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CCR2-Mediated Uptake of Constitutively Produced CCL2: A Mechanism for Regulating Chemokine Levels in the Blood

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Abstract

C-C chemokine receptor 2 (CCR2) is a key driver of monocyte/macrophage trafficking to sites of inflammation, and has long been considered a target for intervention in autoimmune disease. However, systemic administration of CCR2 antagonists is associated with marked increases in CCL2, a CCR2 ligand, in the blood. This heretofore unexplained phenomenon complicates interpretation of *in vivo* responses to CCR2 antagonism. We report that CCL2 elevation after pharmacological CCR2 blockade is due to interruption in a balance between CCL2 secretion by a variety of cells, and its uptake by constitutive internalization and recycling of CCR2. We observed this phenomenon in response to structurally diverse CCR2 antagonists in wild-type mice, and also found substantially higher CCL2 plasma levels in mice lacking the CCR2 gene. Our findings suggest that CCL2 is cleared from blood in a CCR2-dependent but G protein ($G\alpha_i$, $G\alpha_s$ or $G\alpha_{q/11}$)-independent manner. This constitutive internalization is rapid: on a given monocyte the entire cell-surface CCR2 population is turned over in <30 minutes. We also found that constitutive receptor internalization/recycling and ligand uptake are not universal across monocyte-expressed chemokine receptors: for example, CXCR4 does not internalize constitutively. In summary, we describe a mechanism that explains the numerous preclinical and clinical reports of increased CCL2 plasma levels following *in vivo* administration of CCR2 antagonists. These findings suggest that constitutive CCL2 secretion by monocytes and other cell types is counteracted by constant uptake and internalization by CCR2-expressing cells. The effectiveness of CCR2 antagonists in disease settings may be dependent upon this critical equilibrium.

Introduction

The C-C chemokine receptor 2 (CCR2) is a G protein-coupled receptor that mediates the migration of leukocytes, most notably monocytes, into inflammatory sites (1). The interaction between CCR2 and its signature ligand, monocyte chemoattractant protein-1 (MCP-1, also known as CCL2), has been thoroughly studied in humans and rodents, and has

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long been considered an important clinical target for various chronic inflammatory disorders and as a novel approach for multiple forms of kidney disease (2–6). More recently, high levels of CCR2 were identified on subsets of monocytic-myeloid-derived suppressor cells (M-MDSC) (7), which are major components of the tumor microenvironment that prevent cytotoxic T cells from killing tumor cells. The possibility that CCR2 antagonists could prevent entry of M-MDSC into tumors prompted clinical trials in pancreatic cancer, which yielded promising results (8, 9).

Studies evaluating CCR2 antagonists in both clinical and preclinical settings have revealed a consistent and unexplained phenomenon in which CCL2 becomes elevated in the blood of patients, primates or rodents after treatment with CCR2 antagonists (3, 6, 10, 11). This increased concentration of CCL2 in the plasma could potentially counteract the effects of CCR2 blockade (3, 6), thus limiting the effectiveness of the drug. In the current study, we sought to understand the mechanism by which treatment with CCR2 antagonists results in increased levels of CCL2 in the blood. We used two structurally distinct CCR2 antagonists, MK-0812 (12, 13) and CCX598 (14), to fully evaluate their effects on plasma CCL2 levels, and compared these findings to plasma levels from mice genetically deficient in CCR2. Further, we performed extensive *in vitro* experiments to identify the cellular sources of elevated CCL2 following CCR2-antagonist treatment, and to determine how cells can continually remove extracellular CCL2 under basal conditions.

Here we report that human monocytes and other cells constitutively secrete CCL2, and that CCR2 is constitutively internalized and recycled, which removes CCL2 from the cellular environment. Conversely, CCL2 levels rise if CCL2 binding to CCR2 is blocked by an antagonist, or if CCR2 is absent. The constitutive internalization and recycling of CCR2 thus provides an effective mechanism for regulating CCL2 levels in the blood or in an inflammatory microenvironment.

Materials and Methods

Isolation and Culture of Monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from leukocyte reduction system (LRS) chambers from a TrimaAccel® blood collector. Blood from LRS chambers was diluted 1:4 (vol/vol) with calcium and magnesium free PBS, and PBMCs were enriched by Ficoll gradient centrifugation. Monocytes were isolated by $CD14^+$ positive selection using a MACS system with human CD14 MicroBeads (Miltenyi Biotec, Germany), according to the manufacturer's protocol. Freshly isolated monocytes were plated into 48-well plates (Thermo Scientific, Denmark), and cultured in a 5% CO_2 incubator at 37° for 24 hour at a density of 10^6 cells/ml in RPMI-1640 containing 0.3 g/L L-glutamine (Cellgro Mediatech; Herdon, VA) supplemented with 10% (v/v) fetal bovine serum (Sigma), 10 mM HEPES (Cellgro Mediatech; Herdon, VA) and 1 mM Sodium pyruvate (Cellgro Mediatech; Herdon, VA).

Cell Culture

HEK 293 cells lacking functional $G\alpha_s$ (Gs KO) or $G\alpha_{q/11}$ (Gq/11 KO), prepared by CRISPR/Cas9 as previously reported (15, 16), and parental control HEK 293 WT cells, were a kind gift of Dr. Asuka Inoue (Tohoku University, Japan). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Gibco) supplemented with 10% fetal bovine serum (FBS) and grown at 37°C with 5% CO_2. Stable CCR2-expressing cells were generated in the parental, Gs or Gq/11 KO HEK 293 lines by transfection of pReceiver-M02-CCR2b plasmid (Genecopoeia), followed by selection with G418 (Life Tech).

In Vivo Studies

Animals were purchased and housed in accordance with ChemoCentryx Institutional Animal Care and Use Committee guidelines and requirements. Female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Female CCR2 KO mice (1) were bred and raised in the ChemoCentryx animal housing facility. C57BL/6 mice were divided into six groups ($n = 5$): MK-0812 or CCX598 were dosed at 0.1 mg/kg, 10 mg/kg and 30 mg/kg PO for MK-0812, or at 0.5 mg/kg, 30 mg/kg and 90 mg/kg PO for CCX598. One group of CCR2 KO mice and one group of C57BL/6 were injected with only vehicle, to serve as negative controls. All the mice RO bled 4 hours after injection of test agent. The mouse plasma was collected from EDTA blood.

In time course experiments, 10 mg/kg MK-0812 or 30 mg/kg CCX598 was administered to C57BL/6 mice (n=5) and blood collected as above for plasma at 2, 4, 6 and 24 hours after dosing. Both CCL2 levels and drug concentration were measured. Drug plasma levels were analyzed by liquid chromatography/mass spectrometry (LCMS) at ChemoCentryx.

In Vitro Experiments: CCL2 Accumulation in Monocyte Cultures in the Presence of CCR2 Antagonists

Freshly isolated monocytes were challenged with increasing doses of CCX598, MK-0812, CCX872, CCX140, INCB3344 or PF04634817 (only CCX598 and MK-0812 were discussed in the text, the remainder are described in Supplemental Figure S1). After 24 hours of incubation at 37°C with 5% CO_2 or at 4°C, treated and untreated monocyte supernatants were collected for evaluation of CCL2 levels. In the signal transduction study, monocytes were pre-incubated with 200 ng/ml pertussis toxin for two hours. After incubation, the cells were resuspended in culture medium and stimulated with the various CCR2 antagonists for 24 hours at 37°C with 5% CO_2. In the cycloheximide study, the monocytes were treated with 50 μg/ml cycloheximide for 2 hours in culture medium and then stimulated with various concentrations of CCR2 antagonists for 24 hours at 37°C with 5% CO_2. After 24 hours of incubation, treated and untreated monocyte supernatants were collected for CCL2 determination. In the *in vitro* experiments in which chemokines were added exogenously, the freshly isolated monocytes were treated with CCX598 alone or in combination with CCR1 inhibitor CCX721(17) followed by addition of 1 nM CCL8, CCL13 or CCL7, then incubated together for 24 hours at 37°C with 5% CO_2. Samples of supernatant were collected after 24 hours of incubation and CCL8, CCL7 and CCL13 levels were determined by ELISA.

