C–C chemokine receptor-7 mediated endocytosis of antibody cargoes into intact cells

Xavier Charest-Morin1, Rémy Pépin2, Angélique Gagné-Henley1, Guillaume Morissette1, Robert Lodge1 and François Marceau1*

1 Centre de Recherche en Rhumatologie et Immunologie, Centre Hospitalier Universitaire de Québec, Québec, QC, Canada
2 Laboratory of Human Retrovirology, Institut de recherches cliniques de Montréal, Montreal, QC, Canada

INTRODUCTION

C–C chemokine receptor-7 (CCR7) is a G protein coupled receptor that has a role in leukocyte homing, but that is also expressed in aggressive tumor cells. Preclinical research supports that CCR7 is a valid target in oncology. In view of the increasing availability of therapeutic monoclonal antibodies that carry cytotoxic cargoes, we studied the feasibility of forcing intact cells to internalize known monoclonal antibodies by exploiting the cycle of endocytosis and recycling triggered by the CCR7 agonist CCL19. Firstly, an anti-CCR7 antibody (CD197; clone 150503) labeled surface recombinant CCR7 expressed in intact HEK 293a cells and the fluorescent antibody was internalized following CCL19 treatment. Secondly, a recombinant myc-tagged CCL19 construction was exploited along the anti-myC monoclonal antibody 4A6. The myc-tagged ligand was produced as a conditioned medium of transfected HEK 293a cells that contained the equivalent of 430 ng/ml of immunoreactive CCL19 (average value, ELISA determination). CCL19-myc, but not authentic CCL19, carried the fluorophore-labeled antibody 4A6 into other recipient cells that expressed recombinant CCR7 (microscopy, cytofluorometry). The immune complexes were apparent in endosomal structures, co-localized well with the small GTPase Rab5 and progressed toward Rab7-positive endosomes. A dominant negative form of Rab5 (GDP-locked) inhibited this endocytosis. Further, endosomes in CCL19-myc- or CCL19-stimulated cells were positive for β-arrestin2, but rarely for β-arrestin1. Following treatment with CCL19-myc and the 4A6 antibody, the melanoma cell line A375 that expresses endogenous CCR7 was specifically stained using a secondary peroxidase-conjugated antibody. Agonist-stimulated CCR7 can transport antibody-based cargoes, with possible therapeutic applications in oncology.

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*Correspondence: François Marceau, Centre de Recherche en Rhumatologie et Immunologie, Centre Hospitalier Universitaire de Québec, Québec, QC, G1V 4G2, Canada
E-mail: francois.marceau@crchul.uquebec.ca

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was tested in intact cells optionally stimulated with CCL19 treatment. Secondly, a recombinant myc-tagged CCL19 construction was exploited along the anti-myc monoclonal antibody 4A6. We are currently developing a platform of bifunctional myc-tagged peptide agonists of GPCRs that are suitable for coupling with both anti-myc monoclonal antibodies (such as the 4A6 clone) and their receptors. The receptor-mediated endocytosis of agonist–antibody complexes in excess of 150 kDa has been demonstrated for the bradykinin B$_2$ receptor (Gera et al., 2013) and the parathyroid hormone PTH$_1$R (Charest-Morin et al., unpublished data), suggesting that the approach can be generalized and exploit toward the functional cargoes. As for parathyroid hormone, but unlike bradykinin, the N-terminal sequence of CCL19 needs to be intact to preserve a good affinity for the cognate receptor (Ott et al., 2004). Thus, we have exploited a CCL19 sequence C-terminally extended with the myc epitope.

