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The Duffy Antigen/Receptor for Chemokines Exists in an Oligomeric Form in Living Cells and Functionally Antagonizes CCR5 Signaling through Hetero-Oligomerization

Aron Chakera, Ruth M. Seeber, Alison E. John, Karin A. Eidne, and David R. Greaves

Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom (A.C., A.E.J., D.R.G.) and the Western Australian Institute for Medical Research and Centre for Medical Research, University of Western Australia, Nedlands, Western Australia, Australia (R.M.S., K.A.E.)

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ABSTRACT

The Duffy antigen/receptor for chemokines (DARC) is an unusual chemokine receptor that binds a large number of inflammatory chemokines of both the CC and CXC families with nanomolar affinity, yet it lacks the ability to signal upon ligand binding. Using bioluminescent resonant energy transfer, we have demonstrated for the first time that DARC exists as a constitutive homo-oligomer in living cells and furthermore that DARC hetero-oligomerizes with the CC chemokine receptor CCR5. DARC-CCR5 interaction impairs chemotaxis and calcium flux through CCR5, whereas internalization of CCR5 in response to ligand binding remains unchanged. These results suggest a novel mechanism by which DARC could modulate inflammatory responses to chemokines in vivo.

Chemokines are recognized to play an important role in constitutive leukocyte trafficking and inflammatory cell recruitment (Moser and Loetscher, 2001). These effects are mediated through binding to chemokine receptors, a subfamily of the rhodopsin-like (family A) seven-transmembrane G protein-coupled receptor (GPCR) group. The Duffy antigen/receptor for chemokines (DARC) is a unique chemokine receptor that binds inflammatory chemokines of both the CC and CXC families (Gardner et al., 2004). Note that DARC lacks the consensus DRY motif on the second intracellular loop common to family A GPCRs, normally required for signal transduction; consequently, there is no evidence that DARC can signal (Lee et al., 2003). Immunostaining for DARC reveals prominent expression on red blood cells and the postcapillary endothelium with endothelial up-regulation in the presence of inflammatory stimuli (Segerer et al., 2000), consistent with a role for DARC in the regulation of chemokine-mediated inflammation.

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CCR5 is a member of the β-chemokine receptor family that has been extensively studied for its role in the internalization of M-tropic strains of human immunodeficiency virus (Cairns and D'Souza, 1998). CCR5 has previously been shown to dimerize by both biochemical and biophysical methods (Vila-Coro et al., 2000; Hernanz-Falcón et al., 2004), and it displays a chemokine-binding profile that overlaps with DARC. Although CCR5 is expressed primarily on leukocytes, work performed in our laboratory and other laboratories has established that endothelial expression of CCR5 occurs (Veillard et al., 2006) and that CCR5 may be coexpressed with DARC on primary endothelial cells (Fig. 1).

Bioluminescent resonant energy transfer (BRET) uses proteins tagged with either a luminescent donor protein [e.g., *Renilla reniformis* luciferase (Rluc)] or a fluorescent acceptor protein, such as enhanced green fluorescent protein (EGFP) to assess protein-protein interactions (Eidne et al., 2002). In the presence of the cell-permeable substrate coelenterazine, which is catalytically degraded by Rluc, there is release of light at ~480 nm. If a suitable acceptor protein, such as EGFP, that has an excitation spectra overlapping with the emission spectra of Rluc is in proximity (<100 Å), then nonradiative transfer of energy from donor to acceptor may oc-

ABBREVIATIONS: GPCR, G protein-coupled receptor; DARC, Duffy antigen/receptor for chemokines; BRET, bioluminescent resonant energy transfer; Rluc, *R. reniformis* luciferase; EGFP, enhanced green fluorescent protein; RANTES, regulated upon activation normal T-cell expressed and secreted; MCP, monocyte chemotactic protein; IL, interleukin; mSDF, murine stromal derived factor; TRH, thyrotropin-releasing hormone; HA, hemagglutinin; FACS, fluorescence-activated cell sorting; PCR, polymerase chain reaction; HEK, human embryonic kidney; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; TRH-R, thyrotropin-releasing hormone receptor.

cur, resulting in fluorescence at the new wavelength of ~510 nm (Xu et al., 2003). The ratio of fluorescence to luminescence thus defines the BRET ratio and serves as a mechanism to detect and quantify protein-protein interactions in living cells (Eidne et al., 2002). Although BRET cannot distinguish between dimers and the formation of higher order oligomers, evidence suggests that dimers are the most likely conformation for GPCRs in vivo (Rios et al., 2001; Liang et al., 2003).

The biological importance of GPCR dimerization has been reviewed in several recent publications (AbdAlla et al., 2000; Hanyaloglu et al., 2002). Examples include a requirement for homodimerization to facilitate signaling of β_2-adrenergic receptors, and to permit trafficking of dopamine receptors to the cell surface (Angers et al., 2000; Lee et al., 2000). Heterodimerization of GPCRs has been demonstrated to increase the potency of agonists to somatostatin receptors, alter the binding properties of opioid receptors, and modulate signaling through adenosine and metabotropic glutamate receptors (George et al., 2000; Rocheville et al., 2000; Ciruela et al., 2001).

We have used BRET to show for the first time that the nonsignaling chemokine receptor DARC homodimerizes and furthermore that it can form constitutive heterodimers with CCR5. The functional result of DARC-CCR5 heterodimerization is inhibition of CCR5 signaling, as measured by two functional assays; chemotaxis and calcium flux, without alteration of CCR5 internalization kinetics. These results suggest that regulation of DARC expression may represent a novel mechanism to modulate the inflammatory response to chemokines in vivo.

Materials and Methods

Materials. Recombinant human chemokines, RANTES (CCL5), MCP-1 (CCL2), IL-8 (CXCL8), LD78 (CCL3L1), and the recombinant murine chemokine mSDF-1α (CXCL12) were from PeproTech EC (London, UK), and they were reconstituted according to the manufacturer's instructions. Thyrotropin-releasing hormone (TRH) was from Bachem (Merseyside, UK). The anti-CCR5 antibody (clone 2D7) was obtained through the National Institutes of Health AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The Fy6 monoclonal antibody to DARC was kindly donated by Karina Yazdanbakhsh (New York Blood Center, New York, NY). Anti-HA antibody was from Covance (Richmond, CA). Primary antibodies for FACS and confocal imaging were used at 10 μg/ml final concentration. Secondary antibodies, with fluorescent conjugates were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Geneticin (G418), pertussis toxin, cycloheximide, and protein A beads were from Sigma-Aldrich (Gillingham, Dorset, UK). Zeocin was from Invitrogen (Paisley, UK). Coelenterazine h, FuraRed, and Fluo-4 were from Invitrogen. Ninety-six-well white Optiplates were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Western blot analyses were performed using 10% SDS-polyacrylamide gels and a MiniProtean III system (Bio-Rad Laboratories, Hertfordshire, UK). The QIFIKIT for receptor quantification was from Dako UK Ltd. (Ely, Cambridgeshire, UK).

