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Differential Recognition and Scavenging of Native and Truncated Macrophage-Derived Chemokine (Macrophage-Derived Chemokine/CC Chemokine Ligand 22) by the D6 Decoy Receptor

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The promiscuous D6 receptor binds several inflammatory CC chemokines and has been recently proposed to act as a chemokine-scavenging decoy receptor. The present study was designed to better characterize the spectrum of CC chemokines scavenged by D6, focusing in particular on CCR4 ligands and analyzing the influence of NH2-terminal processing on recognition by this promiscuous receptor. Using D6 transfectants, it was found that D6 efficiently bound and scavenged most inflammatory CC chemokines (CCR1 through CCR5 agonists). Homeostatic CC chemokines (CCR6 and CCR7 agonists) were not recognized by D6. The CCR4 agonists CC chemokine ligand 17 (CCL17) and CCL22 bound to D6 with high affinity. CCL17 and CCL22 have no agonistic activity for D6 (chemotaxis and calcium fluxes), but were rapidly scavenged, resulting in reduced chemotactic activity on CCR4 transfectants. CD26 mediates NH2 terminus processing of CCL22, leading to the production of CCL22 (3–69) and CCL22 (5–69) that do not interact with CCR4. These NH2-terminal truncated forms of CCL22 were not recognized by D6. The results presented in this study show that D6 recognizes and scavenges a wide spectrum of inflammatory CC chemokines, including the CCR4 agonists CCL22 and CCL17. However, this promiscous receptor is not engaged by CD26-processed, inactive, CCL22 variants. By recognizing intact CCL22, but not its truncated variants, D6 expressed on lymphatic endothelial cells may regulate the traffic of CCR4-expressing cells, such as dendritic cells.

Materials and Methods

Reagents
Recombinant human chemokines CCL2, CCL3, CCL5, CCL17, CCL19, CCL22, and CXC chemokine ligand 8 (CXCL8) were purchased from DictaGene (Epalinges, Switzerland). CCL1, CCL3L1, CCL4, CCL7, CCL8, CCL11, CCL13, and CCL14 were purchased from R&D Systems (Minneapolis, MN). CCL22 (3–69) and CCL22 (5–69) were produced as previously described (11). 125I-labeled human CCL2 and CCL4 (~2000 Ci/mmol)

Abbreviations used in this paper: CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; DC, dendritic cells; HA, hemagglutinin; MELC, mouse lymphatic endothelial cell.

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were purchased from Amersham Pharmacia Biotech (Little Chalfont, U.K.). The anti-D6 mAb and the anti-CCR2 biotin mAb were obtained from R&D Systems. The anti-hemagglutinin (anti-HA) tag mAb (clone 12CA5) was purchased from Roche (Basel, Switzerland). The anti-CCR4 mAb was purchased from BD Biosciences (Mountain View, CA). Unless otherwise specified, tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD), and laboratory reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture

The mouse L1.2 lymphoma cell line was grown in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% FCS (HyClone Laboratories, Logan, UT), 10 mM HEPES (pH 7.4), and 50 μM 2-ME. D6/L1.2 and D6/CHO-K1 transfectants were obtained as previously described (10). CCR4/L1.2 were a gift from Dr. D. D’Ambrosio (BioXell, Milan, Italy). Mouse lymphatic endothelial cells (MELC) were obtained following a previously described procedure (12). Clones MELC-2 and D6/MELC-2, selected for these studies, have been described previously (10). CCR4-D6/L1.2 were obtained transfecting CCR4/L1.2 with the plasmid D6/pcdNA6 encoding the HA-tagged human D6 receptor and were selected for the stable expression of both receptors in growing medium in the presence of hygromycin and G418.

Binding assays

CCL2 competitive binding was performed by incubating 7.5 × 10⁵ D6/L1.2 cells with 50 pM 125I-labeled CCL2 in the presence of different concentrations of unlabeled CCL2, CCL4, CCL17, CCL19, or CCL22 in 200 μl of binding buffer (RPMI 1640, 4 mM HEPES (pH 7.4), and 1% BSA) at 4°C for 2 h. After incubation, the cell-associated radioactivity was measured. To estimate the Kᵦ (i.e., the equilibrium dissociation constant) and 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 (PRISM 3.0a; GraphPad, San Diego, CA). Inhibition curves were analyzed using the one-site competitive binding equation (GraphPad PRISM 3.0a) to estimate the IC₅₀ value, from which the Kᵦ value was then calculated according to the Cheng-Prusoff equation (13).

