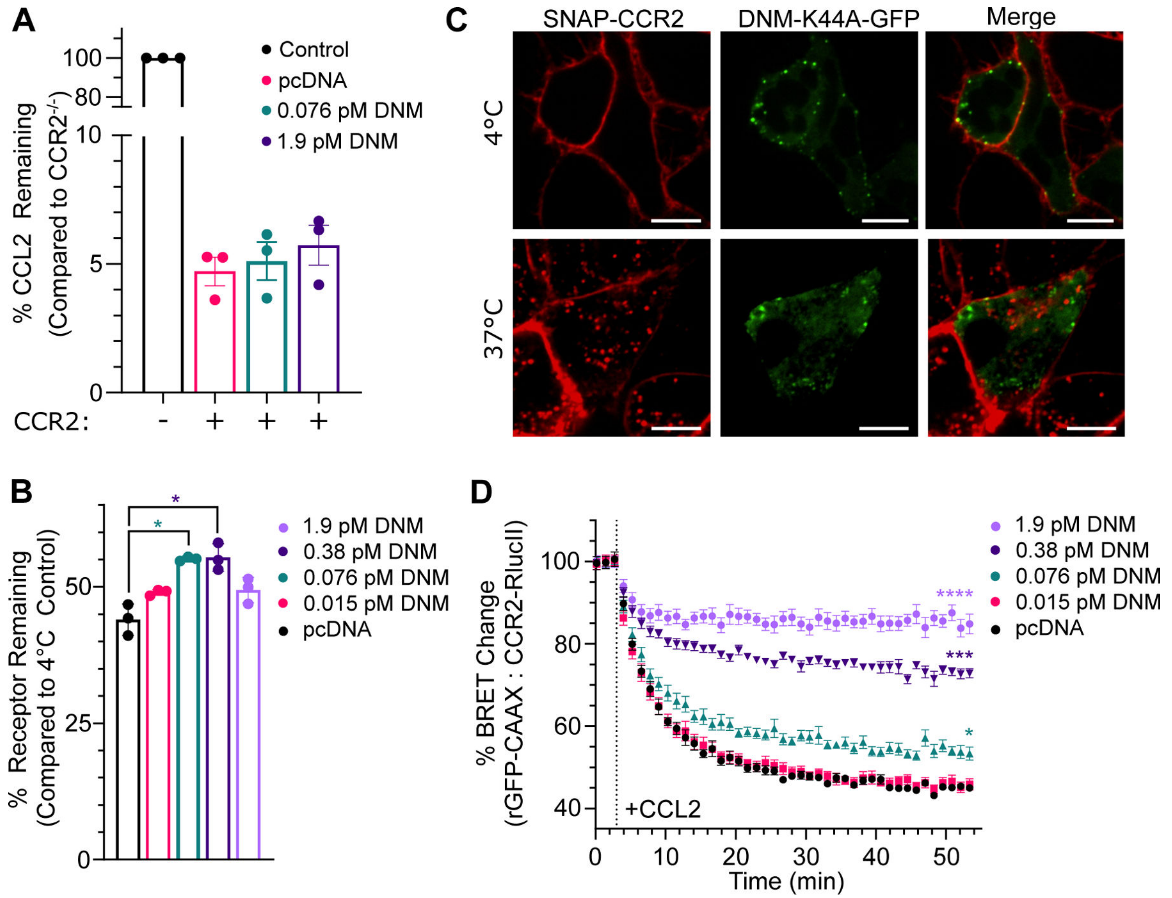

Fig. 5. Clathrin-mediated endocytosis is not required for CCR2 constitutive internalization or scavenging.

(**A**) Non-CCR2 expressing cells and HEK293 cells stably expressing CCR2 were transfected with two different concentrations of a dynamin dominant-negative mutant (DNM2-K44A) or left untransfected. The cells were cultured in media containing 5 nM CCL2 for 16 h. The remaining levels of CCL2 were determined by ELISA and interpolated from CCL2 standards and are presented as percentages of the respective non-CCR2 expressing cells. (**B**) Constitutive internalization of CCR2 was assessed by pre-label flow cytometry. HEK293 cells expressing CCR2 were transfected with pcDNA or an increasing amount of DNM2-K44A. Data are presented as percentages of surface receptor remaining as compared to non-internalized control. (**C**) Constitutive internalization in the presence or absence of DNM2-K44A was visualized by fluorescence confocal microscopy in HEK293 cells expressing SNAP-CCR2 and DNM2-K44A-GFP. SNAP-CCR2 was labeled with cell impermeable SNAP-Surface Alexa Fluor 649 at 4°C for 1 h and then either held at 4°C for 45 min (top panel) or transferred to 37°C for 45 min (bottom panel) before being analyzed. Scale bars, 10 μm. (**D**) Cells transfected with CCR2-RlucII, rGFP-CAAX, and pcDNA or increasing amounts of DNM2-K44A were incubated in the absence or presence of 100 nM CCL2. CCR2 internalization was assessed by BRET. The BRET ratio changes on agonist treatment are expressed as percentages of the BRET ratio observed in the untreated controls. Data are expressed as the means ± SEM of n 3 independent experiments. *$P < 0.05$, **P <

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0.01, ***P < 0.001, ****P < 0.0001 compared to controls by one-way analysis of variance (ANOVA) with Dunnet’s multiple comparison test.

