Identification of a Novel Chemokine (CCL28), which Binds CCR10 (GPR2)*

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We report the identification and characterization of a novel CC chemokine designated CCL28 and its receptor CCR10, known previously as orphan G-protein-coupled receptor GPR2. Human and mouse CCL28 share 83% identity at the amino acid and 76% at the nucleic acid levels. We also identified the mouse homologues of CCL28 and of CCR10, which map to mouse chromosomes 13 and 11, respectively. CCL28 is expressed in a variety of human and mouse tissues, and it appears to be predominantly produced by epithelial cells. Both human and mouse CCL28 induce calcium mobilization in human and mouse CCR10-expressing transfectants. CCL28 desensitized the calcium mobilization induced in CCR10 transfectants by CCL27, indicating that these chemokines share this new chemokine receptor. In vitro, recombinant human CCL28 displays chemotactic activity for resting CD4 or CD8 T cells.

Chemokines and their receptors play a pivotal role in lymphocyte trafficking, recruiting, and recirculation (1). Additionally, recent evidence suggests a critical role for chemokines in a variety of pathophysiological processes (e.g. infectious and autoimmune diseases, allergic responses, modulation of angiogenesis, chronic and acute inflammation, tumor growth, and proliferation of hematopoietic progenitor cells). Chemokines are a superfamily of small (8–16 kDa) proteins sharing 20–70% homology in amino acid sequences. According to the relative position of their cysteine residues, chemokines can be subdivided into four families, including CXC (α-chemokine), CC (β-chemokine), C (γ-chemokine), and CX3C (δ-chemokine). A new nomenclature has recently been proposed for the chemokine ligands, which we will use in this paper (1).

The CXC chemokines, with the first two cysteines separated by one amino acid residue, are subdivided into two subclasses, based upon the presence or absence of the amino acid motif ELR (glutamic acid-leucine-arginine) at the N terminus. Most ELR chemokines, including CXCL8/IL-8, CXCL1/Groα, CXCL5/ENA-78, CXCL7/NAP-2, and CXCL6/GCP-2, are strong neutrophil chemoattractants. However, non-ELR chemokines, exemplified by CXCL10/IP-10, CXCL9/MIG, CXCL11/I-TAC, CXCL12/SCF-1, and CXCL13/BLC, recruit predominantly lymphocytes (2). The second family, the CC chemokines, attract predominantly monocytes, basophils, eosinophils, and T-cells but not neutrophils. Typical CC chemokines include CCL5/RANTES, CCL3/MIP-1α, CCL4/MIP-1β, CCL2/MCP-1, CCL11/eotaxin, CCL21/6ckine, CCL20/MIP3α, and CCL19/MIP3β. The C and CX3C families are comprised of one member each. XCL1/lymphotactin and CX3CL1/fractalkine (neurotactin), respectively. XCL1 is a potent chemoattractant for T and natural killer cells (3, 4). CX3CL1 is the only membrane-bound chemokine and attracts monocytes and T lymphocytes but not neutrophils (5).

Within an organism, cells are exposed to a complex pattern of chemokine signals that must be integrated such that cells arrive at their designated destination in the target tissue or lymphoid organ. These signals are mediated by the interactions of chemokines with seven-transmembrane G-protein-coupled receptors (GPCR) located on the cell surface. Chemokine receptors transduce their signals through heterotrimeric G proteins, which are sensitive to pertussis toxin (6). Based on their ligand specificity, chemokine receptors can be divided into several families. Thus far, five CXC receptors (CXCR1 through CXCR5) (7) and nine CC receptors (CCR1 through CCR9) have been identified (6). Recently, the receptors for XCL1 (8) and CX3CL1 (9) have been described. Additionally, a number of orphan GPCRs (STRL33, CRKX/HRK, GPR15, and GPR2) have been identified whose sequences cluster with other defined chemokine receptors, but as yet no ligand has been identified for these receptors (10, 11).

