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Opposite Fate of Endocytosed CCR7 and Its Ligands: Recycling versus Degradation

Carolina Otero, Marcus Groettrup, and Daniel F. Legler

The chemokine receptor CCR7 and its ligands CCL19 and CCL21 play a crucial role for the homing of lymphocytes and dendritic cells to secondary lymphoid tissues. Nevertheless, how CCR7 senses the gradient of chemokines and how migration is terminated are poorly understood. In this study, we demonstrate that CCR7(-GFP) is endocytosed into early endosomes containing transferrin receptor upon CCL19 binding, but less upon CCL21 triggering. Internalization of CCR7 was independent of lipid rafts but relied on dynamin and Eps15 and was inhibited by hypertonic sucrose, suggesting clathrin-dependent endocytosis. After chemokine removal, internalized CCR7 recycled back to the plasma membrane and was able to mediate migration again. In contrast, internalized CCL19 was sorted to lysosomes for degradation, showing opposite fate for endocytosed CCR7 and its ligand. The Journal of Immunology, 2006, 177: 2314–2323.

Lymphocyte traffic is fundamental for immune regulation and, hence, is highly coordinated. Tissue- and microenvironment-selective leukocyte homing is the basis for this organization. It is now well established that cell migration is orchestrated mainly by chemokines which, together with adhesion molecules, deliver the key signal that allows leukocytes to transmigrate from the bloodstream into the tissue (1–3). All chemokines act through signaling via seven-transmembrane domain G protein-coupled cell surface receptors, but migration properties vary greatly among different cell types. The central role of the chemokine receptor CCR7 and its ligands CCL19 (ELC, Exodus-3, MCP-3β, and CKβ11) and CCL21 (SLC, Exodus-2, 6Ckine, and TCA-4) in the homing to secondary lymphoid organs is undisputed. CCR7 is highly expressed on naïve T cells and to a lower level on B cells. A transient increase in CCR7 expression is found upon T cell activation (4), whereas T cell differentiation toward effector cells is accompanied by the down-regulation of the receptor on the cell surface (5). However, in dendritic cells, CCR7 expression is induced upon maturation (6–8). Mice lacking CCR7 show delayed kinetics in Ab responses, delayed-type hypersensitivity reactions, and morphological abnormalities in secondary lymphoid organs as a consequence of an impaired homing of mature dendritic cells and lymphocytes (9, 10). The fact that CCR7 ligands are mandatory for the homing to secondary lymphoid organs has been demonstrated in plc/plt mice lacking CCL19 and CCL21 (11–14).

Although CCR7 and its ligands are essential for eliciting a potent cellular immune response, CCR7 signaling and its regulation is still sparsely investigated (15–20). In particular, information on how CCR7-mediated migration is stopped after a cell has arrived at its final destination within the lymph node has remained unclear. CCL19 and CCL21 are both produced by stroma cells within the T cell zone (12). Remarkably, CCL21 is transcytosed to high endothelial venules (HEV) (21) and mediates LFA-1-mediated arrest of the recruited T lymphocytes (22–24). Thus, T lymphocytes and dendritic cells that home to the T zone of lymph nodes seem first to be recruited to HEV by CCL21, but then the CCL21 signal must be overcome by an attraction signal provided by CCL19/ CCL21 derived from the T zone. B cells within the lymph node that have seen an Ag migrate directionally toward the B zone–T zone boundary along a gradient of CCL21 (and eventually CCL19) to encounter T cells (25).

One way of rendering a cell unresponsive to chemokines is receptor internalization. Chemokine receptor endocytosis is best described for the HIV coreceptors CCR5 and CXCR4 but follows distinct mechanisms (26). Remarkably, binding of CXCL12 to CXCR4 leads to the ubiquitylation of the receptor followed by its degradation in lysosomes (27, 28). In contrast, endocytosed CCR5 is recycled back to the plasma membrane (29–31). Strikingly, CCR7 internalization was observed by CCL19 triggering, but not by stimulation with CCL21 (32), although binding affinities and G-protein activation are comparable (4, 18). Of note, CCR7 desensitization through receptor phosphorylation and β-arrestin binding was enhanced by CCL19 stimulation, compared with CCL21 (18), whereas T cell polarization mediated by the chemokines was indistinguishable (17). However, up to now, the mechanism of CCR7 signaling and trafficking remains largely unclear, and there is currently no information on the fate of CCL19 after CCR7 endocytosis.

Cell surface receptors can be internalized by two segregated pathways: clathrin-dependent and clathrin-independent, lipid raft/caveolae-dependent endocytosis (33, 34). The classical clathrin-dependent pathway is well characterized. Clathrin-coated pits at the plasma membrane bud and pinch off in a dynamin- and adaptor protein (such as Eps15)-dependent manner to form clathrin-coated vesicles. After endocytosis, clathrin-coated vesicles are uncoated.
and fuse with the early endosomes, the central control organelles for sorting receptors. Either receptors recycle back to the plasma membrane via recycling endosomes or are directed to late endosomes and lysosomes for degradation. Alternatively, receptors can be endocytosed in a lipid raft/caveolae-dependent manner. This pathway is ill defined but largely depends on cellular cholesterol (33–35).

