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CCR6 Colocalizes with CD18 and Enhances Adhesion to Activated Endothelial Cells in CCR6-Transduced Jurkat T Cells

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CCR6 is expressed by memory T cells (mTC) and is a requirement for efficient arrest of a subset of mTC to activated human dermal microvascular endothelial cells (HDMEC) under physiologic shear stress. We now address whether CCR6 alone is sufficient to induce arrest of a model T cell line (Jurkat) that shows low expression of all CCRs tested (CCR1–10). Herein, we transduced Jurkat (JK) T cells expressing fucosyltransferase VII with a chimeric chemokine receptor consisting of CCR6 fused to enhanced green fluorescent protein. In contrast to the starting JK lines, the resulting cell line (JK fucosyltransferase VII-CCR6) migrated 6-fold better to CCL20 in chemotaxis assays, arrested in response to CCL20 that was immobilized to plastic, and demonstrated a 2.5-fold increase in adhesion to activated HDMEC ($p = 0.001$). Adhesion was blocked by anti-CD18 mAb ($p = 0.005$) but not by anti-CD49d mAb ($p = 0.3$). After arrest on recombinant substrates, CCR6 clustered on the surface as detected by real-time observation of enhanced green fluorescent protein fluorescence. Dual-label confocal microscopy revealed that LFA-1 (CD18 and CD11a), but not CXCR4, colocalized with clustered CCR6 in the presence of immobilized CCL20. Thus, the functional expression of CCR6 is sufficient to provide the chemokine signaling necessary to induce arrest of a JK T cell line to activated HDMEC. Clustering of CCR6 and coassociation with critical integrins may serve to strengthen adhesion between T cells and activated endothelial cells. ***The Journal of Immunology,*** **2002, 169: 2346–2353.**

The migration of leukocytes from the bloodstream to tissue is a key event in the process of inflammation. Chemokines and their receptors have been shown to contribute to this event and play critical roles in leukocyte trafficking (1). Indeed, T cell subsets migrate to different sites of the body because of their differential expression of specific adhesion molecules and chemokine receptors (2).

Attachment of leukocytes to the blood vessel wall occurs initially via transient (rolling) interactions mediated by selectins (3). For instance, E-selectin, is up-regulated by inflamed dermal endothelial cells and is thought to mediate the binding of a subset of skin-homing memory T cells (mTC)[2] that express specific carbohydrate-based E-selectin ligands located on a protein backbone of P-selectin glycoprotein ligand 1 (4). Enzymes such as α(1,3)fucosyltransferase VII (FT7) are essential for the T cells to decorate P-selectin glycoprotein ligand 1 with appropriate E-selectin carbohydrate ligands (5, 6). The E-selectin-binding epitopes have been termed cutaneous lymphocyte-associated Ag (CLA) when detected by the HECA452 mAb (7).

In the multistep model of leukocyte recruitment, T cell rolling is followed by chemokine-triggered, integrin-mediated firm adhesion (3). Several chemokines, including CXCL12 (stromal cell-derived factor-1), CCL21 (secondary lymphoid-tissue chemokine), CCL17 (thymus and activation-regulated chemokine), and CCL20 (liver and activation-regulated chemokine) have been shown to induce arrest of T cells to recombinant adhesion molecules in vitro (8–10).

Chemokines trigger changes in both LFA-1 (CD11aCD18 integrin) affinity and avidity, both of which may contribute to integrin-mediated firm arrest (11). Notably, inhibitors of integrin lateral mobility (i.e., calpeptin) block adhesion of lymphocytes to ICAM-1 at low, but not high, site densities (11). Also, chemokine receptors on T cells polarize in response to ligand and cluster at the leading (migrating) edge of the cell (12), although neutrophils do not show this response and may redistribute intracellular molecules such as the AKT protein kinase to the migrating edge of the cell (13).

CCR6 is involved in dendritic cell trafficking to lymphoid organs (14, 15) and is also exclusively expressed by memory, but not naive, T cell subsets (16). CCR6 may be involved in skin homing as suggested by its up-regulation in the inflammatory skin condition known as psoriasis (17) and by apparent skin-homing defects in T cells from CCR6-null mice (15). CCL20, a ligand for CCR6, is strongly up-regulated in both HUVEC and human dermal microvascular endothelial cells (HDMEC) by inflammatory cytokines such as TNF-α (10, 18). Our previous study showed that inhibition of CCR6 effectively prevented the arrest of mTC on TNF-α activated HDMEC under shear stress, suggesting that CCR6 was required for efficient arrest of a arrest of a subset of mTC on acutely inflamed vascular endothelium (10).

Herein, we transduce Jurkat (JK) T cells with a retroviral vector that expressed a fusion protein consisting of CCR6 linked to enhanced green fluorescent protein (EGFP). Using these transduced

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[2] Abbreviations used in this paper: mTC, memory T cell; FT7, α(1,3)fucosyltransferase VII; EGFP, enhanced green fluorescent protein; PTX, pertussis toxin; HDMEC, human dermal microvascular endothelial cell; CCL, CC chemokine ligand; CLA, cutaneous lymphocyte-associated Ag; CXCL, CXC chemokine ligand; JK, Jurkat; Ct, cycle threshold number.

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JK cells, we demonstrate that the expression of CCR6 results in a 2- to 3-fold increase in T cell adhesion to activated endothelial cells. Moreover, we take advantage of the EGFP-tagged CCR6 protein in order to follow the reorganization of CCR6 following arrest in real time and in order to detect CCR6's localization with respect to β_2 integrin. We show that with respect to chemokine receptors, CCR6 alone is sufficient to increase arrest of JK cells on activated microvascular endothelial cells and suggest that CCR6-mediated clustering of selected integrins may play a role in strengthening adhesion of T cells to endothelial cells.

