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Arrestin 3 Mediates Endocytosis of CCR7 Following Ligation of CCL19 but not CCL21

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Abstract

Internalization of ligand bound G protein-coupled receptors (GPCRs), an important cellular function that mediates receptor desensitization, takes place via distinct pathways, which are often unique for each receptor. The C-C Chemokine Receptor 7 (CCR7) GPCR is expressed on naïve T cells, dendritic cells and natural killer cells and has two endogenous ligands, CCL19 and CCL21. Following binding of CCL21, 21 ± 4% of CCR7 is internalized in the HuT 78 human T cell lymphoma line, while 76 ± 8% of CCR7 is internalized upon binding to CCL19. To determine whether arrestins mediated differential internalization of CCR7/CCL19 vs. CCR7/CCL21, we used siRNA to knock down expression of arrestin 2 or arrestin 3 in HuT 78 cells. Independent of arrestin 2 or arrestin 3 expression, CCR7/CCL21 internalized. In contrast, following depletion of arrestin 3, CCR7/CCL19 failed to internalize. To examine the consequence of complete loss of both arrestin 2 and arrestin 3 on CCL19/CCR7 internalization, we examined CCR7 internalization in arrestin $2^{-/-}$/arrestin$3^{-/-}$ murine embryonic fibroblasts. Only reconstitution with arrestin-3 GFP but not arrestin 2-GFP rescued internalization of CCR7/CCL19. Loss of arrestin 2 or arrestin 3 blocked migration to CCL19 but had no effect on migration to CCL21. Using immunofluorescence microscopy, we found that arrestins do not cluster at the membrane with CCR7 following ligand binding, but cap with CCR7 during receptor internalization. These are the first studies that define a role for arrestin 3 in the internalization of a chemokine receptor following binding of one but not both endogenous ligands.

Keywords

T Cells; Chemokine; Cell Activation

Introduction

G protein coupled receptors (GPCRs) are a diverse group of seven transmembrane receptors that are activated by multiple sensory and chemical stimuli including light, odors, tastes, chemokines and neurotransmitters (as reviewed by (1)). Activation of these receptors by their ligands induces a conformational change, which leads to activation of distinct downstream signaling events (2–4). Following activation by agonist binding, the GPCRs couple to heterotrimeric GTP-binding (G) proteins triggering an exchange of GDP for GTP

on the Gα-subunit and the resultant release of the α subunit from the β/γ-dimer (5). Both of these subunits can regulate numerous effectors including adenylyl cyclase and phospholipase Cβ (6, 7). The receptor is then phosphorylated at its C-terminus, which provides a substrate for high affinity binding by arrestin 2 (also known as β-arrestin 1) and/or arrestin 3 (also known as β-arrestin 2), and the receptor is then internalized. Two of the four known arrestins, arrestin 2 and arrestin 3, are expressed ubiquitously in mammalian cells (8–10). Receptor internalization is an important immunological function that controls signaling which controls immunological responses, receptor fate and receptor desensitization (11). Yet it is unclear how distinct receptors are shuttled to the necessary internalization machinery to differentially control the signaling and fate of each internalized receptor.

Arrestins can regulate GPCR internalization, degradation and/or recycling. Arrestin binding can block association of G proteins with phosphorylated GPCRs and thus plays an important role in phosphorylation-dependent desensitization (9, 12). For instance, arrestins mediate internalization of the β2 adrenergic receptor (10, 13). Arrestins can also serve as adaptors between the receptors and the endocytic machinery. In such cases, arrestin binding targets activated receptors to clathrin coated pits via interactions with clathrin, the clathrin adaptor AP-2 and the intracellular transport ATPase *N*-ethylmaleimidesensitive factor (14–16). Arrestins can also regulate other signaling pathways including activating Src extracellular signal-regulated kinase c-Jun NH2-terminal kinase (17–19). Alternatively, while the human FPR does not require arrestins to internalize, arrestins are required for *FPR* recycling (20). In addition, arrestins mediate trafficking of GPCRs to specific sites in the cell, where the receptors are degraded (8, 21, 22).

The C-C Chemokine receptor seven (CCR7) is a GPCR that is expressed on B-lymphocytes, natural killer (NK) cells, naive and central memory T-lymphocytes (23–27). CCR7 is activated by two known endogenous ligands, C-C ligand 19 (CCL19, MIP-3β, ELC, CKβ −11, Exodus-3) and C-C ligand 21 (CCL21, 6Ckine, SLC, TCA4, Exodus-2) (26, 28–31). CCL21 is expressed on the luminal side of high endothelia venules, while both ligands are found within the stromal region of the T cell-rich lymph node areas (32). The CCL19 ligand is expressed on dendritic cells, and is found at lower levels in the spleen and lymph nodes than CCL21 (33). The expression of these ligands in different regions of the immune system implies that each ligand might have a unique role in the regulation of this system and as a result differentially regulate signaling in T cells. Indeed, each ligand has been uniquely associated with different autoimmune diseases. While CCL21 has been associated with Sjogren's syndrome, and lymphoproliferative disorders (34–36), CCL19 has been linked to Crohn's (37, 38). It is unclear, however, how each of these ligands mediates distinct effects following association with CCR7.

CCR7 binds CCL19 and CCL21 with equal affinities (30), yet each ligand has distinct signaling pathways and modulation of cell migration. In one study, ectopic expression of CCL19 in pancreatic islets resulted in the development of small infiltrates of lymphocytes and dendritic cells, whereas ectopic expression of CCL21 resulted in larger, more organized infiltrates. From these observations it was inferred that CCL21 might be more effective than CCL19 in promoting the recruitment of T cells (33). It was surprising therefore, when it was reported that only CCL19 mediates emigration of newly generated lymphocytes from the

neonatal thymus (39). These observations provide further support that distinct signal cascades are initiated following ligand/receptor association, which control different cellular behaviors. The signal transduction events triggered by GPCRs are regulated both spatially and temporally (4).

To begin to unravel the differential regulation of the signaling events between CCL19 and CCL21, we examined the internalization of CCR7 when bound to CCL19 and CCL21. Receptor internalization can initiate signaling, processing and often desensitization of a GPCR. After we observed differences in receptor internalization following engagement of CCL19 and CCL21, we examined the role of arrestins in regulating the differential internalization of CCR7 following ligand binding. To this end, we examined CCR7 internalization following depletion of arrestin 2 or arrestin 3 using siRNA in HuT78 human T cells. In addition we examined the effect of loss of each arrestin on migration of HuT78 cells. Next, we examined internalization of CCR7 in murine embryonic fibroblasts (MEFs) that are null for arrestin expression. We report that arrestin 3 but not arrestin 2 is required for internalization of CCR7 when bound to CCL19. In contrast, arrestins are not required for internalization of CCR7/CCL21. In addition both arrestins control migration of HuT78 cells. Although signaling is typically associated with receptor clustering, we observed that arrestins do not appear to cluster at the membrane with CCR7 following ligand binding. Instead, arrestin 3 but not arrestin 2 co-localizes with CCR7 at peri-nuclear and nuclear regions following ligand association. This is the first description of differential binding of arrestins that is regulated by two distinct endogenous chemokines, which bind to the same receptor.

Materials and Methods

Materials—

Recombinant human and murine CCL19 and CCL21, and PE-conjugated anti-human CCR7 antibody (FAB197P) were purchased from R&D Systems (Minneapolis, MN). M2 antibodies (Catalog # F1804) were purchased from Sigma-Aldrich. Anti-β-arrestin 1/2/3 was purchased from SantaCruz Biotechnology Inc. (Santa Cruz, CA). PE-conjugated anti-mouse was purchased from Jackson Immunolabs. The cDNA encoding N-terminally tagged murine CCR7 was a generous gift from James J. Campbell (Harvard Medical School, Boston, MA). Plasmids encoding bovine arrestin2-GFP and arrestin3-GFP have been described (20) and were generously provided by Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA). The plasmid encoding the human *N*-formyl peptide receptor (FPR) has been described (40) and was generously provided by Dr. Eric Prossnitz (University of New Mexico Medical School, Albuquerque, NM).

Cell lines:

HuT 78 and Jurkat e.6 cell lines were purchased from the American Type Culture Collection, and were maintained in cRPMI (10% fetal bovine serum/90% RPMI/2mM L-glutamine) (Invitrogen) in a humidified atmosphere of 5% CO2/air at 37°C. The arrestin2$^{-/-}$/arrestin3$^{-/-}$ murine embryonic fibroblasts (MEF WT and MEF arr2$^{-/-}$/arr3$^{-/-}$) cell lines were a generous gift from Dr. Robert Lefkowitz (Duke University, Durham, NC).

Murine CCR7 was transiently transfected into the MEF arr2$^{-/-}$/arr3$^{-/-}$ cell lines with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions, with the following modification: we halved all recommended amounts. pCDNA3.1 (Invitrogen) vector was transfected as the vector control. MEF cell lines were maintained in 10% fetal bovine serum (Hyclone)/90% Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2mM L-glutamine at 37°C /5% CO2 in a humidified incubator. Cells were certified to be free of mycoplasma by Charles River laboratories.