ELISA Analysis

Chemokine levels in the supernatants of cultured cells were measured using commercially available ELISA kits (CCL2 DuoSet ELISA (R&D Systems; Minneapolis, MN, USA); CCL8, CCL7 and CCL-13 Quantikine human kits (R&D Systems)), following manufacturer's instructions, and read with a FlexStation-3 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Chemotaxis Assay

In supplemental Figure S2, freshly isolated monocytes were pretreated with 200 ng/ml pertussis toxin or 50 μg/ml cycloheximide for 2 hours. Chemotaxis assays were carried out by using ChemoTX chemotaxis chambers (NeuroProbe, Gaithersburg, MD). Cells were harvested by centrifugation and resuspended in chemotaxis buffer consisting of HBSS with 0.1% BSA at a density of 5×10^6 cells/ml. CCL2, was added to the bottom of the chambers and covered with a 5-μm pore-sized polycarbonate membrane filter while the monocytes were added to the top of the filter. After 60 min incubation at 37°C, the assay was terminated by removal of cell drops from the top of the filter. Migration signal was determined by adding 5 μl of CyQUANT solution (Invitrogen) to each well in the lower chemotaxis chamber and measuring the intensity of fluorescence on a Spectrafluor Plus plate reader (Tecan, Grödig, Austria).

Receptor Internalization Assay

CCR2 receptor expression can was determined by flow cytometry which measures the number of labeled CCR2 molecules remaining on the cell surface before (pre-label) or after (post-label) 30 min of incubation at 37°C. In the pre-label experiments, monocytes were labeled with mouse anti-hCCR2 (Clone # K036C2, BioLegend) or anti-hCXCR4 (Clone# 12G5, R&D system) or its isotype-matched control for 30 min on ice and protected from light. Unbound antibody was washed away with wash buffer (HBSS, 1% FBS). Cells were further stimulated with 1 μM MK-0812, 1 μM CCX598 or vehicle control (DMSO) for 30 min at 37°C or pre-treated with pertussis toxin or PBS as described above. After incubation, cells were transferred to wet ice and the remaining surface receptor was labeled with anti-mouse antibody conjugated to APC (715–136-151, Jackson Immunoresearch). CCR2 or CXCR4 expression was analyzed on a FACS LSRFortessa™ flow cytometer (BD bioscience). Alternatively, in the post-label internalization experiment setup, cells were labeled after 30 min of incubation at 37°C. To establish the time course for constitutive internalization, cells were labeled with CCR2 primary antibody prior to incubation at 37°C for 5, 10, 15, 30, 45 or 60 minutes. The internalization was stopped by transferring the tubes to wet ice. The relative amount of receptor remaining on the cell surface [100(MFI$CCR2_{T2}$-MFIiso_{T2})/(MFI$CCR2_{T1}$-MFIiso_{T1})] at each time point was determined using BD LSRFortessa™ and analyzed using FlowJo software.

cAMP Assay

HEK 293 cells stably expressing hCCR2 were plated at a density of 30,000 cells per well in a 96-well tissue culture treated plate. After an 18 hour incubation at 37°C, the cells were stimulated with 100 μM forskolin, 10 μM MK-0812 or 1 μM hCCL2 in the presence of 1

mM IBMX at 37°C for 15 min. Cells were then lysed and cAMP was measured with the cAMP-Screen Direct Immunoassay system (Life Technologies, CA) according to the manufacturer's instructions.

Transient Transfection

HEK 293 cells were used at 70% confluence in T175 flasks. Plasmid DNA (15 μg) from the indicated constructs were incubated with 400 μl Opti-MEM™ I Reduced Serum Medium (Gibco 31985) and electroporated using a Gene Pulser™ (Bio-Rad) with the voltage set at 0.25 kU. After electroporation, cells were transferred to new T175 flasks and cultured in 25 ml DMEM supplemented with 10% (v/v) fetal bovine serum (Sigma).

CCR2 siRNA Knockdown

HEK 293 cells stably expressing hCCR2 were transfected with small interfering RNA (siRNA) for CCR2 using the DharmaFECT™ 1 transfection reagent (Dharmacon, Colorado): 0.2 μl of 5 μmol of siRNA and 0.5 μl of DharmaFECT transfection reagent was added to each well of a 96-well plate containing HEK 293 hCCR2 cells. ON-TARGET*plus* non-targeting control pool siRNA was used as the negative control (Dharmacon, Colorado). Knockdown efficiency was determined by using the QuantiGene Plex Gene Expression Assay from ThermoFisher Scientific following manufacturer's instructions.

Reagents

The following small molecules were synthesized by the Medicinal Chemistry Department, of ChemoCentryx (Mountain View, CA): CCX140, CCX872, CCX598, CCX507, CCX9588, MK-0812, INCB3344 and PF04634817. Cycloheximide, IBMX and forskolin were obtained from Sigma (St. Louis, MO). Pertussis toxin was purchased from List Biological Lab, (Campbell, CA. Mouse and human CCL2 were both purchased from R&D systems (Minneapolis, MN). YM-254890 was purchased from Wako Chemicals, USA. Anti-human CCR2 antibody (clone# K036C2) and mouse IgG2a isotype control antibody were obtained from BioLegend, San Diego, CA. Anti-human CXCR4 antibody (clone# 12G5) was obtained from R&D systems. SMARTpool siRNA for knockdown of CCR2 and non-targeting siRNA were obtained from Dharmacon Inc, Colorado.

Results

We measured the blood plasma concentration of CCL2 in 4–6 week-old female wild-type C57BL/6 mice (n = 5), and found it to be approximately 60 ± 20 pg/ml (Fig 1A, *left*). This concentration was nearly 6-fold higher in gender- and age-matched CCR2-deficient mice of the same strain (Fig 1A, *left*). Dosing WT mice with the CCR2 antagonists CCX598 (14) or MK-0812 (12, 13, 18, 19) caused plasma CCL2 levels (measured at the 4 hour time point) to approach those of CCR2-deficient mice, plateauing near 300 pg/ml in CCX598 and MK-0812 dose response experiments (Fig 1A, *middle*). The corresponding plasma concentrations of CCX598 and MK-0812 at this same 4 hour time point are shown in Fig 1b). A time-course for assessing the concentration of each CCR2 antagonist versus CCL2 concentration in the plasma indicates that the peak CCL2 level lagged 2–4 hours behind the peak antagonist level for both compounds (Fig 1C).

To investigate immune cells as a potential source of CCL2, we cultured freshly isolated human peripheral blood monocytes in serum-containing medium for 24 hours (Fig 2). We found that CCL2 did not accumulate in the medium under these conditions (Fig 2A). However, consistent with the *in vivo* data (Fig 1), CCL2 accumulated in the medium when monocytes were cultured in the presence of CCR2 antagonist (Fig 2A, *left*). The amount of CCL2 that accumulated in the medium correlated positively with the antagonist concentration (Fig 2A, *right*). The magnitude of this effect for each antagonist paralleled its potency in a CCL2-mediated assay of monocyte chemotaxis (*see* supplemental data Fig S1C *for a table of potency of each antagonist in migration assays*). The antagonist-driven accumulation of CCL2 was temperature dependent, occurring at 37°C cultures but not at 4°C (Fig 2B). Time-course experiments revealed that the majority of CCL2 accumulated between 4 and 24 hours (Fig 2C). CCL2 accumulation in the medium was dependent upon new protein synthesis by the monocytes, as it did not occur in the presence of the protein synthesis inhibitor cycloheximide (Fig 2D).