Plasmids. Human DARC and CCR5 were cloned from genomic DNA using primers to incorporate 5′ BamH1 and 3′ XhoI sites. These constructs were then inserted into a pcDNA3.1 (Invitrogen) backbone containing a Zeocin resistance gene. The resultant sequence was confirmed by DNA sequencing using T7 (forward) and BGH (reverse) primers. DARC sequence matched GenBank accession number AAU47282 and the CCR5 sequence-matched accession number BC038398. For BRET assays, DARC and CCR5 constructs without their stop codons were generated by PCR amplification from the pcDNA3.1 templates and subcloned into a pcDNA3 vector containing an in-frame 3′ *R. reniformis* luciferase or EGFP, inserted between the XhoI and XbaI sites of the polylinker as described previously (Kroeger et al., 2001). For coimmunoprecipitation studies, receptors were subcloned in a similar manner into a pcDNA3.1 vector containing either an in-frame HA or myc tag. Repeat sequencing confirmed that no new mutations had occurred during the cloning process.

Cell Culture and Transfection. HEK-293 cells were from American Type Culture Collection (Manassas, VA), and they were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin solution. The murine preB cell line L1.2 (wild-type L1.2 and L1.2 CCR5 stable cells) was a kind gift from Massimo Locati (University of Milan, Milan, Italy). L1.2 cells were maintained in RPMI 1640 media supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 10 mM

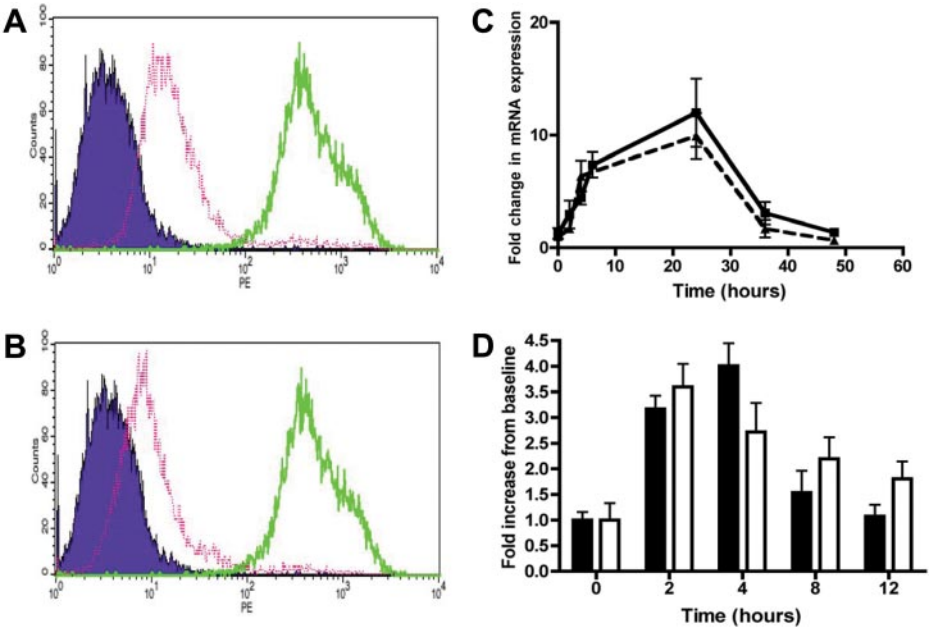

Fig. 1. Expression of DARC and CCR5 on primary endothelial cells. Primary human umbilical vein endothelial cells were stimulated overnight with 20 ng/ml TNF-α, and then they were analyzed for surface expression of DARC and CCR5 by FACS. A, expression of DARC (pink) compared with an isotype control (solid blue) and platelet/endothelial cell adhesion molecule (green). B, expression of CCR5 (pink) with the same controls. C, time course of mRNA induction of DARC (■) and CCR5 (▲) after treatment with 20 ng/ml TNF-α. D, -fold increase in cells expressing cell surface DARC (black bars) and CCR5 (white bars) as measured by FACS. Results are expressed as the mean ± S.E.M.

HEPES, 1% penicillin-streptomycin solution, and 50 μM 2-mercaptoethanol. L1.2 CCR5 cells were maintained in media containing G418. Primary human umbilical vein endothelial cells from pooled donors were from PromoCell (Heidelberg, Germany). L1.2 cells stably expressing DARC were created by electroporation using a Bio-Rad GenePulser II (960 μF; 350 V; 15 μg of pcDNA3.1 Zeocin plasmid; 4×10^7 cells). Transfected cells were selected in 100 μg/ml Zeocin-containing media. Dual selection with both 0.8 mg/ml G418 and Zeocin was used for L1.2 CCR5-DARC-cotransfected cells. To enhance the selection of stable cells with the receptors of interest, magnetic activated cell sorting was performed twice on each cell line according to the manufacturer's protocol (Miltenyi Biotec, Surrey, UK). After QIFIKIT quantification of receptor numbers, MoFlow sorting was performed to select for clones of L1.2 CCR5 and L1.2 CCR5-DARC cells that expressed equivalent numbers of receptors, at levels comparable with those seen on primary cells (Reynes et al., 2001). HEK-293 cells were transiently transfected for BRET assays using GeneJuice (EMD Biochemicals, Madison, WI) or FuGENE (Roche Diagnostics, Basel, Switzerland) according to the manufacturers' instructions. FACS and confocal microscopy with CCR5- and DARC-specific monoclonal antibodies was used to confirm surface expression and functionality of the transfected proteins.