Flow Cytometry:

10^6 HuT78 cells were isolated from culture, rinsed twice with serum free RPMI and resuspended directly in 10μl of phycoerythrin (PE) directly conjugated anti-human CCR7 antibody or the PE directly conjugated isotype control (R&D). Cells were allowed to incubate on ice for 30 minutes, and then were rinsed three times with serum free RPMI and analyzed immediately by flow cytometry on a LSRII (Becton Dickenson).

RT PCR:

10^6 HuT78 or Jurkat e.6 cells were isolated from culture, rinsed twice with serum free RPMI, and total RNA was isolated as per the manufacturer's instructions (Qiagen) and quantified. 5μg of RNA was mixed with 50 ng random hexamers (Invitrogen), 10mM dNTPs, ddH2O, Superscript II (Invitrogen) and 5X First Strand buffer, 10mM DTT, RNasin (Promega). The reaction was mixed at room temperature and incubated at 42°C for 60 minutes. The Superscript II was inactivated by heating the mixture to 70°C for 15 minutes in the presence of NaOH. Of the resultant cDNA, 5μl was mixed with 10X PCR buffer, 200mM dNTPs, 1.5mM MgCl2, ddH2O, 0.5μM of the forward primer 5'GGAAGCTTGGGATCGATGCCATGGACCG GGGAAACCAATGAAAAGCG3' and 0.5μM of the reverse primer 5'GCGGCCGCATGGGGAG AAGGTGGTGGTGG3'. The following conditions were used: a) 95°C for 5 minutes (1cycle); b) 95°C for 30 seconds, 55°C annealing 30 seconds, 72°C extension 1 minute (30 cycles); c) 72°C terminal extension 10 minutes (1 cycle); and d) 4°C infinite hold on a Perkin Elmer 9600 cycler. Products were separated by agarose gel electrophoresis; the gel was stained with ethidium bromide and imaged (Kodak Gel Logic 1000).

Receptor Internalization Assays:

MEF Arr2$^{-/-}$/arr3$^{-/-}$ cells were transiently co-transfected with arrestin-2 GFP, arrestin-3 GFP or pEGFP-N1 and muCCR7 using Lipofectamine 2000 per the manufacturer's instructions using ½ of the recommended amounts of reagents and DNA. Twenty-four hours following transfection, MEF Arr2$^{-/-}$/arr3$^{-/-}$ cells were harvested from dishes by trypsinization and re-plated in larger dishes. Following the same time interval, HuT 78 suspension cells were collected from flasks, counted and washed in PBS. Cells were re-suspended in serum free RPMI (HuT 78) or serum free DMEM (*sf*DMEM)-(MEF Arr2$^{-/-}$/arr3$^{-/-}$) at 5 × 10^6 cells per ml, and 100μl was used for each time point. Following a 10-minute pre-incubation at 37°C, cells were treated with 200 nM CCL19 or CCL21 for the indicated time periods. Internalization was arrested by adding 10 volumes of ice-cold *sf*RPMI HuT 78 or *sf*DMEM MEF Arr2$^{-/-}$/arr3$^{-/-}$. Human ligands were used with the HuT 78 cells, while the murine ligands were used with murine CCR7 in the MEF cell lines.

Receptors on the surface of the HuT 78 cells were stained with PE-conjugated anti-human CCR7 antibodies for 30 minutes on ice (as per the manufacturer's instructions). FLAG-tagged murine CCR7 was detected by incubating the MEF Arr2$^{-/-}$/arr3$^{-/-}$ cells with anti-FLAG (M2) antibody on ice for 30 minutes, followed by three rinses with ice cold PBS and incubation with fluorescein isothiocyanate (FITC) anti-mouse secondary for 30 minutes on ice. Cells were rinsed three times in serum free media and assayed immediately or fixed in 2% paraformaldehyde and stored at 4°C for up to four days. Receptor levels were assayed by flow cytometry on a Becton Dickenson LSRII with DIVA software. Antibody binding was not inhibited by the presence of either CCL19 or CCL21 (data not shown). Level of receptor remaining of the surface was calculated as [mean channel fluorescence of PE-anti-CCR7 or FITC-anti-mouse at time-point]/[mean channel fluorescence of PE-anti-CCR7 at time=0]. Statistical significance was determined using a Paired *T* test. Curves or linear regressions were fit using Prism 4 software package (Graphpad).

Receptor Recycling Assays:

HuT 78 suspension cells were collected from flasks. Cells were counted and washed in PBS. Cells were resuspended in serum free DMEM (*sf*DMEM) at 5×10^6 cells per ml and 100μl was used for each time point. Following a 10-minute pre-incubation at 37°C, cells were treated with 200 nM CCL19 or CCL21 for 20 minutes. Ligand was added again following 20 minutes of receptor internalization to prevent ligand depletion. At 30 minutes, cells were rinsed three times in ice-cold serum free RPMI. Receptor recycling was initiated by addition of pre-warmed 37°C serum free RPMI. Cells were collected at the indicated time points. Recycling was arrested by addition of 10 volumes of ice-cold serum free RPMI. Receptors on the surface of the cells were stained with PE-conjugated anti-human CCR7 antibodies for 30 minutes on ice (as per the manufacturer's instructions). Cells were rinsed three times in serum free RPMI and assayed immediately or fixed in 2% paraformaldehyde and stored at 4°C for up to four days. Receptor levels were assayed by flow cytometry on a Becton Dickenson LSRII with DIVA software. Level of receptor remaining of the surface was calculated as [mean channel fluorescence of PE-anti-CCR7 at time-point] /[mean channel fluorescence of PE-anti-CCR7 at time=0]. Curves were fit using Prism 4 software package (Graphpad). Statistics were determined using a paired *T* test.

si RNA:

FITC labeled, double stranded siRNA, with 19-nucleotide duplex RNA and 2-nucleotide 3'-dTdT over-hangs were purchased from Invitrogen. The siRNA sequences targeting human beta-arrestin1 and beta-arrestin2 were 5'-AAAGCCUUCUGCGCGGAGAAU-3' and 5'-AAGGACCGCAAAGUGUUUGUG-3' corresponding to positions 439–459 and 148–168 relative to the start codon, respectively. A non-silencing RNA duplex (5'-AAUUCUCCGAACGUGUCACGU-3'), as the manufacturer indicated, was used as a control. These siRNAs have been described (41). 20 pmol of siRNAs were transfected using Oligofectamine (Invitrogen) per the manufacturer's directions. Cells were Nucleofected (AMAXA) using solution R (see below) or transfected with Oligofectamine (Invitrogen) to introduce the siRNA. Briefly, on the day of the transfection, cells were plated at ~500,000/80μl serum free RPMI in a 24-well dish. 1μl of a 20μM stock of each double stranded siRNA was combined with 16μl Opti-MEM®I reduced serum medium.

Oligofectamine was inverted several times to mix. In a separate Eppendorf tube labeled “B”, 0.8μl of Oligofectamine was diluted with 2.2μl Opti-MEM®I reduced serum medium. The mixtures in tubes “A” and “B” were incubated separately at room temperature for 10 minutes, combined into one tube and allowed to incubate at room temperature for an additional 20 minutes to form siRNA/Oligofectamine complexes. The 20μl of complexes were added to the cells and incubated at 37C in a humidified, CO2 incubator for 4 hours. 50μl of RPMI/30%FBS/L-glutamine was added to each of the 24 wells. Following treatment with Oligofectamine, cells were allowed to incubate for 72 hours and then assayed for internalization and arrestin expression. Changes in arrestin 2 and/or arrestin 3 expression levels were confirmed by Western blotting.

Western Blot assays:

10^6 HuT 78 cells were harvested for each condition (cells pre-treated for 72 hours with non-specific, arrestin 2 or arrestin 3 siRNA) from flasks, counted and washed in PBS. Cells were pelleted, and the media was replaced with RIPA lysis buffer (50mM HEPES pH→7.5, 150 mM NaCl, 5mM EDTA, 1mM EGTA, 1mM NaF, 2mM Na3VO4, 1% NP40, 0.1% SDS, 1% deoxycholate, 1mM DTT with Protease Inhibitor cocktail from Sigma). Lysates were separated by SDS-PAGE on 10% acrylamide gels, transferred to PVDF and probed for arrestins with a rabbit anti-β-Arrestin-1/2/3 (H-290) Antibody (Santa Cruz). Immune complexes were probed with a rabbit-HRP secondary antibody (Pierce), and visualized by Super Signal West Pico Substrate (Pierce) and exposed membranes to X-ray film or to the Fuji LAS-4000 and bands were quantified.

