Liver-Expressed Chemokine/CC Chemokine Ligand 16 Attracts Eosinophils by Interacting with Histamine H4 Receptor[1]

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Liver-expressed chemokine (LEC)/CCL16 is a human CC chemokine that is constitutively expressed by the liver parenchymal cells and present in the normal plasma at high concentrations. Previous studies have shown that CCL16 is a low-affinity ligand for CCR1, CCR2, CCR5, and CCR8 and attracts monocytes and T cells. Recently, a novel histamine receptor termed type 4 (H4) has been identified and shown to be selectively expressed by eosinophils and mast cells. In this study, we demonstrated that CCL16 induced pertussis toxin-sensitive calcium mobilization and chemotaxis in murine L1.2 cells expressing H4 but not those expressing histamine receptor type 1 (H1) or type 2 (H2). CCL16 bound to H4 with a K_d of 17 nM. By RT-PCR, human and mouse eosinophils express H4 but not H3. Accordingly, CCL16 induced efficient migratory responses in human and mouse eosinophils. Furthermore, the responses of human and mouse eosinophils to CCL16 were effectively suppressed by thioperamide, an antagonist for H3 and H4. Intravenous injection of CCL16 into mice induced a rapid mobilization of eosinophils from bone marrow to peripheral blood, which was also suppressed by thioperamide. Collectively, CCL16 is a novel functional ligand for H4 and may have a role in trafficking of eosinophils. *The Journal of Immunology,* **2004, 173: 2078–2083.**

Chemokines play important roles in innate and acquired immunity by inducing directed migration of various types of leukocytes through interactions with a group of seven-transmembrane G protein-coupled receptors (GPCRs)[3] (1). Liver-expressed chemokine (LEC)/CCL16 (2), also known as novel CC chemokine 4 (2), human CC chemokine 4 (3), lymphocyte and monocyte chemoattractant (4), and liver-specific CC chemokine 1 (5), is selectively expressed in the liver and present in the normal plasma at high concentrations (6). CCL16 was shown to be induced in monocytes by IL-10 (3) and chemotactic for monocytes and lymphocytes (3, 4). In addition, this chemokine was shown to have a potent myelosuppressive activity comparable to that of MIP-1α/CCL3 (4) and to induce tumor rejection (7). Previously, we have shown that CCL16 is a low-affinity functional ligand for CCR1, CCR2, and CCR5 (6). Separately, Howard et al. (8) reported that CCL16 induced chemotaxis and cell adhesion to matrix proteins of human monocytes via CCR1 and CCR8. However, its physiological role still remains mostly unknown.

Histamine receptor type 4 (H4) is the most recently identified subtype of histamine receptors (9, 10). H4 is selectively expressed by cells such as eosinophils and mast cells and mediates histamine-induced chemotaxis and calcium mobilization in these types of cells (11, 12). Chemoattractant receptors including those for chemokines often interact with quite diverse molecules. For example, CCR6, the receptor for CCL20 (1), is also a receptor for β-defensins (13). HIV-1 Tat protein attracts monocytes via CCR2 and CCR3 (14). Histidyl-tRNA synthetase and asparaginyl-tRNA synthetase, the autoantigens in myositis, attract T cells, IL-2-activated monocytes, and immature dendritic cells via CCR5 and CCR3, respectively (15). Formyl peptide receptor-like 1, the low-affinity receptor for the bacterial chemotactic peptide fMLF, is also a receptor for lipoxin A_4 and acute phase protein serum amyloid A (16, 17). Given such versatility in the chemoattractant receptors in terms of ligand-specificity, we tested a large panel of human chemokines on histamine receptors H1, H2, and H4 (18). In this study, we report that CCL16 is a functional ligand for H4 and induces an efficient migration of human and mouse eosinophils via H4.

Materials and Methods

Materials

All recombinant human chemokines were purchased from R&D Systems (Minneapolis, MN). Histamine and thioperamide were purchased from Sigma-Aldrich (St. Louis, MO). Pertussis toxin (PTX) was purchased from Invitrogen (Carlsbad, CA). A murine L1.2 pre-B cell line was kindly provided by E. Butcher (Stanford University School of Medicine, Stanford, CA).

Stable expression of histamine receptors

The coding regions of human histamine receptors H1, H2, and H4 were amplified from a cDNA library generated from PHA-stimulated PBMC by PCR. The cDNAs were cloned into a retroviral vector pMX-IRES/EGFP (19) and recombinant retroviruses were obtained. L1.2 cells were infected with the recombinant viruses and stable transfectants expressing green fluorescence were selected by FACS.

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[3] Abbreviations used in this paper: GPCR, G protein-coupled receptor; LEC, liver-expressed chemokine; PTX, pertussis toxin.

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Calcium mobilization assay

Cells were suspended at 10^6 cells/ml in HBSS containing 1 mg/ml BSA and 10 mM HEPES (pH 7.4), and loaded with 3 μM Fura 2-AM fluorescence dye (Molecular Probes, Eugene, OR). After washing, cells were placed in F2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) and stimulated with recombinant human chemokines or histamine. Emission fluorescence at 510 nm was measured upon excitation at 340 and 380 nm to obtain the fluorescence intensity ratio (R340/380).

Chemotaxis assay

This was conducted using Transwell plates with 5-μm pore polycarbonate membrane filters (Costar, Acton, MA) as described previously (20). Migrated cells were quantitated using PicoGreen dsDNA quantitation reagent (Molecular Probes) (20).

Binding assay

Recombinant human CCL16 was radiolabeled to a specific activity of 1.2×10^7 cpm/μg by using ^{125}I-labeled Bolton and Hunter reagent (Amersham Biosciences, Piscataway, NJ). For binding experiments, 1×10^6 cells were incubated with ^{125}I-labeled CCL16 without or with increasing concentrations of competitors in 200 μl of RPMI 1640 containing 20 mM HEPES (pH 7.4) and 0.1% BSA. After incubation at 15°C for 1 h, cells were washed five times and counted in a gamma counter. The binding data were analyzed using GraphPad PRISM (GraphPad Software, San Diego, CA).

RT-PCR analysis

This was conducted as described previously (20). In brief, total RNA was prepared from cells using TRIzol reagent (Life Technologies, Gaithersburg, MD) and further purified using RNeasy (Qiagen, Hilden, Germany). Total RNA samples prepared from human and mouse tissues were also purchased from BD Biosciences (Mountain View, CA). Total RNA (1 μg) was reverse transcribed using oligo$(dT)_{18}$ primer and SuperScript II reverse transcriptase (Life Technologies). Resulting first-strand DNA (equivalent to 20 ng of total RNA) and original total RNA (20 ng) were amplified in a final volume of 20 μl containing 10 pmol of each primer and 1 U of Ex-*Taq* polymerase (Takara Shuzo, Kyoto, Japan). The amplification conditions were denaturation at 94°C for 30 s (5 min for the first cycle), annealing at 60°C for 30 s, and extension at 72°C for 30 s (5 min for the last cycle) for 36 cycles for human and mouse H3 and H4 and 27 cycles for human and mouse GAPDH. The amplification products (10 μl each) were separated by electrophoresis on 2% agarose and stained with ethidium bromide. The primers used were: +5′-CCACTGTATGTACCCTACGTGCTG-3′ and −5′-ATGCTGAGGTTAAAGAAGGTGACG-3′ for human H3; +5′-AGCTATGACCGATTCCTGTCAGTC-3′ and −5′-CCTCTGGATGTTCAGGTAGATGCT-3′ for mouse H3; +5′-ATGCCAGATACTAATAGC-3′ and −5′-TTAAGAAGATACTGACCG-3′ for human H4; +5′-TGTGGTGGACAGAAACCTTAGACA-3′ and −5′-AAGAATCTGAAGCCAGAATCATCG-3′ for mouse H4; and +5′-GCCAAGGTCATCCATGACAACTTTGG-3′ (+) and −5′-GCCTGCTTCACCACCTTCTTGATGTC-3′ (−) for human and mouse GAPDH.

Preparation of human and mouse eosinophils

Human eosinophils were prepared from peripheral blood of healthy volunteers with no history of allergy as previously described (11). In brief, buffy coat cells were obtained from venous blood by dextran T500 sedimentation. Eosinophils were isolated by density centrifugation on Percoll (1.088 g/ml; Pharmacia Biotech, Uppsala, Sweden). The eosinophils were further purified by negative selection using anti-CD16-bound micromagnetic beads and a magnetic-activated cell sorter column (Miltenyi Biotec, Bergisch Gladbach, Germany). After negative selection, the purity of eosinophils was consistently >99%, and their viability was consistently >95%. Mouse eosinophils were separated from spleens of mice that had been injected i.m. with DNA of an IL-5 expression plasmid as previously described (21). In brief, female BALB/c mice, 8 wk old, were purchased from CLEA (Tokyo, Japan) and kept in specific pathogen-free conditions for at least 1 wk before experiments. Mice were injected into the bilateral soleus muscles with 50 μl of 0.25% bupivacaine (Sigma-Aldrich) using a disposable insulin syringe. At 3 and 10 days, mice were injected with 75 μg of pCAGGS-IL-5 plasmid DNA in 50 μl of PBS into bupivacaine-injected sites. After 2–3 wk, splenic eosinophils were purified from mice that had >50% eosinophils in the blood. Spleen cells were layered onto a discontinuous Percoll density gradient consisting of 1.06, 1.07, and 1.09 g/ml and were centrifuged at $250 \times g$ for 20 min. Eosinophils were layered between 1.07 and 1.09 g/ml. Eosinophils were further purified by two times negative selection using anti-CD90 (Thy1.2), anti-CD45R (B220), and anti-CD8α (Ly-2)-bound micromagnetic beads and IMagnet (BD Biosciences). After negative selections, the purity of eosinophils was consistently >90%, and their viability was consistently >95%.

