Expression of L-CCR in HEK 293 cells reveals functional responses to CCL2, CCL5, CCL7, and CCL8

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Abstract: It has become clear in the past years that chemokines and chemokine receptors are pivotal regulators of cellular communication and trafficking. In addition to the ~20 chemokine receptors that have been cloned and described, various orphan receptors with a chemokine receptor-like structure are known. We have investigated the orphan mouse chemokine receptor (L-CCR) in HEK 293 cells, a receptor that was originally described in a mouse macrophage cell line. Cells expressing this receptor show pertussis toxin-sensitive chemotaxis and small intracellular calcium transients in response to the chemokines CCL2, CCL7, CCL8, and CCL5. Biotinylated CCL2 binds to L-CCR-expressing cells, and transfection experiments with an L-CCR–green fluorescent protein fusion protein showed L-CCR expression in the membranes of recombinant HEK 293 cells. Although radioligand binding was not detected, it is suggested that L-CCR is a functional chemokine receptor. *J. Leukoc. Biol.* 74: 243–251; 2003.

Key Words: *chemokine receptors · chemotaxis · calcium signals · GPCR*

INTRODUCTION

Chemokines are small chemotactic cytokines of ~10 kDa, which orchestrate the inflammatory response by attracting leukocytes to sites of inflammation and by controlling the homing of dendritic cells, T cells, and B cells (for review, see refs. [1–3]). Recent studies indicate that chemokines not only facilitate leukocyte migration and positioning but are also involved in other processes such as leukocyte degranulation, angiogenesis, T cell differentiation, and functioning (for review, see ref. [4]). As a result of their various functions, the involvement of chemokines in diseases has been studied extensively (for review, see ref. [5]). Depending on conserved cysteine residues, chemokines and their receptors, which are all G-protein-coupled, are subdivided into four families: CXC, CC, C, and CX3C chemokines [3, 4, 6]. In humans, more than 40 chemokines and 20 chemokine receptors have been cloned [3, 4, 6, 7]. The large number of chemokines compared with the number of chemokine receptors indicates that chemokine receptors respond to various chemokines. Indeed, chemokine signaling can be promiscuous, and a variety of chemokines activate more than one chemokine receptor [7–9]. Furthermore, it is likely that some of the currently known orphan chemokine-like receptors will further contribute to the complexity of chemokine signaling [8].

Expression of the orphan chemokine receptor L-CCR has been originally described in lipopolysaccharide (LPS)-stimulated RAW cells [10]. Recently L-CCR expression was also detected in mouse glial cells, where it was only found under proinflammatory conditions [11]. To investigate if L-CCR is a functional chemokine receptor, we cloned and expressed L-CCR in HEK 293 cells.

MATERIALS AND METHODS

Chemicals

The following chemicals were used: Dulbecco's modified Eagle's medium (DMEM) from Gibco-BRL Life Technologies (Breda, Netherlands); TA vectors pCR2.1 and pCRII from Invitrogen (Leek, Netherlands); Taq polymerase from InViTek (Berlin, Germany); Fugene from Roche Molecular Biochemicals (Mannheim, Germany); recombinant mouse chemokines from PeproTech EC (London, UK); G418 from Calbiochem (Darmstadt, Germany); biotin rmJE [monocyte chemoattractant protein-1 (MCP-1)] fluorokine kit from R&D Systems (Minneapolis, MN); Fura-2 AM and all other chemicals from Sigma-Aldrich (Bornhem, Belgium). CCR2-blocking antibodies (DOC 3 and MC-21) were kindly provided by Dr. Matthias Mack (Medizinischen Poliklinik, Munich, Germany).

Cell cultures

HEK 293, Chinese hamster ovary (CHO), and RAW 264.7 cells

All cell lines were maintained in DMEM containing 10% fetal calf serum with 0.01% penicillin and 0.01% streptomycin in a humidified atmosphere (5% CO_2) at 37°C.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Cells or brain tissue were lysed in guanidinium isothiocyanate/mercaptoethanol buffer, and total RNA was extracted with slight modifications according to Chomczynski and Sacchi [12]. RT: Total RNA (1 μg) was transcribed into cDNA as described [13]. Potential contamination by genomic DNA was checked by running the reactions (35 cycles) without RT and using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers in subsequent PCR amplifications. Only RNA samples, which showed no bands after that procedure, were used for further investigation. PCR: The RT reaction (2 μl) was

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used in subsequent PCR amplifications as described [13]. The following primers were used in PCR for L-CCR: 5′-CTGGCGGTGTTTATCTTGGT and 5′-AACCAGCAGAGGAAAAGCAA, resulting in a 489-bp PCR product. Primers for GAPDH PCR were: 5′-CATCCTGCACCACCAACTGCTTAG and 5′-GCCTGCTTCACCACCTTCTTGATG, resulting in a 346-bp PCR product.

Cloning and expression of L-CCR

Primers to amplify the full-length sequence for L-CCR have been chosen according to the sequence for L-CCR (accession number AB009384). The full-length L-CCR coding sequence was amplified from cDNA derived from LPS-stimulated microglia with the following primers: forward, 5′-TATCAAGCAACCTGCCTCAA; reverse 5′-TGGCATAAAACAATGTGAAGAGA.

The resulting PCR product was cloned in pCR2.1 (Invitrogen) for sequencing and subcloned into the *Bam*HI–*Not*I sites of pcDNA 3.1 (Invitrogen) for transfection. The plasmid (1 μg) was transfected with 6 μl Fugene® (Roche Molecular Biochemicals) in HEK 293 cells according to the manufacturer's instructions. Stable transfected cells were selected with G418 500 μg/ml for approximately 2 weeks, and the resulting cell clones were checked by RT-PCR for L-CCR mRNA expression. Mock transfections were performed with empty or green fluorescence protein (GFP)-containing pcDNA 3.1.