Immunoprecipitation and Western Blotting. Transfected cells were lysed in radioimmunoprecipitation assay buffer (0.15 M NaCl, 0.05 M Tris, pH 7–7.5, 0.1% SDS, 1.0% deoxycholate, 1.0% Triton X-100, and protease inhibitors) for 20 min at 4°C, and then they were centrifuged at 14,000*g*. Protein concentrations were determined using a bicinchoninic acid assay (Pierce Chemical, Northumberland, UK). Samples (200 μg) were added to 40 μl of prewashed protein A beads, and then they were incubated for 1 h at 4°C to preclear. Twenty-five microliters of prewashed c-myc agarose beads was blocked in 1 ml of 2% bovine serum albumin/PBS for 1 h at 4°C, then washed and added to the sample/protein A bead mix, and incubated for a minimum of 2 h at 4°C. This combined mixture was then washed five times in ice-cold radioimmunoprecipitation assay buffer and boiled before resuspending in Laemmli buffer supplemented with 0.1 M dithiothreitol. Proteins were separated in a 10% SDS-polyacrylamide gel electrophoresis, and then they were transferred to nitrocellulose membranes. For Western blot analysis membranes were blocked in PBS/5% (w/v) milk powder before the addition of the anti-HA primary antibody. Membranes were washed in PBS/0.1% Tween 20, before the addition of an anti-murine horseradish peroxidase-conjugated secondary antibody. After washing, labeled proteins were identified using the enhanced chemiluminescence system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Bioluminescence Resonance Energy Transfer Assay. BRET assays were performed as described previously (Kroeger et al., 2001). In brief, transfected HEK-293 cells were detached 48 h after transfection with PBS/0.05% trypsin, and then they were washed twice in PBS. A proportion of these cells were then analyzed by FACS to confirm expression of the GFP-tagged proteins, to determine transfection efficiency and surface expression of the receptors using specific antibodies. The remaining cells were transferred to 96-well white Optiplates at $\sim 5 \times 10^4$ cells/well. Coelenterazine h (5 μM final concentration) was then added, and baseline recordings of luminescence and fluorescence were performed for 2 to 5 min. Ligand or PBS (5 μl) was then added to samples, and recording was continued for a further 5 to 10 min. BRET ratios were calculated as the ratio of fluorescence to luminescence of the sample minus the ratio of fluorescence to luminescence from the Rluc construct alone expressed in the same experiment (Eidne et al., 2002). All BRET experiments were performed at 37°C using a custom-designed BRET instrument (Berthold, Bundoora, Australia), allowing sequential integration of signals detected in the Rluc and EGFP windows.

^{125}I-Chemokine Binding Assays. ^{125}I binding studies were performed to confirm that the ligand binding affinity of the C-terminally tagged BRET construct receptors was equivalent to native receptors. In brief, HEK-293 cells were transiently transfected with native DARC or CCR5 constructs, or the BRET fusion protein constructs. Cells were lysed 48 h after transfection, and membrane fractions were obtained by ultracentrifugation. Membranes were incubated with ^{125}I-chemokines in binding buffer (25 mM HEPES, 1 mM $CaCl_2$, and 5 mM $MgCl_2$, adjusted to pH 7.4) for 90 min at room temperature before harvesting onto polyethyleneimine-precoated Millipore Multiscreen 1.2-μm filter plates with GF/C filters (Millipore Corporation, Billerica, MA). Once dry, Microscint 20 scintillation liquid (Perkin-Elmer Life and Analytical Sciences, Waltham, MA) was added, and radioactivity was measured with a Packard scintillation counter.

Real-Time PCR Analysis. RNA was isolated from primary cells using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, followed by DNase digestion with RQ1 RNase-free DNase. Five micrograms of total RNA was reverse transcribed in a 25-μl volume using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and oligo(dT) (Invitrogen). mRNA expression was determined by real-time PCR in 1 to 2.5 μl of cDNA on a Rotor-gene 3000 thermal cycler (Corbett Research Ltd., Sydney,

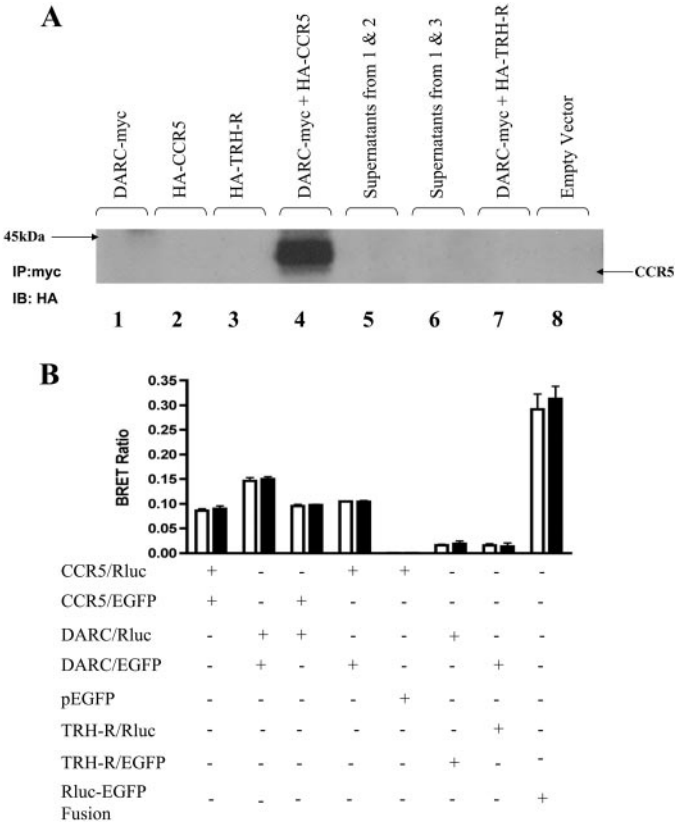

Fig. 2. Dimerization of DARC and CCR5. A, myc-tagged DARC was coimmunoprecipitated from transiently transfected HEK-293 cells with anti-myc-conjugated agarose beads. Immunoblots for HA-tagged CCR5 were performed with an anti-HA antibody. Lane 1, cells transfected with DARC-myc alone; lane 2, cells transfected with HA-CCR5 alone; lane 3, cells transfected with TRH-R alone; lane 4, cells transfected with both DARC-myc and HA-CCR5; lane 5, mixed supernatants from lanes 1 and 2; lane 6, mixed supernatants from lanes 1 and 3; lane 7, cells transfected with both DARC-myc and HA-TRH-R; and lane 8, empty vector. B, BRET ratios from HEK-293 cells transiently transfected with the BRET constructs. CCR5/Rluc + CCR5/EGFP, a known homodimer and the Rluc-EGFP fusion protein are shown as positive controls. CCR5/Rluc + pEGFP and DARC/Rluc + TRH-R/EGFP and DARC/EGFP + TRH-R/Rluc are negative controls. The BRET ratio was assessed before (white bars) and after (black bars) the addition of ligand. The ligands used were RANTES (50 ng/ml final concentration) and thyrotropin-releasing hormone (10^{-6} M). There was no effect on the BRET ratio after the addition of PBS instead of ligand (data not shown). Where both DARC and TRH-R were expressed in the same cell, both ligands were assessed with no significant difference in the BRET ratios. Results are expressed as the mean ± S.E.M of three experiments.