Eosinophil mobilization in mice

Female BALB/c mice, 6 wk old, were purchased from CLEA and kept in specific pathogen-free conditions for at least 1 wk before experiments. Mice were injected i.v. with 100 μl of PBS without or with 0.5 nmol of CCL16. At various time points, mice were given anesthesia by inhalation of diethyl ether and peripheral blood was collected. After blood collection, mice were sacrificed by cervical dislocation and femurs were obtained. The ends of femurs were removed and bone marrow cells were recovered by flushing with 1 ml of PBS. Smears were stained with May-Grünwald and Giemsa solutions. Differential cell counts were performed for at least 500 cells/slide. All animal experiments were performed in accordance with the guideline of the Center of Animal Experiments, Kinki University School of Medicine.

Results

CCL16 induces calcium mobilization via H4

Histamine receptors H1, H2, and H4 are variably expressed by hemopoietic cells, while H3 is selectively expressed by neurons in the CNS (10–12, 18). To examine whether any chemokines act as an agonist for the histamine receptors, we performed calcium mobilization assays using murine L1.2 pre-B cells stably expressing H1, H2, or H4 (L1.2-H1, L1.2-H2, and L1.2-H4, respectively). Among 32 recombinant human chemokines tested at 100 nM (CC chemokines: CCL1, 2, 3, 4, 5, 7, 8, 11, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 24, 25, 26, 27, and 28; CXC chemokines: CXCL*A*, 8, 9, 10, 12, 13, and 16; XCL1; CX3CL1), CCL16 was found to induce vigorous calcium mobilization in L1.2-H4. Fig. 1*A* shows that, while histamine induced calcium mobilization via H1, H2, and H4, CCL16 dose-dependently induced calcium mobilization via H4 but not via H1 or H2. Furthermore, H4-mediated calcium mobilization induced by CCL16 was completely suppressed by PTX, supporting that H4 couples with the Gαi class of G proteins (9, 10). Fig. 1*B* shows that CCL16 dose-dependently desensitized histamine-induced calcium mobilization via H4. Conversely, histamine desensitized CCL16-induced calcium mobilization via H4. Furthermore, thioperamide, an antagonist for H3 and H4 (10, 22), completely suppressed CCL16- and histamine-induced calcium mobilization via H4. Collectively, CCL16 is a novel agonist for H4.

CCL16 induces chemotactic responses via H4

We next examined chemotactic responses of L1.2-H1, L1.2-H2, and L1.2-H4 to CCL16. As shown in Fig. 2*A*, both CCL16 and histamine induced vigorous cell migration in L1.2-H4 with a typical bell-shaped dose-response curve. In contrast, neither CCL16 nor histamine induced any significant migration in L1.2-H1 or L1.2-H2. Thus, these histamine receptors are not coupled with the Gαi class of G proteins in L1.2 cells. As shown in Fig. 2*B*, thioperamide, an antagonist for H3 and H4 (10, 22), dose-dependently suppressed migratory responses of L1.2-H4 to 100 nM CCL16 with an IC_{50} of 50 μM. As shown in Fig. 2*C*, CCL16 and histamine were additive in inducing migration of L1.2-H4 cells. As shown in Fig. 2*D*, a checkerboard-type analysis confirmed that CCL16 induced mostly chemotaxis, not chemokinesis, in L1.2-H4. As shown in Fig. 2*E*, PTX, the inhibitor of the Gαi class of G proteins, suppressed CCL16-induced migratory responses of L1.2-H4.

Specific binding of CCL16 to H4

We next examined binding of CCL16 to H4. As shown in Fig. 3*A*, ^{125}I-labeled CCL16 specifically bound to L1.2-H4. Scatchard analysis revealed a single class of binding sites with a K_d of 17 nM and 41,000 sites/cell (Fig. 3*B*). Furthermore, binding of ^{125}I-labeled CCL16 at 10 nM was completely inhibited by unlabeled CCL16, histamine, and thioperamide with an IC_{50} of 8.8, 4.1, and 57 nM, respectively (Fig. 3*C*).

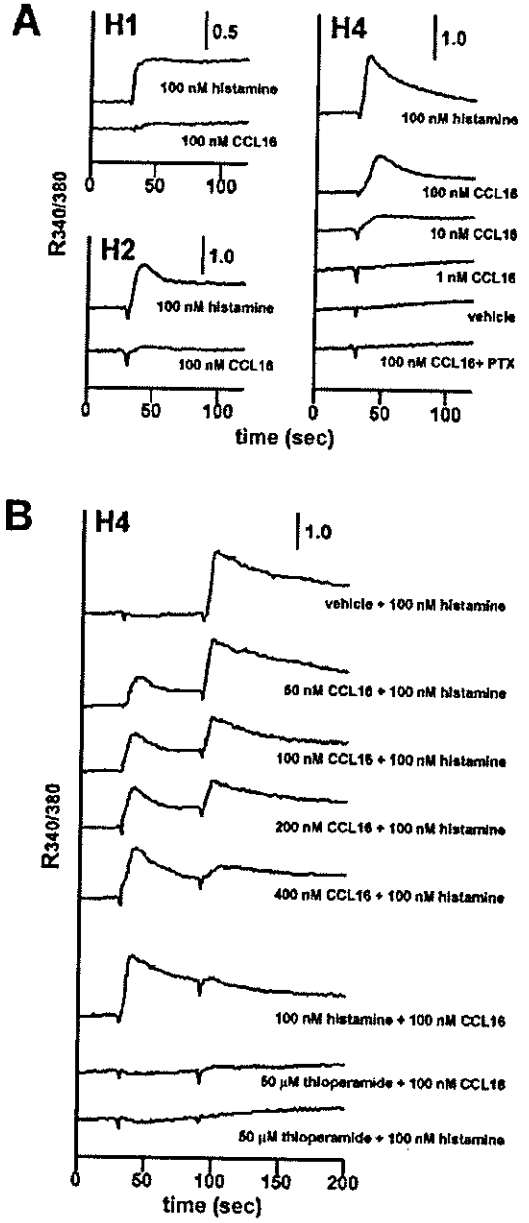

FIGURE 1. Induction of calcium mobilization in H4-expressing L1.2 cells by CCL16. *A*, H4-specific calcium mobilization by CCL16. L1.2 cells stably expressing H1 (L1.2-H1), H2 (L1.2-H2), or H4 (L1.2-H4) were loaded with Fura 2-AM. Cells (5×10^5/assay) were stimulated with CCL16 or histamine at indicated concentrations. Intracellular calcium mobilization was measured on a fluorescence spectrophotometer. Pretreatment of L1.2-H4 cells with PTX at 500 ng/ml was performed at 37°C for 30 min. Representative results from three separate experiments are shown. *B*, Mutual desensitization of CCL16 and histamine in calcium mobilization via H4. L1.2-H4 cells were loaded with Fura 2-AM and stimulated with CCL16 or histamine as indicated. Intracellular calcium mobilization was measured on a fluorescence spectrophotometer. Thioperamide was used at 50 μM. Representative results from three separate experiments are shown.

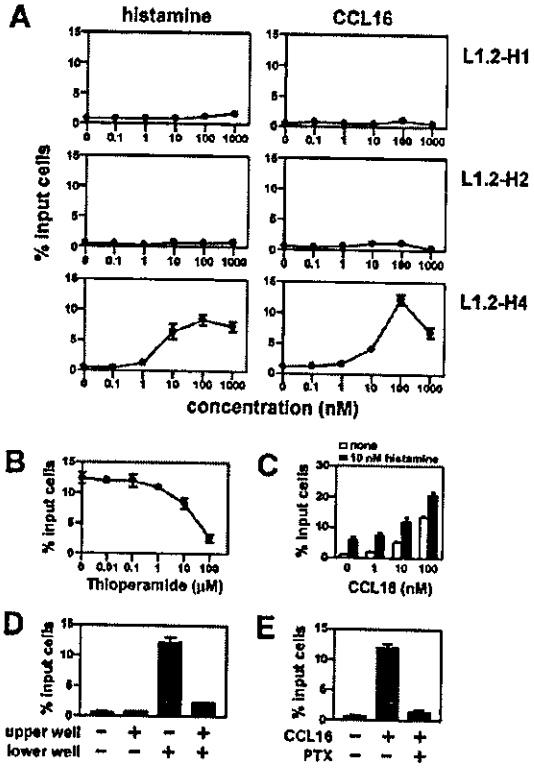

FIGURE 2. Induction of chemotaxis in H4-expressing L1.2 cells by CCL16. *A*, Dose-response experiments. Chemotactic responses of L1.2 cells stably expressing H1 (L1.2-H1), H2 (L1.2-H2), and H4 (L1.2-H4) to indicated concentrations of histamine and CCL16 were examined. *B*, Inhibition of CCL16-induced migration of L1.2-H4 cells by thioperamide. Chemotactic responses of L1.2-H4 cells to CCL16 at 100 nM were determined in the presence of indicated concentrations of thioperamide. *C*, Additive effects of histamine and CCL16. Chemotactic responses of L1.2-H4 cells to indicated concentrations of CCL16 without (□) or with 10 nM of histamine (■) were determined. *D*, A checkerboard-type analysis. In chemotactic assays using L1.2-H4 cells, CCL16 was added to upper and/or lower wells at 100 nM as indicated. *E*, Inhibition by PTX. L1.2-H4 cells were pretreated at 37°C for 30 min without or with PTX at 500 ng/ml and examined for chemotactic responses to CCL16 at 100 nM. All assays were done in duplicate and numbers of cells migrated to lower wells were expressed by percent input cells. Results are shown as mean ± SEM from three separate experiments.