CCR2 antagonist-induced accumulation of CCL2 was not dependent on signaling through $G\alpha_i$, as PTX treatment of monocytes did not affect CCL2 concentration in the culture medium (Fig 3A), despite its effectiveness in preventing chemotaxis of monocytes to CCL2 (see Supplemental Material, Fig S2). The $G\alpha_q$ inhibitor, YM-254890 (20, 21), had no appreciable effect on CCX598-mediated increases in CCL2 concentration (Fig 3B). CCR2 antagonists did not cause cAMP to accumulate, suggesting that CCR2 antagonists did not induce signaling via $G\alpha_s$ (Fig 3C). Consistent with these results, we found that HEK 293 cells lacking functional $G\alpha_{q/11}$ (Gq/11 KO) or $G\alpha_s$ (Gs KO) (15, 16)) secreted CCL2 into the medium at levels comparable to WT cells, but CCL2 was not detected in these KO lines if stably expressing CCR2 (Fig 3D).

Although monocytes cultured with CCR2 antagonists did not secrete CCL7, CCL8 or CCL13 into the culture medium (Fig 4A), monocytes were capable of clearing these CCR2 ligands from the medium if the ligands were added exogenously (Fig 4B–D). This removal of exogenously-added CCR2 ligands from the medium was inhibited by CCR2 antagonists in a dose-dependent manner (Fig 4B–D), except in the case of CCL7 (Fig 4B): Unlike CCL8 and CCL13, CCL7 is a ligand of both CCR2 and CCR1, which are both expressed by monocytes. As such, CCR2 antagonism alone was not able to prevent CCL7 clearance from the medium. However, the addition of a CCR1 antagonist, CCX721 (17), in combination with CCX598, blocked the clearance of CCL7 (Fig 4B), consistent with a prior report that CCR1 also removes its chemokine ligands from the medium (22).

Secretion of CCL2 into culture medium was not restricted to monocytes or immune cells. For example, the transformed human embryonic kidney cell line HEK 293 (23) constitutively secreted CCL2, but not the other known CCR2 ligands CCL7, CCL8 or CCL13 (Fig 5A). When HEK 293 cells were transfected with hCCR2, accumulation of CCL2 was greatly reduced in the medium (Fig 5B). However, addition of CCR2 antagonists to CCR2-expressing HEK 293 cells caused CCL2 to accumulate in a dose-dependent manner, similar to that demonstrated for monocytes in Figure 2 (Fig 5B). To eliminate potential artifacts implicit in comparing a single transfected HEK 293 clone to a single WT clone, we created transient CCR2 and CCR5 transfectants (Fig 5C). CCL2 did not

accumulate in the medium of transient CCR2 transfectants unless a CCR2 antagonist was present (Fig 5C). CCR5 transfectants secreted CCL2 independently of whether a CCL2 antagonist was present (Fig 5C, *right panel, red bars*). Both CCR2 and CCR5 transient transfectants expressed CCL2 mRNA at equal levels whether or not CCR2 antagonist was present (Fig 5C, *left panel*). We further assessed the direct role of transfected CCR2 in preventing CCL2 accumulation by inhibiting CCR2 mRNA expression via siRNA in HEK 293 cells (Fig 5D, *left panel*). Consistent with earlier results, CCL2 accumulated in the medium of siRNA-treated CCR2 transfected cultures but not in the absence of siRNA treatment, or with empty siRNA vector treatment (Fig 5D, *right panel*).

A clue towards the mechanism of chemokine clearance by CCR2 is provided by certain “atypical chemokine receptors”. By constitutive internalization, these receptors transport the chemokine into the cell for degradation and recycle back to the surface for further rounds of chemokine depletion (*recently reviewed in* (24)). We therefore investigated the trafficking behavior of CCR2 and its kinetics. Using a “post-label” protocol for detecting surface levels of CCR2, where antibody is added at various time points after treatment, we observed that CCR2 levels on the monocyte surface remained constant, whether the samples were incubated at 4°C (internalization is inhibited) or 37°C (permissive to internalization). Treatment with CCR2 antagonists or PTX showed no difference from control with medium alone (Fig 6A, *left panel*). By contrast, using a “pre-label” protocol, in which antibody labeling is done once at the start of the experiment, we observed only <5% of the labeled receptor remaining on the cell surface following 30 min incubation at 37°C (Fig 6A, *right panel*). The presence of CCR2 antagonist did not affect this internalization, nor did the inactivation of $G\alpha_i$ via pertussis toxin treatment (Fig 6A, *right panel*). A time course showed that constitutive internalization of CCR2 reached its maximum at 30 minutes (Fig 6B). Together these data suggest that CCR2 undergoes constitutive internalization (Fig 6A, *right panel*) concurrent with constitutive recycling or replenishment of surface receptor from intracellular stores (Fig 6A, *left panel*). This ensures a constant level of cell surface receptor and provides a robust mechanism for chemokine depletion from the medium. Although antagonists have no effect on constitutive internalization and recycling of CCR2, inhibition of chemokine binding prevents uptake and leads to accumulation of CCL2 in the extracellular space.

We next investigated whether constitutive internalization is a general characteristic of chemokine receptors on monocytes, or restricted to a subset, such as CCR2 and CCR1 (22) (Fig 7). CXCR4 is a chemokine receptor expressed along with CCR2 on the cell surface of monocytes, and it’s only known ligand, CXCL12, induces monocyte migration (25). CXCL12 did not accumulate in the culture medium of fresh human monocytes in the presence or in the absence of the CXCR4 antagonist AMD 3100 (Fig 7A). We found that unlike CCR2 ligands, monocytes did not remove appreciable amounts of the CXCR4 ligand from the culture medium, even in the absence of antagonist AMD 3100 (Fig 7B). We took advantage of the fact that saturated staining of human monocytes with anti-CXCR4 MAb yields MFI similar to that of anti-CCR2 (Fig 7C), allowing us to directly compare internalization of CCR2 and CXCR4 ligands within a given population of cells. By “pre-labeling” cell-surface CXCR4 with fluorescent MAb prior to incubation at 37°C (as done for

CCR2 in Figure 6), we observed that monocyte cell-surface CXCR4 does not undergo constitutive internalization (Fig 7 C and D).

Discussion

Human clinical trials and preclinical models demonstrate in multiple species that *in vivo* treatment with CCR2 antagonists leads to a significant, reproducible increase in plasma CCL2 (3, 6, 10, 11). Several putative explanations for this observation have been proposed, including: CCR2 antagonists simply displace existing CCL2 already bound to CCR2 (6); antagonists block clearance of CCL2 from the blood via atypical chemokine receptor 1 (ACKR1) (3, 10); CCR2 antagonists induce increased production of CCL2 by activating alternative signaling pathways from CCR2 (6, 10); or that CCR2 itself controls homeostasis of CCL2 (11). In terms of alternative signaling pathways, we ruled out the dependence of antagonist-induced accumulation of CCL2 on $G\alpha_i$, the main G protein that couples to CCR2, as well as $G\alpha_s$ and $G\alpha_{q/11}$. The finding that $CCR2^{-/-}$ mice inefficiently clear excess CCL2 from the blood (at rates comparable to those of antagonist-treated WT mice (Fig 1 and (26))) renders the possibility of ACKR1 involvement improbable, as ACKR1 expression is unlikely to be affected by CCR2 gene deletion (their genes are located on different chromosomes (27)). Finally, the lag time between antagonist dosing and peak CCL2 plasma levels (2–4 hours, Fig 1C) is not consistent with simple displacement of CCL2 from its receptor.