Australia) using gene-specific primers and probes labeled with 5′-6-5-carboxyfluorescein and 3′ 5-carboxytetramethylrhodamine (Eurogentec, Seraing, Belgium). Reactions were incubated for 2 min at 50°C, denatured for 10 min at 95°C, and then subjected to 40 two-step amplification cycles with annealing/extension at 60°C for 1 min followed by denaturation at 95°C for 15 s. Primers and probes for human hypoxanthine phosphoribosyltransferase labeled with 5′ Yakima Yellow and 3′ 5-carboxytetramethylrhodamine (Eurogentec) were used as an internal control for quantification of the total amount of cDNA used in the reaction. Results were normalized to hypoxanthine phosphoribosyltransferase expression and presented as -fold increase in mRNA expression compared the untreated samples at each individual time point.

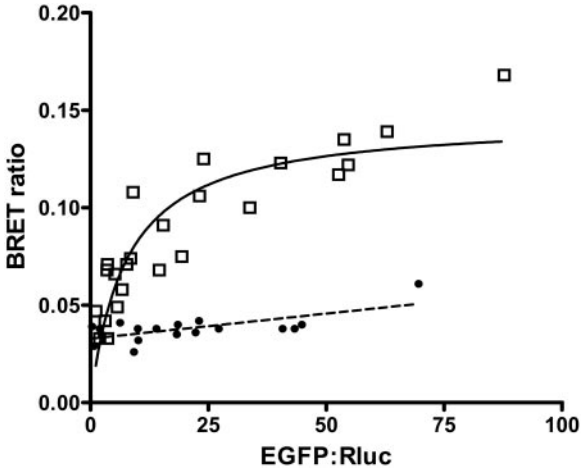

Fig. 3. Increasing acceptor to donor ratios confirms the specificity of the DARC-CCR5 interaction. BRET saturation experiments were performed by transfecting increasing ratios of acceptor to donor tagged DARC and CCR5 into cells, expressed on the abscissa as the ratio of fluorescence output/luminescence output. Specific interactions between tagged proteins result in a corresponding hyperbolic increase in BRET signal, as shown for DARC and CCR5 (□). In contrast, random interactions are characterized by a linear increase in BRET as demonstrated by the DARC-TRH-R interaction (●) that fails to saturate even at a ratio of 70:1.

Chemotaxis. Migration of L1.2 cells stably transfected with CCR5, DARC, or CCR5 and DARC was evaluated using Corning Costar 12-well plates (Fisher Scientific, Leicestershire, UK), with 3-μm pore size inserts. Before chemotaxis, cells were pretreated overnight in 5 mM butyric acid. Chemotaxis was performed in 1 ml of complete media with a range of chemokines (0–100 ng/ml) added to the lower chamber. Approximately 5×10^5 cells were placed on the upper side of the membrane in 500 μl of complete media, and chemotaxis was allowed to proceed at 37°C in a humidified incubator with 5% CO_2 for 4 h. Migrated cells were then resuspended, and they were counted by FACS.

Ca^{2+} Flux. Measurement of Ca^{2+} flux was performed using a ratiometric approach. Cells were resuspended at 5×10^6/ml, and they were loaded with the calcium indicators FuraRed (6 μg/ml) and Fluo-4 (3 μg/ml) for 30 min at 37°C in serum-free media. Samples were then washed twice in serum-free media, before resuspending in complete media. Cells were analyzed on a FACSort or FACSCalibur machine (BD Biosciences, Oxford, UK) at 37°C using a heated cuff. The ratio of FL-1 (Fluo-4) to FL-3 (FuraRed) was calculated using the Flow Cytometry Standard Press program (Ray Hicks, University of Cambridge, Cambridge, UK). Response to the calcium ionophore ionomycin (1 μg/ml) was assessed as a positive control and to ensure equivalent dye loading. For pertussis toxin pretreatment experiments, cells were incubated for 3 h in media containing 200 ng/ml pertussis toxin before indicator loading.

Receptor Internalization Assay and Calculation of Surface Receptor Expression. L1.2 CCR5 and L1.2 CCR5-DARC stable cells were pretreated with 10 μg/ml cycloheximide for 1 h before addition of RANTES, IL-8, or PBS control to assess the ligand-mediated internalization of CCR5. Ligand was added for the stated

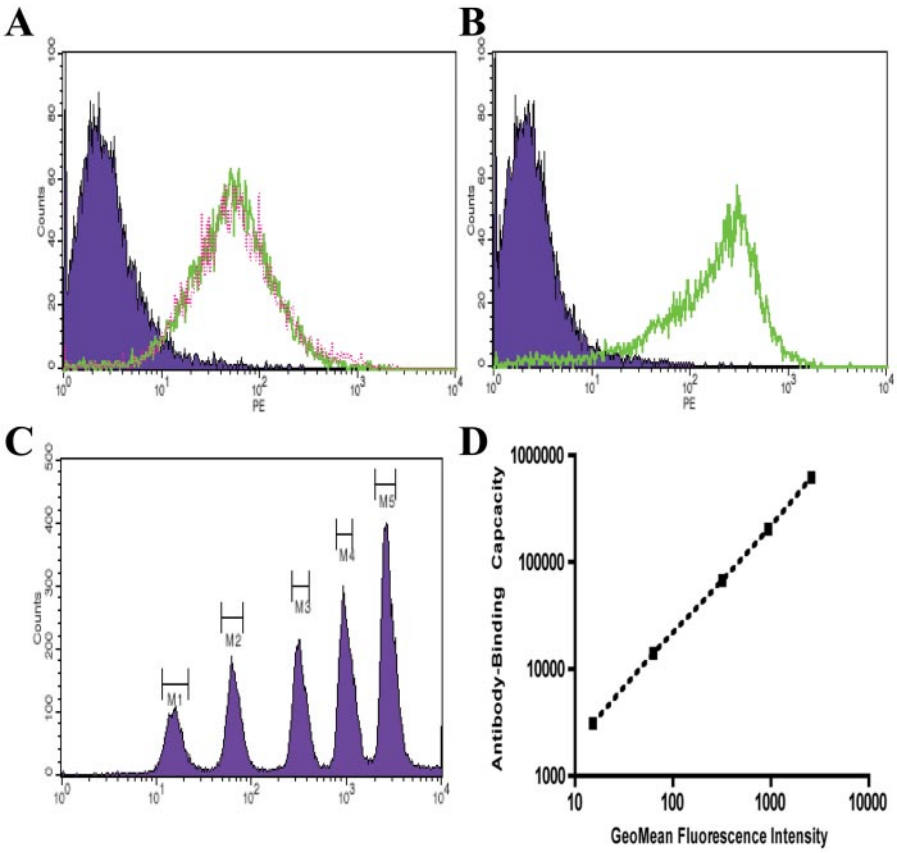

Fig. 4. L1.2 stably transfected cells express the same surface level of CCR5. Stably transfected L1.2 CCR5 and L1.2 CCR5-DARC cells were MoFlow sorted to select for populations expressing equal and physiologically relevant levels of CCR5. A, CCR5 detection on stably transfected cells post-MoFlow sorting (L1.2 CCR5, pink; L1.2 CCR5-DARC, green). B, surface DARC expression (green) on L1.2 CCR5-DARC cells. C and D, QIFIKIT quantification of surface receptor numbers. The standard curve yielded a correlation coefficient of $r^2 = 0.9988$ and the equation $y = 239.42x - 6725$, where y is receptor number and x is GeoMean fluorescence. There were an average of ~6000 CCR5 and 10,000 DARC receptors per cell, respectively.