In this study, we investigated the route of internalization and the trafficking of CCR7 by monitoring a newly generated GFP-tagged CCR7. In addition, we tracked CCL19 after receptor binding by a chemokine-Fc chimera. Analysis of CCR7 endocytosis and investigations on the routes of CCL19 and CCL21 after receptor triggering is critical for a better understanding of how immune cells, such as dendritic cells and lymphocytes, sense a chemokine gradient originating in secondary lymphoid organs.

Materials and Methods

Abs and reagents

Abs were obtained from the following sources: PE-Cy5-labeled rat anti-human CCR7 (clone 3D12; BD Pharmingen), goat anti-human IgGHRP (Santa Cruz Biotechnology); streptavidin-Cy3 and streptavidin-FITC (Jackson ImmunoResearch Laboratories); mouse anti-phospho ERK-1/2 (Santa Cruz Biotechnology); streptavidin-Cy3 and streptoavidin-FITC (provided by Dr. K. Scherrer, Paris, France). Alexa Fluor 546-labeled transferrin and lystateck Red DND-99 were from Molecular Probes. Human chemokines CCL19 and CCL21 were purchased from PromoCell. Monobiotinylated human CCL19 was from RMF Dictagene. Streptavidin-peroxidase, filipin III, methyl-β-cyclodextrin (MCD), sucrose, chloroximidine, chloroquine, and protein A-Sepharose were obtained from Sigma-Aldrich. Fluo-3/AM was purchased from Calbiochem.

Cells and transfection

The human embryonic kidney cell line, HEK293, was grown in DMEM (Invitrogen Life Technologies) with 10% (v/v) FBS. HEK293 cells were stably transfected in 10-cm dishes by the calcium phosphate procedure. Cell clones were established by limiting dilution as described (4, 36). The human T cell line CEM was transfected by electroporation, and clones were achieved by limiting dilution as described (4, 36). The human CCR7 (clone 3D12; BD Pharmingen), goat anti-human IgG1HRP (DakoCytomation); and mouse anti-proteasome subunit C7 (a; provided by Dr. K. Scherrer, Paris, France). Alexa Fluor 546-labeled transferrin and lystateck Red DND-99 were from Molecular Probes. Human chemokines CCL19 and CCL21 were purchased from Promocell. Monobiotinylated human CCL19 was from RMF Dictagene. Streptavidin-peroxidase, filipin III, methyl-β-cyclodextrin (MCD), sucrose, chloroximidine, chloroquine, and protein A-Sepharose were obtained from Sigma-Aldrich. Fluo-3/AM was purchased from Calbiochem.

Construction of expression plasmids

The entire open reading frame of human CCR7 was amplified by PCR from SRopuro-CCL7 (4) using the primers CCR7/3F (5'-ATA GGA TTC CTT CAG CAT GTA CCT GGA CTT GAA AC; restriction site underlined) and CCR7/3R (5' - TAT GCC GCC GCT GGG GAG AAG GTG GTG) and subcloned into the EcoRI/NotI sites of pcDNA3 (Invitrogen Life Technologies). Enlarged GFP (EGFP) was fused to the N terminus of CCR7 by PCR amplifying GFP from pEGFP-N1 (Clontech) and subcloning into the Xhol/Xbal sites of pcDNA-3-CR7 using the primers EGFP-F (5' - AAA TTC CAG CAG TGA CAG GAG GGA) and EGFP-R (5' - AAA TT AAG TAC AGA CTA CTT GAG CTC TGC GTC). A VSVG-tagged CCR7 was cloned by PCR amplification of CCR7 using the primers CCR7/3F (5'-TAT GAA TTC TAC GAG CTG GGG AAA CCA ATG AAA GAC) and CCR7/3R (5'-TAA TTA TAC AGA CTA CTG TGG GAA GAA GTT GGT G) and subcloning into the EcoRi/Xbal sites of pCR3-VSV (MT044; provided by M. Thome, University of Lausanne, Epalinges, Switzerland). The CCR7-CHA construct was made by replacing the GFP (Xhol/Xbal) with the annealed oligonucleotides

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5' - TAA TTA TAC AGA CTA CTG TGG GAA GAA GTT GGT G
5' - TAT GAA TTC TAC GAG CTG GGG AAA CCA ATG AAA GAC
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and subcloning into the EcoRi/Xbal sites of pCR3-VSV (MT044; provided by M. Thome, University of Lausanne, Epalinges, Switzerland). The CCR7-CHA construct was made by replacing the GFP (Xhol/Xbal) with the annealed oligonucleotides

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5' - TAA TTA TAC AGA CTA CTG TGG GAA GAA GTT GGT G
5' - TAT GAA TTC TAC GAG CTG GGG AAA CCA ATG AAA GAC
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and subcloning into the EcoRi/Xbal sites of pCR3-VSV (MT044; provided by M. Thome, University of Lausanne, Epalinges, Switzerland).

Flow cytometry

Cells were washed twice with FACS buffer (PBS containing 2% FBS and 5 mM EDTA) and, where required, incubated with the respective Abs for 30 min at 4°C. Cells were washed twice and fluorescence was acquired by a FACScan II using CellQuest software (BD Biosciences). Data were analyzed with the FlowJo software (Tree Star).