In the current study, we discovered that this phenomenon can be recapitulated *in vitro* in cultures of freshly isolated human monocytes. CCL2 accumulates in the culture medium in a dose-dependent manner in the presence of CCR2 antagonists, but not in the absence of such antagonists. The same is true for the HEK 293 cell line, which constitutively secretes CCL2 into the culture medium. However, when HEK 293 cells are stably or transiently transfected to express CCR2, CCL2 ceases to accumulate. Thus, the combined effects of constitutive CCL2 secretion by monocytes and other cell types, and constitutive CCL2 uptake by CCR2 can parsimoniously explain both *in vivo* and *in vitro* observations. Consistent with this uptake, we found that CCR2 constitutively internalizes and recycles, and that its turnover is dynamic and rapid, with the entire cohort of CCR2 molecules on the surface of a given monocyte being replaced every 30 min. Although constitutive internalization of CCR2 is not affected by the presence of CCR2 antagonists, these antagonists do prevent CCR2 from transporting CCL2 into the cell.

The notion of homeostatic chemokine internalization and clearance from the blood and microenvironments has been a familiar concept in the realm of atypical chemokine receptors (ACKRs) for many years, and is thought to constitute a major role for such receptors in immunology and cell trafficking (24). Interestingly constitutive internalization and chemokine clearance has also been observed for some G protein-coupled chemokine receptors such as CCR1 (22). CCR2 has also been reported to take up chemokine from the medium (28) Additionally, because knockout mice of CXCR3 and CXCR2 show elevated serum levels of their respective ligand(s), these receptors may have a similar propensity to clear their ligands from the extracellular space (29) By contrast, we found that CXCR4 is not constitutively internalized, and does not mediate the clearance of its ligand, CXCL12 (Fig

7). Thus it is unclear why some chemokine receptors show this behavior, while others do not, but we speculate that it has to do with the need for tight regulation of the extracellular concentrations of their cognate ligands. If true, this would indicate that the body places a high premium on maintaining very low blood levels of CCL2, perhaps so that monocytes and other CCR2-expressing cells can sense subtle CCL2 gradients, and migrate over long distances to sites of inflammation or injury without CCR2 desensitization. This also suggests that CCR2 effectively acts as a dual function receptor that is capable of promoting cell migration through G protein-mediated signaling pathways, as well as chemokine clearance through G protein-independent constitutive internalization and recycling.

In conclusion, we have demonstrated that the most likely explanation for the phenomenon of antagonist-induced increases of CCL2 *in vivo* in the circulation is the constitutive secretion of CCL2 by monocytes and other cell types, whose uptake by CCR2 is blocked by the administered antagonist, in a manner that does not require receptor signaling. Since the other CCR2 ligands are not constitutively secreted, the overall mechanism may be specific to the CCL2/CCR2 axis, which may have clinical implications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key Points

Chemokine ligand CCL2 is cleared from the blood in a CCR2-dependent manner.

CCR2-dependent clearance of CCL2 is G protein ($G\alpha_i$, $G\alpha_s$ or $G\alpha_{q/11}$) independent.

Equilibrium between secretion of CCL2 and its uptake by CCL2 determines blood levels.

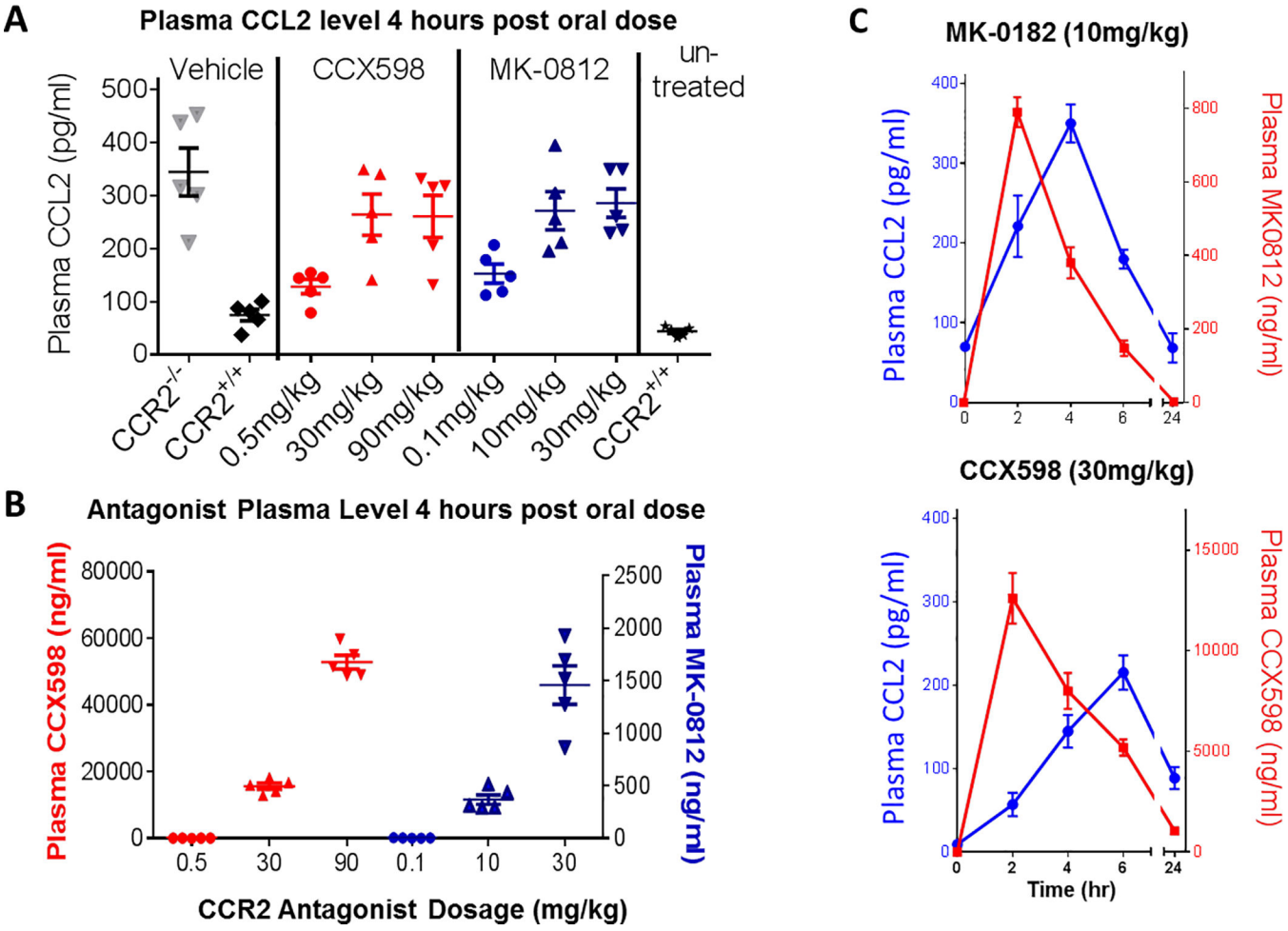

Figure 1: Antagonism of CCR2 and deletion of the gene encoding CCR2 both lead to elevated CCL2 levels in plasma.