Eosinophil responses to CCL16

H4 is selectively expressed by eosinophils and mediates eosinophil migration to histamine (10, 11). By RT-PCR, we confirmed that human and mouse eosinophils expressed H4 but not H3 (Fig. 4*A*). We, therefore, examined induction of eosinophil migratory responses by CCL16. As shown in Fig. 4*B* (*left*), CCL16 dose-dependently induced a highly efficient migration of human eosinophils. In fact, many more eosinophils migrated to CCL16 at 100 or 1000 nM than to eotaxin/CCL11 at its optimal concentration of 10 nM. CCL16 also induced less efficient but still vigorous migration in mouse eosinophils (Fig. 4*B*, *right*). Furthermore, thioperamide, an antagonist for H3 and H4 (10, 22), dose-dependently suppressed migratory responses of human and mouse eosinophils to CCL16 (Fig. 4*C*). Fig. 4*D* further shows induction of calcium mobilization in mouse eosinophils by CCL16 and histamine as well as their mutual desensitization. Again, thioperamide completely suppressed CCL16-induced calcium mobilization in mouse eosinophils.

Eosinophil mobilization from mouse bone marrow by CCL16

Expression of H4 was consistently seen in the human, rat, mouse, and guinea pig bone marrow (Fig. 4*A*) (10, 22). Since CCL16 is selectively expressed in the liver and present in the normal plasma

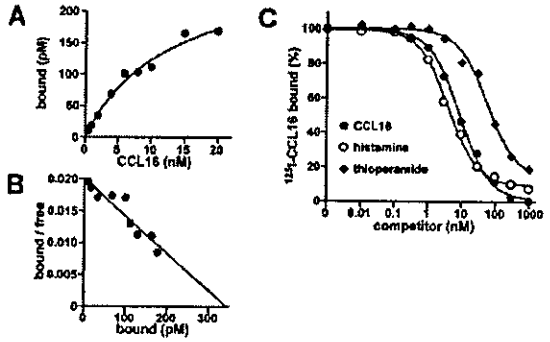

FIGURE 3. Specific binding of CCL16 to H4. *A*, Saturable binding of ^{125}I-labeled CCL16 to L1.2-H4. Nonspecific bindings were determined in the presence of 100-fold molar excess unlabeled CCL16 and subtracted from total bindings. Representative results from three separate experiments are shown. *B*, Scatchard analysis of the binding data in *A*. The calculated K_d is 17 nM. *C*, Displacement experiments. L1.2-H4 cells were incubated with ^{125}I-labeled CCL16 at 10 nM in the presence of various concentrations of unlabeled CCL16 (●), histamine (○), or thioperamide (♦). Representative results from three separate experiments are shown. The calculated IC_{50} is 8.8 nM for CCL16, 4.1 nM for histamine, and 57 nM for thioperamide.

at relatively high concentrations (6), it may be involved in mobilization of eosinophils from the bone marrow. To test this possibility, we injected PBS without or with CCL16 into mice via the tail vein. As shown in Fig. 5*A* (*left*), eosinophil counts but not those of lymphocytes or neutrophils in the peripheral blood were rapidly increased in CCL16-injected mice with a peak at 60 min. Reciprocally, eosinophil counts but not those of lymphocytes or neutrophils in the bone marrow were rapidly decreased in CCL16-injected mice (Fig. 5*A*, *right*). Furthermore, thioperamide effectively suppressed CCL16-induced increases in blood eosinophils and decreases in bone marrow eosinophils, without affecting the numbers of lymphocytes or neutrophils (Fig. 5*B*). Collectively, CCL16 indeed induced a selective mobilization of eosinophils from the bone marrow via H4.

Discussion

Histamine is an important biogenic amine and one of the major products of mast cells. Histamine exerts diverse physiological functions via four subtypes of histamine receptors, namely, H1, H2, H3, and H4, which are all GPCRs (18). These receptors are differentially expressed in various types of cells and mediate diverse effects by coupling with different classes of G proteins (10–12, 18). For example, H1 triggers responses such as smooth muscle contraction and vascular permeability and plays an important role in allergy, while H2 mediates gastric acid secretion. H3 is expressed in the CNS and controls release of histamine and neurotransmitters by neurons (18). Notably, H1, H2, and H3 share less protein sequence identity with each other than with other biogenic amine receptor family members, suggesting that they may have evolved from different ancestral genes (Fig. 6). H4 is the most recently identified subtype of histamine receptor with an amino acid identity of ~38% to H3 (9, 10). H4 has been shown to be primarily expressed by eosinophils and responsible for histamine-induced migration of eosinophils (10, 11). In the present study, we have demonstrated that CCL16, which is a low-affinity ligand for CCR1, CCR2, CCR5, and CCR8 (6, 8), is also a novel agonist for H4. CCL16 induced vigorous calcium mobilization and chemotaxis through H4 (Figs. 1 and 2) and specifically bound to H4 with a K_d of 17 nM (Fig. 3). At the molar basis, CCL16 and histamine

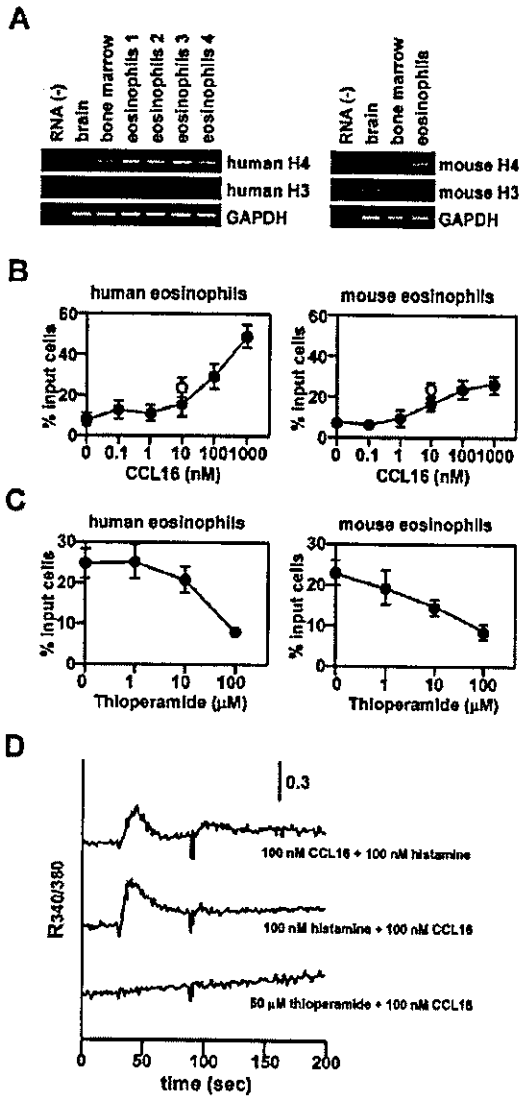

FIGURE 4. Induction of migration and calcium mobilization in human and mouse eosinophils by CCL16 via H4. *A*, RT-PCR analysis for expression of H3 and H4 in human and mouse eosinophils. Human eosinophils were obtained from four donors. cDNA samples from brain and bone marrow served as positive controls for H3 and H4, respectively. Representative results from two separate experiments are shown. *B*, Chemotactic responses of human and mouse eosinophils to CCL16. Chemotactic responses of human (*left*) and mouse (*right*) eosinophils to CCL16 at indicated concentrations (●) and to eotaxin/CCL11 at 10 nM (○) were determined. Each point represents mean ± SEM from four separate experiments. *C*, Effect of thioperamide on eosinophil migration to CCL16. Chemotactic responses of human and mouse eosinophils to CCL16 at 100 nM were determined in the presence of indicated concentrations of thioperamide. Each point represents mean ± SEM from three separate experiments. *D*, Induction of calcium mobilization in mouse eosinophils by CCL16. Mouse eosinophils preloaded with Fura 2-AM were stimulated with CCL16 or histamine at indicated concentrations (5×10^6 cells/assay). Intracellular calcium mobilization was measured on a fluorescence spectrophotometer. Thioperamide was used at 50 μM. Representative results from three separate experiments are shown.

are almost equivalent in potency as an agonist for H4 (Figs. 2 and 3). Fig. 6 shows the phylogenic relationships of the chemokine receptors (for the sake of simplicity, only four members are