Construction of the L-CCR–enhanced GFP (EGFP) fusion protein

L-CCR was coupled to EGFP by cloning into pEGFP-N2 (Clontech, Palo Alto, CA). Full-length L-CCR was amplified using cDNA from L-CCR-transfected HEK 293 cells with the following primers: forward, 5′ ATA CTC GAG ATG GAT AAC TAC ACA GTG GCC; reverse, 5′ ATA GGA TCC A TAT TAT ATC CTG CCT TTG ATG CAA ATT. These primers introduced a *Xho*I restriction site at the 5′ end and a *Bam*HI restriction site at the 3′ end of L-CCR and omitted the stop codon in the receptor sequence. The resulting PCR product was cloned into pCRII (Clontech) by TA cloning. The resulting vector (5 μg) was restricted with *Bam*HI (Promega, Madison, WI) and *Xho*I (Promega); the excised band was purified using gel electrophoresis and gel purification (MinElute™ gel extraction kit, Qiagen, Valencia, CA) and subsequently cloned into the *Bam*HI/*Xho*I sites of pEGFP-N2 (Clontech). Sequence and the orientation of the insert were verified by sequencing. Transfection of HEK 293 cells was performed as described above. Mock transfections were performed with empty or GFP-containing pcDNA 3.1. A Zeiss confocal laser microscope was used to analyze the expression of the L-CCR–EGFP construct in transfected HEK 293 cells.

Chemotaxis assay

Cell migration in response to chemokines was assessed using a 48-well chemotaxis microchamber (NeuroProbe, Gaithersburg, MD). Chemokine stock solutions were prepared in phosphate-buffered saline (PBS) and further diluted in medium for use in the assay. Culture medium without chemokines served as a control in the assay. The chemoattractant solution or control medium (27 μl) was added to the lower wells; the lower and upper wells were separated by a polyvinylpyrrolidone-free polycarbonate filter (8 μm pore size), and 30,000 cells per 50 μl were used in the assay. Chemotaxis was distinguished from chemokinesis by adding the same chemokine concentration to the upper wells or to the lower and the upper wells. Determinations were done in hexaplicate. The chamber was incubated at 37°C, 5% CO_2, in a humidified atmosphere for 120 min. At the end of incubation, the filter was washed, fixed in methanol, and stained with toluidine blue. Migrated cells were counted with a scored eyepiece (three fields, 1 mm^2, per well), and migrated cells per well were calculated. The data are presented as mean values ± SD and were analyzed by Student's *t*-test. *P* values ≤ 0.01 were considered significant. To prevent bias, chemotaxis filters were occasionally counted after double-blinded experiments.

Determination of intracellular calcium

For calcium measurements, cells were cultured on poly-L-lysine-coated glass coverslips. To load the cells with Fura-2 AM, the cells were incubated for 30 min at 37°C in loading buffer containing (in mM) NaCl 120, HEPES 5, KCl 6, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5, $NaHCO_3$ 22, and Fura-2 AM 0.005, pH 7.4. The coverslips were fixed in a perfusion chamber (37°C) and mounted on an inverted microscope. Fluorimetric measurements were done using a sensicam charged-coupled device camera supported by Axolab® 2.1 imaging software. Digital images of the cells were obtained at an emission wavelength of 510 nm using paired exposures to 340 and 380 nm excitation wavelength sampled at a frequency of 1 Hz. Fluorescence values representing spatial averages from a defined pixel area were recorded on-line. Increases in intracellular calcium concentrations were expressed as the 340:380 ratio of the emission wavelengths. Compounds were administered using a pipette positioned at a distance of 100–300 μm from the cells.

F-Actine staining

L-CCR expressing HEK 293 cells were cultured on glass coverslips in six-well chambers. Prior to the experiment, the medium was refreshed, and cells were stimulated with 10 nM CCL2. After various time-points (1, 5, 10, and 60 min), the medium was swiftly removed, and the cells were fixed in 4% paraformaldehyde for 10 min. Subsequently, the cell-containing glass coverslips were washed twice for 10 min in PBS, followed by a 10-min wash step in PBS + 0.1% Triton and two final washing steps for 10 min in PBS, after which, they were incubated for 30 min in PBS containing 0.1 M tetramethyl rhodamine isothiocyanate–phalloidin (Sigma-Aldrich). The coverslips were then washed for 10 min in PBS and mounted for confocal fluorescence microscopy analysis.

Binding of biotinylated CCL2

Binding of biotinylated CCL2 to cultured cells was determined according the manufacturer's instructions. In brief, cells were harvested with a cell scraper and were washed two times with PBS. Then, cells were seeded (50,000/well) in 6 mm wells on Teflon-coated object glasses (Cel-Line Associates, Nutacon, Leimuiden, The Netherlands). In some cases, RAW 264.7 cells have been stimulated overnight (12–16 h) with LPS (100 ng/ml) before the binding experiment. Cells were incubated for 60 min at 4°C in a humidified atmosphere in a total volume of 30 μl with 20 nM biotinylated recombinant mouse CCL2 (biot-rmCCL2). This was followed by a 30-min incubation with fluorescein-conjugated avidin to detect the bound biot-rmCCL2. All samples were viewed using a Zeiss Axioskop 2 with a Plan-NEOFLUAR 40× or 60× objective for observation or quantification, respectively. Fluorescence intensity was quantified using Zeiss KS 300 software and calculated as pixel intensity per cell. Background fluorescence was measured with biotinylated soybean trypsin (negative control, supplied by the manufacturer). During the measurements, conditions of aperture, pinhole, brightness, contrast, and exposure time were maintained constant. Approximately 50 cells per well were measured.

RESULTS

Effect of various chemokines on chemotaxis and intracellular calcium signaling of transient L-CCR-transfected HEK 293 cells

L-CCR was cloned from cDNA derived from LPS-treated mouse microglia [11], and subsequently, the receptor was expressed in various cell lines. Sequencing of the glial L-CCR revealed 99.6% identity with the sequence previously published for the orphan receptor [10]. To exclude that the observed sequence differences were a result of PCR errors, five different clones have been sequenced with all showing identical differences. L-CCR cloned from cultured microglia showed two differences (in position 39, an arginine in place of glutamine, and in position 292, an alanine in place of valine were found in microglial L-CCR) in its amino acid sequence compared with the published sequence of L-CCR (accession number AB009384).