**MATERIALS AND METHODS**

**CELL CULTURE, TRANSFECTION, AND ANALYSIS**

A subclone of HEK 293 cells, called HEK 293a, originally obtained from Sigma-Aldrich was used in many experiments. This cell type was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin–streptomycin stock solutions (100 μg/ml; R&D Systems, Minneapolis, MN, USA). The human melanoma A375, originally obtained from ATCC, was tested in intact cells optionally stimulated with a sole vector coding for β-arrestin–green fluorescent protein (GFP; gift from Dr. Michel Bouvier, Université de Montréal). Stimulations for microscopic or cytofluorometric experiments were based on the CM of the CCL19-myc construction supplemented with AlexaFluor-488-conjugated monoclonal antibodies (clone 4A6, Millipore, dilution 1:1000 corresponding to a final antibody concentration of approximately 3.3 nM in the culture medium). Cells were generally treated for 30 min with stimulants (incubation carried out at 37°C in humidified atmosphere containing 5% CO$_2$), rinsed three times with phosphate buffered saline, observed in microscopy for epifluorescence, and photographed using an Olympus BX51 microscope coupled to a CoolSnap HQ digital camera (filters for GFP and AlexaFluor-488 excitation 460–500 nm, emission 510–560 nm; for Cherry fluorescent protein: excitation 525–555 nm, emission 600–660 nm). The objective lens was generally the 100× oil Planapo (Olympus).

Other transfected HEK 293a cells were detached using the protease-free Cell Dissociation Buffer (Invitrogen), incubated in DMEM without serum at 37°C for 30 min under agitation in the presence of a stimulant, rapidly centrifuged (30 s, 15,000 g) and resuspended in phosphate buffered saline. Then, the fluorescence of the cell suspensions was assessed using the BD SORP LSR II cell analyzer (BD Biosciences, Franklin Lakes, NJ, USA) for the uptake of a green fluorophore as a function of stimulation and transgene expression; results were analyzed using the BD FACS DIVA software.

**MELANOMA CELLS**

The human melanoma A375, originally obtained from ATCC, was a gift from Dr. Faez Aoudjit (CHU de Quebec, Quebec, Canada). The cells were cultured in RPMI 1640 medium supplemented with 10% FBS and antibiotics at 37°C in a 5% CO$_2$ humidified atmosphere. This line is originally derived from a metastatic site (Shields et al., 2007a) and is tumorigenic in immunodeficient mice (McMahon et al., 2001). To evidence the presence of CCR7, A375 cell mRNA was isolated with the Trizol reagent (Invitrogen) and treated with 10,000-fold for this test. The concentration of CCL19-myc was estimated using a commercial ELISA kit for human CCL19 (Sigma-Aldrich, catalog no. RAB0052). The CM samples were diluted 1000- to 10,000-fold for this test. The second CCR7 visualization strategy involved a recipient HEK 293a cells were grown and transiently transfected as described (Bawolak et al., 2011) with a vector coding for CCR7 (wild type in pCDNA3, Otero et al., 2006; gift from Dr. Daniel F. Legler, University of Konstanz, Germany). Using these cells, a first CCR7 imaging strategy was based on staining intact and live HEK 293a cells previously transfected with the vector encoding for the receptor with carboxyfluorescin-conjugated monoclonal anti-human CCR7 (CD197; clone 150503, final concentration 5 μg/ml; R&D Systems, Minneapolis, MN, USA). The cell distribution of the receptor was assessed after a 30- min stimulation period with the agonist CCL19 (incubation at 37°C).