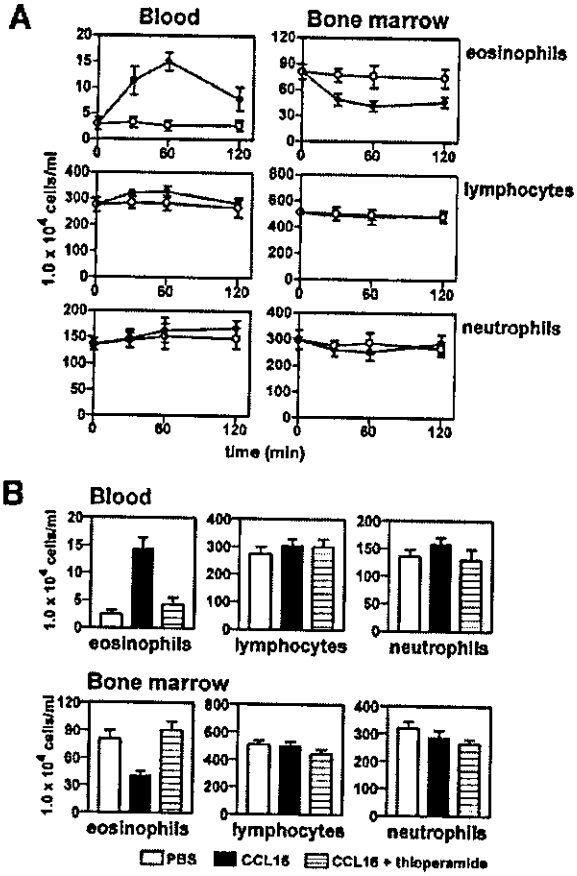

FIGURE 5. Mobilization of mouse bone marrow eosinophils by CCL16 via H4. *A*, Rapid mobilization of eosinophils by CCL16. Mice were injected i.v. with 100 μl of PBS (○) or PBS containing 0.5 nmol of CCL16 (●). At indicated time points, differential cell counts were made for peripheral blood and bone marrow. Each point represents mean ± SEM from five separate experiments. *B*, Suppression of CCL16-induced eosinophil mobilization by thioperamide. Mice were injected i.v. with 100 μl of PBS (□), PBS containing 0.5 nmol of CCL16 (■), or PBS containing 0.5 nmol of CCL16 and 250 nmol of thioperamide (▨). After 60 min, differential cell counts were made for peripheral blood and bone marrow. Each point represents mean ± SEM from five separate experiments.

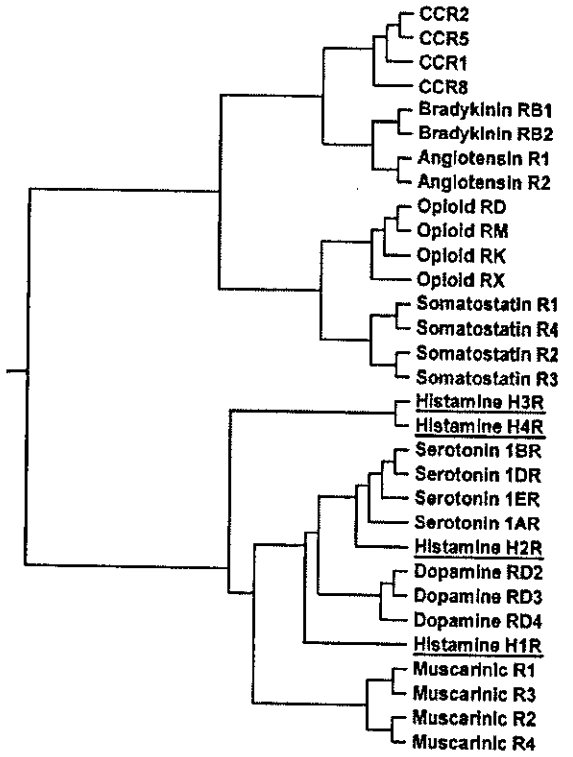

FIGURE 6. Phylogenic relationships of histamine receptors with other GPCRs. A phylogenic tree was constructed from the amino acid sequences of indicated GPCRs. Evolutionary distances were generated using the program of Clustal W (http://clustalw.genome.ad.jp). Histamine receptors H1, H2, H3, and H4 are underlined.

shown) and the histamine receptors. Chemokine receptors are closely related to GPCRs for peptide ligands, while histamine receptors H1 and H2 are the members of GPCRs for biogenic amines. However, H3 and H4, which are most closely related to each other, are only remotely related to other biogenic amine receptors including H1 and H2 and have a closer phylogenic relationship with GPCRs for peptide ligands than other biogenic amine receptors. At any rate, this is the first time of a demonstration of an interaction of a chemokine with a GPCR quite different from the chemokine receptors. Given the versatility of the GPCR system, however, such unexpected interactions may be more frequent than anticipated.

In consistence with the selective expression of H4 in eosinophils (Fig. 4*A*) (10, 11), CCL16 induced vigorous migration in human and mouse eosinophils in vitro (Fig. 4) and rapid mobilization of bone marrow eosinophils into peripheral blood in mice (Fig. 5). Furthermore, thioperamide, an antagonist for H3 and H4 (10, 22), effectively suppressed all of those responses of eosinophils to CCL16 (Figs. 4 and 5). H3 is known to be selectively expressed in the CNS and primarily by neurons (18). By using RT-PCR, we confirmed that human and mouse eosinophils express H4 but not H3 (Fig. 4*A*). Furthermore, human eosinophils do not express CCR1, CCR2, CCR5, or CCR8 (23). Thus, it can be safely concluded that H4 mediates eosinophil responses to CCL16. Since CCL16 is constitutively expressed by the liver parenchymal cells and present in the normal plasma at relatively high concentrations (average, 50 ng/ml) (6), CCL16 may have a role in mobilization of eosinophils from the bone marrow (Fig. 5). In addition, CCL16 is known to be induced in monocytes by IL-10 (3). Therefore, CCL16 may be produced by monocytes and tissue macrophages in Th2-dominant inflammatory conditions and attract eosinophils into inflammatory sites. Furthermore, CCL16 and histamine, whose effects on H4 are additive (Fig. 2), may cooperatively promote eosinophil migration in allergic conditions. CCL16 may also play a significant role in certain liver diseases such as drug-induced liver damages that are known to be associated with liver infiltration of eosinophils and/or blood eosinophilia (24–27). Furthermore, CCL16 may also play a role in migration of other hemopoietic cells such as dendritic cells and mast cells that also express H4 (12, 28). Even though CCL16 is effective only at relatively high concentrations, the versatility of CCL16 as a ligand for CCR1, CCR2, CCR5, CCR8, and even H4 may allow it to have a substantial role in trafficking of various types of hemopoietic cells. These possibilities remain to be seen.

