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CUTTING EDGE

Cutting Edge: Scavenging of Inflammatory CC Chemokines by the Promiscuous Putatively Silent Chemokine Receptor D6[1]

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In an effort to define the actual function of the promiscuous putatively silent chemokine receptor D6, transfectants were generated in different cell types. Engagement of D6 by inflammatory CC chemokines elicited no calcium response nor chemotaxis, but resulted in efficient agonist internalization and degradation. Also in lymphatic endothelium, where this receptor is expressed in vivo, D6 did not elicit cellular responses other than ligand internalization and degradation. In particular, no evidence was obtained for D6-mediated transcytosis of chemokines in the apical-to-basal or basal-to-apical directions. These results indicate that D6 acts as an inflammatory chemokine scavenging nonactivatory decoy receptors and suggest that in lymphatic vessels D6 may function as a gatekeeper for inflammatory CC chemokines, by clearing them and preventing excessive diffusion via afferent lymphatics to lymph nodes. *The Journal of Immunology,* **2003, 170: 2279–2282.**

Chemokines are a superfamily of inflammatory cytokines that guide the recruitment and positioning of leukocytes in tissues by interacting with 7-transmembrane-domain receptors (1–3). Chemokine receptors comprise a distinctive subfamily of the G protein-coupled heptahelical receptor superfamily, characterized by unique structural motifs (4). Structural similarity, binding to chemokines, signaling via G proteins, and activation of chemotaxis are general features of known chemokine receptors. However, members of this family with defective signaling function have been identified (5–7). Among these, D6 is a promiscuous chemokine receptor with significant homology to CC chemokine receptors, which binds with high affinity several inflammatory CC chemokines, including CC chemokine ligand (CCL)[4] 2/monocyte chemotactic protein (MCP)-1, CCL4/macrophage inflammatory protein (MIP)-1β, CCL5/RANTES, CCL7/MCP-3, and CCL3L1/MIP-1αP, a MIP-1α variant with a proline residue in position 2 (6, 8). The D6 primary sequence presents substitutions in conserved elements generally essential for chemokine receptor signaling (4), such as an aspartic acid (D) residue substituted with an asparagine (N) in the second transmembrane segment and the DRYLAIV motif substituted with the DKYLEIV sequence in the second intracellular loop. As expected on the basis of these structural features, D6 does not induce calcium fluxes nor chemotaxis in the experimental systems described so far (6, 8). The D6 receptor is weakly expressed in circulating cells and selected hemopoietic lines, but it is expressed at high levels in placenta as well as lymphatic, but not vascular, endothelium (9). The function of D6 is at present unknown. In this study, we provide evidence that D6 functions as a chemokine-scavenging decoy receptor in diverse cellular contexts, including lymphatic endothelium.

Materials and Methods

Reagents

The recombinant human chemokines CCL2 and CCL4 and the vascular endothelium growth factor receptor 3 (Flt-4/VEGFR3) goat anti-mouse polyclonal Ab were purchased from R&D Systems (Minneapolis, MN). ^{125}I-labeled human CCL2 (^{125}I-CCL2) (~2000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Unless otherwise specified, reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cloning of human D6

The human D6 coding sequence was amplified by PCR from genomic DNA using standard methodology and cloned into the pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA). The insert was fully sequenced and shown to be identical to Gene Bank sequence XM_046265 except for an A to C substitution at position 1118 from the starting codon, causing a Tyr to Ser substitution. This previously described single base polymorphism (dbSNP:

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[4] Abbreviations used in this paper: CCL, CC chemokine ligand; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; Flt-4/VEGFR3, vascular endothelium growth factor receptor 3; MELC, mouse lymphatic endothelial cell; CHO, Chinese hamster ovary; ^{125}I-CCL2, ^{125}I-labeled CCL2.

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2228468, a variation to Gene Bank XM_046265) was present in 11 alleles in a panel of 14 genomic samples tested.