Materials and Methods

Reagents and cells

Anti-human β_1 integrin mAb (33B6) (19) was a gift of Dr. S. I. Simon (University of California, Davis, CA). Anti-human CD11a mAb (R3.1) (20) was provided by Dr. R. Rothlein (Boehringer-Ingelheim, Ridgefield, CT). All other anti-human mAb were purchased from BD PharMingen (San Diego, CA) unless otherwise indicated. Recombinant human chemokines were purchased from PeproTech (Rocky Hill, NJ). Human E-selectin/Ig chimera (21) was provided by Dr. S. Rosen (University of California, San Francisco, CA). Soluble human ICAM-1 and a nonfunction-blocking mouse anti-human ICAM-1 mAb (P79, mouse IgG1) were gifts of Dr. T. Kishimoto (Boehringer-Ingelheim). FT7-transfected JK T cell line (JKFT7) (22) and subsequent transduced lines were cultured in complete RPMI 1640 medium with 10% FCS (cRPMI). Recombinant human VCAM-1/Ig chimera was purchased from R&D Systems (Minneapolis, MN). HDMEC were obtained from the Emory University School of Medicine Dermatology Department cell culture facility (Atlanta, GA), cultured as described (23), and used in passages 3–6.

Construction of CCR6-EGFP fusion vector

Using a CCR6 cDNA clone provided by Dr. J. Farber, (National Institute of Allergy and Infectious Diseases, Bethesda, MD), we PCR-amplified the CCR6 open reading frame using the forward primer (5′-AAA CTC GAG ATG AGC GGG GAA TCA ATG A-3′) and the reverse primer (5′-AGC AAG CTT CAT AGT GAA GGA CGA CGC AT-3′). This amplified CCR6 fragment was digested with *Xho*I and *Hind*III and inserted into retroviral vector pLEGFP-N1 at complementary sites (Clontech, Palo Alto, CA). Thus, after construction, the PCCR6-LEGFP-N1 vector contained an open reading frame for EGFP that was located at 57 bp downstream of the 3′ end of the CCR6 cDNA.

Transduction and selection of transduced cells

GP-293 packaging cells (Clontech) were cultured in a 150-mm culture dish 1 day after transfection using FuGene6 reagent (Roche Molecular Biochemicals, Indianapolis, IN). After cells reached 50–70% confluency, FuGene6 (60 μl) was added to 1.5 ml serum-free medium and gently mixed with 10 μg of pCCR6-EGFP (or pLEGFP) together with 10 μg pVSVG viral coat protein vector. After incubation at 20°C for 30 min, this mixture was added to the GP-293 packaging cells and incubated at 37°C for 3 days.

Virus was harvested from cultured supernatants at 48 and 72 h after transfection, and filtered through a 0.45-μm polyvinylidene difluoride filter (Millipore, Bedford, MA). The viral supernatant was concentrated by repeated centrifugation at 80,000 $\times$ *g* for 2 h at 4°C. The resulting pellet was resuspended in 3 ml of fresh RPMI 1640 medium with polybrene (8 μg/ml; Sigma-Aldrich, St. Louis, MO) and then added 0.5 ml/well to JKFT7 cells that had been subcultured into six-well culture dishes coated with fibronectin. Seven days after transduction, EGFP-positive JKFT7 cells were initially isolated by FACS using a water-cooled argon-ion laser emitting at 488 nm for excitation and a 530/30-nm narrow bandpass filter for emission detection (FACSVantage SE; BD Biosciences, Mountain View, CA). One week later, further purification of CCR6-EGFP-positive cells was carried out using FITC-labeled anti-CCR6 mAb (R&D Systems) followed by anti-FITC-coated magnetic beads and positive magnetic selection (Miltenyi Biotec, Auburn, CA).

Flow cytometric analysis

Expression of chemokine receptors on JKFT7 cells was confirmed by staining cells with PE-labeled CCR6-specific or PE-labeled anti-CXCR4 mAb (both from BD PharMingen) in PBS with 1% BSA. After incubation at 4°C for 30 min, cells were washed twice with PBS with 0.1% BSA and analyzed using a FACScan (BD Biosciences) flow cytometer. Anti-integrin Abs were used as unlabeled primary mAb (anti-CD29) followed by labeling with a PE-conjugated secondary Ab or were used as described above as PE-conjugated primary mAbs (anti-CD11a, CD18, and CD49d).

Chemotaxis assay

Microchemotaxis chambers (ChemoTx no. 101-8, 8-μm pore size; NeuroProbe, Gaithersburg, MD) were used for chemotaxis assays as described (24). cRPMI (29 μl) containing chemokines were loaded in the lower wells. JKFT7 cells transduced with EGFP alone (JKFT7-EGFP) or CCR6-EGFP (JKFT7-CCR6) were stained with calcein-AM for 15–30 min at 37°C (Molecular Probes, Eugene, OR) followed by washing with PBS and resuspension in the cRPMI. Cells in 25 μl of cRPMI were applied to top of the filter in triplicate and allowed to incubate for 3 h at 37°C. The contents of the lower wells were then carefully pooled together, brought to a final volume of 300 μl and then counted on a flow cytometer for 90 s.

In vitro flow assays

E-selectin/human IgG (100 μl at 1 μg/ml) and an anti-human ICAM-1 mAb (p79, 1 μg/ml) were applied overnight to nontreated plastic culture dishes in TBS as described (10). After a brief rinse with TBS, chemokines (40 μl, 10 μg/ml) in TBS were applied for 1.5 h at room temperature. After rinsing twice in TBS, soluble ICAM-1 (50 μl, 10 μg/ml) in PBS with 1% BSA was applied to the plate for 1.5 h at room temperature. Both JKFT7 cells which expressed EGFP only or expressed CCR6-EGFP were collected and resuspended at 0.5×10^6/ml in cRPMI in 12-ml syringe, which was fixed to a precision syringe pump (Harvard Apparatus, Holliston, MA). A parallel plate flow chamber apparatus (Glycotech, Rockville, MD) was affixed to the tissue culture plate and flow was adjusted to achieve a shear stress of 1.5 dyne/cm^2. Six minutes after the initiation of flow, four randomly selected fields were videotaped for 10–20 s under phase microscopy and subjected to computer-aided video analysis. NIH Image 1.62 with macros provided by K. Tangemann (Novartis, Vienna, Austria) was used for quantification of rolling and arrested cells as described (10, 25).