time period (0–60 min), and then cells were placed on ice and washed in a PBS/1% bovine serum albumin/2 mM NaN_3 solution. FACS analysis using receptor-specific monoclonal antibodies, and a phosphatidylethanolamine-conjugated secondary antibody was used to quantify surface receptor expression. The level of receptor expression on transfected and stimulated primary cells was determined using a QIFIKIT (Dako UK Ltd.). In brief, beads with defined surface levels of receptors were used to create a standard curve (under saturating concentrations of antibody), relating GeoMean fluorescence to receptor numbers. GeoMean fluorescence values for transfected cells were calculated using DARC- and CCR5-specific monoclonal antibodies. Receptor numbers were interpolated from the standard curve including a correction for the fluorescence of the isotype control as described in the manufacturer's instructions.

Data Analysis. Data from BRET assays, ^{125}I-chemokine binding, chemotaxis, and calcium flux were analyzed and prepared using Prism 4.0 (GraphPad Inc., San Diego, CA).

Results

Coexpression of DARC and CCR5 on Primary Endothelial Cells. Primary human umbilical vein endothelial cells were assessed for the presence of DARC and CCR5 at both the mRNA and protein level. After treatment with TNF-α, human umbilical vein endothelial cells were shown to express both DARC and CCR5 at the cell surface by FACS (Fig. 1, A and B). In addition, the time course of transcript up-regulation and the percentage of cells expressing DARC and CCR5 over time were assessed (Fig. 1, C and D). These experiments confirm previous published data on the presence of CCR5 on endothelial cells. Furthermore, they demonstrate that CCR5 and DARC may be simultaneously expressed on primary endothelial cells.

Homodimerization and Heterodimerization of DARC and CCR5. Given the interest in GPCR dimerization, we decided to investigate whether DARC might interact with the CC chemokine receptor CCR5, a receptor that is known to form homodimers (Hernanz-Falcón et al., 2004) and is expressed on endothelial cells and shares equivalent affinity for the chemokine RANTES. After initial coimmunoprecipitation studies that supported an interaction between these receptors (Fig. 2A), we used BRET to study the dynamics of this interaction in real-time in live cells (Fig. 2B). Our data confirm that CCR5 homodimerizes and show for the first time that DARC spontaneously forms homodimers in living cells, with dimer formation being independent of the presence of ligand. In addition, we have demonstrated that DARC and CCR5 constitutively heterodimerize. The small difference noted in the BRET ratio of the CCR5-DARC heterodimer for the two different tagging orientations; DARC-Rluc + CCR5-EGFP (0.084 ± 0.003) compared with CCR5-Rluc + DARC-EGFP (0.115 ± 0.001) was consistent throughout the experiments, and it may reflect the slightly longer predicted C-terminal tail of CCR5 compared with DARC, which in turn may increase the flexibility of this region such that the larger Rluc protein (~35 kDa) may more easily interact with EGFP (~27 kDa) in this conformation. Note that there were no significant interactions seen between DARC and the control, thyrotropin-releasing hormone receptor (TRH-R), which has been previously validated in BRET experiments (Hanyaloglu et al., 2002).

DARC-CCR5 Heterodimerization Is Specific. To confirm the specificity of the DARC-CCR5 heterodimer, a series of transfections were performed using increasing ratios of EGFP-tagged to Rluc-tagged constructs (acceptor to donor). Specific interactions are characterized by a rapid nonlinear increase in the BRET ratio, which plateaus at higher ratios of acceptor to donor, such that the relationship is defined by a hyperbolic function (Milligan and Bouvier, 2005). In contrast, where the interactions between proteins are random, increasing the ratio of acceptor to donor results in a linear increase in BRET ratio (Milligan and Bouvier, 2005). Figure 3 shows the profile of the DARC-CCR5 interaction (squares), compared with DARC-TRH-R (circles).

DARC Reduces CCR5-Mediated Chemotaxis. Having observed a specific interaction between DARC and CCR5 at the cell surface, we sought evidence for a functional correlate of heterodimerization, by assessing the responses to chemokines in cells coexpressing DARC and CCR5. L1.2 stably transfected cells were developed to express approximately equal numbers of CCR5 and/or DARC receptors (at levels equivalent to those present on primary cells) before undertaking migration studies (Fig. 4). Stably transfected cells were challenged with either RANTES (Fig. 5A) or the CCR5-specific ligand LD78β (Fig. 5B) in modified Boyden chambers. Cells expressing DARC alone were unable to migrate to stimulus. L1.2 CCR5 cells yielded the expected bell-shaped curves, with maximal responses at ~30 ng/ml RANTES and 1 to 10 ng/ml LD78β, consistent with published data (Baltus et al., 2003). L1.2 CCR5-DARC-coexpressing cells demonstrated reduced chemotaxis at all concentrations tested.

DARC Inhibits CCR5 Signaling. To confirm the findings of the chemotaxis assays, additional functional studies were undertaken to assess calcium flux to various ligands. L1.2

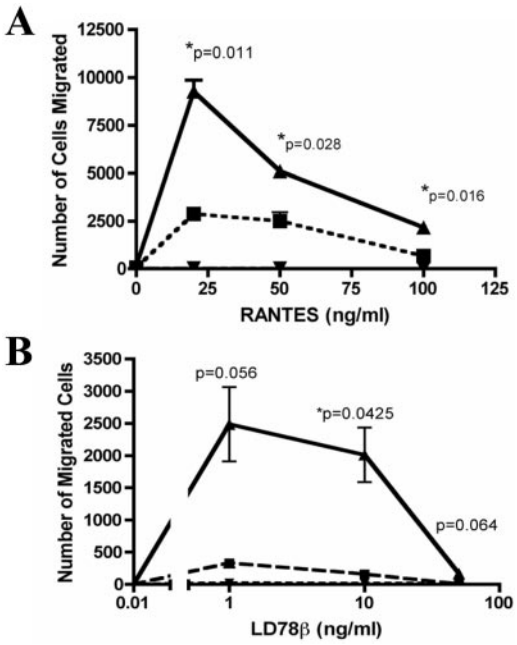

Fig. 5. CCR5-DARC dimerization impairs CCR5-mediated chemotaxis. A modified Boyden chamber assembly was used to assess chemotaxis to RANTES and LD78β in L1.2 stably transfected cells (▲, CCR5; ▼, DARC; ■, CCR5-DARC). Cells were incubated on the upper side of a semipermeable membrane (3-μm pores), with varying concentrations of chemokine in complete media present in the lower chamber. Migration was allowed to proceed for 4 h at 37°C in a humidified incubator. Cells that had migrated into the lower chamber were resuspended and counted by FACS. All chemotaxis assays were repeated in at least triplicate. The figure shows a representative result.