Other HEK 293a cells were used as producer cells for a secreted protein coded by vector purchased from OriGene Technologies (Rockville, MD, USA): myc-DDK-tagged prepro-CCL19, that directs the secretion of the mature human CCL19 sequence extended at its C-terminus with two epitopes in tandem, myc, and DDK (catalog number RCM06523). The latter construction will be conventionally designated as CCL19-myc. Confluent producer cells (70%) were transfected with a given vector using either the Electroporation (Fermentas) or TurboFect (Thermo Scientific) reagents used as directed. Conditioned medium (CM) was collected after 4 days of culture. The concentration of CCL19-myc was estimated using a commercial ELISA kit for human CCL19 (Sigma-Aldrich, catalog no. RAB0052). The CM samples were diluted 1000- to 10,000-fold for this test. The second CCR7 visualization strategy involved a recipient HEK 293a cells were grown and transiently transfected as described above with the vector coding for CCR7 and optionally co-transfected with the fusions proteins Rab5-Cherry, Rab5-GTP-locked-Cherry fluorescent protein, Rab7-GDP-locked-CherryFP (given by Dr. M. J. Tremblay, Université Laval, Canada; Charest-Morin et al., 2013), or β-arrestin–CherryFP (kind gift from Dr. J.-M. Beaulieu, Université Laval, Canada). Other cells were transfected with a sole vector coding for β-arrestin–green fluorescent protein (GFP; gift from Dr. Michel Bouvier, Université de Montréal). Stimulations for microscopic or cytofluorometric experiments were based on the CM of the CCL19-myc construction supplemented with AlexaFluor-488-conjugated monoclonal antibodies (clone 4A6, Millipore, dilution 1:1000 corresponding to a final antibody concentration of approximately 3.3 nM in the culture medium). Cells were generally treated for 30 min with stimulants (incubation carried out at 37°C in humidified atmosphere containing 5% CO$_2$), rinsed three times with phosphate buffered saline, observed in microscopy for epifluorescence, and photographed using an Olympus BX51 microscope coupled to a CoolSnap HQ digital camera (filters for GFP and AlexaFluor-488 excitation 460–500 nm, emission 510–560 nm; for Cherry fluorescent protein: excitation 525–555 nm, emission 600–660 nm). The objective lens was generally the 100× oil Planapo (Olympus).

Other transfected HEK 293a cells were detached using the protease-free Cell Dissociation Buffer (Invitrogen), incubated in DMEM without serum at 37°C for 30 min under agitation in the presence of a stimulant, rapidly centrifuged (30 s, 15,000 g) and resuspended in phosphate buffered saline. The agonist-stimulated uptake of the anti-myc antibody was tested as follows. A375 cells were incubated for 30 min (37°C) in the CCL19-myc or control CM supplemented with the non-labeled 4A6 monoclonal antibody (Millipore, final concentration ≈ 3.3 nM). Then, cells were fixed, permeabilized, and stained with the Tyramide Signal Amplification (TSA) Kit containing horseradish peroxidase (HRP) conjugated goat anti-mouse IgG antibodies and AlexaFluor-488-tyramide as a fluorescent co-substrate of the reaction (Invitrogen kit.
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IMMUNOBLOTS

The agonist action of CCL19-related agonists was investigated using the expression of the transcription factor c-Fos, a distal response to the stimulation of various receptor-ligand systems (Glauser and Schlegel, 2007). Total A375 cell extracts were immunoblotted to detect c-Fos expression using the K-25 rabbit polyclonal antibodies (Santa Cruz Biotechnology; dilution 1:50,000).

DATA ANALYSIS

Numerical values are reported as means ± SEM. Non-normally distributed groups of values were compared using ANOVA followed by Dunn’s multiple comparison test. Normal sets of values were compared using the χ² test (InStat 3.05 computer program, GraphPad Software, San Diego, CA, USA).

RESULTS

CCL19-INDUCED INTERNALIZATION OF CCR7-ANTI-CCR7 IN HEK 293a CELLS

Whether a commercial anti-CD197 antibody could support imaging of CCR7 expressed in intact HEK 293a cells has been tested (Figure 1). In resting cells, the associated fluorescence was sharply defined at the level of the plasma membrane with no intracellular signal, as expected from the exclusion of the antibody from the cytosol. There was no signal in non-transfected cells, suggesting that the antibody reacts with extracellular domain(s) of the non-denatured CCR7. Adding CCL19 concentration-dependently determined the translocation of a fraction of the cell surface fluorescence to ill-defined cytosolic structures, consistent with agonist-induced receptor internalization (Figure 1).

CHARACTERIZATION OF THE CMs

The CM of producer HEK 293a cells transfected with the CCL19-myc vector contained the equivalent of 430 ng/ml of immunoreactive CCL19 (average of three values: 150, 300, and 840 ng/ml). Three control CM of untransfected cells contained none.