For flow assays on cultured HDMEC, cells were grown to confluence in 35-mm tissue culture dishes and stimulated with 10 ng/ml recombinant human TNF-α for 4–5 h at 37°C. In some cases, JK cells were incubated with mouse anti-human CD18 or CD49d mAb (10 μg/ml for 30 min) prior to initiation of the flow assay. Other cells were either untreated or treated with pertussis toxin (PTX; 100 ng/ml; Sigma-Aldrich) for 1.5 h at 37°C. All cells were labeled with calcein-AM (1 uM for 20 min at 37°C). After washing with PBS, cells were resuspended in cRPMI at 0.3×10^6 cells/ml and then allowed to flow over recombinant human TNF-stimulated HDMEC at a shear stress of 1.5 dynes/cm^2. Five minutes after the initiation of flow, images were captured under fluorescent illumination (excitation 485 nm, emission 515 nm) with an exposure time of 2 s. Under these conditions, only cells that had firmly arrested on the endothelial monolayers would generate a single-cell image. Rolling cells appeared as streaks because of movement during the long exposure time as previously described (10). Statistical analyses were performed with Microsoft Excel using two-sided Student *t* tests.

Confocal microscopy

JKFT7 cells expressing CCR6-EGFP were allowed to flow over plastic plates coated with E-selectin/Ig, ICAM-1, and CCL20 as described above. Washing was then carried out by pumping cRPMI over the attached cells for an additional 5 min at 1.5 dynes/cm^2. The attached cells were stained with 5 μg/ml unlabeled mouse anti-human integrin chain mAb on ice for 30 min. After carefully washing twice with PBS/0.1% BSA, the cells were fixed with Cytofix buffer (BD PharMingen, San Diego, CA) on ice for 30 min followed by washing with BD PharMingen staining buffer containing FBS (BD PharMingen). Cells were then sequentially labeled with biotin-labeled goat anti-mouse IgG (Caltag Laboratories, San Francisco, CA) for 20 min on ice and streptavidin-Cy3. The cell surface distribution of CCR6-EGFP and integrins were then analyzed with a Nikon PCM2000 confocal microscope (Nikon, Melville, NY).

Isolated mTC were also allowed to flow over TNF-α-activated HDMEC as previously described (10). After 5 min of flow followed by washing, attached cells were fixed with Cytofix and stained with anti-CCR6 mAb (R&D Systems), a biotin-labeled secondary Ab, and streptavidin-Cy3.

Quantitative real-time RT-PCR

RNA extraction and reverse transcription were performed as described (26). Real-time quantitative PCR (PerkinElmer ABI7700; PerkinElmer, Wellesley, MA) was performed with duplicate samples using SybrGreen

dye and human primer pairs (see Table I) derived from Genbank sequences. All primer pairs were designed to give products between 65 and 75 bp in length, gave rise to single band products, and had similar efficiencies of exponential amplification. Cycle threshold numbers (Ct) were derived from the exponential phase of PCR amplification.

Results

Characterization of FT7-expressing JK T cells

For effective rolling and arrest of mTC to activated HDMEC under shear stress conditions, we have found that human mTC must express E-selectin ligand as detected by mAb recognizing the CLA marker. In conjunction with this, E-selectin must be expressed by the activated HDMEC. As shown in Fig. 1*A*, total mTC depleted of CLA^+ cells showed ~70% less arrest compared to total mTC. Likewise, treatment of the endothelial cells with a function-blocking anti-E-selectin mAb markedly reduced mTC arrest. Thus, in contrast to large vessel endothelial cells (e.g., HUVEC), which have been reported to support the rolling of $CD4^+$ T cells via VCAM-1 and P-selectin and are not dependent on E-selectin expression (27), E-selectin appeared to be critical for rolling and, indirectly, arrest of mTC on HDMEC. This important difference may be due in part to differential regulation of E-selectin and VCAM-1 expression on HUVEC vs HDMEC (28, 29).

To test the hypothesis that CCR6 was sufficient to stimulate arrest of a T cell on activated endothelial cells in the presence of obligatory adhesion molecules, we required a T cell line that was largely devoid of chemokine receptors and that was also capable of rolling and arresting on activated HDMEC in an E-selectin-dependent manner. Isolated peripheral blood T cells expressed several chemokine receptors including CCR4 and CCR7 and, thus, were unsatisfactory for our objectives. We then characterized the JK T cell line for chemokine receptor expression and found that they expressed little mRNA for CCR 1–10, including CCR6 (Fig. 1*B*). They did express CXCR4, the receptor for stromal cell-derived factor-1 (CXCL12) (Fig. 1*B*). JK T cells also did not express detectable CLA epitope by flow cytometry (data not shown), and were unable to effectively roll on E-selectin-coated plates in vitro under shear stress (Fig. 1*C*), but stable transfection of these cells with FT7 allowed the resulting transfected JK cells (JKFT7) to express CLA (22) and to efficiently roll on E-selectin-coated plates (Fig. 1*D*).

Table I. *Primers used for analysis of the expression of chemokine receptors in JK cells by quantitative RT-PCR*

Receptor	Forward/ Reverse[a]	Primer Sequences
CCR1	F	5′-GACGGAGGTGATCGCCTACA-3′
	R	5′-CGGAACCTCTCACCAACGAA-3′
CCR2	F	5′-TGAGTAACTGTGAAAGCACCAGTCA-3′
	R	5′-GCAGTGATTCATCCCAAGAGTCT-3′
CCR3	F	5′-TCGTTCTCCCTCTGCTCGTT-3′
	R	5′-GCCGGATGGCCTTGTACTTT-3′
CCR4	F	5′-CACTACTGTGGGCTCCTCCAA-3′
	R	5′-TCCATGGTGGACTGCGTGTA-3′
CCR5	F	5′-TGGCCTGAATAATTGCAGTAGCT-3′
	R	5′-TGCTTCATCCCAAGAGTCTCT-3′
CCR6	F	5′-GGACCGGTACATCTCCATT-3′
	R	5′-TGCTGCGCGGTAGTGTTCT-3′
CCR7	F	5′-GCTCCAGGCACGCAACTTT-3′
	R	5′-ACCACGACCACAGCGATGA-3′
CCR8	F	5′-GGGTCCCATTCAACGTGGTT-3′
	R	5′-GGCTTATGCTACATCCATCCAAG-3′
CCR9	F	5′-GACGCCTATGCCATGTTCAT-3′
	R	5′-TGGGTGACCTGGAAGCAGAT-3′
CCR10	F	5′-GGGGATGAAGAGGACGCATACT-3′
	R	5′-CCTGGACATCGGCCTTGT-3′
CXCR4	F	5′-TATGCCTTTCCTTGGAGCCAAA-3′
	R	5′-GCTGGACCCTCTGCTCACA-3′
CXCR5	F	5′-CCCAAGAGAACCAAGCAGAAA-3′
	R	5′-GAATCCCGCCACATGGTAGA-3′

[a] F, Forward; R, reverse.