Our laboratory has been searching for novel chemokines and their receptors to understand more about their participation in homeostatic and pathological processes. Here, we describe the identification and characterization of a novel CC chemokine, which we have designated CCL28, according to the recently proposed nomenclature for chemokine ligands (1). CCL28 shares most homology with another recently described chemokine, known previously as orphan G-protein-coupled receptor GPR2, which shares 76% identity at the amino acid and 68% at the nucleic acid levels with CCL28. Additionally, CCL28, which we have designated CCL28, according to the recently proposed nomenclature for chemokine ligands (1), shares most homology with another recently described chemokine, known previously as orphan G-protein-coupled receptor GPR2, which shares 76% identity at the amino acid and 68% at the nucleic acid levels with CCL28.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF229238 and AF229239 (mouse and human CCL28, respectively) and AF208238 (mouse GPR2/CCR10).

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The abbreviations used are: GPCR, G-protein-coupled receptor; HGS, Human Genome Sciences; EST, Expressed Sequence Tags; bp, base pair(s); IL-3, interleukin-3; kb, kilobase(s); PCR, polymerase chain reaction; HBSS, Hank’s balanced salt solution.

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kine (CTACK/CCL27) whose receptor has been identified as the previously orphan G-protein-coupled receptor GPR2 (11), which has now been renamed CCR10 (12). In this study, we show that CCL28 also binds CCR10, making it the second ligand of this new chemokine receptor. Their expression pattern suggests that this ligand/receptor pair may be particularly important in the homeostasis or inflammatory responses of the gastrointestinal system.

MATERIALS AND METHODS

Identification of CCL28—TLBASTN searches of the Human Genome Sciences (HGS) and GenBank dbEST data base using sequences of known β-chemokines, identified several Expressed Sequence Tags (ESTs) for human CCL28. Human CCL28 cDNA, IMAGE consortium clone 136910 (GenBank™ accession number R04595), was obtained from Genome Systems as an EcoRI/NorI insert in the pT77T-PacD vector. The nucleotide sequence was confirmed by automated sequencing. The signal peptide cleavage site was predicted using the SignalP server.

Mouse CCL28 was cloned from a kidney cDNA library derived from Rag-1−/− mice using the full-length human CCL28 as a probe. Prehybridization and hybridization were carried out at 60 °C in 0.5 M Na2HPO4, 7% SDS, 0.5 mM EDTA, pH 8. Filters were washed four times at 60 °C in 1× SSC, 0.1% SDS and twice in 0.5 × SSC, 0.1% SDS. After two rounds of screening, several positive clones were identified. These clones were sequenced, and their sequences were analyzed using Sequencer (Genecodes, Ann Arbor, MI) and aligned using CLUSTAL W.

Cloning of Mouse CCR10 (GPR2) and Production of Stable Transfectants—The full-length cDNA encoding mouse CCR10 was isolated from a TCROq+CD4−CD8− double negative thymocyte cDNA library, using a 920-base pair (bp) EcoRI/NorI probe isolated from an IMAGE consortium clone (1311245, Genome Systems) previously identified as the mouse homologue of human CCR10 (GPR2) (11) (12). Library screening and sequencing of identified clones was performed as described for mouse CCL28.

To generate stable transfectants, full-length expression constructs were made in the mouse interleukin-3 (IL-3)-dependent B-cell line, BaF3. Full-length mouse CCR10 (GPR2) was cloned into the expression vector pFLAG-CMV3 (Sigma), containing an N-terminal M2 FLAG fusion sequence, which also permitted the detection of cell surface expression using an anti-M2 antibody (Sigma). BaF3 cells were transfected by electroporation according to a standard protocol. All transfectants were selected in the presence of 1 μg/ml G418 and 1 mg/ml hygromycin B. Stable cell lines were maintained by fluorescence-activated cell sorting using the M2 antibody to the N-terminal FLAG tag. The production of human CCR10 transfectants has been described (12).