Chemotaxis Assays:

As described (42, 43). Briefly, siRNA transfected cells were resuspended in assay buffer (Hank’s Buffered Saline Solution (Cellgro) + 10mM HEPES pH→7.4 (Invitrogen) with 0.5g Bovine Serum Albumin (A790 Sigma V)). Cells were diluted to 4×10^6 cells/ml in complete RPMI and kept at room temperature during preparation of the chemotaxis chambers. Polycarbonate membranes (8μm pore size) were pre-wet with serum-free RPMI. Chemokines were diluted to 200nM in 1%BSA/PBS, and pipetted into lower chamber of a Neuroprobe chemotaxis chamber. Chambers were fitted with filters, gaskets and lid. 50μl of cells (100,000 cells/well) were loaded and cells allowed to migrate for 2 hours at 37C in a 5% CO2 humidified incubator. Following incubation the membranes were removed and cells that migrated to the lower chamber were counted.

Confocal Microscopy:

2×10^6 HuT78 cells were transiently nucleofected with arrestin 2-GFP, arrestin 3-GFP or pEGFP-N1 using the AMAXA R kit per the manufacturer’s instructions. Briefly, cells were centrifuged for 10 minutes at 200g and resuspended in 100μl Solution R. DNA was added to an Eppendorf and cells resuspended in Solution R were layered on top. Prior to nucleofection, cells/DNA/SolutionR was transferred to an Amaxa cuvette and cells were nucleofected on program V-01. Immediately after nucleofection, 800 μl of cRPMI was added and cells were transferred back into the Eppendorf tube with the transfer pipette included in the Amaxa kit. Cells were then plated in complete RPMI and allowed to recover overnight. Twenty-four hours following transfection, HuT 78 cells were collected,

stimulated with 200nM ligand for four or eight minutes and fixed with 2% formaldehyde (30 minutes on ice). Cells were permeablized with 1% Nonidet-P 40/1 × PBS for 15 minutes at RT and labeled with mouse anti-CCR7 (1:100) (Pharmingen #550937) in 3% BSA/PBS for one hour. The cells were rinsed three times and labeled with Texas red (1:250) directly conjugated to anti-mouse (Jackson Immunological Labs). Cells were rinsed three times in PBS, and plated on glass slides with Prolong Gold anti-fade (Molecular Probes). Immunofluorescence was visualized on a Nikon 90i upright microscope via mercury fluorescent excitation then confirmed via confocal scanning (Nikon C1 series confocal scan head). Lasers used for emission detection were a 488 Multi line Argon (green IR) and 561 Diode laser (red IR) acquisition via the Nikon EZ-C1 series software (3.60).

Results

Characterization of CCR7 Expression and Internalization in HuT 78 cells

To examine the expression of endogenous CCR7 in human T cells, we used reverse transcriptase PCR (RT-PCR) (Figure 1) to look for expression of CCR7 in two human cell lines. In line with previous observations, HuT 78 cells expressed CCR7 mRNA, in contrast with Jurkat cells, which failed to express CCR7 (44, 45) (Figure 1A). To verify surface localization of expressed CCR7 protein, we used fluorescence activated cell sorting (FACS) (Figure 1B). We confirmed that CCR7 was expressed on the surface of HuT 78 cells.

Receptor internalization is an important cellular function that mediates receptor desensitization (11, 46). To confirm that the HuT 78 cell line internalized and recycled in a manner similar to the CCR7 expressed in peripheral blood cells (44), we used FACS based internalization and receptor recycling assays. CCR7 is activated by two endogenous ligands, CCL19 and CCL21 (25, 31, 47). Initially, to identify an optimal concentration of ligand for our assays, we carried out a dose response (Figure 2A) and found that 200nM CCL21 and 200nM CCL19 provided maximal internalization for CCR7. We did not observe increased internalization levels at higher concentrations, possibly due to loss of ligand solubility. Therefore, we selected 200nM ligand to carry out further studies. We compared the extent of internalization and rates of CCR7 internalization in the HuT 78 cells following binding to 200nM CCL19 and CCL21 ligands (Figure 2 B–C). Similar to published observations for peripheral blood leukocytes (44, 45), 76 ± 9 % of CCR7 was internalized with a $t_{1/2}$ of 0.46 minutes following exposure of the HuT 78 cells to 200 nM CCL19. In contrast, only 22 ± 4 % of CCR7 was internalized with a $t_{1/2}$ of 0.28 minutes following exposure of HuT 78 cells to 200 nM of CCL21. Thus, there was a more rapid internalization of CCR7/CCL21, although the total amount of CCR7/CCL19 internalization was greater.

When a receptor internalizes, the extent of this process is a balance between receptor internalization and recycling. Therefore, we hypothesized that the enhanced rate of CCR7/CCL19 internalization was due to a reduced rate of recycling. To test this hypothesis, we compared CCR7 recycling following receptor internalization with CCL19 or CCL21. The difference in the receptor recycling, between the two ligands, was significant (p=0.02). We observed the CCL19 internalized CCR7 recycled slowly, (Figure 3) while the CCL21 internalized CCR7 recycled within 10 minutes (data not shown).

Role of arrestins in CCR7 internalization

Receptor internalization is a key cellular function that controls the duration of signaling from a receptor. As such, the cellular mechanisms that are activated during receptor internalization play pivotal roles in controlling the fate of the internalized receptor and in T cells regulate the immune response (11). Arrestins regulate internalization of many GPCRs, such as the β2 adrenergic receptor, the V2 Vasopressin receptor, and the angiotensin II type 1A receptor, but are not required for internalization of the human N-formyl peptide receptor (FPR), or the PAR-1 receptor (8, 20, 48–51). Thus, we questioned whether arrestin binding regulated the differential internalization of CCR7 following binding to either CCL19 or CCL21. For this purpose, we used a well-characterized combination of siRNAs (41, 52, 53) to knock down the expression level of arrestin 2 or arrestin 3 in the HuT 78 cells (Figure 4C). We used western blots to confirm that arrestin 2 and arrestin 3 levels were knocked down (Figure 4C). When normalized to the β-actin loading control, arrestin 2 siRNA reduced levels of arrestin 2 by 75%, and arrestin 3 siRNA reduced levels of arrestin 3 by 58%. However, arrestin 3 siRNA also reduced arrestin 2 expression levels by 96%. Independent of a reduction in the expression levels of either arrestin 2 or arrestin 3, CCR7 was internalized following binding to CCL21. In contrast, the extent of internalization of CCR7/CCL19 was significantly reduced ($p=0.0013$) in comparison to cells treated with non-specific siRNA, following treatment with arrestin 3 siRNA.

Although these siRNA's have been used to specifically knockdown arrestin 2 or arrestin 3 in human cells (41, 52, 53), in the HuT 78 cells used in this study, both arrestin 2 and arrestin 3 levels were reduced by the siRNA for arrestin 3. Therefore, it was unclear whether loss of internalization of CCR7/CCL19 in the arrestin 3 si RNA treated cells was due to a reduction in the levels of arrestin 3 alone, or due to the loss of both arrestin 2 and arrestin 3 combined. An additional consideration was that since siRNA can have off target effects (54), which can knockdown the levels of non-targeted mRNAs in the cell, it was not clear whether the observed effects on CCR7/CCL19 internalization were due only to depletion of arrestins.

Characterization of CCR7 internalization in murine embryonic fibroblasts

To examine internalization of CCR7/CCL19 and CCR7/CCL21 in the complete absence of arrestins, we examined internalization in the murine embryonic fibroblasts (MEFs), which lack arrestin (MEF $Arr2^{-/-}/arr3^{-/-}$ cells). This MEF $arr2^{-/-}/arr3^{-/-}$ cell line failed to internalize the β2 adrenergic receptor, which is an arrestin dependent process (51). Since MEF cells lack endogenous CCR7, we transiently transfected MEF cells with a murine N-terminally FLAG-tagged murine CCR7. To facilitate detection with the M2 anti-FLAG antibody, the murine CCR7 was fused at the N-terminus to the FLAG tag (28). The MEF transient transfectants were allowed to internalize in the presence of 200nM ligand and assayed for receptor remaining on the surface of the cells by FACS (Figure 5A–B). Ligand binding did not affect antibody detection of the FLAG tag, as ≥100% of the receptor remained detectable on cells that were labeled with antibody, on ice, in the presence of ligand.

Since internalization of the CCR7/CCL19 was blocked by knocking down arrestin 3 expression (Figure 5), we hypothesized that reconstitution of expression of arrestin 3 but not

arrestin 2 would restore CCR7/CCL19 internalization in MEF Arr2$^{-/-}$/arr3$^{-/-}$ cells. Therefore, we co-transfected the MEF cells with muCCR7 and GFP, or muCCR7 and arrestin 2-GFP (Arr2-GFP), or muCCR7 and arrestin 3-GFP (Arr3-GFP). This allowed us to detect and gate on only the GFP or arrestin-GFP expressing cells during FACS for the internalization assay. MEF arr2$^{-/-}$/arr3$^{-/-}$ cells internalized 23 ± 4% of CCR7/CCL21 within 30 minutes (Figure 5B) independent of arrestin expression, while CCR7/CCL19 failed to internalize in the absence of arrestin. When the MEF arr2$^{-/-}$/arr3$^{-/-}$ were reconstituted with arrestin 3, the maximum level of CCR7/CCL19 receptor internalized was 32± 7%. When compared to internalization of CCR7/CCL19 in the presence of GFP alone, internalization of CCR7/CCL19 was significantly increased in the presence of arrestin 3, ($p=0.0051$), but not in the presence of arrestin 2 ($p=0.1532$). This observation suggests that the role of arrestin 3 is not redundant with the role of arrestin 2 during internalization of the CCR7/CCL19 receptor/ligand complex. In addition, this is the first observation of ligand dependent regulation of internalization of a chemokine receptor by arrestin.

