Human samples
These studies were approved by the Institutional Review Board at Case Western Reserve University/University Hospitals, Case Medical Center. After informed consent was obtained, blood was drawn into heparin-coated tubes. Peripheral blood mononuclear cells (PBMCs) were purified from whole blood by Ficoll-Paque centrifugation.

GHOST (3) cells
GHOST (3) cells and transfected GHOST (3) cells expressing high levels of human CCR5 (Hi-5)[1] were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Cells were grown in complete medium consisting of 90% DMEM (4 mM L-Glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate; Hyclone Laboratories, Logan, Utah) and 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA) supplemented with 500 µg/ml Geneticin (Invitrogen, Grand Island, NY), 100 µg/ml Hygromycin B (Invitrogen, Carlsbad, CA), Penicillin/Streptomycin (both 10,000 Units/mL; BioWhittaker Lonza, Walkersville, MD). Puromycin Dihydrochloride, 1 ug/mL (Calbiochem EMD Bioscience, La Jolla, CA) was added to the GHOST (3) Hi-5 cell cultures.

Antibodies
The following fluorescent antibody conjugates were utilized for identifying and sorting T cells: anti-CD3 (Clone SK7 Mouse IgG1, BD Biosciences, San Jose, CA) conjugated to both peridinin chlorophyll-a protein (PerCP) and fluorescein isothiocyanate (FITC); anti-CD4 (Clone OKT4 Mouse IgG2b, EBioscience, San Diego, CA) conjugated to Pacific Blue (PB); anti-CD8 (Clone SK1 Mouse IgG1, BD Biosciences, San Jose, CA) conjugated to phycoerythrin-Cy7 (PE-Cy7).

Fluorescent anti-CCR5 antibodies were obtained from BD Biosciences (San Jose, CA). These were clones 2D7 and 3A9 (mouse IgG2a) conjugated with allophycocyanin (APC), phycoerythrin (PE), FITC, or allophycocyanin-Cy7 (APC-Cy7). For each CCR5 antibody used, a mouse IgG2a fluorochrome labeled control antibody (BD Biosciences) was used to establish gates. AlexaFluor 647 conjugated anti-CCR5 (HEK/1/85a) and corresponding control (Rat IgG2a) were obtained from Biolegend (San Diego, CA).

For Western blotting, the following products were used: rat anti-human CCR5 clone 1/85a (AbD Serotec, Raleigh, NC), rabbit polyclonal antibody to beta-actin (Abcam Inc., Cambridge, MA), horseradish peroxidase (HRP)-conjugated polyclonal goat anti-rabbit immunoglobulin (Dako North American Inc., Carpinteria, CA), goat anti-rat IgG ECL-HRP–linked whole antibody (Amersham GE Healthcare, Piscataway, NJ), and Precision Protein StrepTactin HRP-conjugate (Bio-Rad Laboratories, Inc., Hercules, CA).

For confocal microscopy, the following products were used: CCR5 clone 3A9 or 2D7 or Mouse IgG2a Isotype (BD Biosciences #555571) and/or CCR5 clone 1/85a AlexaFluor 488 (Biolegend, #313710, San Diego, CA) or Rat IgG2a Isotype (Biolegend # 400525). For the 3A9 antibody, a secondary Donkey–anti-Mouse IgG Rhodamine Red X (RRX) antibody was used to visualize primary antibody binding (Jackson Immunoresearch Laboratories Inc., West Grove, PA). Nuclear staining of cells for confocal microscopy was done with 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes, Inc., Eugene, OR) at a concentration of 0.4 µg/ml in

0.1% Triton X-100/PBS. For the 2D7 antibody, a secondary anti-Mouse IgG AlexaFluor 488 antibody was used to visualize primary antibody binding (Molecular Probes, Inc.).

Flow cytometry

For surface staining, cells were pelleted then resuspended in flow wash buffer (1% BSA in phosphate buffered saline (PBS) containing 0.1% sodium azide) to which antibodies were added. Cells were incubated for 15 minutes at 4°C then washed and stored at 4°C until examined on an LSR II Flow Cytometer (Becton Dickinson, San Jose, CA) with Firmware Version 1.8 using FACSDiva software version 6.1.1 (BD Bioscience, San Diego, CA). For intracellular staining, cells were treated with BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences Pharmingen #554714, San Diego, CA). Cells were vortexed and resuspended in Fixation/Permeabilization solution (#554722) for 20 minutes at 4°C. Cells were then washed and incubated for 15 minutes at 4°C with antibody then washed and examined as above. To ensure complete permeabilization, cells were incubated in propidium iodide (BD Biosciences #556463) and examined for dye uptake. A minimum of $2.5 \times 10^4 – 1 \times 10^5$ cell events were collected. The isotype control was gated to identify 2% of cells as positive.