Mapping Mouse CCL28 and CCR10 (GPR2)—Mouse CCL28 and CCR10 were mapped as described previously (3) except that, in this case, a radiolabeled probe of ~1-kilobase fragment from mouse mouse CCL28 or CCR10 was used for Southern blotting, respectively. For CCL28, fragments of 4.2 and 2.9 kb were detected in SacI-digested C57BL/6J DNA and fragments of 5.4, 3.4, and 2.2 kb were detected in SacI-digested Mus spretus DNA. The presence or absence of the 5.4-, 3.4-, and 2.2-kb SacI M. spretus-specific fragments, which cosegregated, was followed in backcrossed mice. Recombination distances were calculated using Map Manager, version 2.6.5. The gene order was determined by minimizing the number of recombination events required to explain allelic distribution. In a similar manner mouse CCR10 (GPR2) was mapped. In this case a fragment of 4.9 kb was detected in EcoRI-digested C57BL/6J DNA, and a fragment of 28.5 kb was detected in EcoRI-digested M. spretus DNA. The presence or absence of the 28.0-kb fragment was followed in backcrossed mice.

Real Time Quantitative Polymerase Chain Reaction (TaqMan) Analysis of Human and Mouse CCL28 and CCR10 (GPR2) mRNA Expression—Total RNA was isolated using the RNeasy method (Qiagen, Santa Clarita, CA) and treated with 10 units of RNase-free DNase I (Roche Molecular Biochemicals). Total RNA (5 μg) was reverse-transcribed using Superscript II (Life Technologies, Inc.). cDNA (50 ng) was analyzed for the expression of human and mouse CCL28 and CCR10 (GPR2) genes by the Fluorogenic 5′-nuclease polymerase chain reaction (PCR) assay (13), using a Prism 7700 sequence detection system (SDS, ABI/Perkin-Elmer, Foster City, CA). Reactions were incubated for 2 min at 50 °C, denatured for 10 min at 95 °C, and 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. Gene-specific primers and probes, used to generate amplicons, were analyzed with 6-carboxyfluorescein-labeled predeveloped TaqMan assay reagents (Perkin-Elmer). An 18 S ribosomal RNA amplicon was analyzed with a labeled probe (Perkin-Elmer) and used as an internal control for quantitation of the total amount of RNA in a multiplex reaction. Five 10-fold dilutions of plasmids (1 ng/ml) containing chemokine and chemokine receptor cDNA were used to create a standard curve for each gene. RT-PCR products were detected using the SDS software. Values were adjusted for the total amount of target gene cDNA and are expressed as femtograms of cDNA per 50 ng of total input cDNA. cDNA from different libraries were also used to analyze the distribution of CCL28 and CCR10. The specificity of primer/probe combinations was confirmed by crossreactivity studies using a panel of well known human genes, the R2C1R1-CCR10, CXCR1-CXCR5, XCR1, CX3CR1 and orphan (STRL33, GPR15) chemokine receptors, and the following panel of chemokines: CCL3/MIP-1α, CCL4/MIP-1β, CCL15/MIP-1β, CCL19/MIP-3β, CCL20/MIP-3α, CCL21/6Ckine, CCL10/IP-10, CCL9/MIG, CCL11/TARC, CCL14/HCC-1, CCL16/HCC-4, CCL17/GROα, CXCL1/groA, CXCL5/ENA78, CXCL4/F4, CCL22/MDC, CCL13/lymphotactin, CXCL8/IL-8, CCL17/TARC, CCL25/TECR, CCL2/MCP-1, CCL8/MCP-2, CCL7/MCP-3, and CCL3/MCP-4. The specificity of the mouse homologues for both chemokine receptors and ligands was similarly analyzed.

Where limited amounts of RNA precluded direct analysis, expression was assessed using plasmid cDNA libraries prepared using the SuperScript plasmid system for cDNA synthesis and plasmid cloning (Life Technologies, Inc.). 50 ng of cDNA was des signed previously (12). A glyceraldehyde-3-phosphate dehydrogenase RNA amplicon with a labeled probe (Perkin-Elmer) was used as an internal control for quantitation.