Since we have shown that GPCRs can have lowered rates and extents of internalization when over-expressed in cells that do not endogenously express the receptor (20), we questioned if the minimal internalization of CCR7 in the MEF cells was due to absence arrestin 2 or other as yet unidentified factors that are normally found in hematopoietic cells. To address this question, we over-expressed CCR7 in the wild type MEF (WT-MEF) and examined internalization when CCR7 was bound to CCL19 (Figure 5C). There was no significant difference ($p=0.1093$) between levels of CCR7 internalized in the WT-MEF when compared with the Arr3-GFP reconstituted MEF Arr2$^{-/-}$arr3$^{-/-}$ cells (Figure 5).

Functional role of Arrestins in CCR7/CCL19 vs. CCR7/CCL21 migration

Arrestin-3 mediates chemotaxis through the CXCR4 receptor in HeLa and Human Embryonic Kidney cells (55). To determine if arrestins regulate migration of T cells via CCR7, we examined migration of HuT78 cells following knockdown of arrestin 2 or arrestin 3. Since our maximal internalization of ligand was at 200nM, we selected this ligand concentration for our studies. In the presence of a full complement of arrestins, the non-specific siRNA treated cells were capable of migration to CCL19 and CCL21 (Figure 6). Similar to the effects on internalization, loss of arrestin 2 or arrestin 3 had no effect on the migration of HuT 78 cells to 200nM CCL21. In contrast, depletion of either arrestin 2 or arrestin 3 blocked migration to CCL19, when compared to cells treated with non-specific siRNA (Figure 6).

Co-localization of arrestins with CCR7

Receptor internalization has multiple important functions within the cell. Initially internalization moves a receptor out of an area of high ligand concentration on the surface of the cell, to inside the cell. Changes in spatial temporal localization of a GPCR, can control signaling from these receptors. Finally, during internalization, a receptor is trafficked to an area within the cell where the receptor releases its ligands and is recycled or degraded. Generally, when a receptor binds its cognate ligand, this binding leads to a conformational change, and receptor clustering. These clusters form signaling domains (56) and terminate further receptor stimulation by coordinating internalization. Arrestins serve as scaffolds that

recruit signaling proteins to the C-terminal domains of activated GPCRs. To determine whether activation of CCR7 could induce co-clustering with arrestins, we transiently transfected the HuT78 cells with GFP, arrestin 3-GFP, or arrestin 2-GFP and examined the clustering of CCR7 by immunofluorescence microscopy (Figure 7). The FPR was used as a control for clustering of arrestins. The cells were plated on glass coverslips, stimulated for the indicated periods of time with either 200 nM CCL19 or CCL21. Fixed/stained cells were examined for clustering of murine CCR7 and arrestin. In the absence of ligand, at t=0 minutes, we found CCR7 distributed evenly over the cell surface and in the cytoplasm.

Following treatment of HuT 78 cells with 200nM CCL19 or CCL21, in both live (data not shown) and fixed cells (Figure 7A–C), we failed to detect clustering of arrestins at the cell surface during receptor internalization. In addition, unlike the FPR, which we have shown forms multiple distinct clusters at the cell membrane following ligand binding (20) (Figure 7C), we found that CCR7 moved into capped structures with CCL19 or CCL21. In these structures, we observed that CCR7 moved to the same region of the cell as arrestin 3 following receptor internalization with CCL19 at four and eight minutes. In addition, CCR7/CCL21 and CCR7/CCL19 co-localized, weakly, with arrestin 2 at four minutes, but this co-localization was lost at eight minutes. In the absence of arrestin over expression (Figure7B) CCR7/CCL21 appeared more dispersed. We did not observe co-localization of either CCR7 or FPR with GFP. In approximately 10% of the cells we observed movement of CCR7/CCL19 to the nucleus in large clusters with arrestin 3-GFP (data not shown).

Discussion

This report is the first to describe differences in the mechanisms that are used by T cells to internalize CCR7 following CCL19 vs. CCL21 ligand binding. Since mice lacking either CCL19 or CCL21 have not yet been described, it is important to distinguish the physiologically distinct roles that the each ligand plays to better understand the regulation of CCR7 and downstream signaling pathways. We observed that while both CCL19 and CCL21 are capable of mediating internalization of CCR7, the mechanisms controlling the internalization lead to different outcomes. In this report we used a human T cell line, HuT78, which like naïve T cells expresses an endogenous CCR7. While HuT 78 internalized 76% of CCR7/CCL19, only 22% of CCR7/CCL21 was internalized (Figure 2). In addition, we found that while CCL21 internalized receptor is rapidly recycled, CCL19 internalized receptor recycled at a slow rate in HuT 78 (Figure 3). These observations are in line with previously published results describing CCR7 internalization and recycling in human peripheral blood lymphocytes (44, 45). The HuT 78 cell line, however, provided a model that allowed us to manipulate arrestin levels and examine the role of arrestins in the internalization of CCR7 and in the chemotaxis of T cells. Loss of arrestin 3, but not arrestin 2 expression blocked internalization of CCL19/CCR7 but had no effect on internalization of CCL21/CCR7. Therefore we were surprised to find that the association of CCR7 with either arrestin 2 or arrestin 3 mediated chemotaxis of HuT78 cells to CCL19, but had no effect on chemotaxis to CCL21 (Figure 6). Equally important, using confocal immunofluorescence microscopy we found that both arrestin 2 and arrestin 3 polarized or capped with CCR7 following eight minutes of internalization (Figure 7).

The CCR7 GPCR, like the nociceptin GPCR demonstrates ligand dependent differences in receptor internalization and desensitization (57–60). Both GPCRs differentially regulate receptor fate following ligand binding (45). The differential regulation of receptor fate suggests that ligand specific mechanisms are activated at the surface of the cell when each ligand binds that rapidly control ligand/receptor behavior. Unlike our studies in which both CCR7 ligands that are full agonists (61) were maximally internalized within eight minutes (Figure 2), the nociceptin receptor required different ligand-dependent incubation times to mediate receptor internalization. This may reflect the differential functions of the described ligands, since agonists and antagonists were internalized at different rates (57–60).

That CCL19 and CCL21 regulate CCR7 internalization differently may reflect their unique functions in the immune system. While CCL19 is expressed primarily by dendritic cells and within the T cell zone of the lymph nodes, CCL21 is constitutively expressed by the high endothelial venules of lymph nodes and is found within the spleen, Peyer's patches, the thymic medulla and the lymphatic endothelium of many tissues (62, 63). Normally, CCR7 functions to induce migration of cells into the lymph nodes (28, 64–67). Initially, CCR7 expressing cells are exposed to CCL21 in the high endothelial venules of the lymph nodes. During this exposure, if a large number of CCR7 receptors were internalized, this could prevent the cell from reaching the CCL19 expressing dendritic cells. Recently, systemic application of CCL19-Ig, which likely internalized most available CCR7, prevented T cells from forming conjugates with dendritic cells in the lymph nodes, *in vivo* (68). In addition, while in the presence of a CCL19 antagonist, cells were able to migrate to the lymph nodes, they were failed to exit (69). These studies suggested that CCL19 likely contribute to pathways involved in T cells exiting the lymph nodes. The signaling pathway regulated by CCL19 is currently under investigation in our laboratory (Shannon, Byers and Vines, *manuscript in preparation*). In contrast, if the cell fails to encounter a CCL19 expressing dendritic cell, then the low level of internalization and the high rate of CCR7 recycling promoted by the CCR7/CCL21 receptor/ligand complex (Figures 2 and data not shown) would allow the cell to continue to traffic through the lymph node. Since stimulation with CCL19 removes ~80% of the CCR7 from the cell surface, it is not surprising the T cell becomes unable to migrate to the CCL21 stimulus of CCR7 following encounter with a dendritic cell (Figure 4 and (61)). By reducing the numbers of CCR7 receptors on the surface and desensitizing the cell to further stimulation with CCR7 ligands (61), CCL19 may help to retain the T cell in the lymph node once it has formed a T cell/dendritic cell conjugate.

Further evidence of the differential regulation of CCR7/CCL19 and CCR7/CCL21 has been revealed in studies of receptor phosphorylation in human embryonic kidney (HEK293) cells transiently transfected with human CCR7. In these studies, stimulation of CCR7 with CCL19 led to an 83% increase in phosphorylation of the C-terminus of CCR7 over basal levels in contrast with only an 18% increase in phosphorylation levels when activated by CCL21 (61). Phosphorylation of GPCRs is a prerequisite for recruitment of arrestins to the C-terminal domains of the receptors. In our study, we find that in T cells and in murine embryonic fibroblasts, arrestins are required for internalization of the CCR7/CCL19 ligand receptor combination, but not of CCR7/CCL21 (Figures 4–5).