Chemotaxis

Chemotaxis of 300-19 cells was measured by migration through a polycarbonate filter of 5-μm pore size in 24-well Transwell chambers (Corning Costar). Cell culture medium (600 μl) containing indicated concentrations of chemokine, or medium alone as a control for spontaneous migration, was added to the lower chamber; a total of 1 × 10^5 cells in 100 μl was added to the upper chamber. After 3 h of incubation at 37°C, a 500-μl aliquot of the cells that migrated to the bottom chamber was counted by flow cytometry acquiring events for a fixed time period of 60 s using CellQuest software. The number of migrated cells was expressed as percentage of input cells.

Chemokine-mediated changes in intracellular free calcium concentrations

Cells were washed twice with Ca^2+ buffer (145 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, 1 mM MgCl2, 5 mM glucose, 1 mM CaCl2, and 10 mM HEPES (pH 7.5)) and resuspended at a concentration of 1 × 10^6 cells/ml. Cells were loaded with 1.5 μl/μl Fluo-3/AM (4 mM in DMSO) for 30 min at 37°C. Cells were washed, and chemokine-induced calcium mobilization-related fluorescence changes of Fluo-3 were measured by flow cytometry.

Confocal laser scanning microscopy

Transfected HEK293 cells were grown on cover slips. 300-19 cells were incubated for 1 h on coverslips coated with poly-L-lysin (Sigma-Aldrich). If not stated otherwise, cells were treated with 2 μg/ml CCL19 or CCL21, 3 μg/ml biotinylated CCL19, 10 μg/ml CCL19-Fc, or CCL21-Fc, 50 μg/ml transferrin, or 50 nM lysotracker. Cells were washed twice with PBS and fixed for 10 min with 4% paraformaldehyde followed by three washing steps with PBS. For intracellular stainings, cells were permeabilized with 0.2% Triton X-100 (Fluka) for 10 min and washed with 0.2% gelatin in PBS. Ab staining of cells was performed at room temperature for 40 min, and cells were washed five times and mounted on glass slides using Fluormount-G (Southern Biotechnology Associates). Immunofluorescence was analyzed with a confocal microscope (LSM 510; Zeiss) with a ×63 Plan-Apochromat objective (aperture = 1.4). Images were acquired using LSM 510 software (Zeiss).

Western blotting

Cells were lysed with 1% Triton X-100 in 150 mM NaCl, 50 mM HEPES, 0.1 M EGTA, 2 mM MgCl2, 10% glycerol containing leupeptin, aprotonin, and pepstatin (1 μg/ml each; Roche). Proteins from total cell lysates were resolved by SDS-PAGE and transferred to Protran nitrocellulose membrane (Schleicher & Schuell Microscience). Membranes were blocked with PBS containing 5% of low-fat dry milk and incubated with the respective Abs overnight at 4°C or for 1 h at room temperature on a rocking plate. After washing, HRP-conjugated secondary Abs were bound and detected using ECL (Pierce/Socochim).

Results

Differential endocytosis of CCR7 by CCL19 and CCL21

The homing of lymphocytes and dendritic cells largely depends on the attraction of the cells by the chemokines CCL19 and CCL21.
At its final destination, the migratory signal needs to be shut off, which normally occurs by chemokine receptor down-modulation or receptor desensitization. To unravel the mechanism how CCR7 is silenced, we investigated the internalization of CCR7 in IL-2- and PHA-activated human PBL. CCR7 endocytosis by CCL19 was readily observed in a concentration- and time-dependent manner (Fig. 1). Cell surface expression of CCR7 on T cells was already reduced by 15% at a CCL19 concentration of 30 ng/ml. More than 60% of CCR7 was internalized in the presence of 3 µg/ml CCL19 (Fig. 1A). Endocytosis was rapid, because after 2 min of chemokine addition, 30% of CCR7 disappeared from the plasma membrane. Maximal internalization was reached after 30 min of incubation (Fig. 1B). Interestingly, CCR7 endocytosis by CCL21 was observed only at high chemokine concentrations (>300 ng/ml) and reached a maximum of ~25% (Fig. 1A). Our data on CCL19-mediated endocytosis of CCR7 are largely in agreement with a previous study by Bardi et al. (32), although they did not find any internalization by CCL21 in T cells at all.

**Generation of a fluorescent fully functional CCR7**

To further investigate CCR7 localization and trafficking, we fused the enhanced GFP to the C terminus of human CCR7. We stably expressed CCR7-GFP in the murine pre-B cell line 300-19, a cell line that does not respond to CCL19 and CCL21 (4, 36). CCR7-GFP transfected cells readily migrated in response to CCL21, similar to 300-19 cells expressing wt CCR7, whereas CCR7-GFP-positive cells did not migrate in a Transwell chemotaxis assay in the absence of chemokines (Fig. 2A). The phosphorylation of the ERK-1 and ERK-2 is an early and transient event after chemokine triggering (16). To test whether CCR7-GFP is fully functional, we stimulated 300-19-transfected cells for various time points with CCL21 and analyzed the phosphorylation of ERK-1/2 by Western blot analysis. Identical kinetics and potency of ERK-1/2 activation upon CCL21 triggering was observed for cells expressing CCR7-GFP and wt CCR7 (Fig. 2B). 300-19 cells are ideal for testing chemotaxis but not for morphological and trafficking studies. Therefore, we stably transfected the human embryonic kidney cell line HEK293 with CCR7-GFP. CCL21 stimulation of HEK293 cells expressing CCR7-GFP resulted in the phosphorylation of ERK-1/2 (Fig. 2C), comparable to 300-19 transfectedants. As expected, CCR7-GFP mainly localized to the plasma membrane of both transfected cell lines as assessed by confocal microscopy (Fig. 2D). These data provide clear evidence that CCR7-GFP is fully functional.