Construction and characterization of CCR6-EGFP fusion protein

We next constructed a chimeric CCR6 in which CCR6 was fused at its C-terminal end to EGFP (see *Materials and Methods*) such that the resulting receptor could be visualized by fluorescence microscopy or flow cytometry. Similar chemokine receptor-EGFP chimeric receptors appear to be fully functional and comparable to the wild-type receptor (30). After transducing JKFT7 cells with this CCR6-EGFP construct followed by initial sorting and selection, 95% of the resulting transduced cells (JKFT7-CCR6) were EGFP, CCR6, and CXCR4 positive (Fig. 2*A*). However, the expression of CCR6-EGFP tended to decrease slightly over time. Thus, all experiments were performed on cells within 3 wk of transduction and selection when EGFP expression was >70% positive. The CCR6-EGFP fusion protein was evenly distributed on the cell membrane in resting cells (Fig. 2*B*), whereas cells transduced with EGFP alone expressed EGFP uniformly in cytoplasm (data not shown). By flow cytometric analysis, JKFT7-CCR6 cells did not express β_7 integrin, but were uniformly positive for CD18 (β_2), CD11a (α_L), CD49d (α_4), and CD29 (β_1) integrin chains (Fig. 2*C*).

To test whether the transduced CCR6-EGFP fusion protein was functional in JKFT7 cells, nontransduced and JKFT7-CCR6 cells were used in chemotaxis assays as described in *Materials and Methods*. As shown in Fig. 3, JKFT7-CCR6 cells showed a 6-fold specific response to the CCR6 ligand, CCL20, whereas JKFT7 cells showed no migration to this chemokine. Neither line responded to CCL17, but both responded to CXCL12, demonstrating that both lines were capable of chemotactic migration.

Enhanced arrest of JKFT7-CCR6 cells on E-selectinIg/ICAM-1/ CCL-20-coated plastic plates under physiological shear stress

Because the chemotactic response of lymphocytes to a particular chemokine does not always correlate with the ability of that chemokine to induce arrest under shear stress conditions (8), we introduced JKFT7-CCR6 and control JKFT7-EGFP cells into a flow chamber coated with recombinant E-selectin, ICAM-1, and various chemokines. As shown in Fig. 4, control JKFT7-EGFP cells arrested poorly on CCL20-coated plates, but arrested well on CXCL12-coated plates. However, JKFT7-CCR6 cells arrested in the presence of CCL20 to the same level observed with CXCL12. The weak stimulation in adherence in the presence of CCL20 for both cell lines was most likely nonspecific, because adherence in the presence of CCL17 was also marginally increased to the same degree. At shear stresses higher than 1.5 dyn/cm^2, e.g. 2.0, 2.5, and 3.0 dynes/cm^2, the number of rolling and arrested cells decreased; yet at all shear stresses tested, there were greater numbers of arrested JKFT7-CCR6 than control cells (data not shown). Thus, expression of CCR6 stimulated the ability to JK T cells to arrest on recombinant adhesion molecules coated to plastic in the presence of CCL20.

Expression of CCR6 enhances arrest of JKFT7 cells on activated HDMEC

The arrest of mTC to activated HDMEC under shear stress conditions can be blocked by ~70% by saturation of CCR6 with CCL20, by PTX treatment, or by depleting total mTC of the $CCR6^+$ subset of mTC (10). These results suggested that CCR6