For ligand internalization assays and isotype controls, 10^4 to 2×10^4 cells were assayed per sample. The flow cytometry data was reported as percent of total cells to be able to directly compare the peaks. There were equal maximal downshifts for both CCL19 and CCL21 ligands; however, a sub-population of CCL21 treated cells showed no internalization after 8 minutes. These results suggest that HuT 78 cells are heterogeneous in regards to CCL21 internalization, which is not surprising, since HuT78 cells, similar to primary human peripheral blood cells, are not homogeneous, but derived from an heterogeneous human lymphoma (70). In addition, these cells are at different phases of the cell cycle and may respond differently to stimulation with CCL21 depending upon their state in the cell cycle. Like the HuT78 cells, primary cells, which are heterogeneous and found at different stages of the cell cycle internalize ~25% of CCL21 and 60% of CCL19 in flow cytometry (45). While it is beyond the scope of this study to try to identify different subpopulations of primary or HuT78 cells that behave differently in response to CCL21, it is important to note that HuT78 cells have been used to isolate several subclones which display distinct phenotypic differences from others cells in the HuT78 human T-cell lymphoma line (71, 72).

In our studies we used well-characterized siRNAs against arrestin 2 and arrestin 3 in our T cells. These siRNAs have been used successfully to knockdown arrestin 2 or arrestin 3 in human embryonic kidney cells (41). Although the transfection efficiency was ~90%, as measured by FACS we were unable to specifically to knock down levels of arrestin 2 or arrestin 3. The inability to knockdown only arrestin 2 or arrestin 3 in the HuT 78 cells may be due to the high level of conservation between arrestin 2 and arrestin 3 in the regions against which the siRNAs were generated. Unlike previous studies that were able to specifically deplete arrestin 3 with arrestin 3 siRNA, we failed to see specific depletion of arrestin 3, but also observed reduced levels of arrestin 2. This made it difficult to determine if the effects of the siRNA were due to depletion of arrestin 3 alone or due to the concomitant loss of arrestin 2. Loss of arrestin 2 and 3 did not effect CCR7/CCL21 internalization when compared to cells treated with non-specific siRNA (Figure 4 A). Therefore, although arrestin 3 mediated CCR7/CCL19 internalization, neither arrestin 2 nor arrestin 3 regulated CCR7/CCL21 internalization in T cells. In support of this observation, we were able to rescue CCR7/CCL19 internalization in MEF arr2$^{-/-}$arr3$^{-/-}$ cells by transient transfection of arrestin 2 but not of arrestin 3 (Figure 5).

The differential phosphorylation of CCR7 following engagement of CCL19 vs. CCL21, positions the receptors for binding to unique effectors, as we observed with arrestins in this study (Figure 3–6). How the phosphorylation of CCR7/CCL19 and CCR7/CCL21 are regulated, leading to homologous desensitization, is unknown. Basal levels of phosphorylation are mediated by the second messenger kinase, protein kinase C (73). Other kinases, however, such as the G protein-coupled receptor kinases (GRKs), which regulate homologous desensitization of GPCRs, have not yet been identified for CCR7. Studies are currently underway in our laboratory to begin to define the GRKs that differentially regulate phosphorylation of CCR7.

Arrestin 2 and arrestin 3 controlled chemotaxis to CCL19 (Figure 6). This was unexpected, since CCR7/CCL19 is internalized via an arrestin 3-dependent but arrestin 2-independent pathway. However, this was in line with previous studies that showed that internalization of a

GPCR is not required for chemotaxis (74). Signaling through chemokine receptors such as CCR7 can control T cell chemotaxis (75–77) since migration of lymphocytes can be regulated through integrins, heterodimeric adhesion molecules that respond to signals originating at activated GPCRs (78). Therefore, it is curious that although only arrestin 3 regulates receptor internalization, loss of arrestin 2 or arrestin 3 affects migration only to CCL19 and not to CCL21. This observation could suggest some redundancy of arrestin function, in mediating activation of adhesion proteins that are used for migration following activation of CCR7. Although we and others have shown that GPCRs can regulate integrin adhesion (78, 79), the roles of arrestins in the regulation of integrin adhesion/migrations has not yet been defined. In this study we have observed distinct roles for arrestin 3 in the regulation of CCR7/CCL19 internalization but not CCR7/CCL21. Arrestin 2 did not regulate the internalization of CCR7/CCL19 or CCR7/CCL21 but along with arrestin 3 controlled migration to CCR7/CCL19. Since arrestins can serve as scaffolds for many different effectors proteins, we anticipate that this early difference in binding to intracellular signaling proteins can be better defined by further dissecting the proteins that are recruited by arrestins following engagement of CCR7 by its ligands, CCL19 and CCL21.

As we and others have discussed, arrestin recruitment to GPCRs can have different consequences. With the β2 adrenergic receptor, arrestins bind to the phosphorylated form to mediate recruitment of receptors to clathrin-coated pits (13, 20). With the V2 Vasopressin receptor, arrestins prevent recycling by trafficking the receptor to the lysosome (21). In contrast, while the FRP does not use arrestins to mediate receptor internalization (80), we have shown that the FPR uses arrestins to regulate receptor recycling (20) and prevent GPCR mediated apoptosis (81). In these studies, arrestins form distinct small clusters with the GPCRs during internalization and trafficking. Therefore, it was surprising to us that although we observed large areas of co-localization that resembled capping of proteins (82), we failed to detect ligand-bound CCR7 in small clusters with arrestin 2-GFP or arrestin 3-GFP either in video microscopy studies (data not shown) or in fixed cells (Figure 7). We observed that CCR7 recruits arrestin 3 only when it is in association with CCL19, but not with CCL21. (Figure 7A). CCR7 bound to CCL21 fails to cluster with arrestin 3 at 4 minutes and only weakly at 8 minutes. This in contrast to CCR7 bound to CCL19, which clusters with arrestin 3 at both time points. Arrestin 2 forms weak clusters with CCR7 at 4 minutes. These clusters are gone by 8 minutes. The HuT78 cells are capable of clustering arrestins with the FPR, demonstrating that these cells have the machinery to tightly cluster arrestins with GPCRs. In contrast, we were not able to observe the same strong clusters of arrestins with CCR7 in the HuT78 cells, suggesting that the cellular mechanisms used by CCR7 that control internalization are distinct from the mechanisms used by the FPR. Interestingly, we did observe that a few of the cells stimulated with CCL19 showed aggregates of receptor/arrestin 3 in the nucleus (Figure 7D). Developing a more complete understanding of the role of arrestins in the regulation of these internalization and trafficking events is the focus of ongoing investigations in our laboratory.

These are the first studies that define a role for arrestin 3 in the internalization of a chemokine receptor following binding of one but not both endogenous ligands. We have found that internalization of CCR7 is regulated by two distinct mechanisms; an arrestin dependent and an arrestin independent mechanism. In response to binding either CCL19 or

CCL21, CCR7 uses either an arrestin 3 dependent or an arrestin 3 independent mechanism, respectively, to be internalized. This study provides insight into how differential recruitment of effectors may control immunological events that are initiated by CCR7 when it is bound to its two ligands CCL19 or CCL21.