Cell culture and transfection

The mouse L1.2 lymphoma cell line was grown in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 10 mM HEPES, pH 7.4, 50 μM 2-ME. After electroporation with linearized D6/pcDNA3 and selection with 800 μg/ml G418 (Life Technologies), resistant cells were cloned by limiting dilution. Mouse lymphatic endothelial cells (MELC) were obtained following a previously described procedure (Ref. 10 and A. Vecchi, M. Sironi, S. Bernasconi, A.M. Fra, A. Del Prete, A. Vecchi, and A. Mantovani, manuscript in preparation). Briefly, hyperplastic vessels were induced by injection of IFA in DBA/2 mice and were isolated from liver and diaphragm. After collagenase treatment, the single cell suspension was cultured at 37°C and 5% CO_2 in gelatin-treated plastic dishes in DMEM (Life Technologies) supplemented with 10% FCS, 10% sarcoma 180 cell-conditioned medium, 1 mM sodium pyruvate, 100 μg/ml heparin, 100 μg/ml endothelial cell growth supplement, and antibiotics. Cells were cloned by limiting dilutions. Clone MELC-2, selected for these studies, was positive for Flt-4/VEGFR3 in Western blot, FACS analysis, and RT-PCR, and for podoplanin in RT-PCR. The uncloned original population showed similar characteristics. MELC-2 cells had low but appreciable levels of D6 transcripts, as assessed by RT-PCR, but no surface receptor expression as assessed by binding of CCL2 and CCL4. MELC-2 cells were transfected by lipofection with linearized D6/pcDNA3 and selected with 500 μg/ml G418. MELC-2/D6 retained expression of endothelial markers and acquired CCL2 binding. Chinese hamster ovary (CHO)-K1 cells were transfected with D6/pcDNA3 by a standard calcium phosphate procedure, selected with 500 μg/ml G418 and cloned by limiting dilution.

Binding and internalization assays

CCL2 competitive binding was performed by incubating 7.5×10^5 L1.2/D6 cells with 50 pM ^{125}I-CCL2 in the presence of different concentrations of unlabeled CCL2 or CCL4, in 200 μl of binding buffer (RPMI 1640, 4 mM HEPES, pH 7.4, 1% BSA) at 4°C for 2 h. After incubation, the cell-associated radioactivity was measured. To estimate the K_d (i.e., the equilibrium dissociation constant) and the B_{max} (i.e., the maximum number of binding sites), CCL2 homologous competitive binding data were analyzed by nonlinear fitting using the equation of the "homologous competitive binding curve" (GraphPad Prism 3.0a; GraphPad, San Diego, CA). The CCL4 inhibition curve was analyzed using the equation of the "one site competitive binding equation" (GraphPad Prism 3.0a) to estimate the IC_{50} value, from which the inhibition constant (K_i) value was then calculated according to the Cheng-Prusoff equation (11). In internalization assays, L1.2/D6 cells (1×10^7/ml) were incubated in binding buffer at 4°C for 2 h with 0.25 nM ^{125}I-CCL2, washed twice with cold binding buffer, aliquoted, and further incubated for the indicated time periods and temperatures. Cells were then cooled, washed once with binding buffer and then incubated for 1 min in acidic buffer (50 mM glycine, 100 mM sodium chloride; pH 3.0). The internalized ligand was then measured by counting the cell-associated acid-resistant fraction.

Chemokine scavenging

L1.2/D6 cells (1×10^6) were incubated for different time periods at 37°C in 200 μl of binding buffer with 0.4 nM unlabeled CCL2 and 0.1 nM ^{125}I-CCL2. At the end, the radioactivity present in the supernatant and cellular fractions was measured. To determine the fraction of degraded chemokine in the supernatant, proteins were precipitated with 12.5% TCA at 4°C and both soluble and insoluble fractions were counted. For CCL2 scavenging in CHO-K1/D6, 2×10^5 plastic adherent cells were incubated with 0.4 nM unlabeled CCL2 and 0.1 nM ^{125}I-CCL2 in 200 μl of binding buffer, and processed as described above. For MELC-2/D6 transfectants, 1×10^5 plastic adherent cells were incubated with 0.1 nM ^{125}I-CCL2 in the absence of unlabeled CCL2, and processed as described above.