stably transfected cells (5×10^7) were loaded with the calcium indicator dyes FuraRed and Fluo-4, they were challenged with increasing concentrations of ligand (RANTES), and then the calcium flux was measured by FACS (Fig. 6, A–C). A progressive increase in calcium flux was seen with the L1.2 CCR5 cells in response to increasing concentrations of RANTES (Fig. 6A). No response was seen in the L1.2 DARC cells (Fig. 6B), and the L1.2 CCR5-DARC cells (Fig. 6C) displayed a markedly attenuated response, even at the highest doses of RANTES where all available receptors would be saturated with ligand. The responses to LD78β displayed a similar profile (Fig. 6D).

Signaling Occurs through Gα_i, Which Is Functional in All Cell Lines. Upon ligand binding, chemokine receptors signal through interactions with heterotrimeric G proteins, usually Gα_i, resulting in decreased cAMP levels and calcium release. To confirm that this signaling pathway was functional in each transfected cell line, cells were challenged with 50 ng/ml murine SDF-1α, a chemokine that signals through the endogenous chemokine receptor, mCXCR4. Signaling through this receptor was equivalent for all cell lines (Fig. 6E). Furthermore, the calcium flux responses of the L1.2 cell lines to mSDF-1α and LD78β were inhibited by pretreatment with pertussis toxin (Fig. 6F).

Pretreatment with DARC-Specific Ligands Fails to Restore Normal Signaling. To prove that DARC dimerization with CCR5, independently of DARC ligand binding, was responsible for the reduction in signaling observed, we pretreated L1.2 CCR5 cells and L1.2 CCR5-DARC-coexpressing cells with a series of ligands to specifically block the chemokine-binding site of DARC (Fig. 7). Two chemokines, IL-8 and MCP-1,and the DARC-specific monoclonal antibody Fy6 were used. After pretreatment with saturating concentrations of these ligands, there was no increase in calcium flux seen in response to RANTES in L1.2 CCR5-DARC cells (Fig. 7A). The response to mSDF-1α was unaffected by pretreatment (data not shown), as was the response to RANTES in L1.2 CCR5 cells (Fig. 7B).

Internalization of CCR5 Is Unaffected by Dimerization. CCR5 is internalized via a β-arrestin-dependent pathway after ligand binding (Mueller et al., 2002). We therefore examined whether coexpression of DARC with CCR5 would alter CCR5 internalization kinetics. Surface receptor numbers were calculated by FACS on cycloheximide-pretreated cells before stimulation with ligand for varying times. Receptor numbers were then calculated at each time point to establish whether there were any differences between surface receptor numbers in CCR5 stable and CCR5-DARC stable cells (Fig. 8). The CCR5 receptors on both L1.2 CCR5 and L1.2 CCR5-DARC cells show equivalent internalization kinetics after treatment with RANTES (Fig. 8A). In contrast, DARC expressed on L1.2 CCR5-DARC stable cells was not internalized in the presence of ligand (Fig. 8B). This finding is consistent with published data (Mueller et al., 2002) and