**FIGURE 1.** Internalization of CCR7 in human PBL. A, Human PBL were cultured for 4 – 6 days in the presence of IL-2 and PHA and incubated with graded concentrations of CCL19 (circles) or CCL21 (triangles) for 30 min at 37°C. Cell surface expression of CCR7 was determined by flow cytometry using the mAb 3D12 against CCR7 which can still recognize the epitope in the presence of bound ligand. B, The same T cells were incubated for various time points with 3 µg/ml chemokines, and cell surface expression of CCR7 was determined by flow cytometry. Mean values of three independent experiments are shown. Error bars are <1%.

**FIGURE 2.** Characterization of CCR7-GFP. A, Migration of the murine pre-B cell line 300-19 stably transfected with either human CCR7 wt or CCR7-GFP in response to CCL21 (1 µg/ml) was assessed in a Transwell chemotaxis assay. After 3 h of incubation at 37°C, cells in the lower chamber were collected and counted by flow cytometry. Mean values and SD of three independent experiments are depicted as percentage of migrated cells. B, 300-19 cells expressing either CCR7 wt or CCR7-GFP were incubated with 2 µg/ml CCL21 for indicated time points and lysed, and the activation of ERK-1/2 was determined by Western blot analysis using an Ab recognizing the phosphorylated forms of ERK-1 and ERK-2 (pERK-1/2). An Ab against total ERK-2 (tERK-2) was used to ensure equal protein loading. C, The activation of ERK-1/2 was confirmed in HEK293 cells expressing CCR7-GFP. D, Cell surface expression of transfected CCR7-GFP in 300-19 and HEK293 cells was determined by confocal microscopy. Scale bars, 10 µm.

CCRF-GFP colocalizes with early and recycling endosomes but not with lysosomes

To investigate the intracellular trafficking of CCR7, we stimulated HEK293 cells expressing CCR7-GFP with CCL19 and CCL21. After 5 min of CCL19 stimulation, CCR7-GFP was endocytosed and appeared as punctuated structures within the cell (Fig. 3A). Some CCR7-GFP was still present at the plasma membrane, confirming our data from primary T cells. Upon CCL21 stimulation, CCR7-GFP remained mainly at the plasma membrane, although intracellular CCR7-GFP spots were reproducibly observed (data not shown), confirming that CCL19 triggers CCR7 internalization more efficiently than does CCL21. Internalized G protein-coupled receptors are generally degraded in lysosomes or recycled back to the plasma membrane via early and recycling endosomes. To investigate these two possibilities for CCR7, we incubated HEK293-CCR7-GFP cells with CCL19 together with Alexa Fluor 546-labeled transferrin. The trafficking of the iron transport protein
transferrin is one of the best studied processes. Upon ligand binding, transferrin receptor is internalized by clathrin-coated pits giving rise to clathrin-coated vesicles. Endocytosed transferrin receptor, together with transferrin, then fuse with recycling endosomes and are directed back to the plasma membrane (39). Extensive colocalization of transferrin and CCR7-GFP was observed after 5 min and 3 h of CCL19 and transferrin stimulation (Fig. 3, A and B), suggesting that CCR7-GFP localizes in endosomes. To discriminate recycling from early endosomes, HEK293-CCR7-GFP cells were incubated with CCL19 and Alexa Fluor 546-labeled transferrin for 5 min, washed to remove unbound ligands, and further incubated for 15 min at 37°C in the absence of ligands. Confocal microscopy studies revealed that endocytosed CCR7-GFP colocalized with transferrin (data not shown), providing clear evidence that the spotted distribution of CCR7-GFP represents recycling endosomes. Furthermore, we investigated whether CCR7-GFP also resides in lysosomes. To this end, we stimulated CCR7-GFP expressing cells with CCL19 for 3 h. Analysis by confocal microscopy demonstrated that CCR7-GFP did not colocalize with lysotracker, a marker for late endosomes and lysosomes (Fig. 3C). Also, shorter or prolonged incubations with CCL19 (up to 9 h) revealed the same results (data not shown), indicating that CCR7-GFP is not sorted to the degradative pathway.