Chemotaxis—Recombinant mouse and human CCL28 proteins were produced in Escherichia coli and purified as described previously (14). For chemotaxis assays, human blood mononuclear cells were isolated on a Ficoll gradient, and T cell purification was performed using a human T-cell enrichment column kit (R&D Systems, Minneapolis, MN). Chemotaxis assays were performed as described previously (15). Peripheral blood mononuclear cells, peripheral blood lymphocytes, freshly isolated T cells, or anti-CD3-activated T cells were resuspended in RPMI 1640 containing 1% bovine serum albumin and 10 μM HEPES at 1 × 10⁷/ml and were added to the upper chamber of a polycarbonate Transwell culture insert (6.5-mm diameter, 3-μm pore size for lymphocytes and B cells, and 5-μm pore size for monocytes and macrophages) and incubated with the indicated concentrations of purified chemokine in the bottom chamber. Assays were carried out at 37 °C for 1 h with monocytes, 1.5 h with macrophages, 2.5 h with T-cells, and 3 h with B-cells. The number of migrating cells of each subtype was determined by manual cell number flow cytometry using fluorescein-conjugated antibodies (Pharmingen, San Diego, CA) against CD3, CD4, CD8, CD62L, and CD45RO for T-cells, CD19 for B-cells, and CD14 for monocytes. A predetermined amount of 15-μm microsphere beads (Bangs Laboratories, Fisher, IN) was added to each sample before analysis to determine absolute numbers of migrating cells. Additionally, chemotaxis assays, as described above, were also performed using human and mouse CCR10 (GPR2) transfectants. These assays, transfectants and parental lines were added to a chamber with a 3-μm pore size and incubated for 3 h. Each chemotaxis experiment was performed in triplicate a minimum of three times, and a representative experiment is shown.

Immunohistochemistry—A polyclonal antibody against a 20-amino acid peptide (residues 78–98) of mouse CCL28 was prepared in rabbits by Zymed Laboratories Inc. (South San Francisco, CA). Preimmune and immune sera were purified by protein A columns (Pierce). Mouse gut samples were obtained from Rag-1−/− mice (Jackson Laboratories). Tissue sections 5 μm thick were obtained and fixed in 3% formaldehyde in phosphate-buffered saline for 15 min at room temperature, and treated as described previously (14). Briefly, sections were sequentially blocked for endogenous biotin binding using the Vector Blocking Kit (Vector Laboratories, Burlingame, CA) and for endogenous peroxidase activity with 1.5% H2O2, 0.2 M Na3N in Hanks’ balanced salt solution with 0.01 M HEPES (HBSS-HEPES) with 0.1% saponin. Nonspecific binding was blocked with 10% normal goat serum. After incubation with preimmune or immune sera overnight, sections were stained with biotin-labeled goat anti-rabbit IgG (Vector) and then treated with the ABC Elite kit (Vector) according to the manufacturer’s instructions. Slides were washed with HBSS-HEPES and developed with 3,3′-diaminobenzidine tetrahydrochloride substrate (0.5 mg/ml) in 0.05 M Tris, pH 7.4, 0.0075% H2O2 (Sigma).

Calcium Mobilization Assays—To identify a receptor that would respond to human and mouse CCL28, a panel of known human (CCR1, 2, 3, 4, 5, 6, 7, 8, and 9, XCR1, CX3CR, CXCR1, 2, 3, and 5) and orphan (STRL33, GPR15, and GPR2) chemokine receptors was
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tested for signaling using calcium mobilization assays. Additionally, a smaller panel of known and orphan mouse chemokine receptors was also tested. Briefly, human and mouse transfectants as well as appropriate parental cell lines were loaded for 60 min at 37 °C with 3 μM INDO-IAF (Molecular Probes, Eugene, OR). Cells were washed and resuspended in HBSS plus 1% fetal calf serum (Life Technologies, Inc.) to a final concentration of 10^6 cells/ml. Calcium mobilization was measured using a Photon Technology International spectrophotometer with excitation at 350 nm and dual simultaneous recording of fluorescence emission at 400 and 490 nm. Relative intracellular calcium levels are expressed as the 400 nm/490 nm emission ratio. Experiments were performed at 37 °C with constant mixing in a cuvette containing 10^6 cells in 2 ml of HBSS with 20 mM HEPES, pH 7.3, and 1.6 mM CaCl_2. Each calcium mobilization experiment was performed a minimum of three times, and a representative experiment is shown.

Human Tissue Samples—Approximately half of the intestinal samples in this study were colon biopsies obtained from patients undergoing colonoscopy for diagnosis of inflammatory bowel disease at the Instituto Nacional de la Nutrición Salvador Zubirán, México, D.F. Samples from both lesional and nonlesional sites were taken. Additional human small and large intestine samples were obtained from the Cooperative Human Tissue Network and the National Disease Research Interchange from patients undergoing bowel resection.