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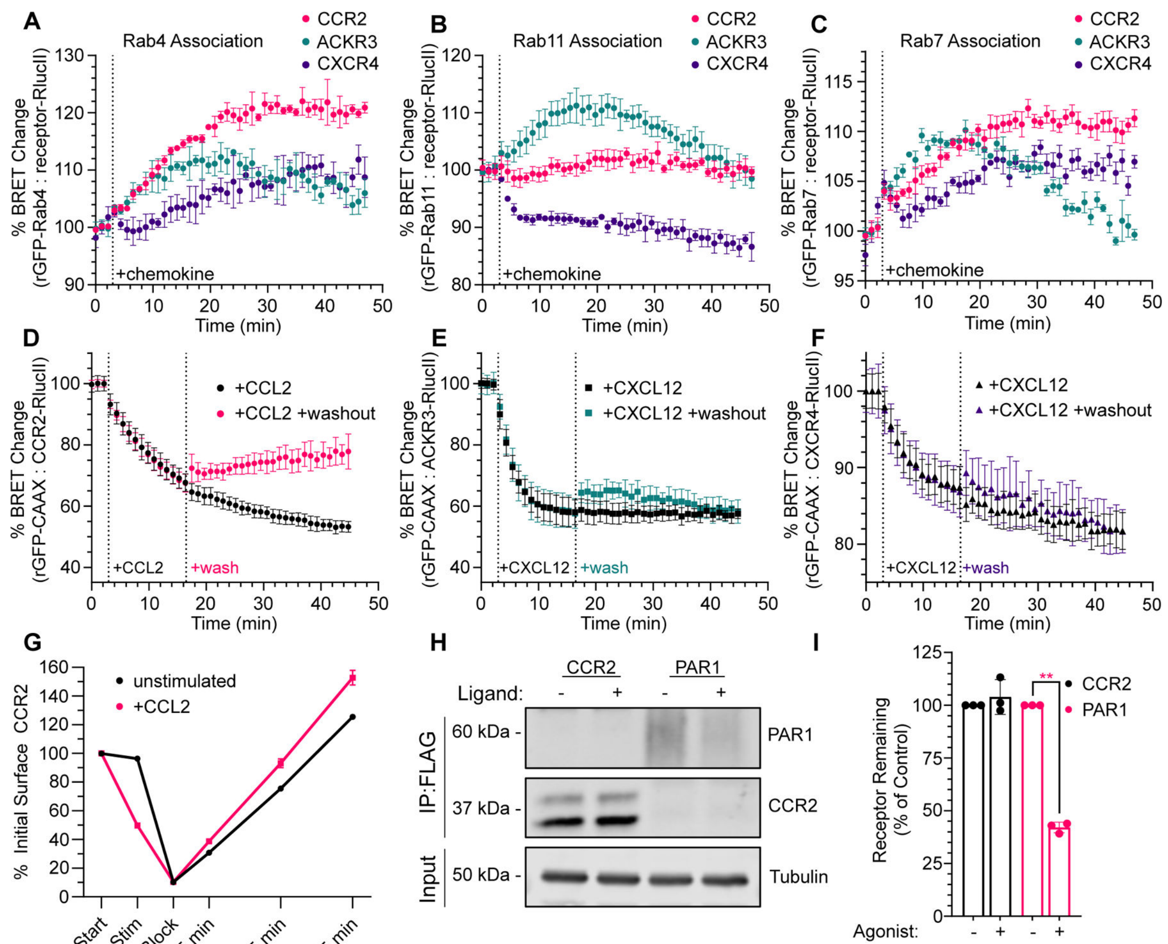