Transfection of CHO cells with L-CCR resulted in concentration-dependent migration in response to CCL2 (**Fig. 1A**) or CCL5 (data not shown). However, as mock-transfected CHO

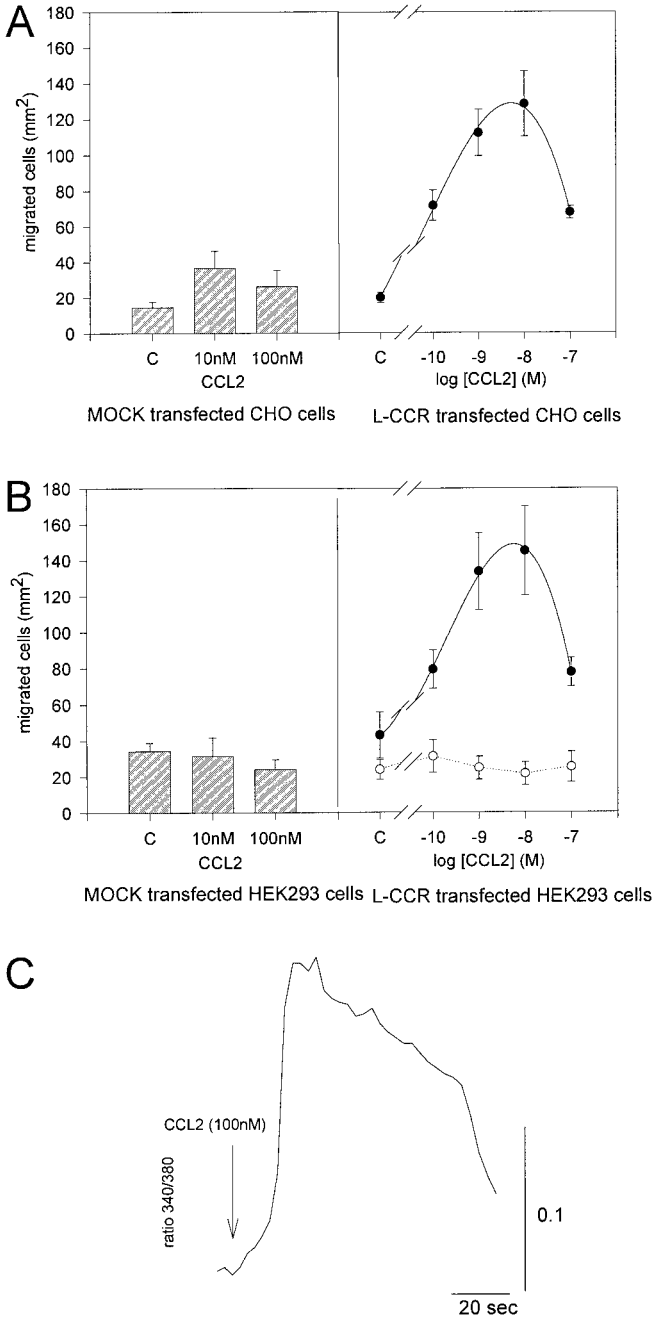

Fig. 1. Effect of CCL2 on chemotaxis and intracellular calcium transients in cells transiently transfected with L-CCR. (A) Stimulation with CCL2 induced pronounced chemotaxis of L-CCR-transfected CHO cells but had also small responses in mock-transfected CHO cells. (B) Chemotaxis of mock-transfected HEK 293 cells was not affected by CCL2, whereas L-CCR-transfected HEK 293 cells migrated in a concentration-dependent manner when stimulated with CCL2 (●). No effect was detected in response to CCL3 (○). (C) Example of a CCL2 (100 nM)-induced intracellular calcium transient in L-CCR-transfected HEK 293 cells. (A and B) The graphs depict the results of a typical chemotaxis experiment performed in hexaplicate for each concentration of CCL2. Data are means ± SEM; similar results were obtained in three independent experiments.

also showed small responses, we did not proceed with CHO cells for further analysis. Similar experiments were performed in HEK 293 cells. In chemotaxis assays, mock-transfected HEK 293 cells did not migrate toward a chemotactic gradient of CCL2, whereas HEK 293 cells transiently transfected with L-CCR migrated in response to CCL2 in a concentration-dependent manner but not to CCL3 (Fig. 1B). Control experiments indicated chemotaxis rather than chemokinesis in our chemotaxis assay. The number of cells per well did not differ from control migration without CCL2 (88±15 cells/mm^2=100±14%) when CCL2 (1 nM) was added to the upper wells alone (105±22%) or in the upper and the lower wells (89±19%). Moreover, CCL2 induced intracellular calcium transients in transiently transfected HEK 293 cells (Fig. 1C). The response of intracellular calcium transients to chemokines was rather weak and detectable in ~10% (12 out of 117) of the HEK cells investigated. Again, CCL3 did not induce intracellular calcium transients in transiently transfected HEK 293 cells (data not shown).

To further investigate the pharmacology of L-CCR monoclonal HEK 293, cells stably expressing L-CCR (HEKL–CCR$_{mc5}$) were used. Of the used chemokines CCL2, CCL3, CCL4, CCL5, CCL6, CCL7, CCL8, CCL11, CCL12, CCL17, CCL19, CCL21, CCL22, CCL25, CXCL9, CXCL10, CXCL12, and CX3CL1 [previously known as MCP-1, macrophage-inflammatory protein (MIP)-1α, MIP-1β, regulated on activation, normal T expressed and secreted, C10, MCP-3, MCP-2, eotaxin, MCP-5, thymus and activation-regulated chemokine, Epstein-Barr-induced 1 ligand chemokine, secondary lymphoid-tissue chemokine, macrophage-derived chemokine, thymocyte-expressed chemokine, monokine induced by interferon-γ (IFN-γ), IFN-inducible protein 10, stromal cell-derived factor-1α, and fractalkine, respectively], only CCL2, CCL5, CCL7, and CCL8 were found to induce chemotaxis of HEKL–CCR$_{mc5}$ (**Table 1**). In **Figure 2**, the concentration-response curves of these four chemokines determined in HEKL–CCR$_{mc5}$ are compared. The EC$_{50}$ values for the four chemotactic chemokines in HEKL–CCR$_{mc5}$ were comparable. The most striking difference between the chemokines of the MCP family (CCL2, CCL7, and