Transcytosis

MELC-2/D6 cells (2×10^4) were seeded onto 6.5-mm, 0.4-μm pore size, Transwell Clear filters (Costar, Cambridge, MA) and grown to complete confluence for 2–3 days. ^{125}I-CCL2 (0.02 pmol) was then added to each Transwell, equivalent to 0.15 and 0.03 nM in the upper and lower chamber, respectively, and, after the indicated time of incubation at 37°C, the radioactivity present in the upper and lower chambers as well as on filters was counted. To determine the fraction of degraded chemokine in both upper and lower chambers, proteins were precipitated with 12.5% TCA at 4°C and both the soluble and insoluble fractions were counted.

Migration

THP-1 migration in the presence of L1.2/D6 cells was evaluated using 5-μm pore size Transwell filters (Costar). THP-1 cells (2×10^6 cells/ml in PBS) were stained with 500 nM CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C. After inactivating the excess dye with 5% FCS, the cells were adjusted to 2×10^6 cells/ml in culture medium. RPMI 1640 (600 μl), supplemented with 3 nM CCL2, was preincubated for 1 h with 4×10^5 of either L1.2 or L1.2/D6 cells. After cell removal by centrifugation, this conditioned medium was placed into the lower chamber of a Transwell filter. One hundred microliters of the THP-1 cell suspension was then placed onto the upper chamber. After 3 h of incubation at 37°C, the upper chamber was removed and the cells in the lower chamber were collected. The number of migrated THP-1 cells was determined in FACS analysis using TruCOUNT tubes (BD Biosciences, Mountain View, CA).

Results

D6-mediated ligand binding and internalization

We cloned human D6 and stably expressed it in the L1.2 lymphoma cell line, a widely used recipient line for chemokine receptors with no specific CCL2 binding sites (Fig. 1*A*, legend). After D6 transfection, several L1.2/D6 clones expressing D6 transcripts and capable of binding CCL2 and CCL4 were isolated. Results presented in subsequent experiments refer to the L1.2/D6 clone 6, characterized in Fig. 1*A*, but similar data were obtained with other clones. When L1.2/D6 transfectants were stimulated with CCL2, no calcium fluxes nor chemotaxis was observed (data not shown). This is in agreement with other previously described D6 polymorphic variants (6, 8). To analyze whether D6 had the ability to internalize its ligands, after binding ^{125}I-CCL2 at 4°C, L1.2/D6 were washed and warmed to 37°C for increasing time intervals to allow endocytosis. At the end of incubation periods, cells were pelleted, the supernatant fraction was collected, and the cell pellet was acid-washed to discriminate between intracellular and membrane-bound radioligand. Radioactivity was measured and plotted as a percentage of total bound CCL2 (Fig. 1*B*). Under these conditions, in