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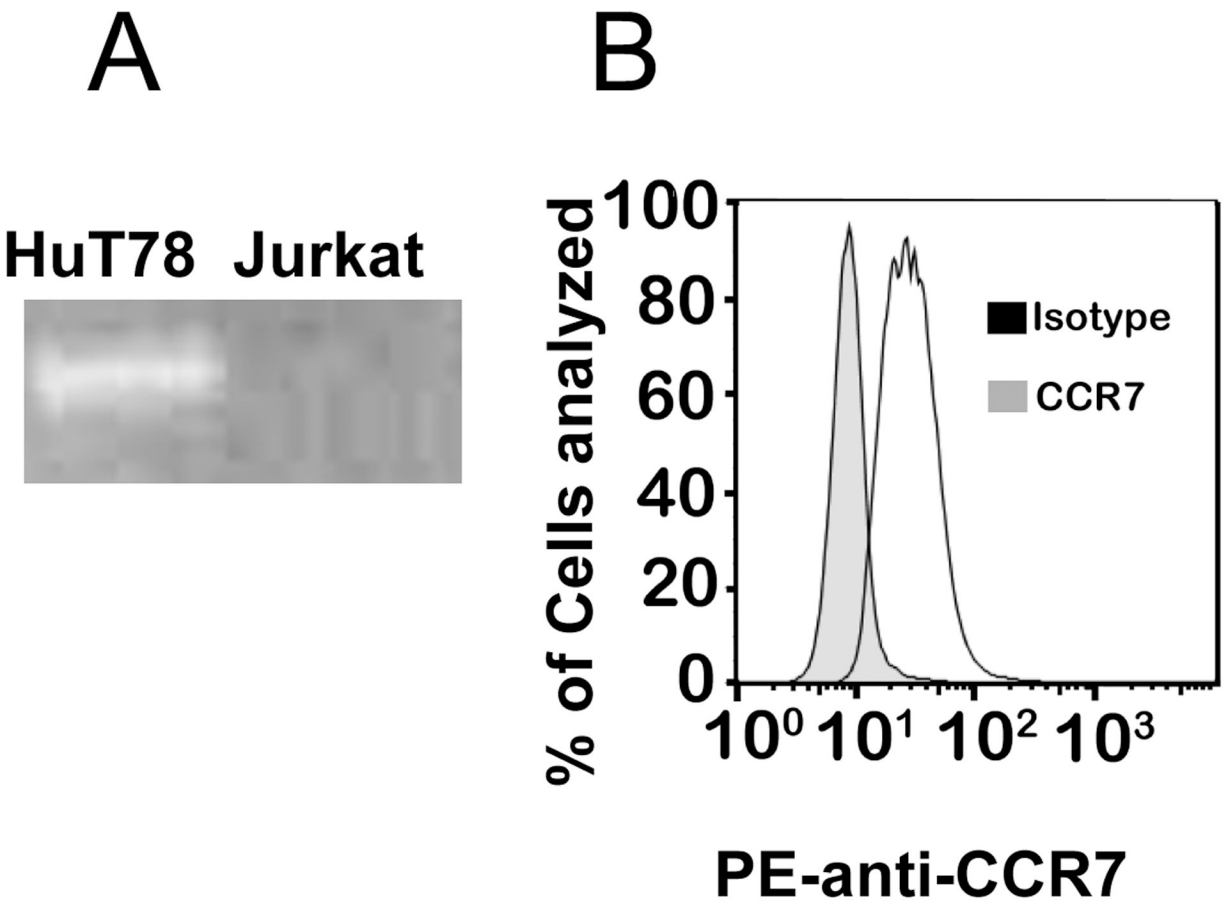

Figure 1.

CCR7 is expressed in HuT 78 cells but not in control Jurkat e.6 cells. A) Total mRNA was isolated from HuT 78 (CCR7+) and Jurkat e6 (CCR7-), reverse transcribed into cDNA and the human CCR7 message was amplified by PCR using CCR7 specific primers. The products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. B) HuT 78 cells were stained with anti-CCR7-PE antibody (↓↓) or with the corresponding isotype control (↓↓) and analyzed by flow cytometry. Each image is representative of a minimum of n=3 independent experiments.

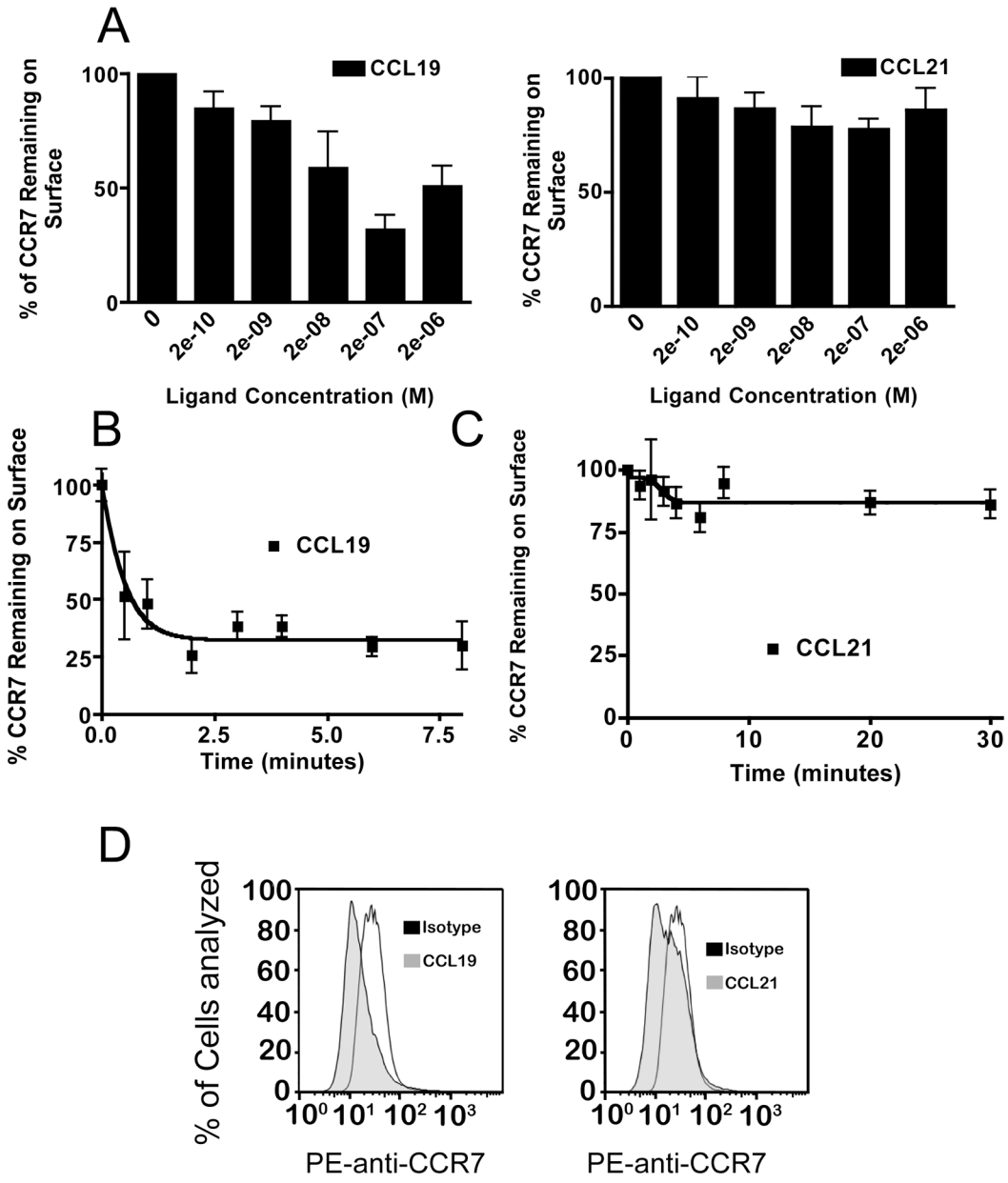

Figure 2.

Internalization of the endogenous CCR7 GPCR in HuT 78 cells is mediated by (A, C, D) CCL19 and (B, C, D) CCL21. HuT 78 cells were incubated at 37°C in the absence (time = 0) or presence of 200 nM ligand for (A and D) 10 minutes or (B-C) the indicated time periods. Internalization was stopped by adding 10 volumes of ice-cold serum free RPMI. Unbound ligand was removed by rinsing. Receptor remaining on the surface was stained with PE-anti-human CCR7 and quantified by FACS. Values are the mean ± SE of a minimum of three independent experiments.

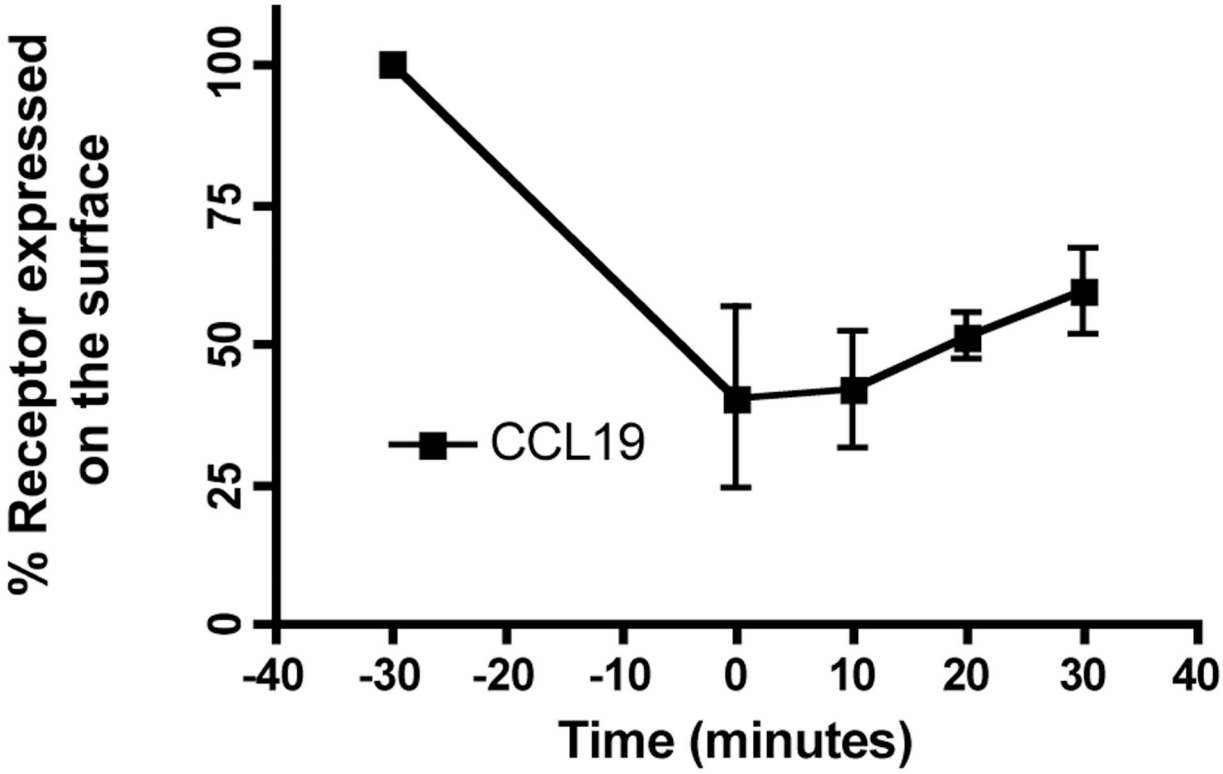

Figure 3.