TABLE 1. Effect of Various Chemokines on Chemotaxis of L-CCR-Transfected HEK 293 Cells

Chemokine (1 nM)	Chemotactic effect on L-CCR-transfected HEK 293 cells
CCL2	+
CCL5	+
CCL7	+
CCL8	+
CCL3	–
CCL4	–
CCL6	–
CCL11	–
CCL12	–
CCL17	–
CCL19	–
CCL21	–
CCL22	–
CCL25	–
CXCL9	–
CXCL12	–
CXCL10	–
CX3CL1	–

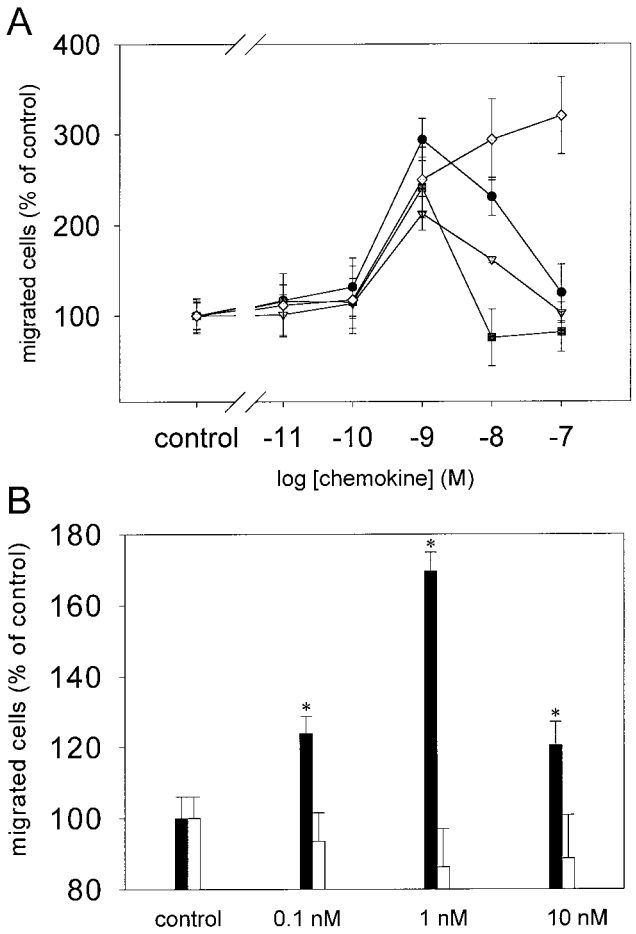

Fig. 2. (A) Effects of various chemokines on the chemotaxis of HEKL–CCR_{mc5}. CCL5 (◆), CCL2 (●), CCL7 (▼), and CCL8 (■) induced chemotaxis of HEKL–CCR_{mc5}. (B) Treatment of HEKL–CCR_{mc5} with pertussis toxin (PTX; 100 ng/ml for 16 h) completely blocked CCL2-induced chemotaxis. Graphs show the results from a typical experiment for each chemokine, performed in hexaplicate for each chemokine concentration. Data given are mean ± SD (n=6) as percent of control. Similar results were obtained in three independent experiments. *, Significantly different from PTX-treated cells; $P < 0.01$ determined by Student's *t*-test.

CCL8) and CCL5 was that the chemotactic response to CCL5 did not decrease at higher concentrations (Fig. 2A). CCL2-induced chemotaxis of HEKL–CCR_{mc5} was completely inhibited by pretreatment of the cells with PTX (100 ng/ml; 16 h; Fig. 2B), indicating that L-CCR, like other chemokine receptors, is coupled to G_i-proteins. This was further corroborated by the findings that CCL2 stimulation inhibited forskoline-induced cyclic adenosine monophosphate (cAMP) synthesis by ~50% in HEKL–CCR_{mc5} (data not shown). The primary receptor for CCL2, CCL7, and CCL8 is CCR2. To exclude endogenous expression of CCR2 in stable-transfected HEK 293 cells, we checked several clones of stable-transfected cells for CCR2 mRNA expression. The primers for human CCR2 have been verified with cDNA derived from human microglia. Whereas in human microglia, CCR2 mRNA expression was detected in stable-transfected L-CCRHEK, no CCR2 mRNA expression was observed (data not shown). Moreover, the presence of CCR2-blocking antibodies did not influence the chemotaxis-inducing effects of CCL2 and CCL5 in L-CCR-transfected HEK 293 cells (data not shown).

L-CCR is expressed in membranes of transfected HEK 293 cells and induces polymerization of actin filaments

Functionality of chemokine receptors requires their localization at the membrane. The translation and protein expression of L-CCR in HEK 293 cells was monitored by fusing an EGFP tag to the COOH terminus of L-CCR. Globally distributed EGFP fluorescence was detected when HEK 293 cells were transfected with EGFP-containing vector without L-CCR (**Fig. 3A**). When cells were transfected with L-CCR–EGFP vector, a different fluorescent staining was detected; the EGFP signal was strictly located to the membrane of the transfected cells (Fig. 3B). Further investigation of L-CCR–EGFP-transfected HEK 293 cells using confocal laser fluorescence microscopy at higher magnification indicated that the majority of the EFGP signal was expressed in tiny cellular processes, as indicated by the arrowheads (Fig. 3B). Stimulation of HEK 293 cells expressing chemokine receptors leads to the rapid formation of lamellipodia based on a polymerization of actin filaments that can be visualized by phalloidin staining [14]. We therefore investigated whether stimulation of CCL2 induced the polymerization of actin filaments in L-CCR–EGFP-transfected HEK 293 cells. Almost no (arrows) or only weak actin filaments (arrowhead) were observed in unstimulated L-CCR–EGFP-transfected HEK 293 cells (Fig. 3C), whereas stimulation for