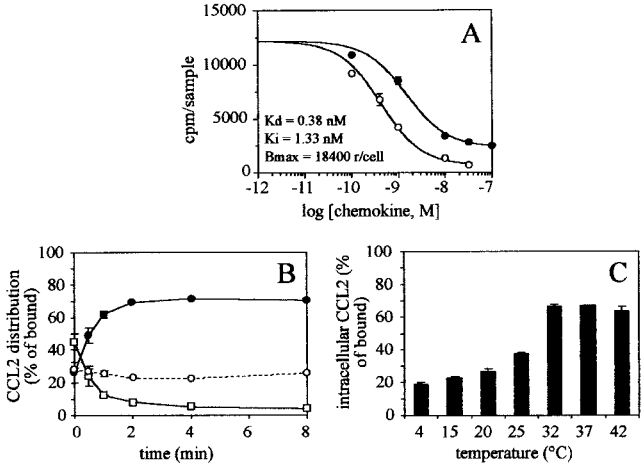

FIGURE 1. D6-mediated ligand binding and internalization. *A*, Competitive binding of ^{125}I-CCL2 (mean ± SD) to L1.2/D6 cells in the presence of different concentrations of unlabeled CCL2 (○) or CCL4 (●). Binding to parental L1.2 cells was 320 ± 59 cpm/sample. *B*, Kinetics of CCL2 internalization. L1.2/D6 cells were bound to ^{125}I-CCL2 at 4°C and then incubated at 37°C for the indicated time periods. Results (mean ± SD) are the percentage of medium released (○), intracellular acid-resistant (●), and cell surface-bound (□) ^{125}I-CCL2 over total bound ^{125}I-CCL2. *C*, Effect of temperature on CCL2 internalization. After binding with ^{125}I-CCL2 at 4°C, cells were incubated for 4 min at the indicated temperatures. Bars (mean ± SD) are the percentage of intracellular, acid-resistant ^{125}I-CCL2 over total bound ^{125}I-CCL2.

2–4 min, ~70% of the bound ligand was internalized, becoming resistant to acid-wash. Less than 5% remained associated to the membrane. About 25% of bound radioligand was released in the supernatant. D6 mediated a temperature-dependent uptake which was almost completely blocked below 20°C and was optimal above 32°C (Fig. 1*C*).

D6-mediated ligand scavenging and degradation

To investigate the fate of the internalized ligand, D6 transfectants of different cellular origin were incubated for 2 h with 0.5 nM trace-labeled CCL2, and the chemokine was then measured in the supernatant and cellular fractions. Supernatants were subjected to TCA precipitation to assess the fraction of chemokine that had undergone degradation. It should be noted that the TCA-precipitable fraction, an approximation of intact chemokine, may actually contain partially processed molecules. As shown in Fig. 2, parental L1.2 (Fig. 2*A*) and CHO-K1 (Fig. 2*B*) cells did not internalize significant amounts of CCL2 over a period of 2 h. The intracellular fraction was negligible (<3%) and most of the agonist (>90%) remained in the supernatant in the TCA-precipitable, presumably intact, form. In contrast, in D6 transfectants, ~30% of total CCL2 was associated to cells. This percentage includes the membrane-bound radioligand fraction, usually <3% (data not shown). Moreover, a substantial fraction of the radioactivity present in the extracellular medium was represented by degraded agonist, as assessed by TCA solubility (31 and 24% of the total for L1.2/D6 and CHO-K1/D6, respectively; Fig. 2). Pertussis toxin treatment did not affect ligand internalization and degradation (P. Signorelli, unpublished results). Treatment with NH_4Cl markedly inhibited the appearance of TCA-soluble radioactivity in the supernatants and caused intracellular accumulation of CCL2. Similar results were obtained when chemokine scavenging was analyzed at different time points (between 0.5 and 3 h) with L1.2 and CHO-K1 D6 transfectants, and by exposing L1.2/D6 cells to unlabeled CCL2 followed by ELISA measurement of the chemokine remaining in the supernatant (data not shown). Taken together, these results show that D6 efficiently clears its ligands, presumably targeting them to endolysosomal compartments where they are degraded. In an effort to assess the functional significance of these observations, the capacity of D6-transfected cells to inactivate the chemotactic activity of CCL2 was evaluated. As shown in Fig. 2*D*, exposure of CCL2 to L1.2/D6 transfectants, but not to parental cells, significantly reduced its chemotactic activity for THP-1 monocytic cells (net THP-1 migration for the parental L1.2-conditioned medium 8.67 ± 0.49, compared with 2.33 ± 0.35 for the L1.2/D6-conditioned medium, $p = 0.001$ by paired Student's t test analysis).