Chemokine scavenging

D6/CHO-K1 (2 × 10⁵), D6/MELC-2 (1 × 10⁵), or D6/L1.2 (1 × 10⁵) cells were incubated for the indicated time periods at 37°C in 200 μl of binding buffer supplemented with 1.2 mM of the indicated chemokine. At the end of the incubation, the chemokine concentration in the supernatant was measured by ELISA. As shown in Fig. 1, among known D6 ligands, the inflammatory CC chemokines CCL5 and CCL11 were scavenged with the highest efficiency (94.3 ± 0.2 and 92.5 ± 2.1% of the initially seeded chemokine, respectively). CCL7, CCL4, CCL2, and CCL3L1 were scavenged with intermediate efficiency (85.1 ± 0.5, 83.9 ± 2.9, 76.5 ± 2.2, and 76.0 ± 6.6% of the initially seeded chemokine, respectively). Interestingly, CCL3 and CCL3L1, which only differ in the presence of a serine or a proline residue in position 2, were scavenged with different efficiencies (29.0 ± 4.1 and 76.0 ± 6.6% of the initially seeded chemokine, respectively), in agreement with previous results reporting that only the CCL3L1 variant is a high affinity D6 ligand (14). CCL11, which does not bind D6 (7), was the only inflammatory CC chemokine tested not scavenged by D6 (3.1 ± 1.4% of the initially seeded chemokine). None of the chemokines tested was scavenged by untransfected CHO-K1 cells. Unlike CC inflammatory chemokines, the homocistemic chemokines CCL19 and CCL20, agonists at CCR7 and CCR6, respectively, were not scavenged (8.2 ± 9.2 and 6.7 ± 12.5% of the initially seeded chemokine, respectively). As expected (7), the CXC chemokine CXCL8 was not scavenged by D6 transfectants (2.4 ± 7.3% of the initially seeded chemokine).

Thus, D6 scavenges with variable efficacy agonists at the inflammatory chemokine receptors CCR1 (CCL3, CCL3L1, CCL5, and CCL7), CCR2 (CCL2 and CCL7), CCR3 (CCL5 and CCL11), and CCR5 (CCL3, CCL3L1, CCL4, and CCL5). Among inflammatory chemokines, the CCR4 agonists CCL22 and CCL17 have not been previously investigated for their ability to interact with D6 (7). As shown in Fig. 2, both chemokines were efficiently scavenged by D6. CCL17 was less efficiently removed than CCL22 by D6/CHO-K1 transfectants after short incubations (69.7 ± 3.0 and 86.4 ± 1.3% of the initially seeded chemokine at 1 h for CCL17 and CCL22, respectively), whereas at longer times of incubation, the two CCR4 agonists were scavenged with comparable efficiencies (81.8 ± 2.6 and 89.6 ± 2.6% of the initially seeded chemokine at

**Statistical analysis**

SDs were calculated, and statistical significance was assessed using paired two-tailed Student's t test. A value of p < 0.05 was considered significant.

**Results**

To better characterize the spectrum of chemokines recognized and scavenged by D6, D6/CHO-K1 transfectants were incubated for 3 h with 1.2 nM of various chemokines. At the end of the incubation, the chemokine concentration in the supernatant was measured by ELISA. As shown in Fig. 1, among known D6 ligands, the inflammatory CC chemokines CCL5 and CCL11 were scavenged with the highest efficiency (94.3 ± 0.2 and 92.5 ± 2.1% of the initially seeded chemokine, respectively). CCL7, CCL4, CCL2, and CCL3L1 were scavenged with intermediate efficiency (85.1 ± 0.5, 83.9 ± 2.9, 76.5 ± 2.2, and 76.0 ± 6.6% of the initially seeded chemokine, respectively). Interestingly, CCL3 and CCL3L1, which only differ in the presence of a serine or a proline residue in position 2, were scavenged with different efficacies (29.0 ± 4.1 and 76.0 ± 6.6% of the initially seeded chemokine, respectively), in agreement with previous results reporting that only the CCL3L1 variant is a high affinity D6 ligand (14). CCL11, which does not bind D6 (7), was the only inflammatory CC chemokine tested not scavenged by D6 (3.1 ± 1.4% of the initially seeded chemokine). None of the chemokines tested was scavenged by untransfected CHO-K1 cells. Unlike CC inflammatory chemokines, the homocistemic chemokines CCL19 and CCL20, agonists at CCR7 and CCR6, respectively, were not scavenged (8.2 ± 9.2 and 6.7 ± 12.5% of the initially seeded chemokine, respectively). As expected (7), the CXC chemokine CXCL8 was not scavenged by D6 transfectants (2.4 ± 7.3% of the initially seeded chemokine).