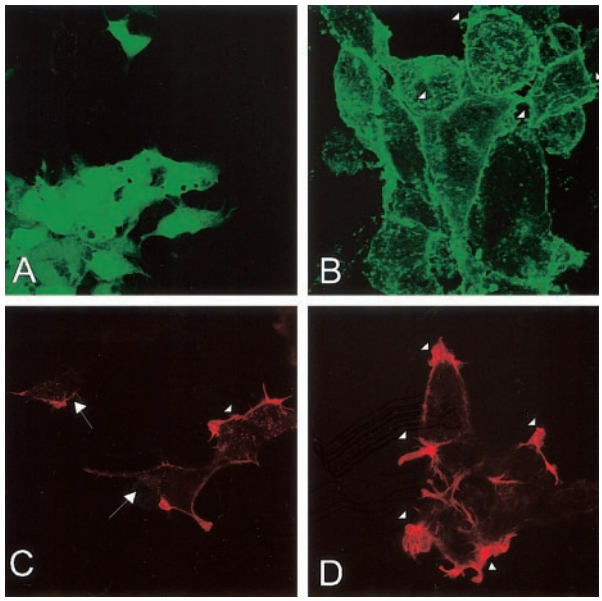

Fig. 3. Expression of L-CCR–EGFP fusion protein in HEK 293 cells. (A) Control transfections with EGFP-containing vector resulted in globally cytoplasmic expression of EGFP. (B) Transfection of HEK 293 cells with L-CCR–EGFP fusion protein gave EGFP fluorescence in the membranes of the cells, indicating a membrane location of L-CCR. Analysis with confocal laser scan microscopy at higher magnification revealed prominent L-CCR expression in tiny processes of the membrane (arrowheads). (C) Almost no (arrows) or only weak formation of actin filaments (arrowhead) were observed in unstimulated L-CCR–EGFP-transfected HEK 293 cells. (D) Stimulation with 10 nM CCL2 induced a pronounced polymerization of actin filaments (arrowheads) in L-CCR–EGFP-transfected HEK 293 cells.

10 min with 10 nM CCL2 induced the formation long actin filaments (arrowheads) in these cells (Fig. 3D). Similar formation of actin filaments was already observed after 5 min and was still detectable after 1 h of stimulation with 10 nM CCL2 (data not shown).

Binding of biot-rmCCL2 to L-CCR-expressing cells

Binding of radiolabeled chemokine ligands to various chemokine receptors has been shown in many publications, and ligand binding, next to signaling, is one of the criteria for a chemokine receptor to obtain an official name [3]. We therefore performed binding experiments using iodine-labeled mCCL2 and mCCL5 (obtained from Perkin Elmer Life Science, Zavebtem, Belgium). Binding experiments were performed using several L-CCR-expressing HEK 293 or L929 cell lines (polyclonal or monoclonal) and LPS-treated primary-cultured mouse glial cells that show L-CCR mRNA expression [11]. Different experimental approaches were tried. Whole-cell radioligand binding was performed at 37°C room temperature and 4°C with different binding buffers. Moreover, we tested the scintillation proximity assay (SPA; wheatgerm agglutinin SPA beads, Amersham Pharmacia Biotech, Little Chalfont, UK) at 37°C, according to the supplier's instructions. Additional lysed cells or membrane-enriched cell fractions were used again with different binding buffers at 37°C. In all these experiments, mCCR2b or mCCR5 expressing CHO cells were used as positive controls that showed binding of iodine-labeled CCL2 or CCL5, respectively. Although we attempted several experimental approaches using different protocols and different L-CCR-expressing recombinant cells, binding experiments with iodine-labeled chemokines did not provide clear, reliable results. There was either no difference in binding of iodine-labeled mCCL2 or CCL5 between L-CCR-expressing cells and controls or the binding to L-CCR-expressing cells was not replaced by unlabeled ligands.

As an alternative, we used biotin-labeled CCL2 (biot-rmCCL2) [15, 16] to detect chemokine-ligand binding to L-CCR-expressing cells. Incubation of HEKL–CCR_{mc5} with 20 nM biot-rmCCL2 revealed an intense fluorescence signal of the membranes of the cells (**Fig. 4A**). This signal was completely absent in mock-transfected HEK 293 cells (Fig. 4B). The intensity of the signal obtained with biotinylated soybean trypsin inhibitor (negative control) in HEKL–CCR_{mc5} was similar to the signal obtained from mock-transfected cells (data not shown). Similar results have been obtained with transiently transfected CHO cells. The intensity of the signal obtained with 20 nM biot-rmCCL2 in mock-transfected CHO was weak (Fig. 4C) but present in L-CCR-transfected CHO cells (Fig. 4D).

As it was published earlier that L-CCR mRNA expression was enhanced in RAW cells by LPS treatment [10], we investigated whether treatment with LPS would influence the binding of biot-rmCCL2 in RAW cells. Weak binding of biot-rmMCP-1 was found using unstimulated RAW cells (Fig. 4E). From Fig. 4, E and F, it is clear that under control conditions, not all cells were labeled with biot-rmCCL2. Stimulation of RAW cells with LPS (100 ng/ml for 12–16 h) increased the intensity of biot-rmCCL2 binding (Fig. 4G), and almost 100% of the cells showed this signal after LPS stimulation (Fig. 4H). Similar results have been found in cultured mouse glial cells [11].