RESULTS

Identification of Novel Human and Mouse CC Chemokine (CCL28)—Using a human chemokine consensus sequence as the basis for a TBLASTN search of the HGS and GenBank® data bases, three ESTs were identified, which encoded a new CC chemokine. The ESTs identified in the HGS data base were derived from human fetal heart and osteoblast cDNA libraries and were not full-length. A single EST from the GenBank® dbEST data base was also identified. This clone comprised the entire open reading frame and encoded a novel CC chemokine of 127 amino acids with a predicted 22-amino acid signal peptide at the N terminus. In the mRNA, the 3'-untranslated region has a long terminal repeat/retroviral element of 244 bp and one mRNA instability sequence (ATTTA) characteristic of cytokine mRNAs.

The mouse homologue was cloned from a Rag-1^−/− kidney cDNA library. Several 2-kb full-length clones for mCCL28, with an open reading frame of 393 bp were identified. The 3'-untranslated region has a LTR/MalR element of 340 bp, two simple repeats and two low complexity repeats, one instability sequence (ATTITAT), and one polyadenylation signal (AATAAA) in the typical position (data not shown). As shown in Fig. 1A, Human and mouse CCL28 are highly conserved, because they share 83 identical amino acids; at the nucleic acid level, hCCL28 is 76% identical to its mouse counterpart in the translated region.

The optimal concentration ranged between 1 × 10^{-7} and 2 × 10^{-7} M and was donor-dependent (Fig. 2). Anti-CD3-activated T-cells did not respond to this chemokine.

Human CCL28 is highly expressed in normal and pathological colon (see Fig. 6 below), so we also tested whether CCL28 chemoatracted β7^+ T-cells. The β7 integrin group includes two members that are believed to be involved in lymphocyte homing into the mucosal tissues: αββ_7 and αββ_7 (16). However, we did not observe a response of these cells to CCL28 (data not shown).

The Orphan GPCR, GPR2, Is the Receptor of CCL28—Once we established that human CCL28 was biologically active, we sought to identify the receptor for this novel chemokine. Initially we examined the ability of CCL28 to induce a calcium flux in all known chemokine receptor transfectants but CCL28 failed to mobilize calcium in any of them. As positive controls, chemokines known to bind to these receptors induced the expected responses in the same assay (data not shown). Known chemokine receptors tested were CXCR1–5, CCR1–9, XCR1, and CX3CR1. We then tested transfectants of the previously known orphan G-protein-coupled receptor GPR2, because we recently found it to be the receptor of CCL27/CTACK (12) and has been renamed CCR10. However, we first sought to identify mouse CCR10. The mouse homologue of GPR2 was isolated from a TCRαβ^+ CD4^−CD8^− T-cell cDNA library. Human and mouse GPR2 encode a putative protein of 363 amino acids and display 87% identity to one another (Fig. 3A). Mouse and human CCR10 are most closely related to human CXCR5 and CXCR3 (Fig. 3B). Mouse GPR2 maps to the distal region of chromosome 11, a region syntenic to human chromosome 17q where human GPR2 has previously been mapped (Fig. 3C).

Stable transfectants of the full-length human and mouse GPR2 were produced in the mouse IL-3-dependent B-cell line, BaF3. This cell line expresses mouse CXCR4 endogenously and is responsive to CXCL12/SDF-1α, which was used as an internal positive control. Human CCL28 was able to produce a robust calcium signal in both human and mouse GPR2 transfectants (Fig. 4A) in a dose-dependent manner. Furthermore, human CCL28 desensitized the calcium mobilization response induced in mouse CCR10 transfectants by mouse CCL28 and vice versa, whereas hCXCL12/SDF-1α did not (Fig. 4B). Similar results were observed when testing human or mouse CCL28 in human CCR10 transfectants (Fig. 4C). In addition, the parental line BaF3 was unresponsive to CCL28 (Fig. 4, B and C) or to CXCR4 transfectants (data not shown). These results indicate that CCR10 is a receptor for CCL28. Finally, the CCL27/CTACK-induced calcium flux in human or mouse CCR10 transfectants can be specifically desensitized by CCL28 (Fig. 4, B and C) and vice versa, indicating that these two chemokines share receptor specificity.