Acknowledgments

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References

1. Yoshie, O., T. Imai, and H. Nomiyama. 2001. Chemokines in immunity. *Adv. Immunol. 78:57.*
2. Shoudai, K., K. Hieshima, S. Fukuda, M. Iio, R. Miura, T. Imai, O. Yoshie, and H. Nomiyama. 1998. Isolation of cDNA encoding a novel human CC chemokine NCC-4/LEC. *Biochim. Biophys. Acta 1396:273.*
3. Hedrick, J. A., A. Helms, A. Vicari, and A. Zlotnik. 1998. Characterization of a novel CC chemokine, HCC-4, whose expression is increased by interleukin-10. *Blood 91:4242.*
4. Youn, B. S., S. Zhang, H. E. Broxmeyer, K. Antol, M. J. Fraser, Jr., G. Hangoc, and B. S. Kwon. 1998. Isolation and characterization of LMC, a novel lymphocyte and monocyte chemoattractant human CC chemokine, with myelosuppressive activity. *Biochem. Biophys. Res. Commun. 247:217.*
5. Yang, J. Y., K. S. Spanaus, and U. Widmer. 2000. Cloning, characterization and genomic organization of LCC-1 (scya16), a novel human CC chemokine expressed in liver. *Cytokine 12:101.*
6. Nomiyama, H., K. Hieshima, T. Nakayama, T. Sakaguchi, R. Fujisawa, S. Tanase, H. Nishiura, K. Matsuno, H. Takamori, Y. Tabira, et al. 2001. Human CC chemokine liver-expressed chemokine/CCL16 is a functional ligand for CCR1, CCR2 and CCR5, and constitutively expressed by hepatocytes. *Int. Immunol. 13:1021.*
7. Giovarelli, M., P. Cappello, G. Forni, T. Salcedo, P. A. Moore, D. W. LeFleur, B. Nardelli, E. D. Carlo, P. L. Lollini, S. Ruben, et al. 2000. Tumor rejection and immune memory elicited by locally released LEC chemokine are associated with an impressive recruitment of APCs, lymphocytes, and granulocytes. *J. Immunol. 164:3200.*
8. Howard, O. M., H. F. Dong, A. K. Shirakawa, and J. J. Oppenheim. 2000. LEC induces chemotaxis and adhesion by interacting with CCR1 and CCR8. *Blood 96:840.*
9. Oda, T., N. Morikawa, Y. Saito, Y. Masuho, and S. Matsumoto. 2000. Molecular cloning and characterization of a novel type of histamine receptor preferentially expressed in leukocytes. *J. Biol. Chem. 275:36781.*
10. Liu, C., X. Ma, X. Jiang, S. J. Wilson, C. L. Hofstra, J. Blevitt, J. Pyati, X. Li, W. Chai, N. Carruthers, and T. W. Lovenberg. 2001. Cloning and pharmacological characterization of a fourth histamine receptor (H_4) expressed in bone marrow. *Mol. Pharmacol. 59:420.*
11. O'Reilly, M., R. Alpert, S. Jenkinson, R. P. Gladue, S. Foo, S. Trim, B. Peter, M. Trevethick, and M. Fidock. 2002. Identification of a histamine H4 receptor on human eosinophils–role in eosinophil chemotaxis. *J. Recept. Signal Transduct. Res. 22:431.*
12. Hofstra, C. L., P. J. Desai, R. L. Thurmond, and W. P. Fung-Leung. 2003. Histamine H4 receptor mediates chemotaxis and calcium mobilization of mast cells. *J. Pharmacol. Exp. Ther. 305:1212.*
13. Yang, D., O. Chertov, S. N. Bykovskaia, Q. Chen, M. J. Buffo, J. Shogan, M. Anderson, J. M. Schroder, J. M. Wang, O. M. Howard, and J. J. Oppenheim. 1999. β-Defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science 286:525.*
14. Albini, A., S. Ferrini, R. Benelli, S. Sforzini, D. Giunciglio, M. G. Aluigi, A. E. Proudfoot, S. Alouani, T. N. Wells, G. Mariani, et al. 1998. HIV-1 Tat protein mimicry of chemokines. *Proc. Natl. Acad. Sci. USA 95:13153.*
15. Howard, O. M., H. F. Dong, D. Yang, N. Raben, K. Nagaraju, A. Rosen, L. Casciola-Rosen, M. Hartlein, M. Kron, K. Yiadom, et al. 2002. Histidyl-tRNA synthetase and asparaginyl-tRNA synthetase, autoantigens in myositis, activate chemokine receptors on T lymphocytes and immature dendritic cells. *J. Exp. Med. 196:781.*
16. Fiore, S., J. F. Maddox, H. D. Perez, and C. N. Serhan. 1994. Identification of a human cDNA encoding a functional high affinity lipoxin A_4 receptor. *J. Exp. Med. 180:253.*
17. Su, S. B., W. Gong, J. L. Gao, W. Shen, P. M. Murphy, J. J. Oppenheim, and J. M. Wang. 1999. A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic cells. *J. Exp. Med. 189:395.*
18. Hill, S. J., C. R. Ganellin, H. Timmerman, J. C. Schwartz, N. P. Shankley, J. M. Young, W. Schunack, R. Levi, and H. L. Haas. 1997. International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacol. Rev. 49:253.*
19. Yoshida, T., D. Izawa, T. Nakayama, K. Nakahara, M. Kakizaki, T. Imai, R. Suzuki, M. Miyasaka, and O. Yoshie. 1999. Molecular cloning of mXCR1, the murine SCM-1/lymphotactin receptor. *FEBS Lett. 458:37.*
20. Nakayama, T., R. Fujisawa, D. Izawa, K. Hieshima, K. Takada, and O. Yoshie. 2002. Human B cells immortalized with Epstein-Barr virus upregulate CCR6 and CCR10 and downregulate CXCR4 and CXCR5. *J. Virol. 76:3072.*
21. Tokui, M., I. Takei, F. Tashiro, A. Shimada, A. Kasuga, M. Ishii, T. Ishii, K. Takatsu, T. Saruta, and J. Miyazaki. 1997. Intramuscular injection of expression plasmid DNA is an effective means of long-term systemic delivery of interleukin-5. *Biochem. Biophys. Res. Commun. 233:527.*
22. Liu, C., S. J. Wilson, C. Kuei, and T. W. Lovenberg. 2001. Comparison of human, mouse, rat, and guinea pig histamine H4 receptors reveals substantial pharmacological species variation. *J. Pharmacol. Exp. Ther. 299:121.*
23. Nagase, H., M. Miyamasu, M. Yamaguchi, T. Fujisawa, K. Ohta, K. Yamamoto, Y. Morita, and K. Hirai. 2000. Expression of CXCR4 in eosinophils: functional analyses and cytokine-mediated regulation. *J. Immunol. 164:5935.*
24. Terasaki, S., Y. Nakanuma, M. Yamazaki, and M. Unoura. 1993. Eosinophilic infiltration of the liver in primary biliary cirrhosis: a morphological study. *Hepatology 17:206.*
25. Nagral, A., Z. Ben-Ari, A. P. Dhillon, and A. K. Burroughs. 1998. Eosinophils in acute cellular rejection in liver allografts. *Liver Transplant. Surg. 4:355.*
26. Larrey, D. 1995. Drug-induced hepatitis: epidemiologic, clinical, diagnostic and physiopathologic aspects in 1995. *Rev. Med. Interne 16:752.*
27. Dansette, P. M., E. Bonierbale, C. Minoletti, P. H. Beaune, D. Pessayre, and D. Mansuy. 1998. Drug-induced immunotoxicity. *Eur. J. Drug Metab. Pharmacokinet. 23:443.*
28. Gantner, F., K. Sakai, M. W. Tusche, W. W. Cruikshank, D. M. Center, and K. B. Bacon. 2002. Histamine H_4 and H_2 receptors control histamine-induced interleukin-16 release from human $CD8^+$ T cells. *J. Pharmacol. Exp. Ther. 303:300.*

CC Chemokine Ligands 25 and 28 Play Essential Roles in Intestinal Extravasation of IgA Antibody-Secreting Cells[1]

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CCL25 (also known as thymus-expressed chemokine) and CCL28 (also known as mucosae-associated epithelial chemokine) play important roles in mucosal immunity by recruiting IgA Ab-secreting cells (ASCs) into mucosal lamina propria. However, their exact roles in vivo still remain to be defined. In this study, we first demonstrated in mice that IgA ASCs in small intestine expressed CCR9, CCR10, and CXCR4 on the cell surface and migrated to their respective ligands CCL25, CCL28, and CXCL12 (also known as stromal cell-derived factor 1), whereas IgA ASCs in colon mainly expressed CCR10 and CXCR4 and migrated to CCL28 and CXCL12. Reciprocally, the epithelial cells of small intestine were immunologically positive for CCL25 and CCL28, whereas those of colon were positive for CCL28 and CXCL12. Furthermore, the venular endothelial cells in small intestine were positive for CCL25 and CCL28, whereas those in colon were positive for CCL28, suggesting their direct roles in extravasation of IgA ASCs. Consistently, in mice orally immunized with cholera toxin (CT), anti-CCL25 suppressed homing of CT-specific IgA ASCs into small intestine, whereas anti-CCL28 suppressed homing of CT-specific IgA ASCs into both small intestine and colon. Reciprocally, CT-specific ASCs and IgA titers in the blood were increased in mice treated with anti-CCL25 or anti-CCL28. Anti-CXCL12 had no such effects. Finally, both CCL25 and CCL28 were capable of enhancing α_4 integrin-dependent adhesion of IgA ASCs to mucosal addressin cell adhesion molecule-1 and VCAM-1. Collectively, CCL25 and CCL28 play essential roles in intestinal homing of IgA ASCs primarily by mediating their extravasation into intestinal lamina propria. *The Journal of Immunology*, 2004, 173: 3668–3675.

Chemokines are a large group of closely related cytokines and transmembrane proteins that play important roles in innate and acquired immunity primarily by inducing directed migration of various types of leukocytes through interactions with a group of seven-transmembrane, G protein-coupled receptors (1). It is now known that chemokines and their receptors are crucial elements in the trafficking and tissue microenvironmental localization of various lymphocyte classes and subsets (1–3). Thus, in accordance with differentiation, activation, and functional maturation, lymphocytes dynamically change their expression profiles of chemokine receptors and shift their migration programs to a new set of chemokines (1–3).

Plasma cells represent the end stage of B cell differentiation and function as the factories of Ab production. Upon antigenic stimulation, naive B cells either migrate to the medullary cords, where they proliferate and rapidly differentiate into short-lived plasma cells producing low-affinity IgM Abs, or migrate to the B cell follicles, where they participate in the germinal center reaction, which leads to differentiation into long-lived plasma cells producing high-affinity IgG and IgA Abs (4). Thus, there are roughly two types of plasma cells, the short-lived ones mostly dying in situ and the long-lived ones homing to a wide variety of organs such as bone marrow and gastrointestinal tract. Furthermore, plasma cells producing IgG Abs tend to home to bone marrow, whereas those producing IgA Abs migrate into lamina propria of gastrointestinal, respiratory, and urogenital tissues (5, 6). Chemokines are likely to play important roles in the migration and tissue localization of plasma cells. In this context, Hargreaves et al. (7) reported that CXCR4 and its ligand CXCL12 were critically involved in the localization of plasma cells within the splenic red pulp and lymph node (LN)[3] medullary cords as well as in their homing to the bone marrow. Wehrli et al. (8) demonstrated down-regulation of chemokine receptors such as CCR7 and CXCR5 in plasmablasts, which presumably allows their migration from the B cell follicles to the medullary cords and into efferent lymphatic vessels. Hauser et al. (9) demonstrated that Ag-specific IgG Ab-secreting cells (ASCs), which appeared in the spleen a few days after secondary immunization and were destined to home to the bone marrow, selectively migrated to CXCR4 and CXCR3 ligands. Bowman et al. (10) reported that IgA ASCs, but not IgG or IgM ASCs, in mice expressed CCR9 and efficiently responded to its ligand CCL25, which is selectively expressed in the small intestine (11, 12). Previously, we have demonstrated that plasma cells in mouse salivary glands as well as those in human bone marrow selectively express CCR10 and respond to its ligand CCL28 (13, 14), which is selectively expressed in certain mucosal tissues and human bone marrow (13–16). Butcher and coworkers (17, 18) have also demonstrated that CCL28 attracts IgA ASCs present in both intestinal and

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[3] Abbreviations used in this paper: LN, lymph node; ASC, Ab-secreting cell; CT, cholera toxin; MAdCAM, mucosal addressin cell adhesion molecule; MLN, mesenteric LN; PP, Peyer's patch.

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nonintestinal mucosal tissues, while CCL25 only attracts a subpopulation of IgA ASCs associated with small intestine. These results suggest that CCL25 and CCL28 play important roles in the intestinal homing of IgA ASCs. However, their exact roles in homing of IgA ASCs in intestinal lamina propria still remain to be defined.