The detection of binding with the used fluorokine kit is based on a nonlinear amplification that occurs between the avidin–fluorescein molecules and the excess biotinylated chemokine present in the reaction. Therefore, this kit is not suitable to determine dissociation constant (K_d) and Bmax values (Frank Mortari, R&D Systems, personal communication). However, to obtain more knowledge on the specificity of the binding-signal competition, experiments with unlabeled chemokines were performed. Competition studies were performed with 20 nM biot-rmCCL2 and multifold concentrations of unlabeled CCL2, CCL5, CCL3, or CCL21 (five-, ten-, 50-, and 100-fold). Unlabeled CCL2 competed with biot-rmCCL2 for binding to HEKL–CCR_{mc5} (**Fig. 5, A–C**, left side). Five-fold excess of unlabeled CCL2 decreased the intensity of the binding signal (**Figs.** 5B and **6**), whereas the higher concentration of unlabeled CCL2 (ten- to 100-fold) completely inhibited the specific binding of biot-rmCCL2 (Figs. 5C and 6). In the presence of a ten- to 100-fold excess of unlabeled CCL2, only nonspecific binding was detectable (Fig. 6). Similar results were also found for an excess of unlabeled CCL5 (Fig. 6). In striking contrast, the presence of unlabeled CCL3 did not influence the binding of biot-rmCCL2 to HEKL–CCR_{mc5} at any concentration (Figs. 5, D–F, and 6). Other chemokines, such as CCL21, which did not have any effect on chemotaxis of HEKL–CCR_{mc5}, did not influence the binding of biot-rmCCL2 (data not shown).

Expression of L-CCR mRNA in various tissues of the mouse

To investigate the expression of L-CCR mRNA, RT-PCR experiments have been performed with cDNAs derived from a set of different mouse tissues. Expression of L-CCR mRNA was found in liver, spleen, heart, lung, kidney, and skeletal muscle (**Fig. 7**). No L-CCR mRNA expression was detectable in lymph nodes, thymus, and whole blood (Fig. 7). Expression of L-CCR mRNA in blood cells, however, was inducible by peritoneal LPS injection (Fig. 7).

DISCUSSION

We have recently described the expression of the orphan chemokine receptor L-CCR in mouse glial cells [11], an orphan chemokine receptor that has been originally identified in a murine macrophage–monocyte cell line RAW 264.7 [10]. To identify potential ligands, L-CCR was cloned and expressed in various cell lines. The following results suggest that L-CCR is a functional chemokine receptor: i) Concentration-dependent chemotaxis and intracellular calcium signals in cells expressing L-CCR were induced by various chemokines. (ii) This chemotactic response was sensitive to PTX treatment. (iii) Expression of L-CCR was localized in membranes of L-CCR–EGFP-transfected HEK 293 cells, and stimulation of L-CCR in these cells led to a rapid polymerization of actin filaments. (iv) Binding of biot-rmCCL2 was found in L-CCR-transfected cells but not in mock-transfected cells. (v) The intensity of biot-

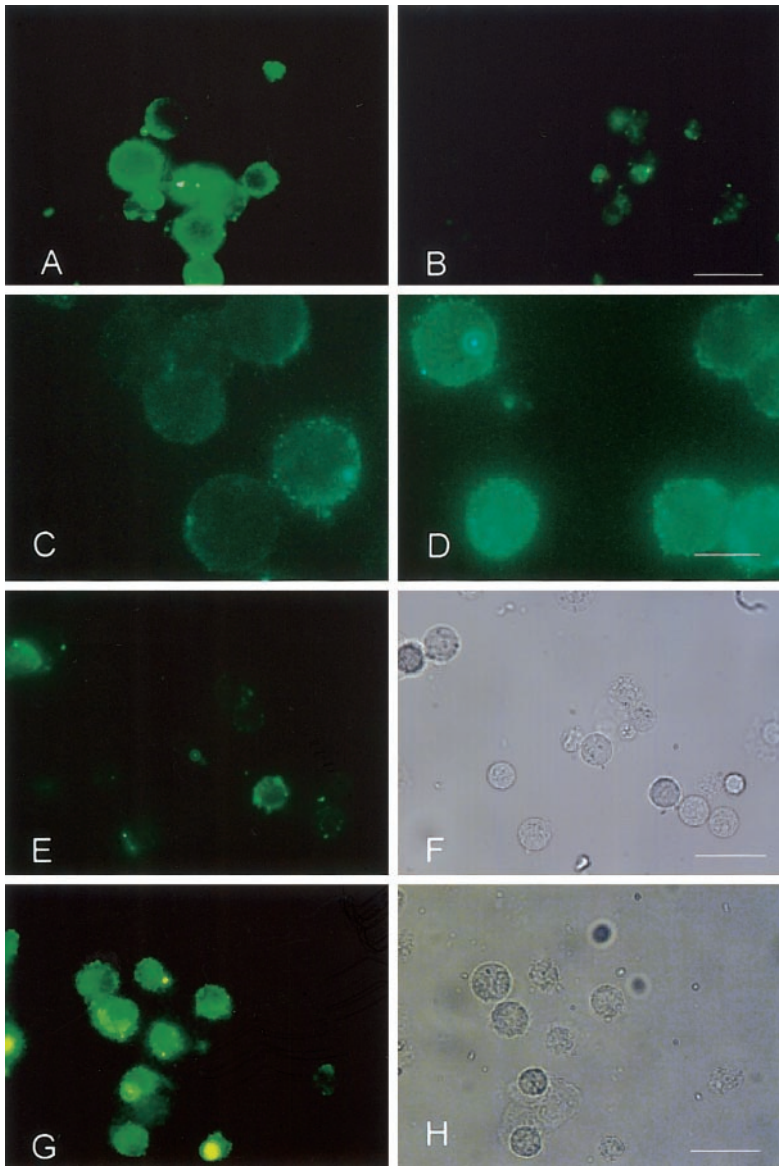

Fig. 4. Binding of biot-rmCCL2 to L-CCR-expressing cells that were incubated with biot-rmCCL2 (20 nM) or biotinylated soybean trypsin inhibitor (negative control), as indicated in Materials and Methods. Incubation of HEKL–CCR_{mc5} resulted in a fluorescent signal at the cell membrane (A); this was completely absent in mock-transfected HEK 293 cells (B). The intensity of the signal obtained with biot-rmCCL2 in mock-transfected CHO was weak (C) but increased in CHO cells transiently transfected with L-CCR (D). The intensity of biot-rmCCL2-binding fluorescence to unstimulated RAW cells was weak (E), and not all cells showed binding of biot-rmCCL2 (cf., E, with the bright-field picture of the same area, F). Treatment of RAW cells with LPS (100 ng/ml for 16 h) increased the intensity of biot-rmCCL2 binding to the membrane of the cells (G). Comparison of the bright-field picture of the same area (H) with the fluorescent picture shows that all cells showed biot-rmCCL2 binding after LPS treatment. Space bar represents 100 μm in A, B, and E–H; in C and D, 20 μm.