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**FIGURE 1.** D6-mediated chemokine scavenging. Untransfected CHO-K1 cells (□) or D6/CHO-K1 cells (■) were incubated for 3 h with 1.2 nM of the various chemokines. At the end of the incubation, the chemokine concentration in the supernatants was measured by ELISA. Results are expressed as the percentage of scavenged chemokine (mean ± SD; three replicates, at least three experiments). *, p < 0.05 compared with untransfected cells.
6 h, respectively). CCL17 and CCL22 scavenging was also analyzed in D6/L1.2 and D6/MELC-2 transfectants, with similar results (data not shown). As ELISA may detect partially degraded forms of chemokines, we tested the effective biological inactivation of CCL22 by analyzing the chemotactic activity of CCL22 preincubated with untransfected CHO-K1 or D6/CHO-K1 transfectants on CCR4/L1.2 transfectants (Fig. 3A). After 3 h of preincubation with untransfected CHO-K1 cells, the chemotactic activity of CCL22 was unaffected, whereas incubation with D6/CHO-K1 transfectants drastically reduced (≥100 times) CCL22-mediated chemotactic activity, in agreement with ELISA results.

To define the functional outcome of coexpression of CCR4 and D6 in the same cell context, L1.2 transfectants stably coexpressing both CCR4 and D6 (CCR4-D6/L1.2) have been generated. Flow cytometric analysis showed comparable expression of the two receptors (data not shown). When tested in a chemotaxis assay, CCR4-D6/L1.2 cells completely lost ability to migrate in response to CCL22 (Fig. 3B). On the contrary, cell migration in response to CXCL12, a chemokine not scavenged by D6, acting on the endogenous CXCR4, was unaffected by D6 coexpression (data not shown).

The interaction of CCR4 agonists with D6 was further investigated on D6/L1.2 transfectants in competition binding experiments with 125I-labeled CCL2 (Fig. 4). D6 binds CCL2 more strongly (Kᵦ = 0.33 nM), similarly to CCL2, whereas it binds CCL17 more weakly (Kᵦ = 2.9 nM), similarly to CXCL4. Similar results were obtained using 125I-labeled CCL22 (data not shown). As expected, CCL19 did not bind to D6. As for some G protein-coupled receptors, the apparent affinity of ligands can vary depending on the tracer. Competition binding experiments have also been performed using 125I-labeled CXCL4, with similar results (data not shown). As for other D6 ligands, treatment of D6/L1.2 transfectants with either CCL17 or CCL22 was unable to elicit any detectable calcium flux (data not shown) or cell migration (Fig. 5).

To determine whether ligand binding would induce D6 internalization, D6/L1.2 and CCR4/L1.2 cells were labeled with the appropriate receptor-specific mAb at 4°C, incubated at 37°C in the presence or the absence of the ligand (60 nM CCL22) for the indicated time period, and labeled with the appropriate secondary
antibody. Receptor expression levels were analyzed by flow cytometry as described in Materials and Methods. As shown in Fig. 6, CCL22 induced a significant and rapid decrease in cell surface CCR4 expression levels, in agreement with previous reports (15). On the contrary, a significant fraction of D6 receptors underwent internalization in both the presence and the absence of the ligand, and treatment with the ligand (60 nM CCL22) was unable to induce any further receptor internalization. Similar results were obtained using an anti-HA mAb, recognizing an HA tag inserted at the N terminus of D6 and CCR4 (data not shown). In addition, D6 underwent rapid recycling, as previously demonstrated (10), and data not shown.