Endocytosed CCR7 is recycled and not degraded

To formally prove that CCR7 is indeed recycled, we incubated HEK293 cells expressing CCR7-GFP with CCL19 or CCL21 for 30 min at 37°C. Subsequently, the excess of chemokine was removed, and cells were incubated for 1 h in the absence of chemokine at 37°C to allow receptor recycling. Cell surface expression of CCR7 was determined by flow cytometry using a CCR7 specific mAb (Fig. 4A). As expected, CCR7-GFP surface expression was reduced after incubation of the cells with CCL19, and to a lesser extent also with CCL21. After washing off chemokines, endocytosed CCR7 reappeared at the plasma membrane (Fig. 4A), demonstrating that CCR7 is either recycled after internalization or de novo synthesized. Similar results also were obtained with 300-19 cells expressing HA-tagged CCR7 (Fig. 4B). To discriminate between recycling and de novo synthesis, we pretreated 300-19-CCR7-HA cells with cycloheximide for 1 h and kept in the presence of cycloheximide for the entire experiment to block de novo synthesis of CCR7 (gray bars). CCR7 endocytosis and recycling in 300-19 cells expressing HA-tagged CCR7 was performed as described in A. In addition, cells were pretreated with 50 μg/ml cycloheximide for 1 h and kept in the presence of cycloheximide for the entire experiment to block de novo synthesis of CCR7 (gray bars). C, CEM T cells were incubated or not for 30 min with 2 μg/ml CCL19 to induce CCR7 endocytosis, washed, and subjected to a Transwell chemotaxis assay. In addition, internalized CCR7 was allowed to recycle back to the plasma membrane by incubation for 1 h in the absence of chemokine before the chemotaxis assay. To prevent de novo synthesis of CCR7, CEM cells were pre-incubated for 1 h and kept in the presence of 50 μg/ml cycloheximide (gray bars). Cells were allowed to migrate in response to 1 μg/ml CCL19 for 3 h and quantified by flow cytometry.
the receptor to recycle back to the plasma membrane for 1 h. As expected, cells with internalized CCR7 did not migrate in response to CCL19, whereas cells with reexpressed CCR7 migrated toward CCL19 (Fig. 4C). Similar results were obtained with 300-19 cells expressing either CCR7-GFP or CCR7-HA (data not shown). Recycled rather than newly synthesized CCR7 was responsible for chemotaxis, because pretreatment of CEM cells with cycloheximide did not hamper migration (Fig. 4C). Furthermore, recycled CCR7 elicited the mobilization of cytosolic free calcium upon CCL19 stimulation (data not shown), providing clear evidence that recycled CCR7 is biologically functional.

To examine the overall rate of CCR7 degradation, potentially by a nonlysosomal pathway, we performed a degradation assay in the presence of both chemokines. HEK293-CCR7-GFP cells were incubated for up to 9 h with CCL19 or CCL21 in the presence or absence of cycloheximide, to monitor on the one hand the steady-state level of the protein and, in contrast, the impact of de novo synthesis. Using flow cytometry, we found no evidence for CCR7 degradation, because the fluorescence derived from CCR7-GFP was not reduced upon chemokine triggering (Fig. 5A). To corroborate these data, we also investigated the degradation of CCR7 by Western blotting. HEK293 cells expressing CCR7-HA were incubated with CCL19 or CCL21 for up to 6 h at 37°C, and the amount of CCR7-HA was determined from total cell lysates using an anti-HA Ab (Fig. 5B). Again, we found no evidence for CCR7 degradation, suggesting that the half-life of the receptor is very long.

**Generation of functional recombinant CCL19-Fc and CCL21-Fc chemokine fusion proteins**

To monitor the fate of CCL19 and CCL21 once they bound to CCR7, we generated expression constructs encoding for chemokines fused to the Fc part of human IgG1 as there are no good Abs against the chemokines available. We expressed human CCL19-Fc and human CCL21-Fc in HEK293 cells and purified the recombinant fusion proteins from the supernatants over protein-A columns. Both proteins were purified with a relative mass of ~40 kDa in a monomeric state after reduction and of ~80 kDa in a nonreduced dimeric form as judged by SDS-PAGE followed by Coomassie brilliant blue staining (data not shown). The biological activity of the chemokine Fc fusion proteins was tested by the ability to mobilize intracellular free calcium and to induce chemotaxis. 300-19 cells expressing CCR7 were loaded with Fluo-3/AM and subsequently exposed to the chemokines, and the calcium-dependent change in fluorescence was measured over time. Challenging 300-19-CCR7 cells with CCL19, CCL21, or the corresponding chemokine-Fc-fusion proteins elicited comparable transient rises in \([\text{Ca}^{2+}]_i\), (Fig. 6A), indicating that both CCL19-Fc and CCL21-Fc are functional. No mobilization of \([\text{Ca}^{2+}]_i\) was observed in parental 300-19 cells lacking CCR7, indicating that the rise in \([\text{Ca}^{2+}]_i\) was specific. Additionally, the chemotactic activity of CCL19-Fc and CCL21-Fc were tested in a Transwell chemotaxis assay. As shown in Fig. 6B, 300-19 cells expressing CCR7 migrated normally in response to CCL19-Fc, CCL19, and CCL21. For an unknown reason, only marginal migration toward CCL21-Fc was observed.

**FIGURE 5.** Internalized CCR7 is not degraded. A, HEK293-CCR7-GFP cells were incubated for different time periods with 2 μg/ml CCL19 (circles) or CCL21 (triangles) in the presence (open symbols) or absence (closed symbols) of cycloheximide. Total GFP-derived fluorescence was measured by flow cytometry. B, HEK293 cells stably expressing CCR7-HA were incubated with 2 μg/ml CCL19 or CCL21 for up to 6 h. Cells were lysed, and the total amount of CCR7-HA was determined by Western blotting using an anti-HA Ab. The α1 proteasome subunit was used as a loading control.