Effect of fixation and permeabilization conditions on CCR5 signals in immunofluorescence experiments

CHO-CCR5 cells or CHO-WT[2] cells were grown on 20-mm glass coverslips for 2 days. Cells were then subjected to either the 'mild' or 'harsh' fixation or permeabilization procedure.

'Mild' fixation: Cells were fixed in 4% paraformaldehyde for 20 minutes at 4°C. After quenching with 50mM NaH_4Cl in PBS for 15 minutes, cells were washed with phosphate-buffered saline (PBS) and then incubated with PBS-bovine serum albumin (BSA) 1% with 0.1% saponin for 20 minutes.

'Harsh' fixation: (Conditions equivalent to those used for flow cytometry experiments by Achour *et al.*): Cells were fixed for 20 minutes with 1% paraformaldehyde, washed with PBS, and permeabilized for 1 hour at 4°C in PBS containing 2% FBS, and 0.2% Triton X-100.

Following fixation with or without permeabilization, cells were incubated with the anti-CCR5 rat antibody 1/85a (1:50) and an anti-TGN38 rabbit antibody (Permeabilization control, 1:200)[3] for 1 hour.

Cells were then incubated for 30 minutes with the Alexa 488 secondary antibody against rat IgG and with Alexa 647 secondary antibody against rabbit IgG (Molecular Probes), and coverslips were mounted in Moewiol[4] and observed with an LSM510 confocal microscope (Carl Zeiss, Feldbach, Switzerland).

Cell sorting

For sorting experiments, T cells were first enriched from whole blood using RosetteSep T (StemCell Technologies, Vancouver, BC Canada) and Ficoll Hypaque density sedimentation. The T-cell–enriched population was incubated for 15 minutes at 4°C with antibody and then washed with flow wash buffer, pelleted and resuspended, placed on ice, filtered through a 0.35

μM filter cap tube (BD Biosciences Labware #352235, Bedford, MA), and sorted using a BD FacsARIA instrument (Becton Dickinson) into CD3+CCR5− and CD3+CCR5+ populations.

Western blot

Cells were resuspended at 10^7/100 μL in lysis buffer (1% Nonidet P-40 (NP-40; Sigma, St. Louis, MO), 10 mM Tris HCl pH 7.5, 2 mM EDTA, 150 mM NaCl supplemented with PhosSTOP phosphatase inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and complete protease inhibitor cocktail (Roche Applied Science) then frozen overnight at −20°C. Tubes were then thawed, incubated on ice for 40 minutes, and centrifuged for 10 minutes at 14000 rpm at 4°C to pellet debris. Supernatants were harvested and protein concentration determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories).

Lysates were denatured in 1× NuPAGE LDS Sample Buffer (4×; Invitrogen), 1× NuPAGE reducing agent (10×; Invitrogen), and deionized water. Samples were loaded on to Nu-Page gels (10% Bis-Tris, 1.0 mm × 12 well; Invitrogen, Carlsbad, CA). Following electrophoresis, the gels were transferred to nitrocellulose membranes (Hybond-ECL Nitrocellulose Membrane; Amersham/GE HealthCare, Piscataway, NJ), and membranes were blocked overnight at 4°C in 5% Sanalac Non-Fat Dry Milk (NFDM; ConAgra Foods, Omaha, NE) diluted in PBS/0.1% Tween-20 (Sigma, St. Louis, MO). Following blocking, membranes were washed in PBS/0.1% Tween-20 and incubated with either anti-CCR5 or anti–beta-actin antibodies, washed again, then incubated with the appropriate developing reagent. Membranes were developed using the Pierce SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Pierce Biotechnology, Rockford, IL) or Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) and exposed to Premium Autoradiography Film (Denville Scientific, South Plainfield, NJ).

Real-time PCR

One to two million cells were lysed in RLT Lysis Buffer (Qiagen, Valencia, CA) containing 1% β-Mercaptoethanol (Gibco Invitrogen Grand Island, NY). RNA was extracted and purified using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's protocol. Preparation of cDNA was carried out using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Complementary DNA was quantitated by Taqman assay using a Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA). Primers and probe used for the Taqman assay were: CCR5 Forward Primer 5′-CAACATGCTGGTCATCCTCATC-3′; 9 μM (10×), CCR5 Reverse Primer 5′-ATGGCCAGGTTGAGCAGGTA-3′; 9 μM (10×), CCR5 Probe 6FAM-TGTCAGTCATGCTCTTCAGCCTTTTGCAG-TAMRA; 1.5 μM (10×), R18 Forward Primer 5′-CGCCGCTAGAGGTGAAATTC-3′; 9 μM (10×), R18 Reverse Primer 5′-CATTCTTGGCAAATGCTTTCG-3′; 9 μM (10×), and R18 Probe VIC-ACCGGCGCAAGACGGACCAGA-TAMRA; 2 μM (10×). At least two different dilutions of each cDNA sample were run and each dilution was run in duplicate. The standard curve for the CCR5 Taqman assay used 10-fold serial dilutions from 5 to 5×10^5 copies per well and the standard curve for the R18 housekeeping gene used 10-fold serial dilutions from 5×10^6 to 5×10^8 copies per well. The run method used for this experiment included a holding stage (50°C for 2 minutes followed by 95°C for 10 minutes) and a cycling stage (95°C for 15 seconds followed

by 60°C for 1 minute for 40 cycles). Wells with results in range were used to calculate a median copy number for each sample.