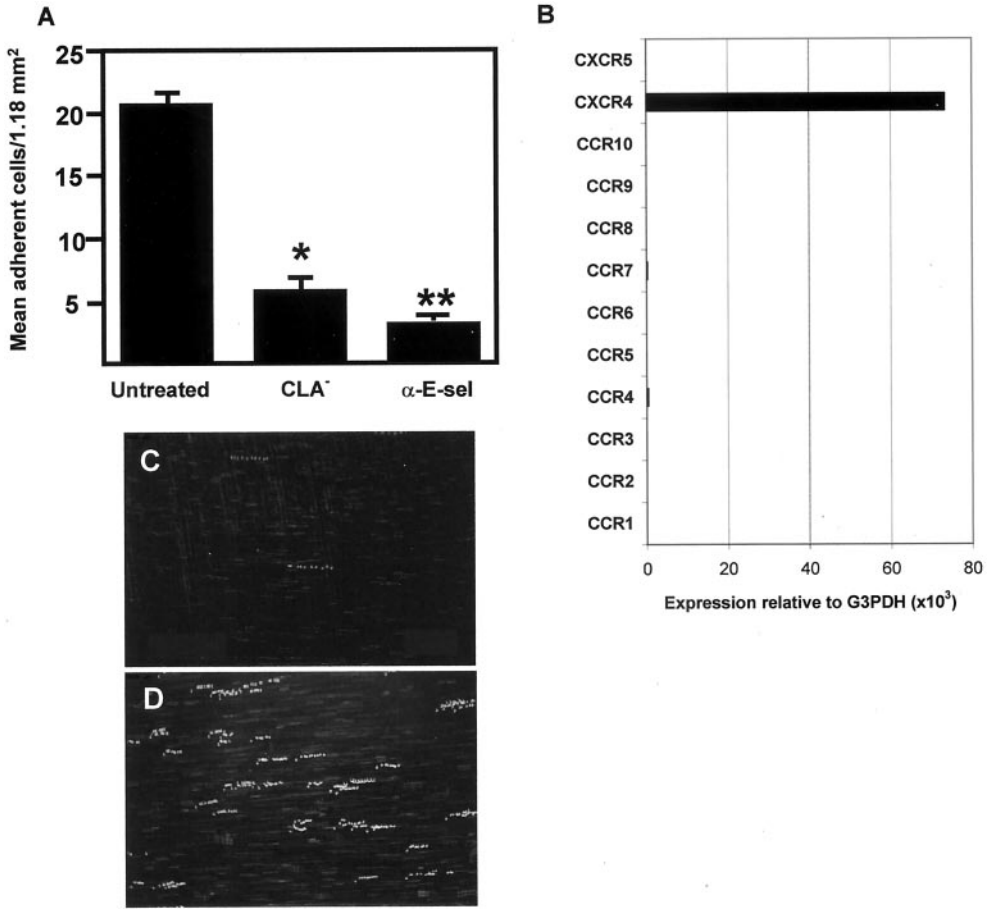

FIGURE 1. CLA-dependent mTC and JK rolling. *A*, Calcein-labeled untreated total mTC (untreated and α-E-sel) or mTC depleted of CLA^+ cells by immunomagnetic bead selection (CLA^-) were introduced at a sheer stress of 1.5 dynes/cm^2 into a flow chamber containing a monolayer of TNF-α-activated HDMEC that were either untreated or treated with 5 μg/ml anti-human E-selectin mAb (α-E-sel) (Endogen, Cambridge, MA) for 30 min prior to the flow assay. Arrested cells were quantified as described in *Materials and Methods*. *, $p < 0.0001$ vs untreated, **, $p = 0.08$ vs CLA^- mTC (student *t* test, two-sided). *B*, Quantitative, real-time RT-PCR of chemokine receptor mRNA expression by JKFT7 cells. Relative expression values were obtained by the equation: relative expression chemokine receptor ($y = 2^{-(Ct(y)-Ct(G3PDH))}$). JK (*C*) or JKFT7 (*D*) cells (2×10^5 cells/ml) were introduced into a parallel plate flow chamber coated with recombinant E-selectin/Ig chimera. Five minutes after introduction of cells, representative fields were video-recorded for 10 s using phase microscopy (1.18 mm^2/field). NIH Image 1.62 was used to superimpose images at 1-s intervals to observe individual tracings of rolling cells (white dots). Note that the spacing between JKFT7 cells is much closer than that observed with JK cells, indicating lower rolling velocities as well as fewer cells in general interacting with the plate.

was necessary for arrest of a subset of mTC to activated HDMEC under in vitro conditions, but we could not demonstrate that, among chemokine receptors, CCR6 was sufficient for effective arrest to occur. Therefore, we introduced JKFT7-CCR6 and control JKFT7-EGFP cells at a physiologic flow rate into a parallel plate flow chamber containing activated HDMEC. Interactions in the absence of TNF-α pretreatment were negligible, and arrested cells were rarely observed (data not shown). The two cell lines were labeled with fluorescent dye, calcein, which allowed us to quantify arrested and rolling cells. After exposure of HDMEC monolayers to TNF-α, calcein-labeled cells were observed to transiently bind, roll, and arrest. In the case of EGFP-transduced JKFT7 cells, a low number of arrested cells were observed at baseline (Fig. 5). This likely indicated the existence of a CCR6-independent cell arrest pathway in JKFT7 cells. CCR6-EGFP expressed in JKFT7 cells significantly enhanced cell arrest on activated HDMEC by 2- to 3-fold (Fig. 5). Interestingly, the enhanced arrest in JKFT7-CCR6 cells was abolished by treating cells with anti-CD18 mAb which targets β_2 integrin, but not by anti-CD49d (α_4 integrin) mAb. PTX, which blocks the G_i-protein-coupled signal transduction pathway, also completely suppressed the enhanced arrest observed with the JKFT7-CCR6 cells. Decreased rolling cells were also observed in both $CCR6^+$ and $CCR6^-$ JK cells that had been treated with PTX and anti-CD18 mAb, a finding which might be explained by a requirement of functional β_2 integrin/ICAM-1 adhesion for optimal selectin-mediated rolling (31). Thus, the expression of CCR6 in JKFT7 cells effectively stimulates arrest that is PTX-sensitive and β_2, but not α_4, integrin-dependent.

Reorganization of CCR6 and colocalization with β_2 integrin in JKFT7-CCR6 cells after exposure to immobilized CCL20

Previous reports have suggested that chemokine receptors on lymphocytes redistribute to the leading edge of polarized migrating cells in response to a soluble, chemotactic ligand (12) and that ligation of chemokine receptors induces the lateral movement of β_2 integrins on lymphocytes (11). Therefore, we hypothesized that chemokine receptors may colocalize with integrins. To test this hypothesis, we took advantage of the fact that EGFP was fused to the C-terminal end of CCR6 in our JKFT7-CCR6 cells, allowing us to follow CCR6 distribution both in real time and after fixation. After JKFT7-CCR6 cells arrested on recombinant substrates and CCL20 as in Fig. 4, we stained the arrested cells in separate plates