We also tested CCL28 on other orphan G-protein-coupled receptors likely to be chemokine receptors, including STRL33 and GPR15 (10, 17) but did not observe any responses in these transfectants (data not shown).

Expression Pattern of Human and Mouse CCL28 and CCR10—Several approaches were used to assess the distribution of human and mouse CCL28 mRNA in a variety of normal and disease tissues as well as in many lymphoid and hematopoietic cell lines (resting and activated). A Northern blot containing mRNA from a variety of human organs and tissues showed that CCL28 is expressed predominantly in prostate, colon, spleen, and to a lesser degree in peripheral blood leukocytes. Message sizes were 0.8, 1, 3, and 6 kb (data not shown). Comparable blots of mouse tissue revealed that CCL28 was mainly expressed in the testis and to a lesser degree in kidney.
FIG. 1. Molecular characteristics of human and mouse CCL28. A, amino acid sequence alignment of human (h) and mouse (m) CCL28. The predicted signal peptide cleavage site is indicated by an arrow. B, amino acid sequence of mouse and human CCL28 aligned with other CC chemokines, hCCL27/CTACK, hCCL25/TECK, hCCL17/TARC, hCCL20/MIP-3α, hCCL19/MIP-3β, and hCCL21/6Ckine. In A and B, boxed residues indicate identical or conserved amino acids. C, dendrogram showing the relationship of CCL28 with other closely related CC chemokines. D, mCCL28 maps to the distal region of chromosome 13. CCL28 was located to mouse chromosome 13 by interspecific backcross analysis. The
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and brain with message sizes of 1, 2, and 5 kb (data not shown). For cell lines and tissues where limited amounts of mRNA precluded Northern analysis, expression in many cDNA libraries was determined by real time quantitative PCR (TaqMan) analyses and normalized to internal standards. Fig. 5 shows the expression of mouse CCL28 and CCR10 in mouse tissues and cell lines, whereas Fig. 6 shows the distribution of human CCL28 and CCR10 in various tissues and cell lines. This analysis indicated that human CCL28 is less abundantly expressed in libraries of lymphoid or hematopoietic origin. Conversely, CCL28 was highly expressed in libraries produced from mRNA obtained from organs, especially in human colon as well as in an ulcerative colitis sample. Human CCR10 exhibited a wider tissue distribution profile with expression in some B- and T-cell clones, monocytes, and a variety of tissues, including normal human colon (Fig. 6). We sought to identify the cells producing CCL28 in the colon. To this end, we performed immunohistochemical analysis and detected a CCL28 signal consistent with its production by epithelial cells (Fig. 7). This pattern also accounts for the organ distribution shown in Figs. 5 and 6, because CCL28 was expressed in organs where epithelial cells are localized. However, because of the expression of CCL28 and CCR10 in colon, we decided to compare the expression of CCL28 and CCR10 in lesional and nonlesional human ulcerative colitis samples comparing them to normal colon controls. As shown in Fig. 8, there was no significant difference in the expression of CCL28 or CCR10 between normal or pathological samples. Normal colon (n = 15) showed in average 256 ± 250 fg/50 ng cDNA for hCCL28, and 4.95 ± 2.09 fg/50 ng cDNA for hCCR10. Nonlesional samples of ulcerative colitis colon (n = 10) showed 393 ± 185 fg/50 ng cDNA for hCCL28 and 3.35 ± 1.12 fg/50 ng cDNA for hCCR10, whereas lesional samples of ulcerative colitis colon (n = 10) showed 358 ± 174 fg/50 ng cDNA for hCCL28 and 3.39 ± 1.68 fg/50 ng cDNA for hCCR10.