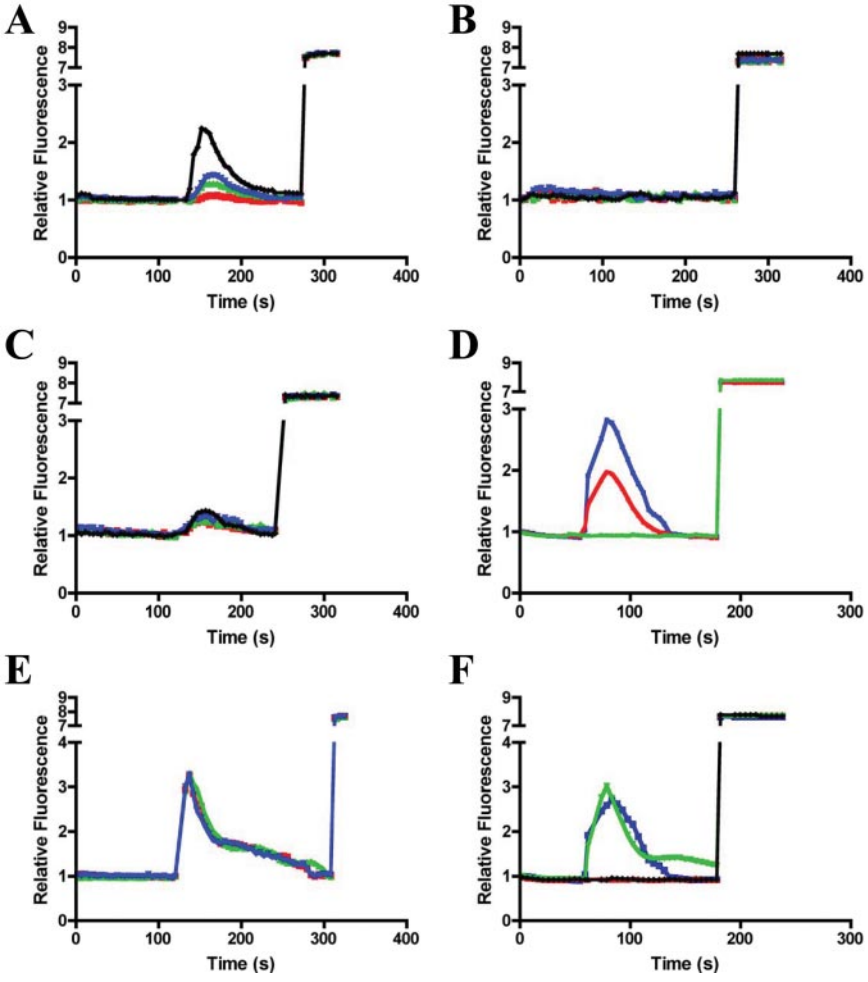

Fig. 6. CCR5-DARC dimerization impairs CCR5-mediated Ca^{2+} flux. A to C, response of L1.2 CCR5 (A) L1.2 DARC (B), and L1.2 CCR5-DARC (C) stably transfected cells to increasing concentrations of RANTES (red, 5 ng/ml; green, 20 ng/ml; blue, 50 ng/ml; black, 500 ng/ml). Calcium flux was measured by FACS using the calcium indicators Fura-Red and Fluo-4. Ionomycin (1 μg/ml) was added at the end of each experiment as a positive control and to confirm equivalent cellular loading with indicators. L1.2 cells (5×10^7) were used for each recording with a flow rate of ~1000 cells/s passing through the analyzer. D and E, response of each cell line to LD78β (L1.2 CCR5, blue; L1.2 CCR5-DARC, red; L1.2 DARC, green) and murine SDF-1α (mCXCL12), respectively. SDF-1α binds to the endogenous chemokine receptor CXCR4. This receptor uses the same signaling pathway for calcium flux as CCR5. F, inhibition of signaling through Gα_i by pertussis toxin pretreatment in L1.2 CCR5 cells. The response to LD78β is shown in blue and red (pertussis toxin), and the response to mDSF-1α is shown in green and black (pertussis toxin). All experiments were repeated in at least triplicate and representative graphs are shown.

our BRET experiments, demonstrating a ligand-dependent interaction between CCR5 and β-arrestins that is not evident for DARC (Fig. 9).

Discussion

The results of this study provide the first direct evidence that the nonsignaling chemokine receptor DARC forms constitutive homodimers in living cells. Furthermore, our experiments show that DARC has the ability to heterodimerize with CCR5. DARC and CCR5 are coexpressed in primary human endothelial cells at the mRNA and protein levels, and using BRET we demonstrated that at physiological levels of expression, coexpression of DARC with CCR5 can result in hetero-oligomers, leading to the functional consequence of impaired chemokine signaling through CCR5, as evidenced by diminished chemotaxis and calcium flux. Despite the effect of DARC coexpression on signaling, internalization of CCR5 upon ligand binding was unaffected.

Our experiments show that the interaction between DARC and CCR5 is constitutive with no increase in BRET signal after the addition of ligand (Fig. 2), consistent with DARC and CCR5 existing as preformed hetero-oligomers, cotrafficking from the endoplasmic reticulum. This is in keeping with literature that has highlighted the importance of cotrafficking, (and heterodimerization), of olfactory receptors with β_2-adrenergic receptors, GABA receptor subtypes, and adrenergic receptor subtypes, for normal expression (Marshall et al., 1999; Hague et al., 2004a,b; Uberti et al., 2005). How DARC interacts with CCR5 and the kinetics of this interaction is unknown. The experiment highlighted in Fig. 3, which was designed to test the specificity of the DARC and CCR5 interaction, also provides some limited data on the equilibrium association between these receptors. The BRET signal at a 1:1 ratio (of fluorescence output/luminescence output) is perhaps lower than would be expected for a constitutive interaction between receptors with similar affinities; however, this is in keeping with other receptors studied in the literature [e.g., the δ-opioid receptors and β-adrenergic receptors (Breit et al., 2004, 2006)]. This may reflect the existence of higher order oligomers of DARC and CCR5, or simply that energy transfer between donor and acceptor is not optimal at this ratio. The ideal baseline acceptor/donor ratio for most BRET experiments is ~4:1, because at lower ratios measuring BRET is difficult because of unfavorable energy transfer (Kroeger et al., 2001).

A further theoretical concern arising from our data is how the functional effects of heterodimerization (Figs. 5–7) can be explained through the formation of DARC-CCR5 dimer pairs. Although the affinity of DARC for CCR5 is greater than CCR5 for itself (but less than that of DARC for itself) (Fig. 2),

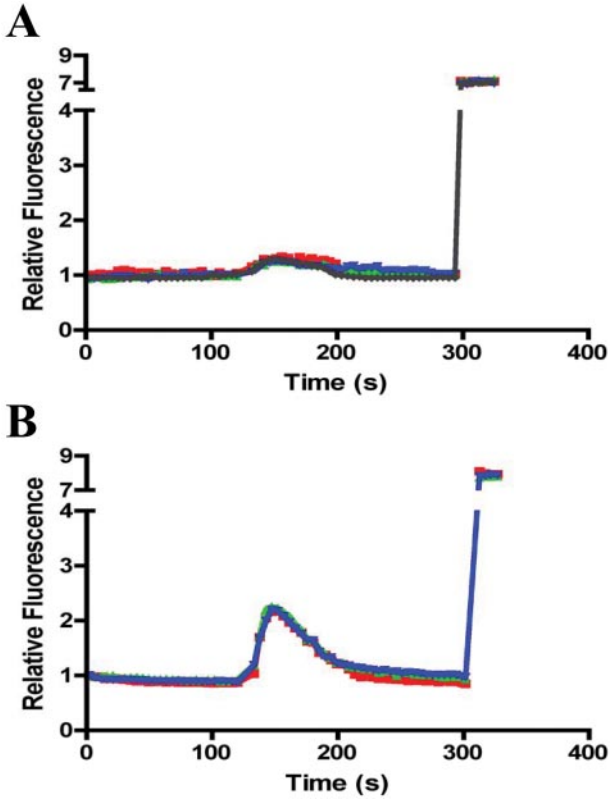

Fig. 7. Blockade of the DARC ligand-binding site does not restore normal signaling. A, Ca^{2+} flux trace from stably transfected L1.2 CCR5-DARC cells pretreated with saturating concentrations of IL-8 (green), MCP-1 (black), or the anti-DARC antibody Fy6 (blue) to a maximal dose of RANTES (500 ng/ml). The red tracing shows the response to RANTES without pretreatment. B, Ca^{2+} flux trace after equivalent treatment of stably transfected L1.2 CCR5 cells. Each experiment was repeated in triplicate, and a representative result is shown.