CCL19 bound receptors fail to recycle. HuT 78 cells were allowed to internalize CCR7 in the presence of 200nM ligand for 30 minutes at 37°C. Cells were rinsed three times with ice-cold RPMI at 4 °C and incubated in pre-warmed serum free RPMI at 37°C for the indicated times to allow for receptor recycling. Recycled receptor was labeled with PE-anti-human CCR7 and cells were assayed by flow cytometry. Values are the mean ± SE of a minimum of three independent experiments.

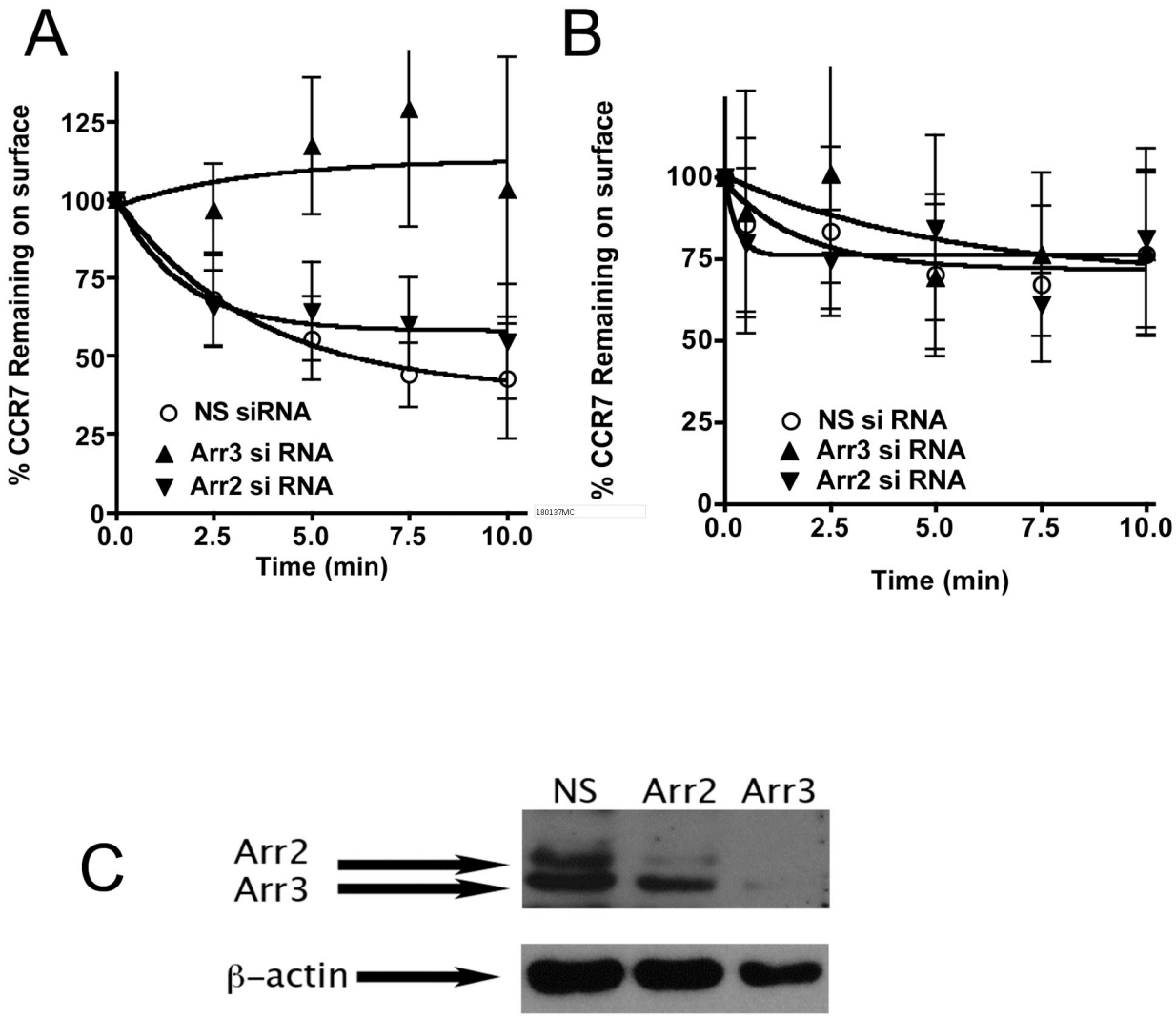

Figure 4.

Internalization of CCR7/CCL19 is blocked by arrestin 3 siRNA but not arrestin 2 siRNA. HuT 78 cells were attenuated of arrestin 2 or arrestin 3 by Oligofectamine transfection of cells with siRNA in the absence of serum for four hours. Serum was replaced, and cells were allowed to recover in the presence of siRNA for 72 hours prior to assay. Subsequently, cells were incubated in the presence of 200nM of A) CCL19 or B) CCL21 for the indicated periods of time, rinsed with ice-cold serum free media and stained with PE-anti human CCR7 or (C) lysed and assayed by Western blot for expression of arrestin 2 or arrestin 3. (A) p=0.0013 for arrestin 3 vs. ns si RNA. There was no significant difference any other values. Values are the mean ± SE of seven independent experiments for CCL19 and four independent experiments for CCL21.

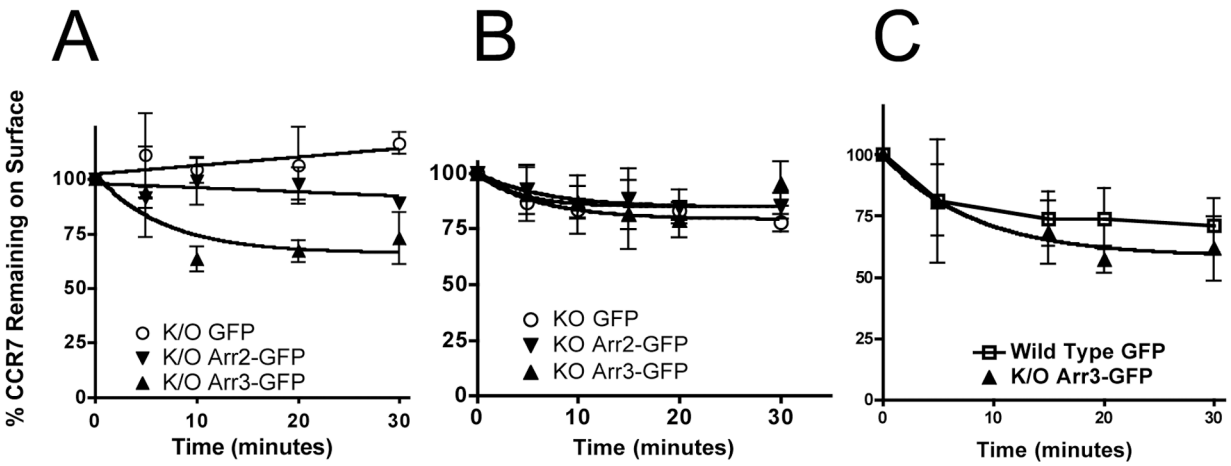

Figure 5.

Expression of arrestin 3-GFP in arr2$^{-/-}$arr3$^{-/-}$MEF (KO) cells restored internalization of CCR7/CCL19 but did not affect internalization of CCR7/CCL21. A) and B) KO or C) WT and KO cells were transiently transfected with mu-FLAG tagged CCR7 and GFP alone, Arr2-GFP or Arr3-GFP. Cells were allowed to internalize CCR7 in the presence of 200 nM A and C) CCL19, or B) CCL21 for 30 minutes at 37°C. Cells were rinsed three times and incubated on ice with M2 anti-FLAG, rinsed, and labeled with PE-anti-mouse secondary. Cells were assayed by FACS. Values are the mean ± SE of three independent experiments.