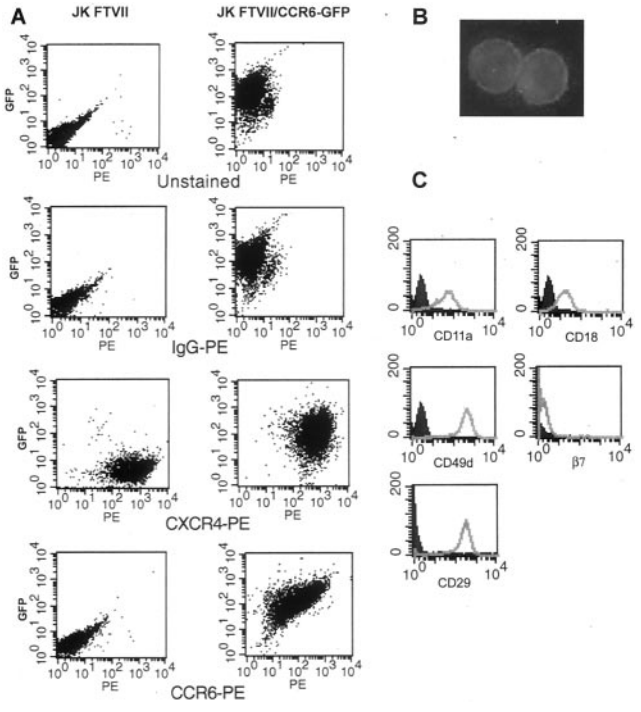

FIGURE 2. Expression of CCR6-EGFP by JKFT7 cells. *A*, JKFT7 and JKFT7-CCR6 cells were either left unstained or stained with the indicated mAb and analyzed by flow cytometry (as described in *Materials and Methods*) with EGFP intensity displayed on the *y*-axis and PE intensity on the *x*-axis. *B*, JKFT7-CCR6 cells in the absence of chemokine demonstrate uniform membrane distribution of the CCR6-EGFP under fluorescence illumination. *C*, Staining of JKFT7-CCR6 with the indicated anti-integrin chain mAb. Gray histogram, Anti-human integrin mAb as indicated. Filled histogram, Isotype-matched control mAb.

with mAb to CD18 (Fig. 6, *A–C*), CD11a (Fig, 6, *D–F*), and CD49d (Fig. 6, *H–J*). With CD18 and CD11a, there was clear colocalization of CCR6 (green) with CD18 and CD11a (red). The overlap of red and green signals can be seen in Fig. 6, *A* and *D* as a yellowish hue. CD49d did not appear to strictly colocalize with CCR6, although we did observe individual cells (Fig. 6, *H–J*, cell on right) that hinted at possible colocalization. Repeated experiments in which VCAM-1/Ig (in addition to ICAM-1 and E-selectin/Ig) was coated onto tissue culture plates revealed that clearer

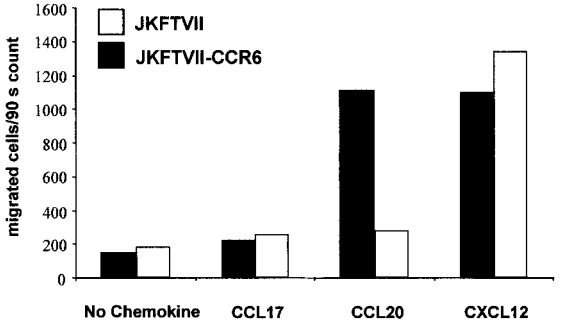

FIGURE 3. CCL20-induced cell migration of CCR6 transformed JKFT7. CCR6 transduced (■) and nontransduced JKFT7 cells (□) were used in microchemotaxis assays as described in *Materials and Methods*. After 3 h, migrated cells from three chambers per condition were pooled and counted for 90 s (representing ~25% of the total volume of the sample) using a flow cytometer. The chemokines tested were present at 100 ng/ml. The experiments shown represents one of at least three independent experiments with similar results.

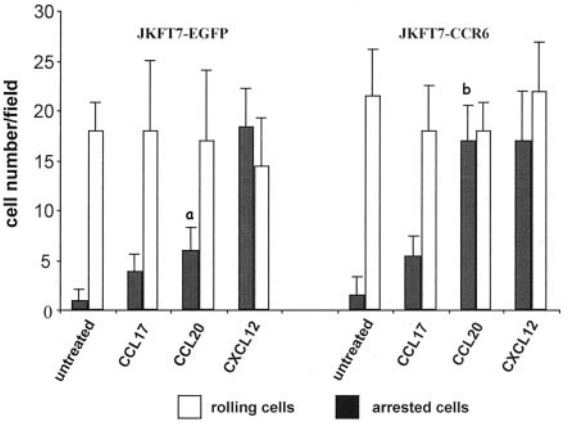

FIGURE 4. CCL20 mediates the arrest of CCR6 transformed JKFT7 cell under flow conditions in vitro. JKFT7 cells expressing either EGFP or CCR6 were allowed to roll and arrest on recombinant protein substrates E-selectin/ICAM-1 with either no chemokine or various chemokine substrates as indicated at a shear rate of 1.5 dynes/cm^2. The mean numbers of rolling and firmly arrested cells per field (1.18 mm^2) averaged from four 20-s video recordings of separate fields ± SD. $p = 0.004$ for a vs b. One of three experiments with similar results.

association of CD49d with CCR6, suggesting that a suitable $\alpha_4\beta_1$ ligand was required for maximal colocalization of the α_4 integrin with CCR6 (data not shown).

To rule out that the clustering of CCR6 was due to an artifact of fixation or Ab staining, we also allowed JKFT7-CCR6 cells to arrest on recombinant substrates and CCL20 as before and recorded the position of CCR6-EGFP fluorescence by conventional fluorescence microscopy immediately after arrest in the absence of fixation. As shown in Fig. 6*G*, CCR6 had clearly redistributed to one area of the cell. Because the fusion of EGFP to the C-terminus

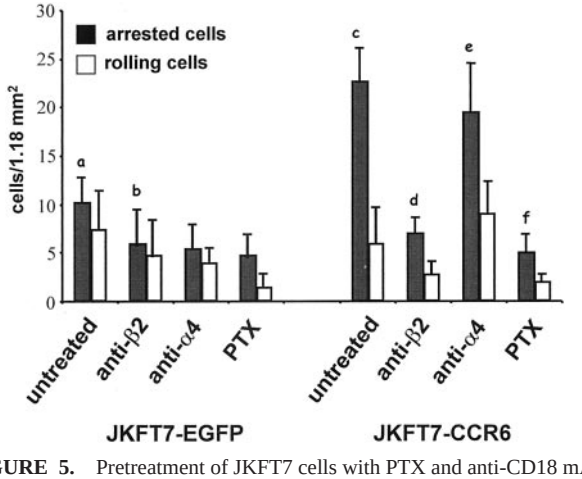

FIGURE 5. Pretreatment of JKFT7 cells with PTX and anti-CD18 mAb inhibits firm arrest on HDMEC under physiological flow conditions. JKFT7 cells expressing EGFP or CCR6 were pretreated with PTX for 1.5 h, pretreated with anti-integrin Abs at 10 μg/ml for 30 min, or left untreated at 37°C. At the end of incubation period, all cells were stained with calcein-AM, resuspended in cRPMI at 3×10^5 cells/ml, and introduced into the flow chamber containing TNF-α activated HDMEC at a shear rate of 1.5 dynes/cm^2 for 5 min. Rolling and adherent cells were then quantified as described in *Materials and Methods*. *p* values were as follows (four fields per condition): a vs b, 0.09; a vs c, 0.001; c vs d, 0.0005; c vs e, 0.307; c vs f, 0.0003. This experiment was one of three experiments with similar results.