(A) C57BL/6 mice were dosed by oral gavage with the indicated doses of CCR2 antagonists CCX598 *(red symbols)* or MK-0812 (*blue symbols*). Mouse blood was collected from the retro-orbital plexus 4 hours after dosing and plasma CCL2 levels were determined by ELISA. WT C57BL/6 control mice receiving only vehicle (1% HPMC, *black diamonds*) were directly compared to gender and age-matched CCL2-deficient mice on the C57BL/6 background (also vehicle-injected, *gray triangles*). Resting plasma CCL2 levels were also measured for untreated C57BL/6 mice (*black stars*). (B) Plasma levels of CCR2 antagonists CCX598 and MK-0812 from the same blood samples used to measure CCL2 concentrations in panel *A* as determined by LC-MS/MS. (C) Time course for CCL2 (*blue lines*) and CCR2 antagonist (*red line*) plasma levels after oral dosing with CCX598 (*bottom panel*) or MK-0812 (*top panel*). Each data point in panel *C* represents mean of 5 mice ± SEM.

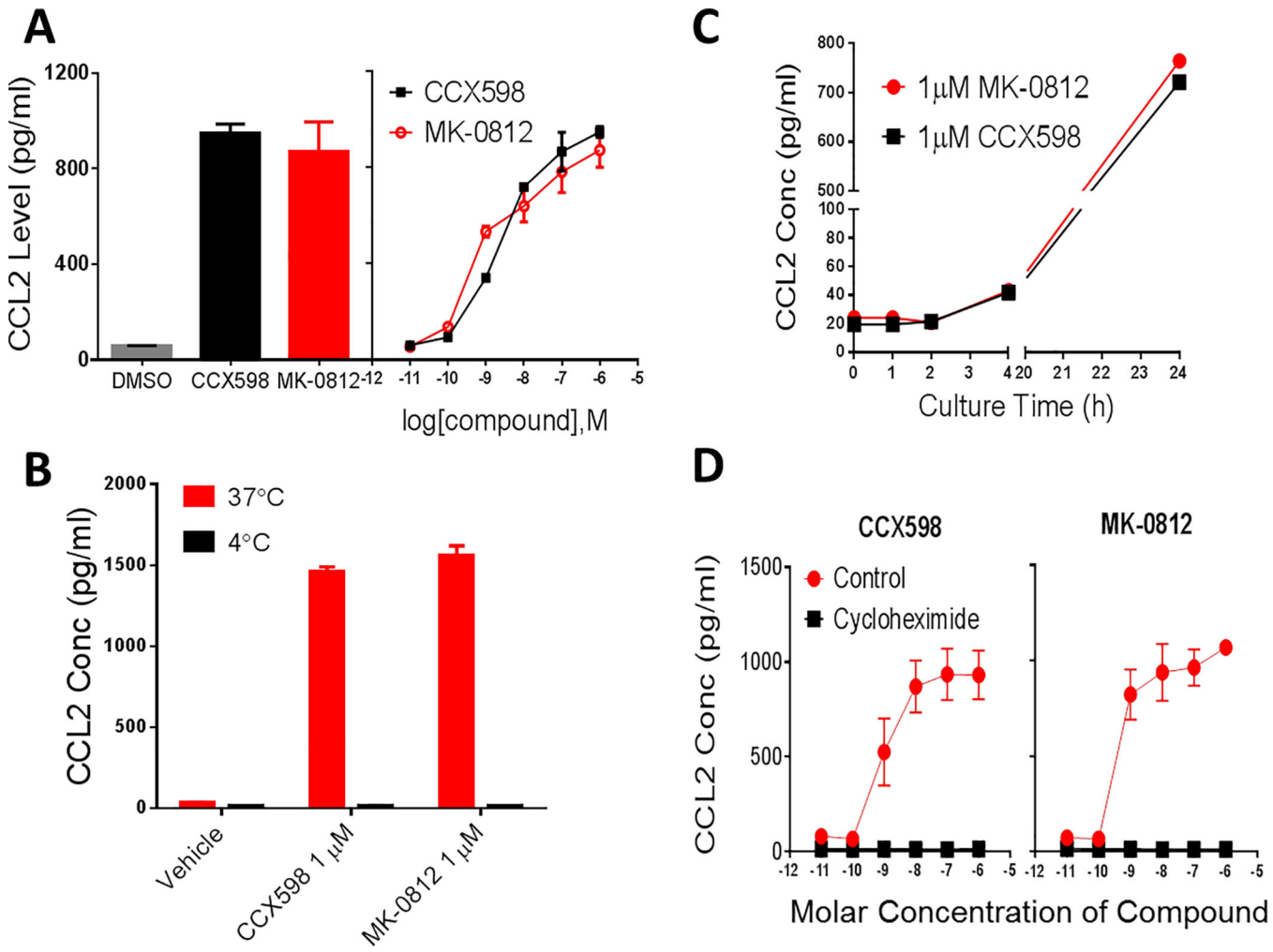

Figure 2: Accumulation of CCL2 following CCR2 antagonist treatment is time and temperature dependent, and requires protein synthesis.

(A) *Left panel:* Fresh human peripheral blood monocytes were incubated with vehicle (DMSO, *gray bar*), CCX598 (1μM) (*black bar*) or MK-0812 (1μM) (*red bar*) at 37°C for 24 hours. *Right panel:* Fresh human peripheral blood monocytes were incubated with increasing concentrations of CCX598 or MK-0812 as indicated on the *x*-axis. The resulting CCL2 concentrations in the conditioned medium are indicated on the *y*-axis. (B) Monocytes were incubated at 37°C (*red bars*) or 4°C for (*black bars*) with 1μM of CCR2 antagonists or vehicle. Data from a single experiment is shown, which used cells from a single donor representative of at least 2 other donors. (C) Monocytes were incubated with CCX598 (1μM) (*black squares*) or MK-0812 (1μM) (*red circles*) at 37°C for the incubation times indicated on the *x*-axis. CCL2 concentration in the conditioned medium is shown on the *y*-axis. (D) Monocytes were incubated as in panels A-C except for a 2 hour pre-incubation with 50μg/ml cycloheximide (*black squares*) or vehicle (*red circles*). Cyclohexamide-treated cells were shown to remain viable by demonstrating their ability to migrate in a gradient of CCL2 (*see* Supplementary Fig S1).