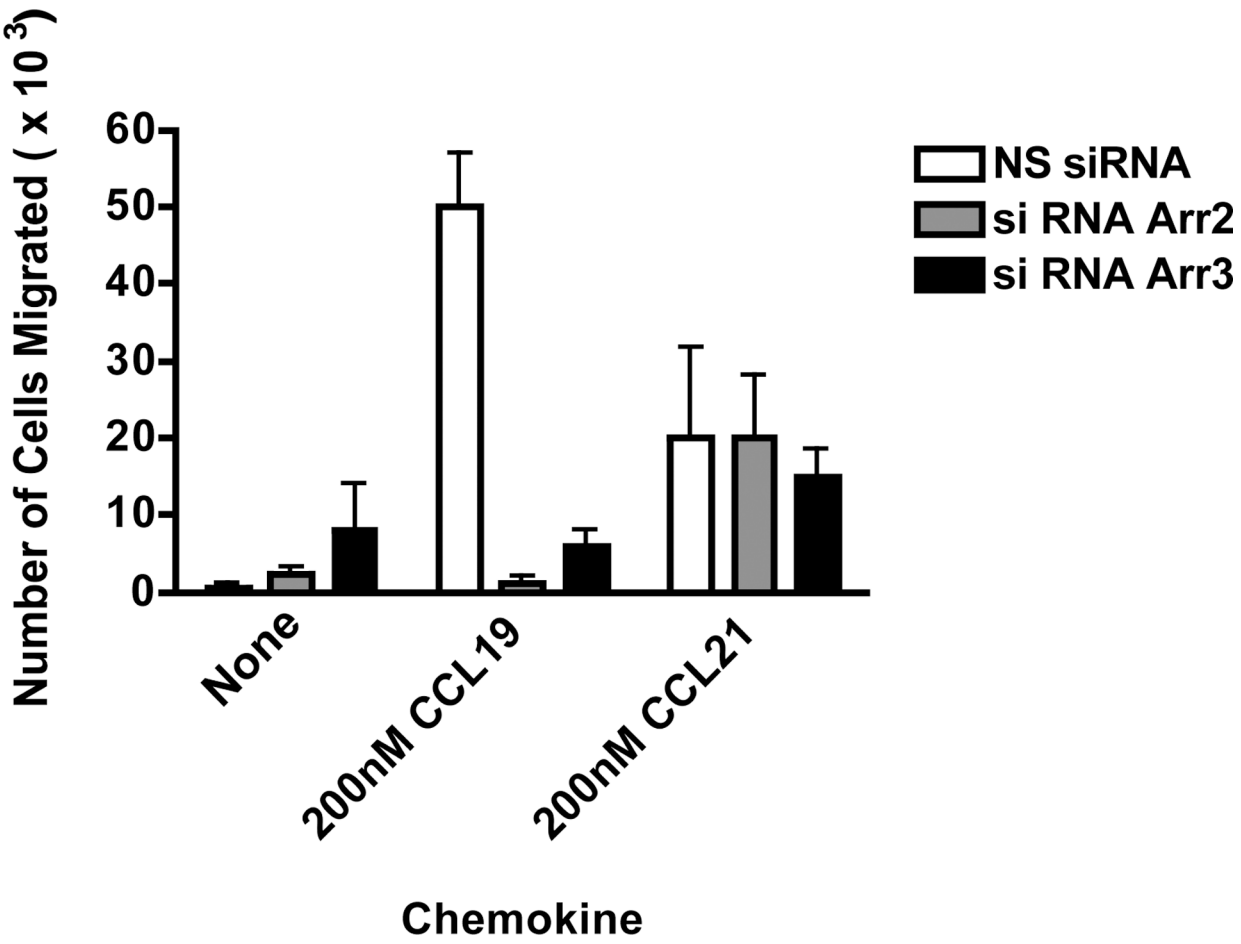

Figure 6.

Knockdown of expression of arrestin2 or arrestin 3 in HuT78 inhibits migration to CCL19 but has no effect on migration to CCL21. HuT78 cells were nucleofected with arrestin 2 siRNA, arrestin 3 si RNA or non-specific siRNA. Cells were allowed to recover for 72 hours prior to assay. 200nM CCL19, CCL21 or carrier (1%BSA/PBS) were transferred to the lower wells of a Neuroprobe chemotaxis chamber, and cells separated by a membrane, were allowed to migrate for 2 hours. Migrated cells in the lower chamber were counted. Values are the mean ± SE of 3 independent experiments.

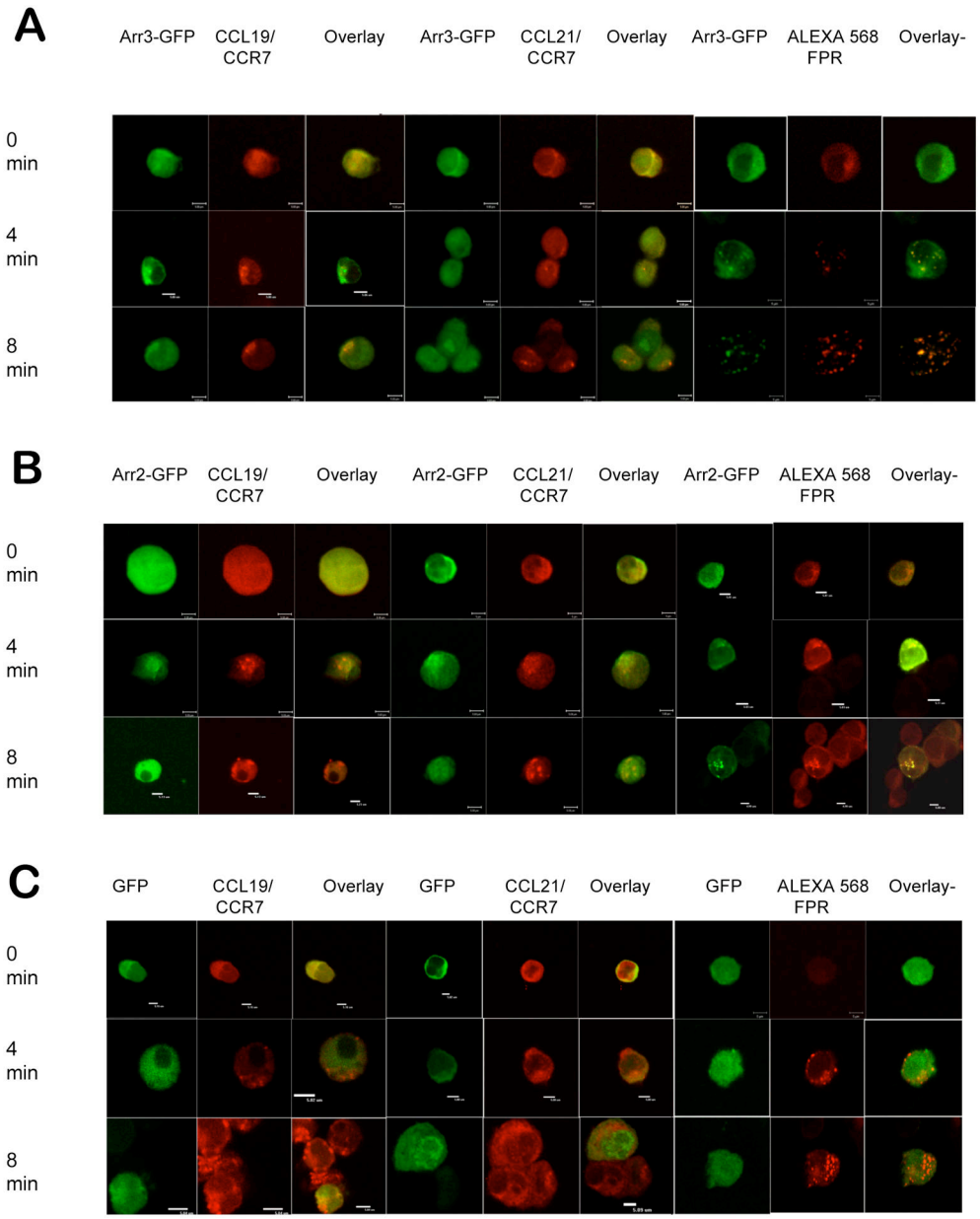

Figure 7.

Internalized CCR7/CCL19 co-localizes with arrestin 3. HuT78 cells were transiently nucleofected with either A) arrestin-3-GFP (Arr3-GFP) alone or Arr3-GFP with the human *N*-formyl peptide receptor B) arrestin-2-GFP (Arr2-GFP) alone or Arr2-GFP with the human *N*-formyl peptide receptor GFP alone or C) GFP with the human *N*-formyl peptide receptor. The cells were then either fixed with 2% paraformaldehyde prior to ligand stimulation (t=0) or stimulated in the presence of 200 nM CCL19 or CCL21 or 10nM ALEXA-568-formly peptide and fixed at four and eight minutes. Cells were permeablized in 0.1% NP-40/PBS, stained with anti-human CCR7 followed by Rhodamine anti-mouse and

imaged by immunofluorescence microscopy. Figures represent images from n=3 independent experiments.