DISCUSSION

In this study, we present a new CC chemokine, CCL28, which is the second chemokine ligand that binds to the previously known orphan G-protein-coupled receptor GPR2, now renamed CCR10 (12). The availability of large data bases of Expressed Sequence Tags (ESTs) has made possible the rapid identification of new members of chemokines based on sequence and structural homologies. We identified a full-length cDNA encoding human CCL28 using a human chemokine consensus sequence. CCL28 has a number of interesting features. Both human and mouse CCL28 have 6 cysteines, which represent 2 extra cysteines in addition to the 4 conserved common cysteines, one of them between the second and the third cysteines, and another after the fourth cysteine. Several chemokines have been identified that contain two additional cysteines in various positions. CCL23/MIPIF-1, CCL6/C10, and CCL9/MIP-1γ have one additional conserved cysteine located between the usual Cys-2 and Cys-3 positions and a second additional conserved cysteine positioned after Cys-4 (1). CCL1/I-309 and its mouse homologue TCA-3 have a similar pattern of additional cysteines, which by CLUSTAL W alignment correlate to the 2 additional cysteines present in mouse and human CCL28 (data not shown). CCL21/6Ckine is another chemokine with 6 cysteines, with the two additional ones located after the 4 conserved chemokine cysteines in the long α-helix tail C terminus (18). It is possible that the 2 additional cysteines form a disulfide bond as has been proposed for CCL15/HCC-2 (19). Another characteristic of mouse and human CCL28 is its long C-terminal tail. It is known that both CC and CXC chemokines possess a C-terminal helix (20, 21). The additional disulfide might join the C terminus to the core of the protein, and therefore may restrict its mobility. Disruption or truncation of the C-α helix eliminates the biological activity of these molecules (4, 22).
One interesting feature of human and mouse CCL28 is their high RNA expression in normal colon, and in tissues derived from mouse models of inflammatory bowel disease or human ulcerative colitis, as well as in mouse gut associated lymphoid tissue. Human CCL28 RNA was highly expressed by an epithelioid tumor cell line, HCT 116, derived from colon carcinoma, as well as by colorectal adenocarcinoma cell lines, DLD-1 (data not shown). These data, along with immunohistochemical staining, suggest that CCL28 is produced by epithelial cells. Thus, it is possible that this chemokine plays a homeostatic role in immunological surveillance in the gut. CCL28 RNA was also present in normal human lung, monkey lung, and in several asthmatic lung tissues (data not shown), suggesting that CCL28 is a homeostatic chemokine but may also participate in inflammatory responses associated to the mucosal immune system (23). Mouse CCL28 was also detected up-regulated in tissues from the IL-10−/− mouse, suggesting that IL-10 down-regulates the expression of this chemokine.

One of the most important aspects of finding a new chemokine is that it may represent the ligand for a previously known receptor. Mouse CCR10 shares 87% identity with human CCR10 at the amino acid level. B, dendrogram showing the relationship of CCR10 with other known chemokine receptors, CCR1–9 and CXCR1–5. CCR10 is most closely related to CXCR3 and CXCR5. C, mouse CCR10 maps to the distal region of chromosome 11, a region syntenic to human chromosome 17q where human CCR10 was previously mapped. (See the legend to Fig. 1 for further details of mapping.)
Calcium mobilization of CCR10 transfectants stimulated with CCL28. A, dose-dependent calcium mobilization response on INDO-1AM-loaded BAF-3-CCR10 cells. Left, BAF-3 cells transfected with mouse CCR10 (BaF/3-mCCR10) were stimulated with 1 nM, 10 nM, and 100 nM mouse CCL28 (mCCL28) chemokine. Right, BaF/3 cells transfected with human CCR10 (BaF/3-hCCR10) were stimulated with 1 nM, 10 nM, and 100 nM human CCL28 (hCCL28) protein. B, calcium mobilization desensitization with BaF/3-mouse CCR10-transfected cells. 200 nM mouse CCL28, human CCL28, mouse CCL27/CTACK, and human CXCL12/SDF-1α were applied sequentially as indicated. C, calcium mobilization desensitization with BaF/3-human CCR10-transfected cells. 200 nM human CCL28, mouse CCL28, human CCL27, and human CXCL12 were applied as indicated. In B and C the parental BaF/3 cell response is presented in the lower tracing.
orphan G-protein-coupled receptor. To this end, we tested CCL28 against a panel of known orphan receptor transfectants. Although it failed to flux any of the known chemokine receptor transfectants, we detected a significant Ca\textsuperscript{2+} flux in transfectants of the previously known orphan receptor, GPR2. We have recently observed that GPR2 is the receptor for the skin-associated chemokine CCL27/CTACK (12), which is also the chemokine that shares the highest homology with CCL28 (Fig. 1B). GPR2 has several structural features that strongly suggest that it is a chemokine receptor, including the presence of a DRY box motif (which is present in most of the other chemokine receptors) as well as strong homology to CXCR3 and other chemokine receptors (Fig. 3B). Accordingly, this receptor has been renamed CCR10 (12). We also identified mouse CCR10 (Fig. 3A). Both human and mouse CCR10 transfectants fluxed Ca\textsuperscript{2+} in response to CCL28. Furthermore, CCL28 desensitized the CCL27 responses, indicating that these chemokines share the CCR10 receptor. Interestingly, the expression of CCL27/CTACK is strongly associated with the skin, whereas the expression of CCR10 is more widespread. This led us to speculate that there are other ligands for this receptor (12). In particular, CCR10 is strongly expressed in the gut where CCL28 is also expressed (Figs. 5 and 6). CCL28 as well as CCR10 expression is already strong in normal gut samples,