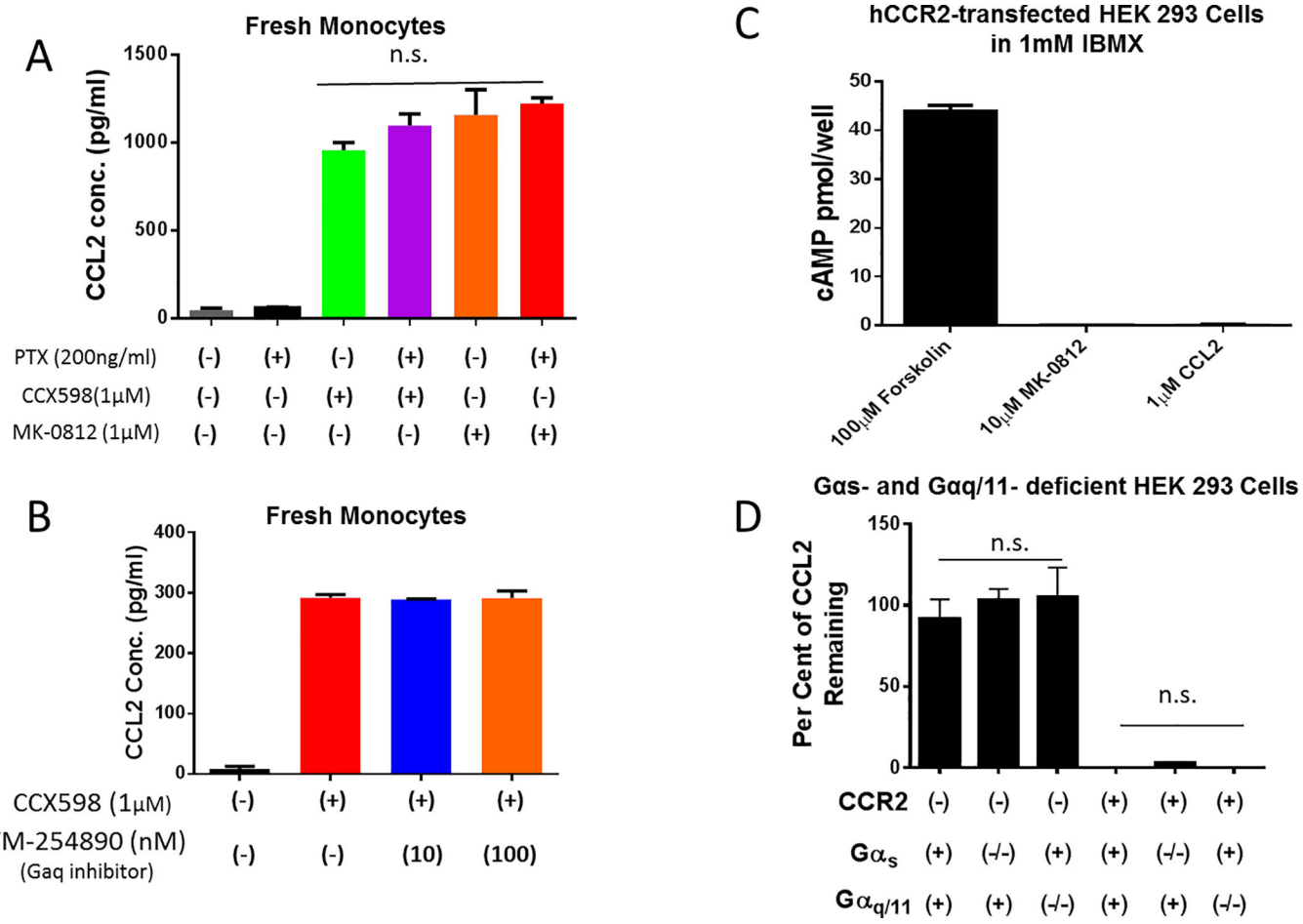

Figure 3: Accumulation of CCL2 following CCR2 antagonist treatment is independent of $G\alpha_i$, $G\alpha_s$ or $G\alpha_{q/11}$.

(A) Fresh human peripheral blood monocytes were incubated for 2 hours with 200ng/ml pertussis toxin (PTX) or vehicle prior to incubation with CCX598 (1μM), MK-0812 (1μM) or vehicle (DMSO) as indicated on the *x*-axis. PTX-treatment of monocytes was demonstrated to be effective by its inhibition of their migration in a gradient of CCR2 ligands (*see* Supplementary Fig S1) (B) Monocytes were treated with vehicle (DMSO) or Gαq inhibitor YM-254890 (10nM or 100nM) for 24 hours in the presence of 1μM CCX598. (C) CCR2-expressing HEK 293 cells (which, like monocytes secrete CCL2 into the medium in response to CCR antagonists, *see* Fig 4) were stimulated with 100μM forskolin, CCR2 ligand CCL2 (1μM) or MK-0812 (10μM) in the presence of IBMX (1mM). The intracellular cAMP was determined by using cAMP-Screen Direct Immunoassay system kit from (Life Technologies, CA) (D) Gαs- and Gαq/11-deficient, CCR2- or vector- transfected HEK 293 cells were incubated with 10μM CCR2 antagonist or vehicle at 37°C for 24 hours. The percent CCL2 remaining in the medium in response to CCR2 antagonist is shown as the ratio of CCL2 concentration in the CCR2 antagonist-treated medium versus vehicle (DMSO)-treated medium on the *y*-axis. The difference between knockout Gαs or Gαq/11-deficient and WT cells was shown to be not significant (*n.s.*) by t-test using GraphPad Prism (GraphPad Software). The origins of the Gαq/11- and Gαs-deficient cell lines are described

in Refs 15 and 16, respectively. The CCL2 concentration in the conditioned medium was assessed by CCL2 ELISA assay from R&D systems.

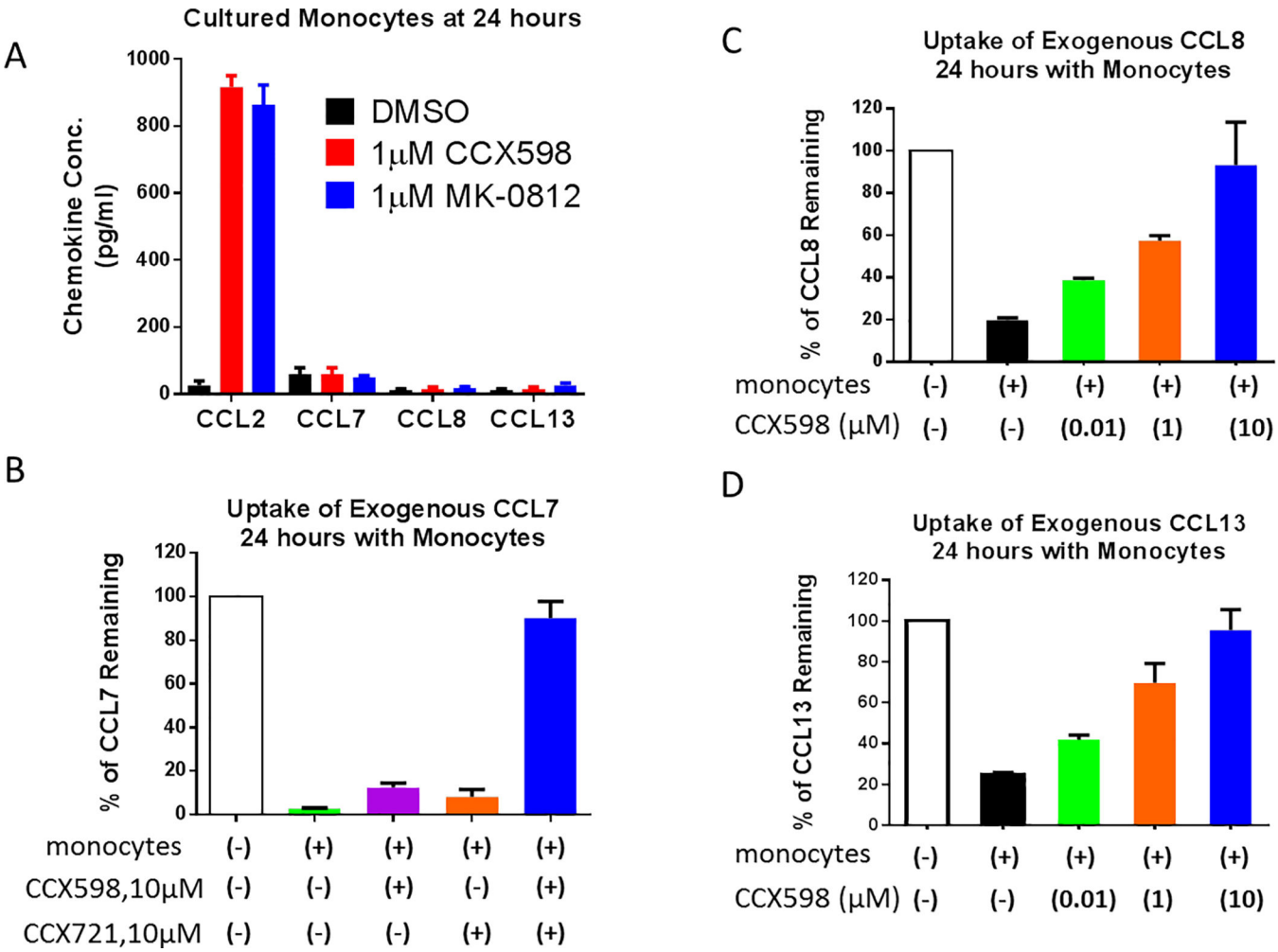

Figure 4: Antagonism of CCR2 on monocytes does not induce accumulation of CCR2 ligands other than CCL2, but monocytes are able to deplete exogenously-added CCL7, CCL8 and CCL13 from culture medium.