INTERNALIZATION OF myc-TAGGED CCL19-ANTI-myc IMMUNE COMPLEXES IN HEK 293a CELLS

The second scheme for the visualization of CCR7 endocytosis was based on the myc-tagged agonist co-incubated with a fluorescent anti-myc antibody in the culture medium (Figure 2). Controls included cells that did not express CCR7, cells transfected with authentic CCL19 (1 μg/ml) or with the CM of untransfected HEK 293 cells. A specific endosomal labeling of recipient cells that expressed the receptor was observed in cells stimulated with CCL19-myc (Figure 2), leading to a significantly higher mean cell fluorescence (Figure 3). These observations provide evidence that the CCL19-myc-4A6 immune complexes formed in the culture medium were recognized as agonists by the fraction of cells that expressed CCR7, and were transported into endosomes. Authentic CCL19 devoid of the myc epitope was not competent to induce the endocytosis (pinocytosis) of droplets of the culture medium containing the fluorescent antibody, providing additional evidence for the specific molecular interaction of the myc-tagged agonist with the antibody. Cells untreated with the fluorescent anti-myc antibody had mean fluorescence intensity identical to the five control conditions in Figure 3, suggesting that the control cells exhibited only the autofluorescence level.

The CCL19-myc/4A6 co-stimulation scheme was used to investigate β-arrestin endosomal co-localization studies (Figure 4). The Cherry-tagged β-arrestin, is expressed as a homogenous cytosolic protein in recipient cells that co-expressed CCR7. Neither authentic CCL19 nor CCL19-myc CM induced significant condensation of β-arrestin-Cherry into endosomal structures 5–30 min after stimulation; however, the immune complexes were effectively detected at this level as the green particles (see statistics

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FIGURE 2 | Endocytosis of the anti-myc monoclonal antibody (clone 4A6, conjugated to AlexaFluor-488, final concentration in the culture medium 3.3 nM) as determined by co-treatment with the CCL19-myc construction in intact HEK 293a cells that optionally and transiently expressed CCR7. A control conditioned medium (CM) or authentic CCL19 were used as control stimuli. The undiluted CM were transferred for a 30-min incubation period before rinsing and observation. Original magnification × 1000.

in Figure 4 legend). The alternate construction β-arrestin2-GFP was co-expressed with CCR7 in other HEK 293a cells and stimulated without the 4A6 antibody (also green light emitting). It was observed that the resting smooth cytosolic distribution of this arrestin isoform was condensed into endosomal structures revealed by treatments with CCL19-myc (5–30 min) or with authentic CCL19 (Figure 5), producing further support for a selective affinity of activated CCR7 for one of the non-visual arrestins.

Other HEK 293a cells that expressed CCR7 were also co-transfected with one of three Rab5-Cherry constructions: the fusion protein consisting of the wild type Rab5, the GTP-locked activated one that causes the formation of giant endosomes where cargo accumulates (Stenmark et al., 1994), or the dominant negative GDP-locked Rab5 (Figure 6). Co-localization was observed in cells treated for 30 min with the CM of CCL19-myc in cells expressing Rab5-Cherry (arrowheads; this concerned 19.1% of green organelles in a sample of 89 from 10 cells). The GTP-locked construction induces the formation of giant vacuoles where the green fluorescence of the CCL19-myc/anti-myc-antibody complexes was extensively trapped (Figure 6; 88.3% of green organelles were circled by a red lining; sample of 43 from 11 cells). The GDP-locked Rab5 construction rather suppressed the endocytosis of immune complexes (average of 2.1 ± 0.9 green specs per cell in 10 cells treated with CCL19-myc CM, comparable to the value of control CM in Figure 5).

Rab5 is a marker of early endosomes, whereas Rab7 rather labels late endosomes and lysosomes. Minor co-localization of the CCL19-myc/anti-myc-antibody complexes was observed after either 30-min (5.3% of green endosomes) or 3-h treatments (12.6% of green endosomes; Figure 7), indicating a slow progress of the antibody cargo in the endosomal/lysosomal tract as these proportions significantly differ (p < 0.05, χ²-test; sample of 11 cells for each treatment duration containing totals of 189 and 175 green endosomes, respectively).