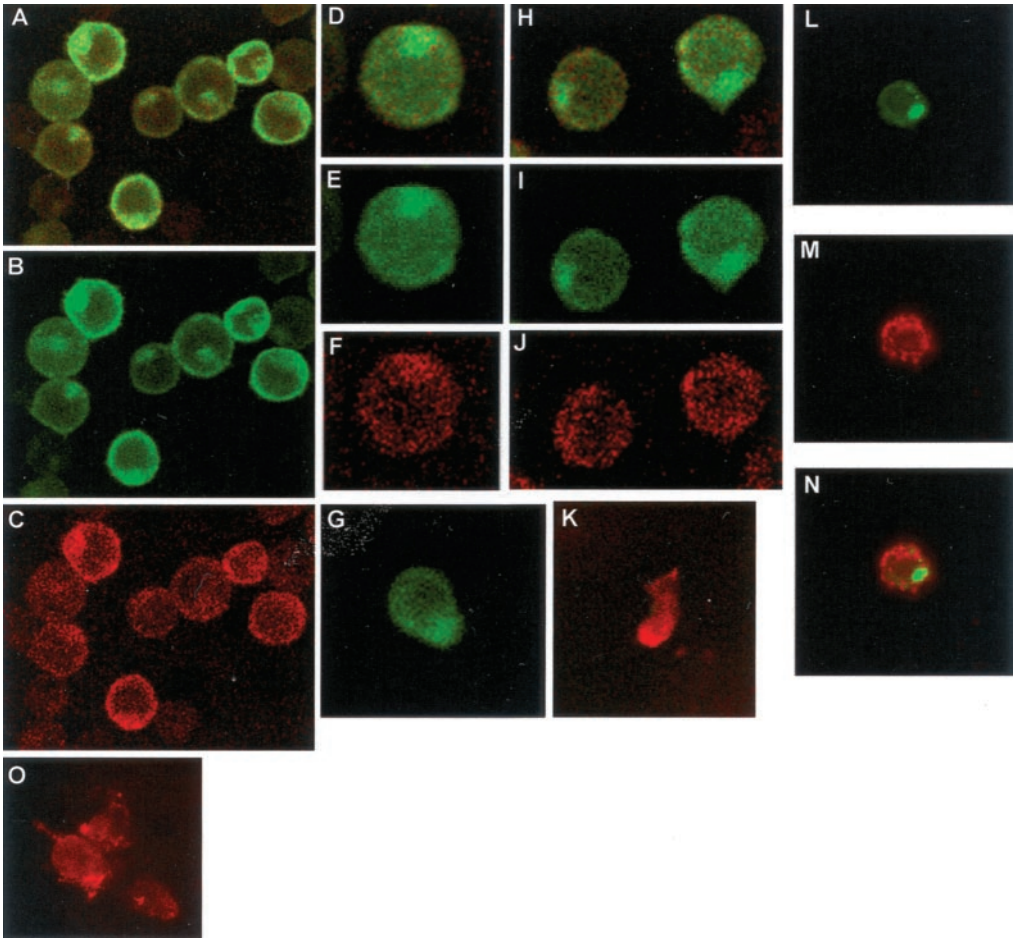

FIGURE 6. CCCR6-ligand interaction triggers reorganization of CCR6 and colocalization of β_2. JKFT7-CCR6 cells were introduced into a flow chamber coated with E-selectin/Ig, soluble ICAM-1, and CCL20 (see *Materials and Methods*), where the cells were allowed to arrest 15 min. After staining for CD18 (*A–C*), CD11a (D–*F*), and CD49d (*H–J*), fixation, and labeling with streptavidin-Cy3 (see *Materials and Methods*) in separate experiments, cells were analyzed with a confocal microscope. *A*, *D*, and *H*, Combined signals from EGFP-CCR6 (green channel) and integrin chain signals (red channel) in which overlap is indicated by a yellowish hue. Isolated red channel signals from CD18, CD11a, and CD49d staining are shown in *C*, *F*, and *J*, respectively, while the green channel signal from CCR6-EGFP is shown in *B*, *E*, and *I*. *G*, JKFT7-CCR6 cells were introduced into the flow chamber coated with recombinant adhesion molecules and CCL20 as above. Immediately following arrest, a fluorescence image was captured of the stationary cell in the absence of fixation or Ab staining. *K*, Unlabeled peripheral blood mTC, isolated as described (10), were introduced into a flow chamber containing TNF-α-activated HDMEC and allowed to accumulate over 10 min at a shear stress of 1.5 dynes/cm^2. Cells were then stained with an anti-CCR6 mAb, a biotinylated secondary Ab, and streptavidin-Cy3 (red) and analyzed by confocal microscopy. *L–M*, JKFT7-CCR6 cells were allowed to roll and arrest on CCL20. Following arrest and washing, the bound cells were stained with biotinylated anti-CXCR4 and streptavidin-Cy3. CCR6-EGFP (*L*, green) and CXCR4 (*M*, red) distributions on a representative cell are depicted singly and in a merged two-color photomicrograph (*N*). *O*, Following arrest on recombinant substrates and plate-bound CXCL12, JKFT7-CCR6 cells were stained with biotinylated anti-CXCR4/streptavidin Cy3 (as above) and photographed (×40 objective) using Cy3-appropriate filter.