D6 in lymphatic endothelial cells

The D6 receptor is expressed in a restricted set of tissues (Ref. 9 and our unpublished data) and most notably in endothelial cells lining some dermal and mucosal lymphatics (9). Therefore, it was important to explore the functional properties of D6 in a physiologically relevant cellular context. The uncloned MELC-2/D6 transfected population was selected for further analysis, but all experiments were repeated with a clonal population with similar results. D6 engagement by CCL2 or CCL4 in MELC-2/D6 cells did not elicit an increase in intracellular free calcium concentrations nor functional responses including cytokine production (CCL2, IL-6) and expression of VCAM-1 (data not shown). However, as observed in L1.2 and CHO-K1 D6 transfectants, MELC-2/D6 cells internalized and degraded the CCL2 ligand (Fig. 2*C*). Given its location in endothelial cells lining lymphatics (9) and the capacity of vascular endothelia to translocate chemokines in the basal-to-apical direction (12), it was important to test the possibility that D6 may act as a carrier for inflammatory chemokines across the endothelial barrier

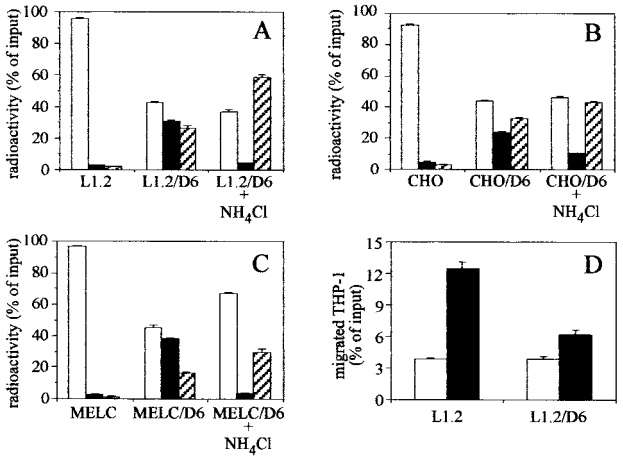

FIGURE 2. D6-mediated ligand scavenging and degradation. *A–C*, CCL2 clearance by L1.2/D6 (*A*), CHO-K1/D6 (*B*), and MELC-2/D6 (*C*) cells. Parental or D6-transfected L1.2 and CHO-K1 cells were incubated for 2 h with 0.4 nM CCL2 and 0.1 nM ^{125}I-CCL2, in the presence or absence of 10 mM NH_4Cl. For parental or D6-transfected MELC-2 cells, the unlabeled CCL2 was omitted. Results (% of input, mean ± SD, three replicates, three experiments) represent radioactivity associated to the cells (▨) and present in the supernatants as TCA-soluble (■) and TCA-insoluble (□). *D*, Migration of THP-1 cells (% of input, mean ± SD, three replicates, three experiments) to culture medium (□) or culture medium supplemented with 3 nM CCL2 (■), preincubated with 4×10^5 parental L1.2 or L1.2/D6 cells.

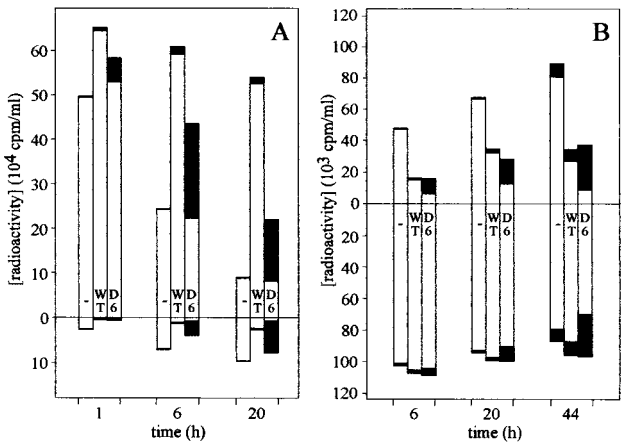

FIGURE 3. Lack of D6-mediated ligand transcytosis. ^{125}I-CCL2 (0.02 pmol) was added either on the apical side (*A*) or on the basal side (*B*) of filters coated with no cells (−), parental MELC-2 (WT), or MELC-2/D6 cells (D6). Radioactivity was measured in both the upper and lower chambers, and separated in TCA-soluble (filled part of the bars) and TCA-insoluble fractions (open part of the bars). Results are the mean of duplicates (range was <2% for all samples) and are expressed as cpm per milliliter. One experiment of two performed with similar results is shown. It should be noted that when the different volumes present in the lower and upper wells (120 and 620 μl, respectively) are taken into account, the total amount of radioactivity recovered was similar among various experimental groups and to the input.