CCR7 IN METASTATIC MELANOMA CELLS

RT-PCR provided evidence for the presence of CCR7-mRNA in a human melanoma cell line, the A375 line (Figure 8A). The
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FIGURE 4 | Epifluorescence microscopy studies in cells co-expressing β-arrestin1-Cherry and CCR7 and stimulated as indicated (stimulant and duration). Stimulation always included the fluorescent anti-myc monoclonal antibody 4A6 (green signal). Either CCL19-myc CM or authentic CCL19 rarely condensed β-arrestin1 at the level of plasma membrane of endosomes (arrowhead indicates possible co-localization with the CCL19-myc-antibody cargo). Cells that expressed well the red-emitting transgene were assumed to co-express co-transfected CCR7 and were evaluated for morphology (n = 16–49 eligible cells per group). The average number of red condensed structures was 0.4 ± 0.4 in cells treated with control CM and did not vary significantly in all other groups (Kruskal–Wallis test). The average number of green condensed structures in cells treated with control CM was 0 per cell and varied significantly according to treatments (p < 10⁻⁴, Kruskal–Wallis test; p values for Dunn’s multiple comparison test for each value vs. that of control CM reported thereafter). There were 8.3 ± 1.7 green specs per cell (p < 0.001), 25.8 ± 3.2 (p < 0.001), 33.1 ± 4.4 (p < 0.001), and 0 (N.S.) in cells treated with CCL19-myc CM for 5, 10, or 30 min, or with recombinant CCL19, respectively.

DISCUSSION

By exploiting two commercially available monoclonal antibodies, we have illustrated that the human recombinant CCR7 receptor can internalize such large proteins when stimulated with the agonist CCL19. The anti-receptor antibody clone 150503 recognized an extracellular epitope in non-denatured CCR7 expressed by intact cells (Figure 1) and, as such, was internalized with the stimulated receptor. Furthermore, the antibody did not inhibit the interaction of CCR7 with its agonist ligand. This experimental system is similar to that consisting of the N-terminally tagged myc-β2 receptor for bradykinin, that bound the anti-myc 4A6 monoclonal antibody and internalized immune complexes built around this antibody at the surface of intact cells upon stimulation with bradykinin (Bawolak et al., RCS19848 and containing the equivalent of ∼180 ng/ml of the hormone).
Data from these studies provide strong evidence that GPCR-mediated endocytosis can transport extremely large MDa cargoes, such as secondary antibodies bound to Qdot nanomaterials.

The second strategy exploited a myc-tagged agonist along with the anti-myc monoclonal antibody 4A6. It was hypothesized that the CCL19-myc construction, but not authentic CCL19, could carry anti-myc antibodies to endosomes. The myc tag is a 10-residue sequence that is widely used in recombinant protein constructs, and also in a synthetic bradykinin homolog that carried the 4A6 antibody into cells that expressed the bradykinin B2 receptor, albeit with low efficacy due to the low receptor affinity of the bifunctional peptide (Gera et al., 2013). The present system is more favorable as the CM of cells transfected with the CCL19-myc vector, which contains an average immunoreactive concentration (430 ng/ml), compares well with concentrations of authentic CCL19 needed to internalize CCR7 (Figure 1).
agonist CCL19 does not support the internalization of the 4A6 antibody, as the non-tagged lent antibody, may be quite large (in excess of 150 kDa) and are not taken up by activated cells by pinocytosis, as the non-tagged antibody cargo.

The immune complexes, composed of a divalent agonist of CCR7 and of the anti-myc antibody. The latter can be transported in cell expressing various types of receptors according to the repertoire of internalized receptors, whether or not GPCRs, expressed by a given malignancy.

FIGURE 9 | Detection of endogenous CCR7 in A375 cells using the Tyramide Signal Amplification (TSA) system that enzymatically generates AlexaFluor488 labeling. Confocal fluorescence and transmission, original magnification: ×1000. Right: proportion of cells with a mean fluorescence intensity above a set threshold ( Photoshop level >50/255) in large photographic records of A375 cells stained as in the left of figure (the number of evaluated cells is indicated next to each histogram). The proportions were compared with that of control cells without antibody (top-most histogram, y² test, *p < 10⁻⁵).