In this study, we have further explored the roles of CCL25 and CCL28 in intestinal homing of IgA ASCs in mice. As reported previously, CCL25 and CCL28 are strongly expressed in the small intestine and colon, respectively (11, 12, 15, 16). We have further shown that CCL28 is also expressed in mouse small intestine. Furthermore, we have revealed by immunohistochemistry that CCL25 and CCL28 are present not only in intestinal epithelial cells, but also in endothelial cells of small venules in intestinal lamina propria. This suggested direct roles of CCL25 and CCL28 in extravasation of IgA ASCs into intestinal lamina propria. Consistently, anti-CCL25 prevented homing of Ag-specific IgA ASCs into small intestine, whereas anti-CCL28 prevented their homing into both small intestine and colon. Furthermore, both CCL25 and CCL28 enhanced α_4 integrin-dependent adhesion of IgA ASCs to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and VCAM-1 in vitro. Collectively, CCL25 and CCL28 play essential roles in intestinal homing of IgA ASCs primarily by promoting their extravasation into intestinal lamina propria.

Materials and Methods

Mice

Female BALB/c mice were purchased from SLC (Hamamatsu, Japan) and maintained in specific pathogen-free conditions for at least 1 wk before experiments. All animal experiments in the present study were approved by the Center of Animal Experiments, Kinki University School of Medicine.

Chemokines and Abs

All recombinant mouse chemokines and human stromal cell-derived factor-1α/CXCL12 were purchased from R&D Systems (Minneapolis, MN). Affinity-purified goat-neutralizing Abs against mouse CCL25 (AF-481-NA), mouse CCL28 (AF533), and human CXCL12 (AF-310-NA) were also purchased from R&D Systems. Control normal goat IgG was purchased from Genzyme-Techne (Mineapolis, MN). We used goat anti-human CXCL12 for detection and neutralization of mouse CXCL12 because human and mouse CXCL12 differ only at a single amino acid residue (1). We confirmed that anti-human CXCL12 reacted equally well with both human and mouse CXCL12 in ELISA and similarly neutralized both human and mouse CXCL12 in a standard chemotaxis assay. Specifically, the neutralizing activities (ED_{50}) of anti-mouse CCL25, anti-mouse CCL28, and anti-human/mouse CXCL12 determined against mouse CCL25 at 1 μg/ml, mouse CCL28 at 2 μg/ml, and mouse CXCL12 at 2 ng/ml, respectively, in a standard chemotaxis assay were 4–12, 5–25, and 0.2–0.6 μg/ml, respectively. The concentrations of chemokines used in these assays were the optimal doses that gave maximal chemotactic responses. The relatively low neutralizing activity of anti-CXCL12 may be in part due to a very high potency of CXCL12 in comparison with CCL25 or CCL28.

Real-time PCR analysis

Real-time PCR was performed using TaqMan assay and 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Template cDNAs were generated from total RNAs extracted from various mouse tissues using guanidinium isothiocyanate (19). Amplification conditions were 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/extension). The primers were as follows: +5′-AGCACAGGATCAAATGGAATGTT-3′ and −5′-GGTTGCAGCTTCCACTCACTT-3′ for CCL25; +5′-TGAGCCCGGCTCCTGAA-3′ and −5′-GCTTGGGAGTGGCTGTCTATAGA-3′ for CCL27; +5′-CAGCCCGCACAATCGTACT-3′ and −5′-ACGTTTTCTCTGCCATTCTTCTTT-3′ for CCL28; and + 5′-TGCCCCTGCCGGTTCT-3′ and −5′-TGTTGAGGATTTTCAGATGCTTGA-3′ for CXCL12. The probes for chemokines were as follows: +5′-TCCGGCATGCTAGGAATTATCACCAGC-3′ for CCL25; +5′-CTTGCCTCTGCCCTCCAGCACTAGCT-3′ for CCL27; +5′-TGAAGCAGTGGATGAGAGCCTCAGAGG-3′ for CCL28; and +5′-CGAGAGCCACATCGCCAGAGCC-3′ for CXCL12. The probes were labeled with the reporter fluorescent dye 6-FAM at the 5′ end. The primers and fluorogenic probes for 18S ribosomal RNA were obtained from TaqMan kit (Applied Biosystems). Chemokine expression was quantified by using Sequence Detector System software (Applied Biosystems).

Isolation of lymphocytes

All tissues were obtained from 8- to 12-wk-old female BALB/c mice. Lymphocytes were prepared from Peyer's patches (PPs) and mesenteric LNs (MLNs) by mincing the tissues in RPMI 1640 containing 10% FCS with scissors and pressing them through a nylon mesh screen with a round-edged spatula. Lamina propria lymphocytes were isolated from small intestine and colon, as described previously (10). Briefly, small intestines that were carefully cleared of PPs and colons were cut open longitudinally, cut into ~5-mm segments, and washed at room temperature with vigorous shaking four times in divalent cation-free HBSS supplemented with 5 mM EDTA, 25 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin to remove epithelial cells and intraepithelial lymphocytes until no more shedding occurred. Intestinal tissues were washed twice in RPMI 1640 containing 10% FCS, 15 mM HEPES, and antibiotics. Then lamina propria lymphocytes were released by shaking intestinal tissues in RPMI 1640 containing 20% FCS, 25 mM HEPES, antibiotics, and 300 U/ml collagenase type VIII (Sigma-Aldrich, St. Louis, MO) for three 40-min sessions. At the end of each 40-min incubation, released cells were immediately washed in RPMI 1640 containing 10% FCS and antibiotics. Isolated lymphocytes were resuspended in RPMI 1640 containing 10% FCS and antibiotics and incubated in 6-cm dishes at 37°C in a CO_2 incubator for 2 h to remove adherent cells and to allow recovery from potential desensitization of chemokine responses.

Flow cytometric analysis

Single cells were isolated from various mouse tissues, as described above. Blood leukocytes were prepared from heparinized blood samples through osmotic lysis of RBC. Cells were suspended in ice-cold PBS containing 3% FCS and 0.1% sodium azide (staining medium). All of the following steps were conducted on ice. Cells were first treated with PBS containing 0.1% BSA, 2% normal mouse serum, and 1 μg/ml anti-mouse CD32/16 (Beckman Coulter, Marseille, France) to block Fc receptors. After washing, cells were incubated with a mixture of FITC-labeled anti-IgA (C10-3) or FITC-labeled isotype-matched control IgG, PE-labeled anti-CD3ε, CyChrome-labeled anti-B220, and biotinylated anti-CD38 (all from BD Pharmingen, Mountain View, CA) for 30 min. After washing, cells were incubated with streptavidin-allophycocyanin for 30 min. For surface staining of CCR9, CCR10, and CXCR4, mouse CCL25-Fc, human CCL27-Fc, mouse CXCL12-Fc, respectively, and control Fc were used, as described previously (13). After staining, cells were immediately analyzed on a FACSCalibur (BD Biosciences, Mountain View, CA).

Chemotaxis assay

This was performed using 8-μm Transwell plates (Corning Costar, Cambridge, MA), as described previously (13). Briefly, cells were placed in upper wells (2×10^6/well), while bottom wells contained medium alone or medium containing chemokines at concentrations that were predetermined to be optimal (300 nM for CCL25, 300 nM for CCL28, and 50 nM for CXCL12) (13, 14). After incubation at 37°C for 4 h, migrated cells in multiple replicate wells (generally 4–5 wells per each condition) were combined. IgA ASCs in original and migrated cell populations were enumerated by ELISPOT.

ELISPOT

Total IgA ASCs and cholera toxin (CT)-specific IgA ASCs were enumerated with ELISPOT following the protocol provided by BD Pharmingen. Briefly, nitrocellulose 96-well plates (Multiscreen 96-Well Filtration Plate; Millipore, Billerica, MA) were coated with 2 μg/ml polyclonal goat anti-mouse IgA (Kirkegaard & Perry Laboratories, Gaithersburg, MD) or CT (Sigma-Aldrich) in PBS at 4°C overnight. After washing and blocking with RPMI 1640 containing 10% FCS, cells suspended in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin were added to wells (1×10^3 to 5×10^5 cells/100 μl/well) and incubated at 37°C overnight in humidified air with 5% CO_2. After washing with deionized water, each well was added with 100 μl of biotinylated polyclonal anti-mouse IgA (Kirkegaard & Perry Laboratories) at 100 ng/ml in PBS containing 10% FCS and incubated at room temperature for 2 h. After washing with PBS containing 10% FCS, each well was added with 100 μl of streptavidin-HRP in PBS containing 10% FCS and incubated at room temperature for 1 h. After washing thoroughly with PBS

containing 0.05% Tween 20, each well was added with 100 μl of chromogen substrate containing 0.3 mg/ml 3-amino-9-ethylcarbazole (Sigma-Aldrich) and 0.015% (v/v) H_2O_2 and incubated for 15–20 min, yielding reddish spots by ASCs. Numbers of spots per well were counted under an inverted microscope.

Immunohistochemistry

Frozen sections were made from small intestine and colon derived from 8-wk-old female BALB/c mice and briefly fixed with periodate-lysine-4% paraformaldehyde. After washing, sections were treated with anti-CD32/16 (Beckman Coulter) supplemented with 10–50% normal rabbit serum, 3% BSA, 0.25% gelatin, 0.025% Nonidet P-40, and 5 mM EDTA, and further treated with Biotin Blocking System (DakoCytomation, Kyoto, Japan). Then sections were reacted with goat anti-mouse CCL25, goat anti-mouse CCL28, goat anti-human/mouse CXCL12, or normal goat IgG. After washing, sections were successively reacted with biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA) and Vectastain ABC/HRP kit (Vector Laboratories). Chromogenic reactions were performed with diaminobenzidine and H_2O_2, resulting in dark brown reaction products in positive cells. Sections were counterstained with hematoxylin, dehydrated, and mounted in nonaqueous mounting medium.