![Identification of CCL28](image-url)
suggesting that this ligand/receptor pair plays a homeostatic role in the gut. We have made similar observations in the skin (12). However, because both of these anatomical sites are also continuously exposed to antigens, it is possible that the expression observed may represent an inflammatory background rather than homeostatic expression. It is known that freshly isolated colon epithelial cells spontaneously secrete CXCL8/IL-8, CCL2/MCP-1, CXCL1/Groα, CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/Rantes (24), which are generally considered inflammatory rather than homeostatic chemokines. We found that CCL28 is expressed by normal and pathological colon, and the protein was detected by immunostaining in epithelial cells from colon or small intestine. It is possible that certain T-cell populations enriched in the gut, like αβ7-positive cells present in lamina propria and/or intraepithelial T-cells or γδ intraepithelial T-cells (25), may be attracted by CCL28. Future experiments will investigate these possibilities.

Typically, CC chemokines mapping outside the CC chemokine cluster act on lymphocytes (1), and CCL28 appears to be no exception, because we observed responses to CCL28 by both CD4 and CD8 T-cells. CCR10 is expressed in some T-cells as well as in some B-cell lines but not in normal B-cells. The latter

**Fig. 6. Human CCL28 and CCR10 expression in various mouse tissue and cells.** Real time quantitative PCR (TaqMan) analyses were performed using plasmid cDNA libraries prepared from human tissue and cells. A glyceraldehyde-3-phosphate dehydrogenase amplicon was used as an internal control for quantitation. Values were adjusted for the total amount of cDNA and expressed as femtograms of cDNA per 50 ng of input total RNA.
observation suggests that normal B-cells may respond to CCL28 under certain circumstances but not in the resting state. It is necessary to perform further studies to characterize the subpopulations of T-cells that express CCR10 and respond to CCL28. Furthermore, it is possible that CCR10 is regulated by the activation state of the T-cells (12). Future studies will

**FIG. 7.** CCL28 is expressed by epithelial cells. Intracellular staining for mouse CCL28 expression with a specific polyclonal antibody was performed as described under "Materials and Methods." Staining with anti-mouse CCL28 polyclonal antibody small intestine (A) and colon (B) from RAG-2⁻/⁻ mice. Staining with preimmune serum as a control small intestine (C) and colon (D). Staining of mouse small intestine with anti-e-cadherin monoclonal antibody (E) or with an isotype control (F).

**FIG. 8.** Expression of human CCL28 and human CCR10 in colon samples by TaqMan analysis. Real time quantitative PCR (TaqMan) analysis was carried out using total RNA derived from human samples (see "Materials and Methods"). Each point represents an individual patient, and the groups represent: 1, normal colon; 2, nonlesional ulcerative colitis colon; 3, lesional ulcerative colitis colon. Values were adjusted for the total amount of cDNA and expressed as femtograms of cDNA per 50 ng of input total RNA. Panel A, levels of hCCL28; Panel B, levels of hCCR10 detected in colon samples.
attempt to clarify the target populations of this novel chemokine.

In summary, we present here a new chemokine ligand/receptor pair, which is potentially involved in both homeostatic and inflammatory responses in the skin and gut.

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