**FIGURE 6.** Generation of functional CCL19-Fc and CCL21-Fc. A, Parental 300-19 cells (bottom panel) or cells stably expressing CCR7 were loaded with Fluo-3/AM and stimulated with 4 μg/ml purified CCL19-Fc or CCL21-Fc and chemokine-mediated changes in intracellular free calcium concentrations were recorded over time by flow cytometry. For comparison, \([\text{Ca}^{2+}]_i\) changes in response to 2 μg/ml untagged CCL19 and CCL21 were measured. The arrowheads indicate the time point of chemokine addition (B). The migration of 300-19 cells expressing CCR7 in response to CCL19, CCL21 (1 μg/ml), and chemokine-Fc fusion proteins (10 μg/ml) was measured in Transwell chemotaxis assays. After 3 h of incubation, cells migrated to the lower wells were collected and counted by flow cytometry. As a control, cell migration in the absence of chemokine was determined.
observed at different concentrations of chemokine (Fig. 6B and data not shown).

**CCL19-Fc is internalized together with CCR7 but then sorted to lysosomes for degradation**

The functional recombinant proteins enabled us to study the intracellular trafficking and interaction of both chemokines with CCR7. CCL19-Fc induced internalization of CCR7-GFP similar to CCL19; and CCL19-Fc colocalized with CCR7-GFP as shown by confocal microscopy (Fig. 7A). Consistent with CCL21, CCL21-Fc also induced some internalization of CCR7 (data not shown). Remarkably, after incubation of HEK293 cells expressing CCR7-GFP with CCL19-Fc for 30 min at 37°C, followed by washing off the chemokine and an additional incubation for 6 h in the absence of the chemokine, most of the CCL19-Fc staining disappeared and vaguely colocalized with CCR7-GFP, which recycled back to the plasma membrane (Fig. 7B). Similar results were obtained with monobiotinylated CCL19 (data not shown). To address the trafficking of endocytosed chemokine, we stimulated CCR7-transfected HEK293 cells with CCL19-Fc together with Alexa Fluor 546-labeled transferrin. After 30 min of incubation, CCL19-Fc partially colocalized with transferrin (Fig. 7C), indicating that CCL19-Fc localizes in early endosomes like CCR7. However, not all intracellular CCL19-Fc spots colocalized with endosomes. To address the origin of these additional compartments, we stimulated CCR7-expressing cells with CCL19-Fc for 8 h in the presence of lysotracker. As depicted in Fig. 7D, CCL19-Fc also partially colocalized with lysosomes. To prove that CCL19-Fc is

**FIGURE 7.** CCL19-Fc is internalized together with CCR7 and colocalizes with both transferrin and lysosomes. A, HEK293 cells expressing CCR7-GFP were incubated with 10 μg/ml CCL19-Fc for 30 min at 37°C. Cells were fixed and permeabilized with Triton X-100. CCL19-Fc was visualized using a biotinylated anti-human IgG Ab and streptavidin-Cy3. The localization of CCR7-GFP and CCL19-Fc was determined by confocal microscopy. B, HEK293-CCR7-GFP cells were stimulated with CCL19-Fc for 30 min as in (A), washed extensively and cultured in the absence of chemokines for additional 6 h. C, HEK293 cells stably transfected with VSV-CCR7 were incubated with CCL19-Fc for 30 min at 37°C in the presence of Alexa Fluor 546-labeled transferrin. CCL19-Fc was monitored with a biotinylated anti-human IgG Ab and streptavidin-FITC. D, HEK293 cells expressing VSV-CCR7 were incubated with CCL19-Fc and lysotracker for 8 h. Representative images are shown. Scale bars, 10 μm. E, HEK293 cells expressing CCR7-HA were incubated with 10 μg/ml CCL19-Fc (upper panel) or 3 μg/ml monobiotinylated CCL19 (lower panel) for 30 min at 37°C in the presence or absence of 200 μM chloroquine. Cells were washed, incubated for indicated time periods in the presence or absence of chloroquine, washed, and lysed, and proteins were separated by SDS-PAGE. CCL19-Fc was detected by Western blotting using an anti-human IgG Ab coupled to HRP. Biotinylated CCL19 was detected using streptavidinHRP.
indeed degraded in lysosomes, we stimulated HEK293 cells expressing CCR7-HA with CCL19-Fc for 30 min in the presence or absence of chloroquine. Chemokines were removed and cells were further incubated for 3 and 6 h in the presence or absence of chloroquine. CCL19-Fc was degraded after 3 and 6 h (Fig. 7E). Treatment of the cells with the lysosomotrophic agent chloroquine and subsequent incubation with CCL19-Fc significantly inhibited chemokine degradation, providing clear evidence for CCL19-Fc degradation in lysosomes (Fig. 7E). To exclude that the degradation was due to the Fc part, we repeated the experiments with a chemically synthesized monobiotinylated CCL19, where a single amino acid was biotinylated. In fact, biotinylated CCL19 also was degraded (Fig. 7E) comparable to CCL19-Fc. Chloroquine treatment abolished the degradation of the biotinylated chemokine leading to an accumulation of CCL19 in intracellular compartments (data not shown).