Fig. 6. CCR2 endosomal trafficking, recycling and lack of degradation contribute to scavenging.
(**A** to **C**) HEK293 cells were transfected with receptor-RlucII (CCR2, ACKR3 or CXCR4) along with either rGFP-Rab4 (A), rGFP-Rab11 (B) or rGFP-Rab7 (C). Cells were stimulated at 37°C with indicated chemokine or left untreated. Data are presented as percentages of BRET values compared to untreated controls. (**D** to **F**) HEK293 cells were transfected with rGFP-CAAX and either CCR2-RlucII (D), ACKR3-RlucII (E) or CXCR4-RlucII (F). Cells were stimulated at 37°C with indicated chemokine and washed with PBS to remove chemokine or left unwashed. Data are presented as percentages of BRET values compared to non-chemokine treated controls. (**G**) HEK293 cells stably expressing SNAP-CCR2 were preincubated for 90 min with 10 mg/mL cycloheximide to block de novo protein synthesis and left untreated or stimulated with 100 nM CCL2 at 37°C for 45 min. The remaining SNAP-CCR2 at the surface was blocked at 4°C with SNAP-Surface block for 45 min. Cells were moved to 37 °C for 15, 45 or 75 min. Receptors were labeled with SNAP-Surface Alexa Fluor 649 at 4°C following each experimental condition and timepoint. Data are displayed as percentages of CCR2 detected relative to initial levels of surface CCR2 prior to stimulation (Start). (**H, I**) HEK293 cells expressing FLAG-CCR2 or FLAG-PAR1 were pretreated with 10 μg/mL cycloheximide for 90 min and left unstimulated (0 min) or stimulated with 100 nM CCL2 (to activate CCR2) or 100 μM TFLLRN (to activate PAR1) for 2 h at 37°C. Equivalent amount of cell lysates were immunoprecipitated with M2 anti-

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FLAG antibody and immunoblotting detection with anti-FLAG antibody. Cell lysates were analyzed for endogenous α-tubulin as controls (H). Receptor degradation was quantified and data (mean ± SEM) shown are expressed as the fraction of receptor remaining compared with untreated control cells as determined from three independent experiments (I). Data are expressed as the means ± SEM of n 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared to controls by one-way analysis of variance (ANOVA) with Dunnet's multiple comparison test or unpaired t test.

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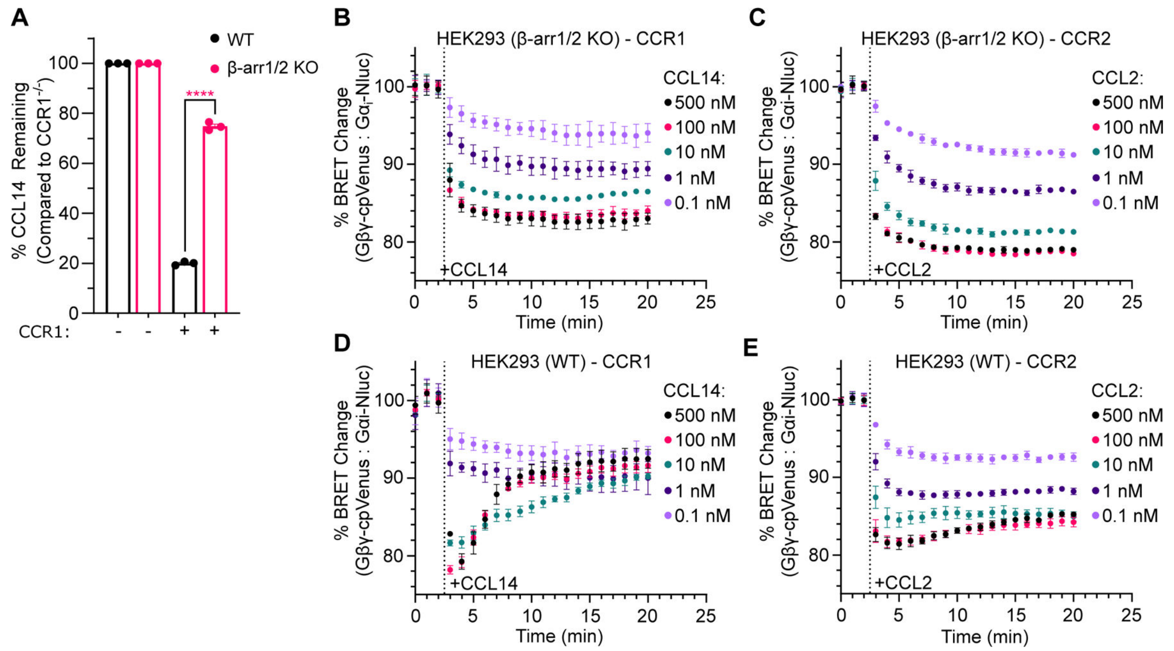

Fig. 7. CCR1 and CCR2 have distinct mechanisms of chemokine scavenging.