(A) Fresh human peripheral blood monocytes were incubated with vehicle (DMSO, *black bars*), CCX598 (1μM) (*red bars*) or MK-0812 (1μm) (*blue bars*). Cell culture supernatant was collected at 24 hours and the level of CCL2, CCL7, CCL8 and CCL13 were measured by ELISA. Data from a single experiment with cells from a single donor are shown, representative of at least 2 other donors. (B) 1nM MCP-3/CCL7 was added to the culture medium with the or without CCX598 (10μM) and/or CCX721 (a CCR1 antagonist) in the presence (*black bars*) or absence (*white bar*) of monocytes. (C, D) 1nM MCP-2/CCL8 or MCP-4/CCL13 with the indicated concentrations of CCX598 were added to the culture medium in the presence (*black bars*) or absence (*white bars*) of monocytes. Medium was collected after 24 hours of culture, and the remaining CCL7, CCL8 and CCL13 were measured by ELISA. The percentage of remaining exogenously-added ligand was normalized to the ligand concentration measured from monocyte-free control medium (*white bars*).

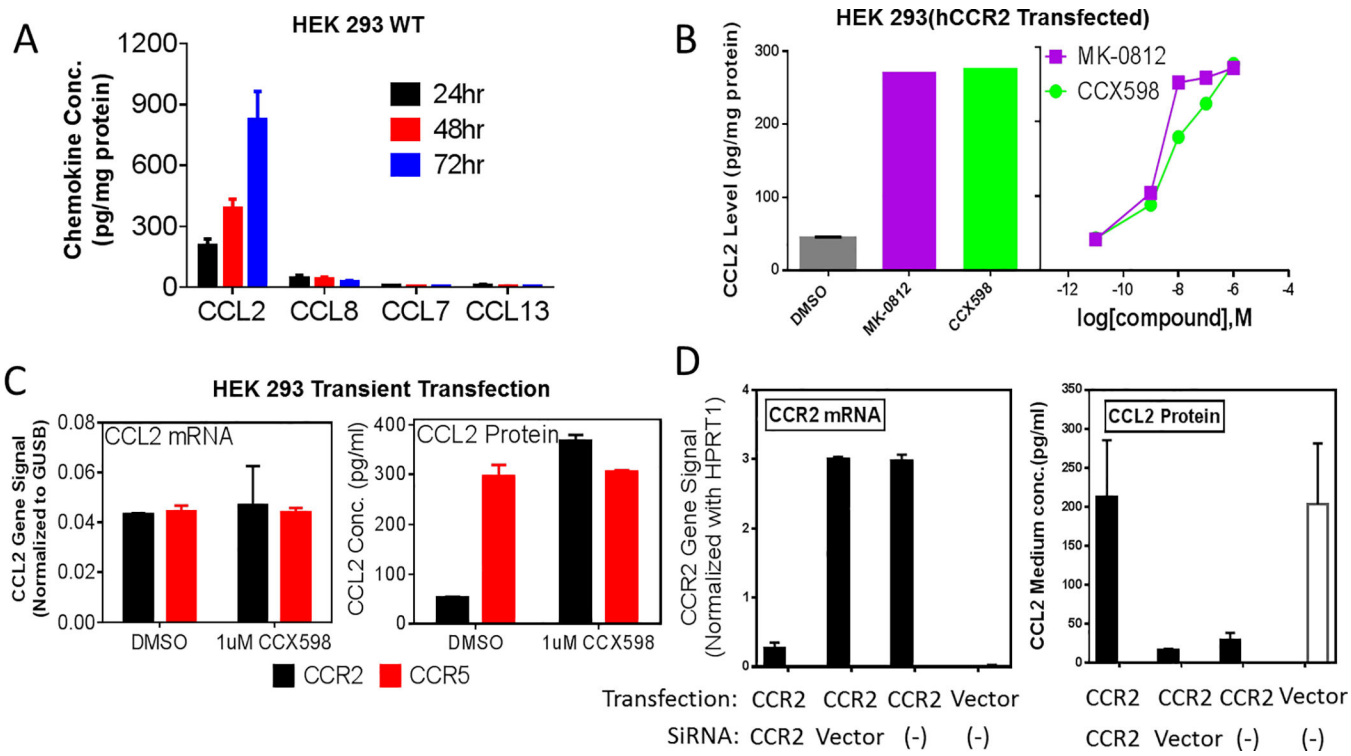

Figure 5: CCL2 is constitutively expressed by WT HEK293 cells, but is cleared from the medium by hCCR2-transfected HEK 293 cells.

(A) WT HEK 293 cells were cultured in DMEM supplemented with 10% FBS for 24 hr *(black bars*), 48 hr (*red bars)* or 72 hr (*blue bars*) hours. The concentration of each of the four known CCR2 ligands in the culture medium (CCL2; CCL7, CCL8 and CCL13) was determined by ELISA. (B, *Left panel*) The same experiment as in panel A (at 24 hours) was performed with hCCR2-expressing HEK 293 cells instead of WT HEK 293 cells, and in the presence of DMSO or CCR2 antagonists. (*Right panel*) Dose-response of CCR2 antagonists MK-0812 (*purple squares*) and CCX598 (*green circles*) on CCL2 concentrations in medium from hCCR2-expressing HEK 293 cells. Data shown in panel B is from a single experiment representative of 3 additional experiments. (C) WT HEK 293 cells were transiently transfected with expression vectors for either CCR2 (*black bars*) or CCR5 (*red bars*). Twenty-four hours after transfection, each cell type was plated (at identical density) in 24 well plates and treated with CCX598 (1μM) or vehicle control (DMSO) for another 24 hours. Forty-eight hours post transfection, the cultured media were collected (to measure CCL2 protein concentration) and cells were collected (to measure CCL2 mRNA expression). Cells were lysed and CCL2 mRNA was measured with the QuantiGene Plex Gene Expression Assay. (D) Stably-transfected hCCR2-expressing HEK 293 cells (or vector transfectants) were transiently transfected with the indicated siRNA and incubated for 48h. mRNA expression levels for CCR2 were determined by the QuantiGene Plex Gene expression assay and normalized to HPRT1 mRNA levels. The CCL2 concentrations in the culture medium 48 hours after siRNA knock down were determined by ELISA.