Immunization

Immunization of mice with CT (Sigma-Aldrich) was performed, as described previously (20). In brief, mice at 8 wk old were inoculated i.p. with 10 μg of CT in 0.2 ml of PBS emulsified with 0.2 ml of CFA. An identical dose of CT without CFA was given i.p. on day 14. On day 21, mice were deprived of food for 6 h and given 0.5 ml of an isotonic solution containing eight parts HBSS and two parts 7.5% sodium bicarbonate by gastric intubation to neutralize stomach acidity. After 30 min, mice were given 0.4 ml of PBS containing 10 μg of CT by gastric intubation, and also given 0.1 ml of PBS containing 10 μg of CT per rectum by intubation. Subsequently, mice were injected i.p. with 0.2 ml of PBS alone or PBS containing 100 μg of goat anti-mouse CCL25, goat anti-mouse CCL28, goat anti-human/mouse CXCL12, or normal goat IgG. On day 27, mice were sacrificed and lamina propria lymphocytes were isolated from small intestine and colon. Total and CT-specific IgA ASCs were enumerated with ELISPOT (see above). In parallel, serum samples and blood leukocytes were prepared. Serum CT-specific IgA and IgG titers were determined by ELISA and CT-specific IgA ASCs in blood leukocytes by ELISPOT.

ELISA

ELISA kits for mouse IgA and IgG were purchased from Bethyl Laboratories (Montgomery, TX). Serum CT-specific IgA and IgG titers were determined by ELISA using microtest plates precoated with CT. The endpoint titers were expressed as the reciprocals of the highest serum dilutions that gave an OD reading above the background level of 0.1.

Cell adhesion assay

Mouse MAdCAM-1-Fc and VCAM-1-Fc were purchased from R&D Systems. Control Fc fragment of human IgG1 was generated, as described previously (13). Cells were prepared from MLNs obtained from BALB/c mice and resuspended in adhesion medium (DMEM containing 0.5% BSA and 2 mM $MgCl_2$). Cells were preincubated without or with anti-mouse CD49d or control IgG (both from BD Pharmingen) for 30 min. After washing, cells were treated without or with CCL25 or CCL28 at 300 nM and immediately added in triplicate to 96-well microtest plates (High-binding; Corning Costar) precoated with 5 μg/ml Fc, MAdCAM-1-Fc, or VCAM-1-Fc at 1×10^6 cells/well. Plates were centrifuged for 15 s at 400 rpm and placed at 37°C for 5 min. Unbound cells were removed by gently washing with DMEM twice. Bound cells were released by incubation in ice-cold PBS containing 2 mM EDTA. IgA ASCs in original and adherent cell populations were then enumerated by ELISPOT.

Results

Differential expression of CCL25, CCL27, CCL28, and CXCL12 in various mouse organs and tissues

Because plasma cells are known to reside in a wide variety of organs, their immediate precursors, morphologically plasmablasts, and functionally ASCs are likely to migrate into various target tissues via locally produced chemokines. CCL25, CCL28, and CXCL12 are the chemokines known to play pivotal roles in plasma cell migration (6, 10, 13, 14, 17, 18). Even though the expression of these chemokines in various mouse tissues was separately examined by previous studies (11, 12, 15, 16, 21), we wished to directly compare their expression levels in various mouse organs and tissues. Therefore, we performed quantitative real-time PCR for CCL25, CCL28, and CXCL12. CCL27 was also examined because it shares CCR10 with CCL28 (22, 23). The results are shown in Fig. 1. CCL25 was selectively expressed in thymus, small intestine, and PP. CCL27, which is known to be selectively expressed in the skin (24, 25), was also expressed in thymus and weakly in brain and axillary LN (a draining LN of skin). CCL28 was strongly expressed in parotid gland, colon, and appendix. CCL28 was also moderately expressed in small intestine, PP, kidney, and brain. CCL28 was weakly expressed in thymus and lung and totally negative in liver, spleen, axillary LN, and MLN. Unexpectedly, CCL28 was also totally negative in mouse bone marrow, even though CCL28 was clearly shown to be expressed in human bone marrow (14). This may indicate a species difference in the role of CCL28 in bone marrow homing of plasma cells. Finally, CXCL12 was widely expressed in all the tissues examined, but its expression was very low in parotid gland and small intestine. Collectively, the highly differential expression of plasma cell-related chemokines in various organs and tissues supports their differential roles in plasma cell homing.

Surface expression of CCR9, CCR10, and CXCR4 on IgA ASCs

We next examined surface expression of CCR9, CCR10, and CXCR4, the receptors for CCL25, CCL28, and CXCL12, respectively, in IgA ASCs present in PP, MLN, small intestine, and colon (Fig. 2). CCR9, CCR10, and CXCR4 were detected by using chemokine-Fc chimera proteins (13). By gating on CD3-negative

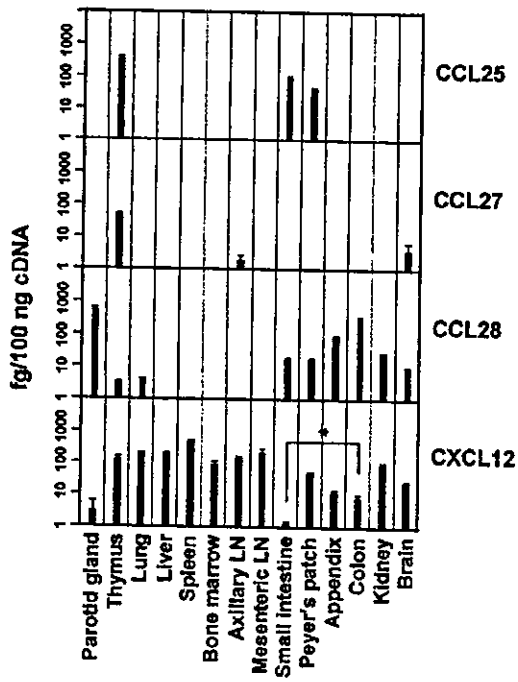

FIGURE 1. Expression of CCL25, CCL27, CCL28, and CXCL12 mRNA in various mouse organs and tissues. Total RNA samples were prepared from indicated mouse organs and tissues. Expression of CCL25, CCL27, CCL28, and CXCL12 mRNA was quantitated by real-time PCR. For details, see *Materials and Methods*. Data represent mean ± SD from three separate experiments. *, $p < 0.05$ (Student's t test).

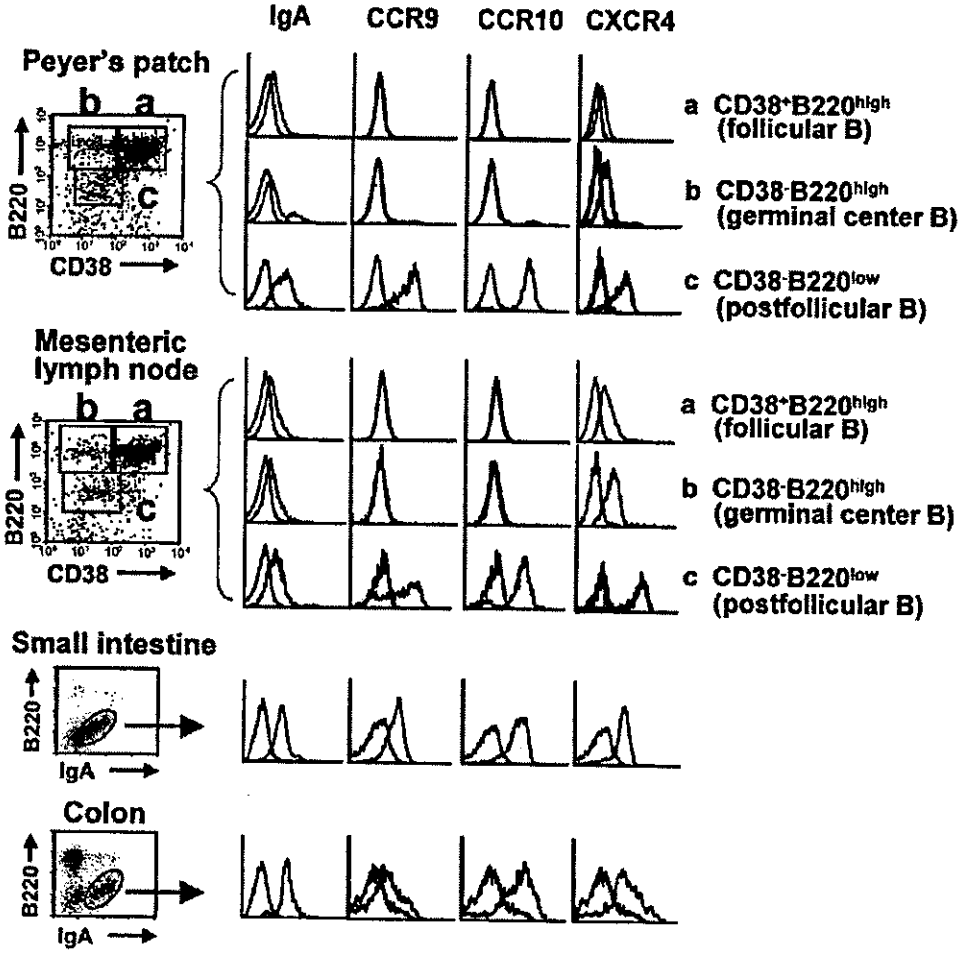

FIGURE 2. Flow cytometric analysis on surface expression of CCR9, CCR10, and CXCR4. Single cells prepared from PPs and MLNs were stained for CD3, B220, CD38, IgA, and CCR9, CCR10, or CXCR4. Single cells prepared from small intestine and colon were stained for CD3, B220, IgA, and CCR9, CCR10, or CXCR4. For surface staining of CCR9, CCR10, and CXCR4, cells were reacted with CCL25-Fc, CCL27-Fc, and CXCL12-Fc, respectively. For negative controls, cells were reacted with control Fc. The data were gated on CD3-negative populations. For details, see *Materials and Methods*. Representative results from at least three separate experiments are shown.