(transcytosis). Lymphatic endothelial cells were grown to confluency on Transwell filters and labeled CCL2 was applied either to the apical (Fig. 3*A*) or to the basal side (Fig. 3*B*). In the absence of cells, CCL2 freely diffused and reached the equilibrium in both experimental conditions. In the presence of parental MELC-2 cells, CCL2 diffusion was significantly delayed, showing that MELC-2 cells created a competent monolayer. As assessed by TCA precipitation, in the absence of D6 most (>90%) CCL2 remained intact (open part of the bars). In contrast, in the presence of MELC-2/D6 cells, a substantial fraction of CCL2 was degraded and released as TCA-soluble degradation products on both sides of the filter (filled part of the bars). In particular, in the apical-to-basal transcytosis experiment shown in Fig. 3*A*, at the equilibrium time point (20 h), 68% of the total CCL2 was degraded to TCA-soluble fragments in MELC-2/D6-containing wells. Similarly, in the basal-to-apical transcytosis experiment shown in Fig. 3*B*, at the equilibrium time point (44 h), 41% of the total CCL2 was degraded to TCA-soluble fragments in MELC-2/D6-containing wells. At no time point was any evidence obtained for a facilitated transfer of intact CCL2 either in the basal-to-apical or apical-to-basal directions in the presence of D6 transfected MELC-2 cells, compared with parental MELC-2 cells. At all time points and in both experimental conditions tested, less intact ligand was found on the side opposite to seeding in the presence of MELC-2/D6 compared with controls. In this series of experiments, the radioactivity associated with cells was also determined. Less than 1% of the total radioactivity remained associated with filters, with no difference between samples with or without cells (data not shown). Therefore, no evidence was obtained for transcytosis and release or presentation of chemokines in D6-transfected lymphatic endothelial cells.

Discussion

Following identification of the type II IL-1 receptor as a decoy (13), decoy receptors (i.e., nonsignaling receptors acting as a molecular trap for agonists and, in some cases, for components of signaling receptor complexes) have been identified in the IL-1, TNF, and IL-10 receptor families (7). Moreover, under certain conditions, signaling receptors for inflammatory chemokines can be uncoupled and act as functional decoy receptors (14). The results presented in this study strongly suggest that D6 is a nonsignaling decoy receptor and scavenger for inflammatory CC chemokines and are consistent with the hypothesis that D6 lacks the classical G protein-dependent signaling properties, while retaining ligand internalization and degradation (15). Consistent with this, preliminary data indicate that D6 internalization involves a clathrin-coated pits-mediated mechanism (P. Signorelli, unpublished results). Receptor internalization could occur in a ligand-dependent fashion, or D6 may shuttle constitutively, similarly to what was recently suggested for a virally encoded chemokine receptor (16–18).

Inflammatory chemokines produced in tissues are transported via lymphatic vessels to lymph nodes where they act as a "remote control" mechanism to recruit leukocytes such as monocytes (19, 20). The D6 receptor is strategically expressed in lymphatic endothelial cells in tissues and afferent lymphatics (9). In a lymphatic endothelial cell context, we found no evidence for D6 receptor-mediated ligand transcytosis. In lymphatic endothelium, as in other cell types, D6 acted as a mechanism of chemokine sequestration and scavenging. These results suggest that D6 expressed on the endothelium of afferent lymphatics may act as a gatekeeper, preventing excessive accumulation of inflammatory chemokines in lymph nodes and uncontrolled, disruptive leukocyte recruitment. A similar function may be played in placenta where D6 is abundantly expressed (Refs. 6 and 8, and our unpublished data).

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