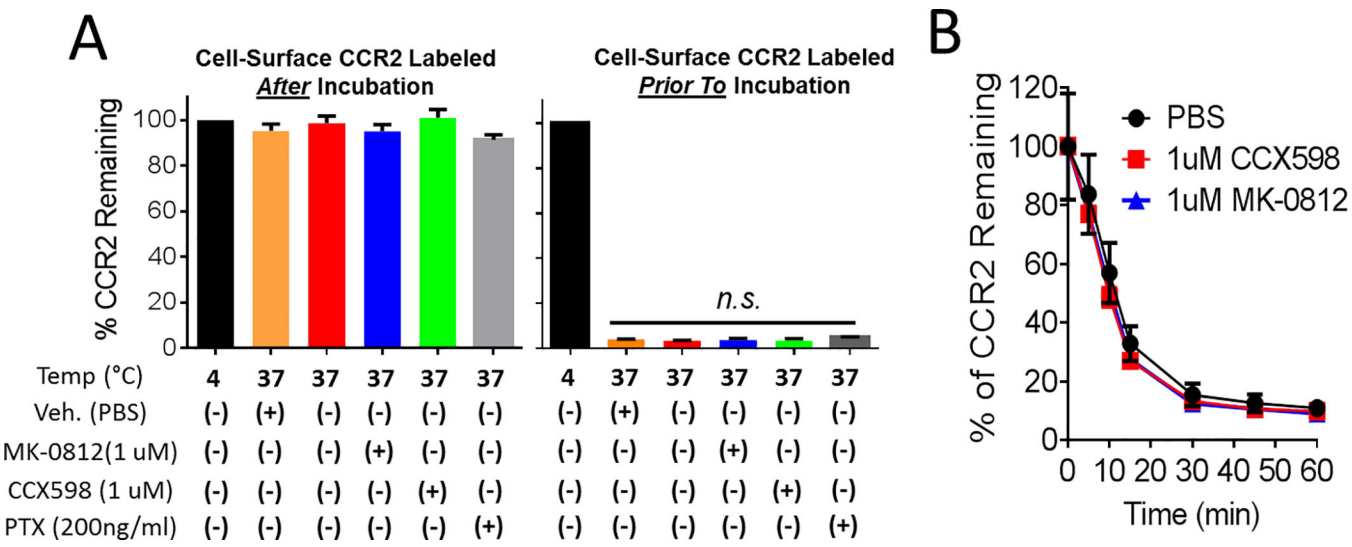

Figure 6: CCR2 is constitutively internalized on human monocytes, and this process is not affected by PTX or by CCR2 antagonists.

(A) (*Left panel*) Fresh human peripheral blood monocytes were incubated for 30 minutes at 37°C with CCR2 antagonists, PBS control or cells were pre-treated with PTX (as indicated in the matrix on the *x*-axis) then labeled for flow cytometry with fluorescently-labeled anti-CCR2 Mab ("post label" protocol). Percent of specific CCR2 MFI on the cell surface with respect to that of cells kept at 4°C is shown on the *y*-axis. (*Right panel*) Experiment identical to that shown in the left panel, except cells were labeled with the anti-CCR2 MAb ***before*** 30min incubation at 37°C ("pre-label" protocol). The variance between the untreated control, PBS, CCX598, MK-0812 and PTX treated cells was shown to be not significant (*n.s.*) by analysis of variance with the t-test using GraphPad Prism (GraphPad Software). The data were generated from three replicates shown as mean± SEM. (B) Cells were labeled with CCR2 antibody on ice as in the right panel of A and pretreated with or without CCX598 (1μM), MK-0812 (1μM) for 1 hour, then incubated at 37°C for the amount of time shown on the *x*-axis. The amount of receptor-specific MFI remaining on the cell surface is shown on the *y*-axis.

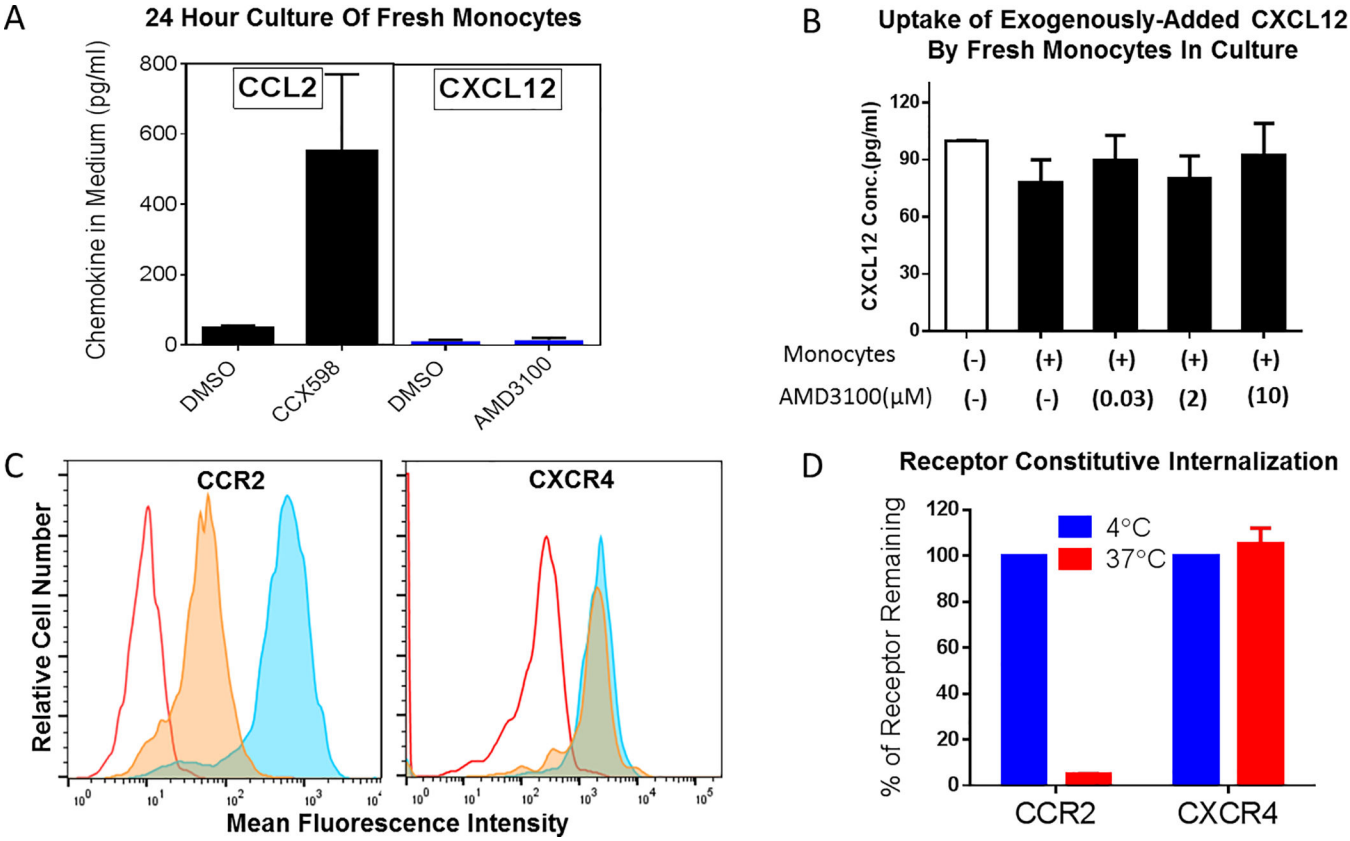

Figure 7: Constitutive internalization of cell-surface receptors, and constitutive uptake of ligand, are not universal characteristics of chemokine receptors.

(A) Freshly isolated monocytes were cultured in RPMI supplemented with 10% FBS in the presence or absence of CCR2 antagonist (CCX598, 1μM, *black bar*), CXCR4 antagonist (AMD3100, 1μM, *blue bar*) or DMSO control for 24 hours. The CCL2 and CXCL12 concentration in the culture supernatant were measured by ELISA (R&D Systems). (B) CXCL12 (1nM) and the indicated concentrations of AMD3100 were added to the culture medium in the presence (*black bars*) or absence (*white bar*) of freshly-isolated monocytes. Media were collected after 24 hours of culture, and the concentration of CXCL12 remaining in the medium was measured by ELISA. The percentage of exogenously-added CXCL12 was normalized to the ligand concentration measured from cell-free control medium. (C) Constitutive internalization of cell-surface CCR2 and CXCR4 on monocytes was measured as described in Fig 6. Representative flow cytometry plots showing initial cell surface CXCR4 or CCR2 (*solid blue histograms*), receptor remaining after 30 minutes of ligand-independent internalization at 37°C (*solid orange histograms*), and isotype-matched control (*open red histogram*). (D) Quantitation of receptor-specific MFI from experiment in panel C, performed in triplicate.