rmCCL2 binding in RAW 264.7 cells correlated to the mRNA expression level of L-CCR. (vi) Binding of biot-rmCCL2 to HEKL–CCR_{mc5} was inhibited by an excess of unlabeled CCL2 and CCL5 but not by an excess of CCL3 of CCL21. (vii) Among several chemokines, only CCL2, CCL5, CCL7, and CCL8 were identified as ligands for L-CCR. The members of the MCP family (CCL2, CCL7, and CCL8) are known to activate CCR2, but CCL5, however, is not a chemokine ligand for CCR2, indicating that the pharmacological profile observed for L-CCR is new and unrelated to the "ligand" profiles of other chemokine receptors.

Sequence similarity studies showed homologies from 48% to 56% between L-CCR and other mouse CC chemokine receptors. A higher homology was found between L-CCR and the human orphan chemokine receptor HCR. Cloning and expression of HCR in HEK 293 cells gave similar results, indicating that HCR is the human homologue to L-CCR (M. W. Zuurman et al., manuscript submitted). RT-PCR analysis of mouse tissues revealed L-CCR mRNA expression in heart, muscle, lung, kidney, spleen, or liver. No L-CCR expression was detected in lymph nodes and thymus. L-CCR expression was also detected in cultured glial cells and in inflamed brain, suggesting a role of L-CCR in neuroinflammation [11].

According to the rules of the chemokine receptor nomenclature committee, only orphan receptors that show signaling and high-affinity radioligand binding will obtain an official name [3]. As L-CCR does not fulfill these conditions, a designation of L-CCR as an official chemokine receptor cannot yet be claimed. The observed binding of biot-rmCCL2 does not allow an exact quantification, which would be needed for a calculation of K_d or Bmax values. Why radioligand binding could not be detected in this study is not clear yet. A conserved amino acid sequence (DRYLAIVHAVF), which seems to be characteristic for CC chemokine receptors, is not present in L-CCR. The first three amino acids of this motive (DRY) are especially highly conserved in class A G-protein-coupled receptors (GPCR) and very important in ligand-binding and signal transduction [17–19]. A few functional class A GPCR with modifications of the DRY motive are known (i.e., QRY in human P2Y6 or PAR2 receptors), but the QGY found in L-CCR is a

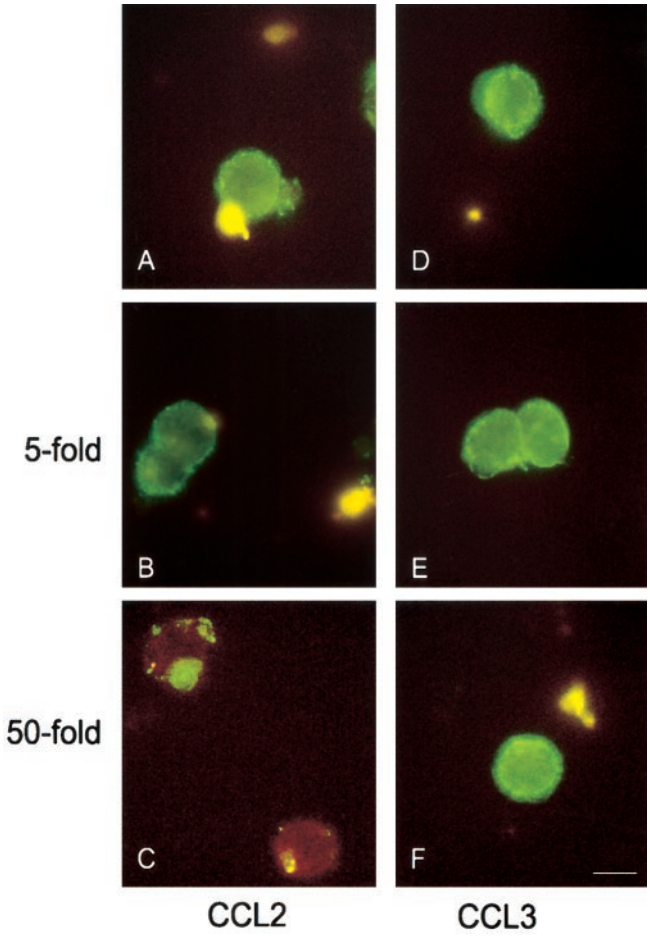

Fig. 5. Effect of an excess of unlabeled CCL2 and CCL3 on biot-rmCCL2 binding to HEKL–CCR_{mc5}. (A–C) Competition of biot-rmCCL2 binding by unlabeled CCL2. (A) Control binding of 20 nM biot-rmCCL2. (B) Decreased binding intensity of biot-rmCCL2 (20 nM) after incubation with a fivefold excess of unlabeled CCL2 (100 nM). (C) The binding of 20 nM biot-rmCCL2 to HEKL–CCR_{mc5} is blocked in the presence of a 50-fold excess of unlabeled CCL2 (1 μM). (D–F) Lack of competition of biot-rmCCL2 binding to HEKL–CCR_{mc5} by unlabeled CCL3. The binding intensity of 20 nM biot-rmCCL2 to HEKL–CCR_{mc5} cells (D) was not influenced by a fivefold excess (E) or a 50-fold excess (F) of unlabeled CCL3. Space bar represents 50 μm.