**CCR7 endocytosis is mediated by clathrin-coated pits**

Two principal pathways of membrane receptor internalization are known (33). The best-studied pathway is clathrin-dependent endocytosis with the respective machinery of adaptor proteins and GTPases. The other pathway depends mainly on cholesterol-rich membrane microdomains, also termed lipid rafts, and referred to as clathrin-independent endocytosis, or in cells expressing caveolin, also caveolae-dependent endocytosis (34). Both pathways can be specifically inhibited. The formation of clathrin-coated pits can be blocked under hypertonic conditions using 0.4 M sucrose (40). Clathrin-independent endocytosis can be inhibited by sequestering cellular cholesterol by MCD or filipin (41–43). The pathway of CCR7 internalization has not yet been investigated. To do so, we pretreated IL-2- and PHA-activated PBL with filipin, MCD, or sucrose; incubated the cells with chemokines for 30 min at 37°C; and measured the surface expression of CCR7 by flow cytometry. Blocking the clathrin-independent pathway by filipin had no effect on CCR7 endocytosis by CCL19 or CCL21 (Fig. 8). These data were corroborated by treatment with MCD (Fig. 8) at a concentration that hampered TCR signaling (data not shown). Inhibition of the clathrin-dependent pathway by sucrose abolished CCR7 endocytosis by CCL19 and CCL21 (Fig. 8), suggesting that CCR7 endocytosis is mediated by clathrin-coated pits.

To further characterize the endocytic pathway, we investigated on the role of dynamin II and Eps15 in CCR7 internalization by confocal microscopy. Dynamin II is involved in the formation of both clathrin-coated and caveolar vesicles (44, 45). As expected, a normal rate of CCR7 endocytosis was detected after CCL19-Fc triggering in HEK293-CCR7 cells transiently transfected with GFP-tagged dynamin II (Fig. 9A). However, expression of a GFP-tagged dominant-negative mutant of dynamin II (dyn II K44A) abolished the internalization of CCR7 (Fig. 9B). In contrast, Eps15 has until very recently (46, 47) been implicated only in clathrin-coated pits assembly (38). Internalization of CCR7 was readily observed after CCL19-Fc-mediated stimulation of CCR7-positive cells transfected with Eps15-GFP (Fig. 9C). Remarkably, overexpressing a GFP-tagged dominant-negative form of Eps15 (Eps15 EΔ95/295) completely inhibited CCR7 internalization (Fig. 9D). These data, in conjunction with the finding that sucrose treatment abolished endocytosis, strongly suggest that CCR7 is internalized through the clathrin-dependent pathway.

Taken together, we demonstrate that CCL19 is more efficient than CCL21 in CCR7 internalization. CCR7 and its ligands are most likely endocytosed together through clathrin-coated pits. In early endosomes, the CCR7-ligand complex may dissociates and CCR7 and its ligand follow different routes. The chemokine, which is no longer used, is eliminated by lysosomal degradation. However, the receptor recycles back to the plasma membrane, ready to bind a new ligand, permitting cell migration toward the source of chemokine within the draining lymph node.

**Discussion**

There is no doubt that CCR7 and its ligands are essential for the homing of dendritic cells and T lymphocytes to lymph nodes, Peyrer’s Patches and the spleen (2, 9, 10, 48, 49). Ag-loaded DCs and circulating T lymphocytes enter secondary lymphoid organs by...
sensing CCL21 presented on HEV. Thereafter, they migrate to the T zone where CCL19, as well as CCL21, are expressed, facilitating the contact between naïve T lymphocytes and Ag-loaded DCs and, hence, the priming of T cells. For this event, fine-tuning of cell migration may be critical.

One important way of modulating chemokine receptor responsiveness is receptor endocytosis after ligand binding. Studies on chemokine receptor endocytosis moved into focus because chemokine-induced internalization of cell surface receptors was a major defense mechanism of chemokine-mediated inhibition of HIV infection. Thus, endocytosis has been studied most intensively on the HIV coreceptors CCR5 and CXCR4. CXCR4 internalization is induced by its ligand CXCL12, but also by phorbol esters (50, 51). CXCL12-mediated endocytosis occurs via clathrin-coated pits and depends on Rab5 and Eps15 (26). After ligand binding, CXCR4 is monoubiquitylated, endocytosed, and subsequently sorted to lysosomes for degradation (27). Interestingly, receptor mutants that are not ubiquitylated internalize normally (27), but CXCR4 ubiquitylation by AIP4 is required for sorting to lysosomes and its degradation (28). However, CXCR4 also was shown to recycle back to the plasma membrane (26, 50). In contrast, CCR5 internalization does not occur by phorbol esters (52), but only by its ligands (26). However, data on the routes of CCR5 internalization are controversial. CCR5 endocytosis was shown to be clathrin dependent (29, 53). Furthermore, intracellular CCR5 colocalized with fluorescent-labeled transferrin (54) and β-arrestin (55, 56). In contrast, cholesterol depletion by nystatin and filipin affected CCR5 endocytosis, and CCR5 was found to colocalize with caveolin, suggesting a role of caveolae/lipid rafts in this process (26, 55). Although different routes of CCR5 endocytosis have been described, there is consensus that CCR5 is recycled back to the cell surface (29, 54, 55).