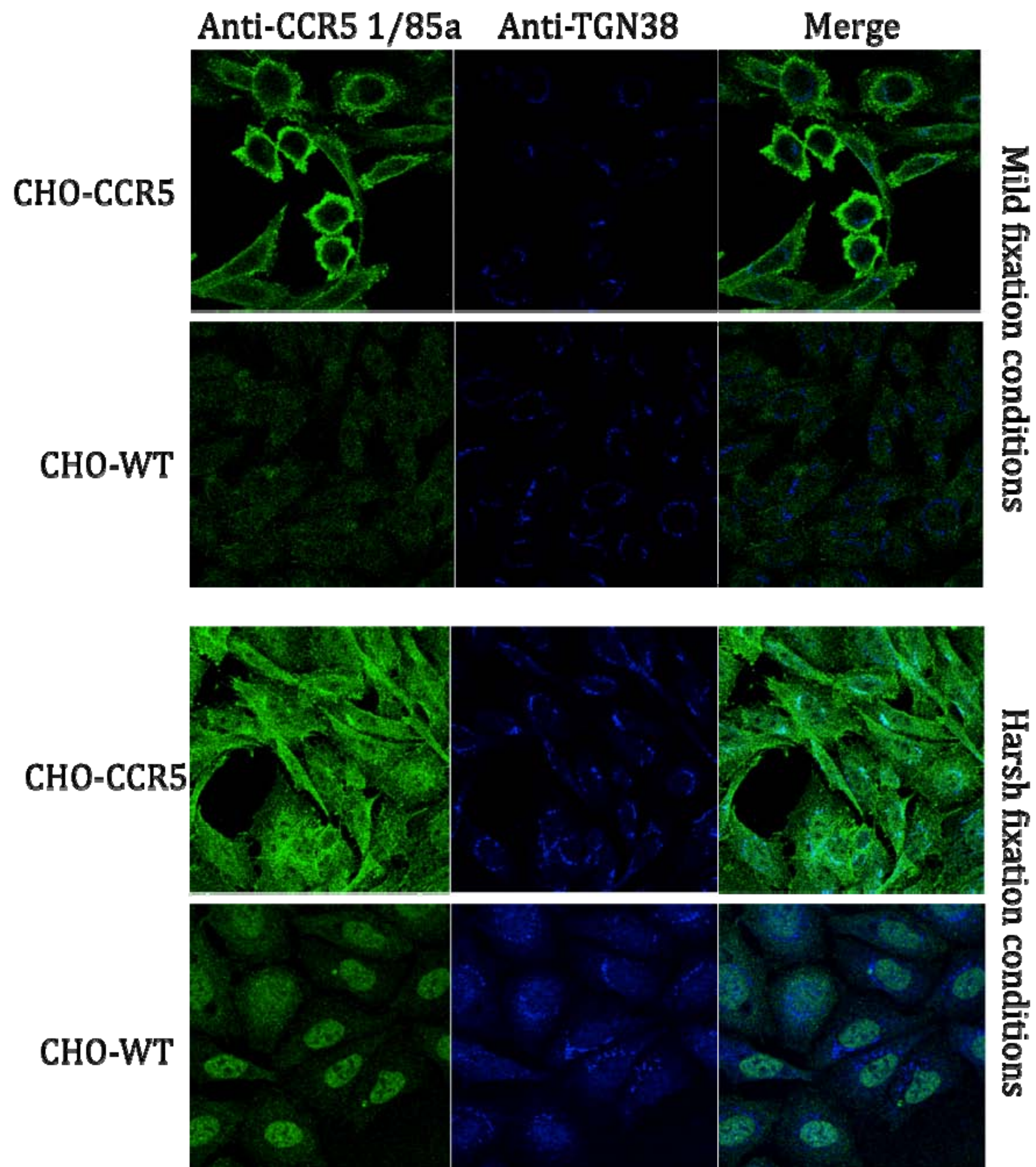

Figure S1. CHO-CCR5 cells or CHO-WT cells were grown on 20-mm glass coverslips for 2 days

Cells were subjected to ‘mild’ or ‘harsh’ fixation and permeabilization procedures (see methods section above for details) then incubated with the anti-CCR5 rat antibody 1/85a (1:50) or an anti-TGN38 rabbit antibody (Permeabilization control, 1:200) for 1 hour and revealed using either Alexa 488 secondary antibody against rat IgG or with Alexa 647 secondary antibody against rabbit IgG (Molecular Probes).

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