of CCR6 may have altered the pattern of CCR6 redistribution following binding to immobilized ligand, we allowed human peripheral blood memory T cells (~40% express CCR6) to arrest on TNF-α-activated HDMEC monolayers under flow conditions as previously described (10), and then stained the cells with an anti-CCR6 mAb (red) (Fig. 6*K*). Again, CCR6 appeared to redistribute to one area of the MTC, demonstrating that there was lateral movement of nonchimeric CCR6 following receptor ligation in human mTC. To determine whether other chemokine receptors redistributed in conjunction with CCR6 when the JKFT7-CCR6 cells arrested in response to immobilized CCL20, we stained arrested cells with anti-CXCR4 and observed the distribution of CXCR4 and CCR6 by two-color fluorescence microscopy. Although CCR6 clustered (green, Fig. 6*L*) following arrest on CCL20, CXCR4 appeared to remain distributed throughout the cell membrane (Fig. 6*M*). In the merged micrograph (Fig. 6*N*), CCR6 distribution (green fluorescence) appears clearly distinct from that of CXCR4 (red fluorescence), suggesting that the clustering of CCR6 is independent of the lateral movement of other chemokine receptors. Finally, we allowed JKFT7-CCR6 to arrest on CXCL12, which resulted in clustering of CXCR4 (Fig. 6*O*) in response to plate-bound ligand. Thus, CCR6 specifically redistributed on the cell membrane in response to immobilized CCL20. β_2 integrins (and to a lesser extent α_4 integrin) appeared to colocalize with CCR6 during this process.

Discussion

Our previous studies demonstrated that desensitization of CCR6 by saturating concentrations of CCL20 inhibited adhesion of mTC to activated HDMEC under shear stress conditions. Although we showed that CCR6 was necessary, we could not demonstrate that CCR6, by itself, was sufficient for mTC arrest (assuming requisite adhesion molecules such as LFA-1 were present) because peripheral blood mTC express multiple chemokine receptors. Herein, we

transduced JK T cells, which showed very low levels of mRNA for all CCRs tested, with CCR6 to address whether this was sufficient as a chemokine receptor for increasing JK arrest on activated HDMEC. Indeed, CCR6 expression significantly increased adhesion of JKFT7 cells to activated HDMEC by 2- to 3-fold. Interestingly, adhesion was CD18-dependent but not CD49d-dependent. Finally, CD18 integrin colocalized with CCR6 as detected by two-color confocal microscopy.

Our data add additional support for the role of CCR6 in the recruitment of CCR6-expressing subsets of mTC to sites of inflammation. We demonstrated that the presence of CCR6 enhances the adhesion of our model T cells to both recombinant substrates and activated microvascular endothelial cells. The lack of CCRs such as CCR1–CCR10 on the starting JK cells provided a system by which we could test the efficacy of CCR6 alone in mediating arrest. Although JK cells expressed CXCR4, activated HDMEC in our previous study produced little or not CXCL12 mRNA by comparison with CCL20 (10). Thus, this receptor is unlikely to play a role in JK attachment to activated HDMEC in our in vitro flow model.

The use of a CCR6-EGFP fusion protein allowed us to determine the distribution of CCR6 in relationship to that of β_2 integrins, which have been reported to cluster following chemokine receptor-mediated signaling (11). Surprisingly, we observed clear colocalization of CD18 and CD11a integrin chains (i.e., LFA-1) with CCR6 (Fig. 6, *A–F*). To our knowledge, this association has not previously been reported in the literature, although chemokines have been reported to induce the redistribution of ICAM-1, CD44, and CD43 to lymphocyte uropods (32) and of LFA-1 (11). We caution that colocalization does not necessarily suggest physical coassociation of CCR6 and β_2 integrin. Immunoprecipitation studies to determine this await suitable Ab reagents. Although not formally addressed, it was possible that the changes in lateral mobility of β_2 integrin observed by Constantin et al. (11) may have coincided with the spatial redistribution of chemokine receptors.

The α_4 integrin forms one chain of CD49dCD29 ($\alpha_4\beta_1$ or very late Ag-4), an integrin capable of mediating both rolling (33) and arrest (34) of lymphocytes under flow conditions. Since β_7 integrin was not expressed by our JKFT7-CCR6 cells (Fig. 2), we presume that most of the α_4 integrin on these cells was associated with β_1 integrin to form very late Ag-4. However, adhesion of JKFT7-CCR6 to activated HDMEC was not inhibited by anti-α_4 mAb. This may have been due to differences in the kinetics and expression of VCAM-1 on HDMEC vs HUVEC (28, 29). Furthermore, α_4 integrin did not appear to colocalize with CCR6 (Fig. 6, *H–J*) to the same extent as did CD11a and CD18 (Fig. 6, *A–F*) on ICAM-1 in the flow chamber, although colocalization of α_4 integrin was clearly enhanced when a recognized $\alpha_4\beta_1$ ligand, VCAM-1/Ig, was coimmobilized on the surface. This suggests that clustering of integrins is at least partially dependent on the presence of a suitable integrin ligand. Although differential regulation of β_1 vs β_2 integrin function in T cells by chemokines is possible under some conditions (35, 36), our experiments demonstrated similar clustering of α_1 and β_2 integrins on the cell surface in response to CCL20 as long as both VCAM-1 and ICAM-1, respectively, were immobilized on the plate.

In summary, we provide additional data that support an important role for CCR6 in the arrest of a subset of mTC to activated endothelium. Secondly, we demonstrate that CCR6 clearly colocalizes with CD11aCD18 (LFA-1) integrin. The clustering of the CCR6 appeared to be a specific response to CCL20 since CXCR4, while similarly showing redistribution in response to plate-bound CXCL12 (Fig. 6*O*), did not redistribute on the membrane when CCL20 was used to induce arrest (Fig. 6, *M–N*). Although we could not follow integrin clustering (i.e., avidity changes) in real time, colocalization of β_2 and β_1 integrins with CCR6 (before, during, or after CCR6 redistribution) may provide a basis for increasing the strength of the interaction between T cells and relevant physiological targets such as activated endothelial cells. As suggested by the work of Constantin et al. (11), increased avidity of LFA-1 may be of particular importance at the early stages of inflammation when ICAM-1 expression is low. Since CCR6 is expressed by both CLA^+ (so-called skin-homing T cells) as well as by $\alpha_4\beta_7^+$ mTC (so-called gut-homing T cells), CCR6 is unlikely by itself to lend absolute homing specificity to one organ vs another. However, because CCR6 is both necessary and sufficient for T cell homing in our in vitro system, pharmacologic inhibitors of CCR6 may be of potential value in preventing undesirable T cell influx in multiple organs.

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