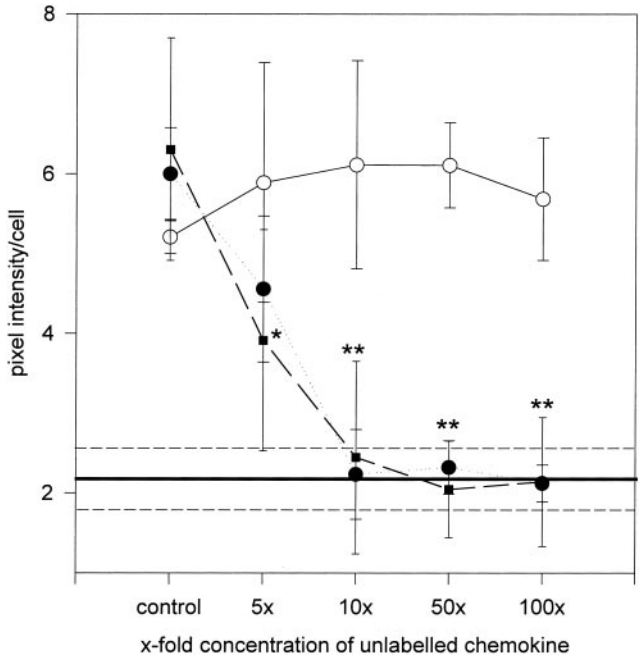

Fig. 6. Quantification of the effects of an excess of unlabeled CCL2 (●), CCL5 (■), and CCL3 (○) on biot-rmCCL2 (20 nM) binding on HEKL–CCR_{mc5}. The binding intensity of biot-rmCCL2 was decreased significantly by a fivefold excess of unlabeled CCL2 and completely inhibited by higher concentrations of unlabeled CCL2. Similar results were obtained for an excess of unlabeled CCL5. Conversely, an excess of unlabeled CCL3 up to 100-fold did not influence the binding intensity of 10 nM biot-rmCCL2 to HEKL–CCR_{mc5}. Data given are means ± SD from approximately 50 cells per chemokine concentration. Bold line and dashed lines represent the mean and the margins of the SD of the intensity of the signal obtained with biotinylated soybean trypsin inhibitor, respectively. *, **, Significantly different from control $P < 0.05$ and 0.01, respectively, determined by Student's *t*-test.

unique motive among all different class A GPCR listed in the GPCR database <www.GPCR.org>.

Functional responses in the absence of high-affinity radioligand binding have already been published for other GPCR such as $5\text{-}HT_{1B}$ receptors [20]. As a potential explanation, it was assumed here that these data are a result of a very efficient receptor–effector coupling at low densities of receptors present in the system [20]. Another explanation might be that imbalance between the functional receptor states R (inactive receptor) and R* (active receptor) occurs. Radiolabeled, high-affinity binding requires a certain number of R* receptors [21]. Constitutive receptor activity results in such an imbalance with more receptors in the R than in the R* state [21, 22]. Accordingly, low histamine binding (K_i: 369 μM) but high efficacy of histamine-induced cAMP production (EC_{50}: 180 nM) was detected for the human H2 histamine receptor expressed in CHO cells, a receptor that showed high constitutive activity [19, 23]. Whether this may account for the observed lack of radioligand binding needs further investigations. Although we have made several, thoroughly controlled attempts, we cannot exclude technical problems in our binding assays. It would for example be possible that the number of receptors expressed in our cells is too low to find radioligand binding and that the signal we detected with biot-rmCCL2 is a result of the amplification of the signal in the reaction.

Several publications suggest the possible existence of an additional CCL2 receptor, different from CCR2. For example,

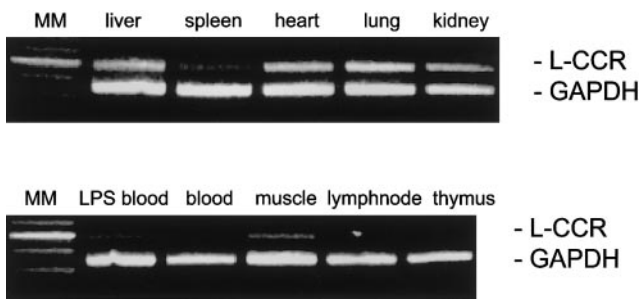

Fig. 7. RT-PCR experiments showing the expression of L-CCR mRNA in various mouse tissues. The numbers of cycles for GAPDH and L-CCR were 28 and 32, respectively. MM, Molecular weight marker; highlighted band, 500 bp. Both PCR products were run in the same gel. Similar results were found in three independent experiments.

responses to CCL2 have been observed in cells that do not express detectable CCR2 mRNA [11, 24]. Moreover, different T helper 2 responses in CCR2-deficient and CCL2-deficient mice have been described [5]. Furthermore, it was recently indicated that a CCL2 receptor different from CCR2 is involved in the development of airway hyper-responsiveness [25]. Accordingly, the identification of a second CCL2 receptor has been an issue in the chemokine field for quite some time. Two previous publications already indicate the existence of another CCL2 receptor [26, 27]. The mouse chemokine receptor D6 has been described as a chemokine receptor with CCL2-binding and signaling properties [25]. However, CCL2 signaling for D6 could not be reproduced [28]. Therefore, this receptor has not been classified as a CCR by the nomenclature committee and currently keeps its orphan name D6 [3]. Another CCL2 chemokine receptor has been described by Schweickart et al. [26], which they designated CCR11. Simultaneously, Gosling et al. [29] described a chemokine receptor with the same sequence but with different ligands, as found by Schweickart and colleagues [26]. Further investigations of the used cell line by Schweickart and colleagues [30] showed that the CCL2 effects described were a result of endogenous expression of CCR2 in their transfection system. Using different transfection systems (HEK 293 and L1.2 cells), Schweickart and colleagues [30] found the same ligand set, as Gosling et al. [29] already published it. The chemokine receptor nomenclature committee [31] has recently disqualified the CCR11 designation for this receptor.

In summary, the orphan chemokine receptor L-CCR has been expressed in HEK 293 cells to identify possible chemokine ligands that define its pharmacological signature. Although L-CCR stimulation induces cellular chemotaxis and other responses, radioligand binding could not be observed. Therefore, L-CCR-deficient mice would be very important tools to investigate whether L-CCR is a physiological receptor in vivo.

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