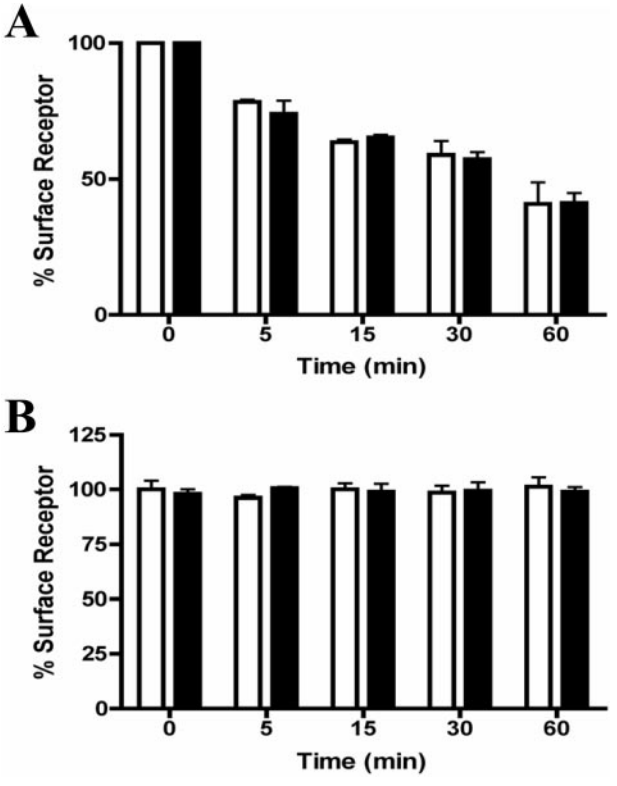

Fig. 8. Assessment of CCR5 internalization by ligand in L1.2 stable cells. A, L1.2 CCR5 (white bars) and L1.2 CCR5-DARC (black bars) stable cells were pretreated with 10 μg/ml cycloheximide for 1 h before addition of 50 ng/ml RANTES. Receptor internalization was assessed by FACS using the 2D7 monoclonal antibody. Because the L1.2 CCR5 and L1.2 CCR5-DARC cells express equivalent numbers of surface CCR5 receptors (Fig. 4), the GeoMean fluorescence at each time point was the same, and results are therefore expressed as a percentage of surface receptor numbers after treatment with IL-8 or the PBS control. Assays were repeated in triplicate, and the mean ± S.E.M. is shown. B, internalization profile for DARC on L1.2 DARC stably transfected cells in response to 50 ng/ml RANTES (white bars) and 50 ng/ml IL-8 (black bars) over the same time period.

the degree of inhibition of signaling is more than would be predicted if the effect is the result of associations between specific receptor pairs. Because we have demonstrated that the internalization of CCR5 is unaffected by the presence of DARC (Fig. 8), DARC-CCR5 interactions may inhibit chemokine signaling by altering the affinity of CCR5 for G proteins, the activity and/or association of G protein receptor kinases, or the responsiveness of CCR5 to ligand, but not its affinity for ligand.

Although DARC was identified more than half a century ago, and its ability to bind inflammatory chemokines was demonstrated more than a decade ago, the function of DARC in vivo still remains elusive (Szabo et al., 1995). That individuals in areas prone to *Plasmodium vivax* malaria have developed erythroid-specific promoter mutations that abolish expression of DARC on red blood cells, yet retain expression of DARC in other tissues argues for an important physiological role for DARC (Tournamille et al., 1995; Zimmerman et al., 1999). However, this evidence needs to be married with data from both DARC knockout mice and individuals who fail to express DARC as a result of rare coding region mutations, yet have an apparently normal phenotype (Mallinson et al., 1995; Luo et al., 2000).

The prominent expression of DARC on red blood cells initially favored the hypothesis that DARC acts simply as a chemokine "sink" in vivo, binding circulating chemokines and preventing them from reaching effector cells (Neote et al., 1993). This view was supported by studies on DARC knockout mice challenged with intraperitoneal LPS that showed an exaggerated inflammatory response (Dawson et al., 2000). However, further studies on another DARC$^{-/-}$ mouse strain looking at a different time point and dose of LPS displayed opposing results (Luo et al., 2000). Rather than sequestering chemokines, recent reports have suggested a role for red blood cell-expressed DARC in maintaining circulating concentrations of chemokines with diminished chemokine levels in persons lacking red blood cell DARC expression and rapid clearance of injected chemokines from mice that lack DARC on their red blood cells (Fukuma et al., 2003).

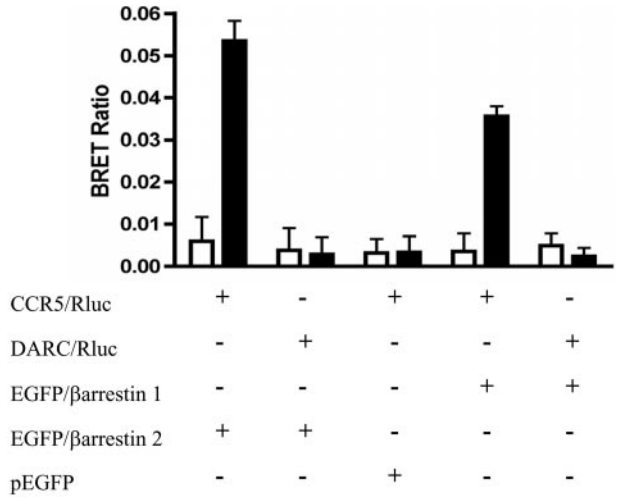

Fig. 9. DARC does not interact with either β-arrestin 1 or β-arrestin 2. HEK-293 cells were transfected with either CCR5/Rluc or DARC/Rluc and EGFP-β-arrestins, and the BRET ratios were assessed before and after addition of ligand (500 ng/ml RANTES). The interaction of CCR5/Rluc with pEGFP is shown as a negative control. Results are expressed as the mean ± S.E.M of three experiments.

A second theory is that DARC may have a role in chemokine presentation and transfer of chemokines across the endothelium (Rot, 2003). Studies have suggested that transport of chemokines from the abluminal to the luminal surface of endothelial cells could be mediated by DARC and that DARC may have a role at intercellular junctions (Lee et al., 2003). Given the selective chemokine-binding profile of DARC, this may also represent a mechanism to modulate inflammation through presentation of a restricted subset of chemokines.

In this study, we provide evidence consistent with a third potential mechanism by which DARC may regulate chemokine signaling, through heterodimerization with other chemokine receptors. In a series of functional studies in a stable cell line coexpressing DARC and CCR5 signaling through CCR5 was attenuated without affecting its internalization. Although novel, these data are not contrary to previous evidence on the role of DARC in vivo, but instead they suggest that the regulation of chemokines and chemokine receptor signaling may be considerably more complex than previously thought. Heterodimerization of active chemokine receptors with nonsignaling receptors may serve to create new patterns of response to CC and CXC chemokines.

Despite the critical role of chemokine receptors in development and the trafficking of immune cells, there are relatively few publications addressing the role that dimerization, particularly heterodimerization, may play in vivo. As evidence accumulates that dimerization of GPCRs may be a common theme in receptor biology, our data suggest that the expression of nonsignaling chemokine receptors, such as DARC, may act as negative feedback regulators to limit the signaling capacity of receptors they associate with, but still permit their internalization and desensitization by chemokine ligands.

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Address correspondence to: Dr. David R. Greaves, Sir William Dunn School of Pathology, University of Oxford, South Parks Rd., Oxford OX1 3RE, UK. E-mail: david.greaves@path.ox.ac.uk