The mechanism of CCR7 endocytosis is poorly investigated. In this study, we demonstrate that CCR7 in PBL is rapidly internalized after binding of CCL19 and to a lesser extent also by its second ligand CCL21 (Fig. 1). This is intriguing because CCR7 is the only chemokine receptor that is able to turn on different signaling pathways depending on different ligands. Our data are essentially in agreement with previous findings by Bardi et al. (32), who observed CCL19- but not CCL21-mediated endocytosis of CCR7 in activated lymphocytes. However, they also found borderline internalization of CCR7 by CCL21 in naive T cells, dendritic cells, and transfectants (32). The differential behavior of CCL19 and CCL21 is striking because both chemokines have similar binding affinities and induce G protein activation, calcium mobilization, and chemotaxis with equal potency (4, 18). To further characterize the trafficking of CCR7, we generated a CCR7-GFP fusion protein that fulfills all functional properties of wt CCR7 (Fig. 2). We provide evidence that CCR7 is internalized by clathrin-coated pits, because overexpression of dominant-negative mutants of dynamin II and Eps15 blocked CCL19-induced endocytosis of CCR7 (Fig. 9). For a long time, Eps15 has been described to be specific for clathrin-dependent endocytosis (33, 38). However, it has been shown recently that Eps15 also may be involved in clathrin-independent endocytosis (46, 47). As hypertonic sucrose treatment also abolished CCR7 internalization (Fig. 8) is it reasonable to assume that CCR7 uses clathrin-coated pits for entering the cells. After endocytosis, clathrin-coated vesicles containing CCR7 fuse with early endosomes, as shown by colocalization with transferrin (Fig. 3). Endosomes are the key control organelles for sorting where the decision is shaped whether receptors are directed to late endosomes and lysosomes for degradation or are recycled back to the plasma membrane (33). Internalized CCR7 followed the route of recycling back to the plasma membrane (Fig. 4 and Ref. 32), and recycled CCR7 was able to mediate chemotaxis and flux calcium in response to CCL19. Nevertheless, we also investigated whether CCR7 is degraded. However, we neither found colocalization with lysosomes nor evidence for degradation as measured by the fluorescence intensity of CCR7-GFP over time and by assessing the protein levels over time by Western blotting (Figs. 3 and 5). Thus, in contrast with CXCR4, which is rapidly degraded and has a half-life of ~3 h (27), CCR7 has a very long half-life and recycles (Fig. 5). This finding is important because, for example, dendritic cells that are infected in the periphery with a virus need to migrate over a significant distance after sensing CCR7 ligands for the first time. Viral infection of dendritic cells is often accompanied by an inhibition of translation (57). Consequently, if CCR7 would be degraded after endocytosis-like CXCR4, infected dendritic cells lacking surface expression of CCR7 would be insensitive to CCL19/CCL21 and would never make it into the lymph node. Under these conditions, an efficient priming of lymphocytes would not occur because the Ags would remain in the periphery.

The fate of chemokines after receptor internalization has remained enigmatic. More than a decade ago, even before the first CC chemokine receptor was cloned, Wang et al. (58) identified the first hint of chemokine degradation after endocytosis. Internalized 125I-labeled CCL2 was progressively released into the culture supernatant of monocytes in a degraded form. CCL2 degradation was inhibited by ammonium chloride, implicating lysosomal degradation (58). Later, it has been postulated, based on indirect evidence, that CCR5, one of the receptors for CCL2, is recycled back to the plasma membrane with APO-CCL5, but not with CCL5 (29). In contrast, iodinated CCL3L1 was shown to be slowly degraded in an ammonium chloride-dependent manner after internalization via CCR5 (59). Interestingly, the membrane-anchored chemokine CX3CL1 is expressed at two different locations within the cell, diffuse at the plasma membrane and punctuated in juxtanuclear compartments, and continuously cycles between the cell surface and the endomembrane storage compartment in a SNARE-dependent manner (60). The situation for the putative chemokine receptor D6 that scavenges a large variety of inflammatory CC chemokines is clearer. Internalized 125I-labeled CCL2 and CCL3L1 by D6 were rapidly degraded in an ammonium chloride-dependent manner (59, 61). Noteworthy, CCL19 is not scavenged by D6 (62), and CCL21 scavenging was not addressed; thus, information about internalized CCR7 ligands is missing. Using a CCL19-Fc fusion protein, we could, for the first time, investigate the trafficking of a CCR7 ligand. CCL19 is internalized together with CCR7 and localized in early endosomes (Fig. 7). There, CCL19 dissociated from the receptor, which was recycled, and was sorted to lysosomes for degradation as shown by confocal microscopy (Fig. 7). Degradation of CCL19 by lysosomes was further supported by the fact that chloroquine treatment abolished CCL19 degradation as assessed by Western blotting (Fig. 7).

Taken together, we provide strong evidence that CCR7 after CCL19 triggering is internalized via clathrin-coated pits and is transported to early endosomes followed by its recycling back to the plasma membrane where it can participate again in chemokine gradient sensing. In contrast, CCL19 dissociates from the endocytosed receptor, presumably in early endosomes, and is sorted to lysosomes for degradation. This may be of fundamental importance for inducing an efficient and potent immune response, because CCR7 is critical for the homing of lymphocytes and dendritic cells to secondary lymphoid organs. The fact that CCR7 is recycled, rather than degraded, may be essential for virally infected dendritic cells to maintain the capacity to sense the chemokine gradient until the cells reach their final destination, even if
viral infection inhibits translation and, hence, neosynthesis of CCR7.

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References