CCL22 is processed by the dipeptidyl-peptidase IV (CD26) to produce the truncated forms CCL22 (3–69) and CCL22 (5–69) that lack, respectively, the first two and four amino acids at the NH₂ terminus and lose their agonist activity on CCR4 transfectants (11, 16). It was important to assess whether the promiscuous CC chemokine D6 receptor was able to interact with processed CCL22. As shown in Fig. 7A, after 3 h of incubation, D6-expressing cells scavenged unprocessed CCL22 (94.6 ± 1.8% of the initially seeded chemokine), but not the processed variants CCL22 (3–69) and CCL22 (5–69) (1.3 ± 3.6 and 3.5 ± 12.0% of the initially seeded chemokines, respectively). To understand whether the lack of scavenging of the NH₂-truncated molecules was due to their lost ability to interact with D6, competition binding analysis on D6/L1.2 transfectants using 125I-labeled CCL2 as tracer were performed. As shown in Fig. 7B, CCL22 (3–69) and CCL22 (5–69), unlike native CCL22, were unable to displace 125I-labeled CCL2, indicating that neither CCL22 processed form was recognized by D6.

Discussion

The results presented in this study confirm and extend previous observations on the promiscuous binding (7) and scavenging (10) of CC chemokines by the D6 receptor. Although the chemokine classification into homeostatic and inflammatory groups is not absolute (17, 18), it was found that D6 only recognizes and scavenges chemokines usually considered as inflammatory mediators, including agonists at the receptors CCR1 through CCR5. Unexpectedly, analysis of receptor internalization revealed that ligand scavenging is mediated by ligand-independent internalization. A similar behavior has been previously described for the human CMV-encoded chemokine receptor US28, which has also been proposed as a chemokine scavenger receptor (19).

Although recognizing and scavenging most inflammatory CC chemokines, D6 did not interact with CC chemokines such as CCL19 (CCR7 ligand) and CCL20 (CCR6 ligand), usually behaving as homeostatic chemokines. Thus, the spectrum of ligands recognized by the silent decoy receptor D6 contrasts with that of the receptor CCX-CKR, which binds the homeostatic chemokines CCL19, CCL21, and CCL25, with no signaling function assigned to date, and not the inflammatory chemokines (20). Whether CCX-CKR internalizes and scavenges homeostatic CC chemokines, as D6 does for inflammatory ones (10), has not been established.

In addition to known D6 ligands, the results presented in this study indicate that the spectrum of chemokines recognized by D6 also includes the CCR4 agonists CCL22 and CCL17. CCL22 and CCL17 are constitutively expressed in lymphoid organs, in particular in the thymus, spleen, lymph nodes, and, to a lesser extent, gut (18). Immature myeloid DC constitutively express low levels of CCL22 (21). However, as inducible chemokines, CCL22 and CCL17 are part of the regulatory circuits of polarized Th1 and Th2 responses. IL-4 and IL-13 induce CCL22 production, whereas IFN-γ inhibits it (22). Moreover, generic inflammatory signals (e.g., LPS) induce or augment CCL22 production (23, 24). Hence, CCL22 and CCL17 belong to both realms of homeostatic and inflammatory chemokines, and their recognition by D6 is therefore consistent with the general preferential interaction of this decoy receptor with inflammatory chemokines.
DC express CCR4 and respond to CCR4 agonists. CCL22 has been suggested to play a role in the trafficking of epidermal Langerhans cells at the inflammatory site (25) and in the formation of T cell-DC clusters in both inflamed skin and lymph nodes (26, 27). D6 is strategically located on endothelial cells lining afferent lymphatics (8) and has been suggested to act as a gatekeeper to prevent excessive transfer of inflammatory chemokines to lymph nodes. By recognizing CCR4 agonists, D6 may regulate DC migration to lymph nodes via afferent lymphatics.

CCL22 is processed by dipeptidyl peptidase IV (CD26) to produce the processed variants CCL22 (3–69) and CCL22 (5–69), which lose the capacity to interact with CCR4 (11, 16). Dipeptidyl peptidase IV is widely expressed in cells and tissues and is more abundant in Th1 than in Th2 cells (18, 28). Interestingly, processed CCL22 forms are not recognized by the promiscuous receptor D6. The selective recognition of CCL22 vs CCL22 (3–69) and CCL22 (5–69) may represent a strategy to focus the decoy function on the CCR4 agonists, without interference from inactive processed forms.

References