(**A**) WT HEK293 and β-arrestin1/2 KO HEK293 cells stably expressing CCR1 and the respective non-expressing cells were cultured in media containing 5 nM CCL14 for 16 h. The remaining CCL14 was quantified by ELISA and interpolated from CCL14 standards and are presented as percentages of the respective non-CCR1 expressing cells. (**B** to **E**) HEK293 β-arrestin1/2 KO cells (B and C) or HEK293 WT cells (D and E) expressing either CCR1 or CCR2 and transfected with an IRES vector encoding $G\alpha_i$-Nluc and Gβγ-cpVenus were stimulated with 0.1–500 nM CCL14 or CCL2, respectively (indicated by the dotted lines). The dissociation of the Gβγ- from the Gα-subunit results in a decrease in BRET ratio upon agonist treatment and is expressed as percentages of the BRET ratio to that observed in the untreated control cells. Data are expressed as the means ± SEM of n 3 independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ compared to controls by one-way analysis of variance (ANOVA) with Dunnet's multiple comparison test or unpaired t test.

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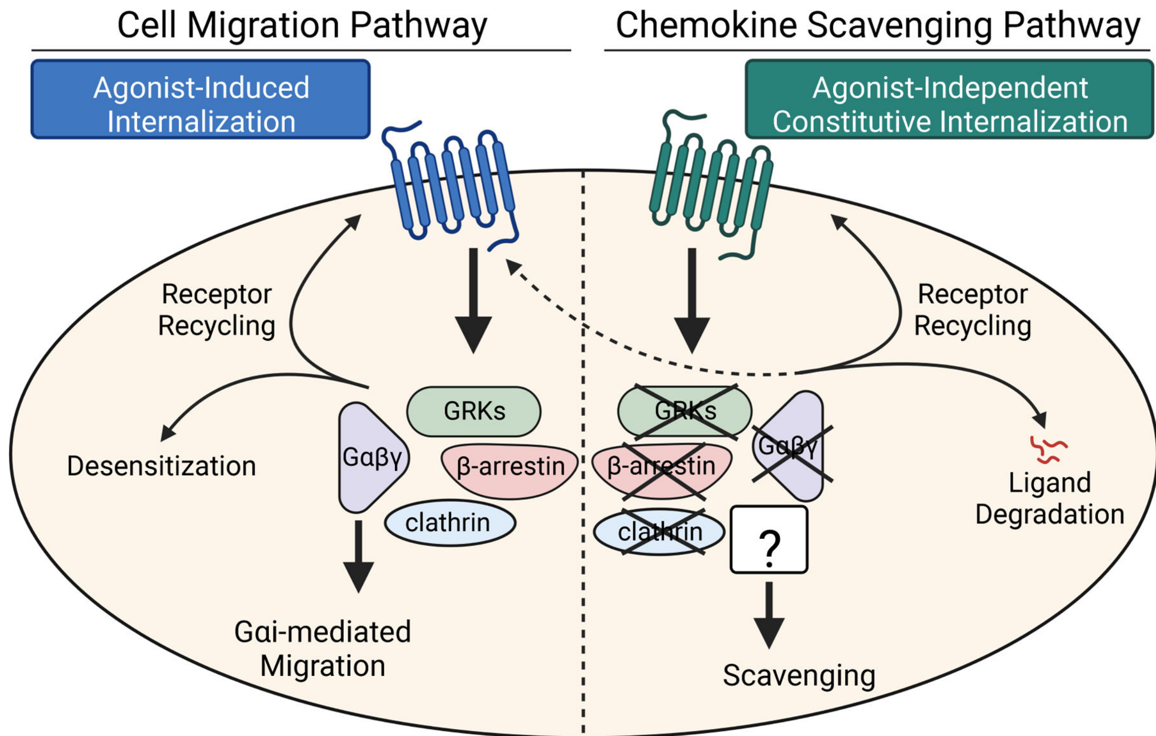

Fig. 8. CCR2 appears to have two distinct functional populations, one that regulates migration and one involved in scavenging.

The canonical GPCR interactors, including Gαβγ, GRKs, β-arrestins, and clathrin are primarily involved in the CCL2-induced internalization mechanisms and are part of the migration-promoting population (left). These same interactors are dispensable for the scavenging population of CCR2 (right), which instead can constitutively internalize while passively sequestering extracellular CCL2. The exact mechanisms and regulatory proteins involved in the scavenging pathway are yet to be determined. A key aspect of both populations is the ability of CCR2 to recycle to the cell surface to continue to drive G protein-dependent migratory functions as well as continue to scavenge excess chemokine. Additionally, the constitutively internalized receptor may provide non-desensitized receptors (dotted arrow) for the G protein-coupled pathway to enable sustained cell migration.