cells, $CD38^+B220^{high}$, $CD38^-B220^{high}$, and $CD38^-B220^{low}$ fractions in PP and MLN represented follicular B cells, germinal center B cells, and postfollicular B cells including plasma cells, respectively (26–28). Follicular B cells and germinal center B cells were essentially negative for CCR9 and CCR10 and weakly positive for CXCR4. In contrast, postfollicular B cells up-regulated surface IgA and were strongly positive for CCR9, CCR10, and CXCR4. Given a homogeneous expression pattern of surface IgA, CCR9, CCR10, and CXCR4 in postfollicular B cells of especially PP, the majority of these cells were likely to be IgA ASCs coexpressing CCR9, CCR10, and CXCR4. In MLN, however, a minor fraction of postfollicular B cells was negative for CCR9 or CCR10. This may indicate down-regulation of CCR9 and/or CCR10 in a fraction of IgA ASCs during migration into MLN. In small intestine, the fraction of $IgA^+B220^{low/-}$ cells represented the majority of lymphocytes in the lamina propria and were homogeneously positive for CCR9, CCR10, and CXCR4. Similarly, the fraction of $IgA^+B220^{low/-}$ cells in colon was again mostly positive for CCR9, CCR10, and CXCR4. These cells were likely to be IgA ASCs in the small intestine and colon (10). However, in comparison with $IgA^+B220^{low/-}$ cells in small intestine, those from colon expressed CCR9 at much lower levels. This may be in part due to selective recruitment of IgA ASCs expressing less CCR9 in the colon (17, 18).

Chemotactic responses of IgA ASCs to CCL25, CCL28, and CXCL12

We next examined chemotactic responses of IgA ASCs derived from PP, MLN, small intestine, and colon to CCL25, CCL28, and CXCL12 (Fig. 3). IgA ASCs were enumerated by ELISPOT. IgA ASCs in MLN vigorously responded to CCL28 and also to CCL25 and CXCL12. IgA ASCs in PP and small intestine responded to CCL25, CCL28, and CXCL12 at similar levels. Notably, however, IgA ASCs in colon responded to CCL28 and CXCL12, but not to CCL25. This was consistent with their low surface expression of CCR9 (Fig. 2). Taken together, in accordance with the surface expression of CCR9, CCR10, and CXCR4 (Fig. 2), IgA ASCs in these tissues were mostly capable of responding to CCL25, CCL28, and CXCL12. Furthermore, the highly efficient responses of IgA ASCs in MLN to CCL28 may indicate an enhanced signaling function of CCR10 in migrating IgA ASCs.

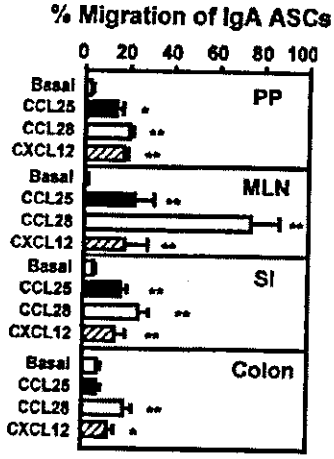

FIGURE 3. Chemotactic responses of IgA ASCs derived from various mouse tissues to CCL25, CCL28, and CXCL12. Single cells prepared from PPs, MLNs, small intestine (SI), and colon were examined for chemotactic responses to CCL25 at 300 nM, CCL28 at 300 nM, and CXCL12 at 50 nM. IgA-specific ELISPOT assay was performed to enumerate IgA ASCs in input and migrated cell populations. For details, see *Materials and Methods*. Data represent mean ± SD from three separate experiments. *, $p < 0.05$; **, $p < 0.01$ (Student's t test).

Immunohistochemical staining of CCL25, CCL28, and CXCL12

To determine cells producing and/or presenting CCL25, CCL28, and CXCL12 in the small intestine and colon, we next performed immunohistochemical staining. As shown in Fig. 4*A*, the epithelial cells of small intestine were positive for CCL25 and CCL28. In contrast, the epithelial cells of colon were positive for CCL28 and CXCL12. Furthermore, as shown in Fig. 4*B*, the venular endothelial cells in small intestine were positive for CCL25 and CCL28, while those in colon were positive for CCL28. These results were highly consistent with the differential expression of CCL25, CCL28, and CXCL12 mRNA in the small intestine and colon (Fig. 1). Furthermore, the endothelial staining of CCL25 in small intestine and CCL28 in both small intestine and colon may indicate their direct roles in extravasation of IgA ASCs by arresting IgA ASCs on the endothelial cell surface and promoting their transendothelial migration into intestinal lamina propria. In contrast, CXCL12 may not be directly involved in extravasation of IgA ASCs into intestinal lamina propria.

Effects of anti-CCL25, anti-CCL28, and anti-CXCL12 on intestinal homing of IgA ASCs

To explore the roles of CCL25, CCL28, and CXCL12 in intestinal homing of plasma cells, we next examined effects of in vivo neutralization of CCL25, CCL28, and CXCL12 on homing of Ag-specific IgA ASCs into small intestine and colon in mice immunized with CT. CT-specific IgA ASCs and total IgA ASCs were enumerated by ELISPOT. As shown in Fig. 5*A*, both anti-CCL25 and anti-CCL28 significantly reduced CT-specific IgA ASCs among total IgA ASCs in small intestine. No such reduction was seen with anti-CXCL12 or control IgG. This supported that CCL25 and CCL28, but not CXCL12, were involved in the extravasation of newly generated CT-specific IgA ASCs into small intestine. Rather unexpectedly, however, the combined treatment with anti-CCL25 and anti-CCL28 did not further reduce CT-specific IgA ASCs in small intestine. In the case of colon, only anti-CCL28 significantly reduced CT-specific IgA ASCs among total IgA ASCs. No such reduction was seen with anti-CCL25, anti-

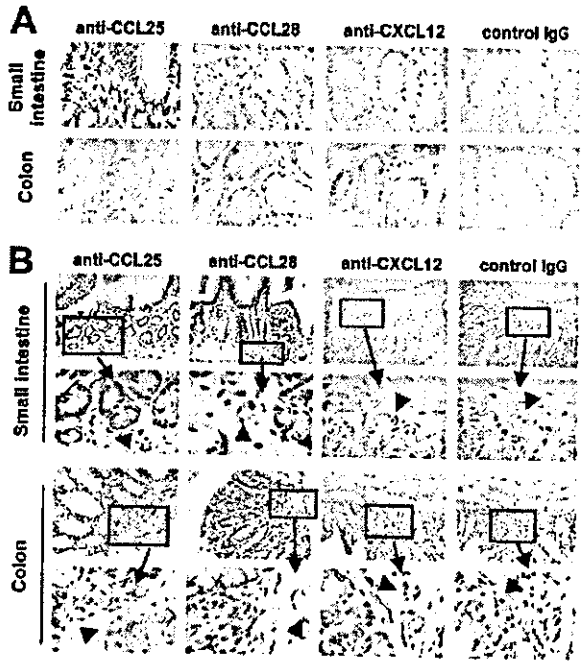

FIGURE 4. Immunohistochemistry of CCL25, CCL28, and CXCL12 in mouse small intestine and colon. Frozen sections of mouse small intestine and colon were fixed with periodate-lysine-4% paraformaldehyde and stained for CCL25, CCL28, and CXCL12 using goat anti-mouse CCL25, goat anti-mouse CCL28, and goat anti-human/mouse CXCL12, respectively. Negative controls were reacted with normal goat IgG. For details, see *Materials and Methods*. Representative results from at least three separate experiments are shown. *A*, Immunoreactivity of epithelial cells. Original magnification: ×400. *B*, Immunoreactivity of endothelial cells. Original magnifications: *upper panels*, ×400; *lower panels*, ×1000. Arrowheads indicate endothelial cells of microvessels.

CXCL12, or control IgG. No further reduction was seen by the combined treatment with anti-CCL25 and anti-CCL28 either. If these results indicated that intestinal extravasation of newly generated CT-specific IgA ASCs was blocked in mice treated with anti-CCL25 or anti-CCL28, there would be reciprocal increases in CT-specific IgA ASCs and titers in the blood of these mice. This was indeed the case. As shown in Fig. 5*B*, mice treated with anti-CCL25 or anti-CCL28, but not those treated with anti-CXCL12, showed significant increases in CT-specific IgA ASCs in the blood. We were unable to compare CT-specific IgG ASCs because of their very low numbers in the blood of these mice (data not shown). Furthermore, as shown in Fig. 5*C*, serum CT-specific IgA titers, but not CT-specific IgG titers, were significantly increased in mice treated with anti-CCL25 or anti-CCL28, but not in mice treated with anti-CXCL12. In both parameters, no further increases were seen by the combined treatment with anti-CCL25 and anti-CCL28. Rather unexpectedly, however, there were no significant differences in increase of CT-specific IgA ASCs and titers between mice treated with anti-CCL25 and those treated with anti-CCL28, even though the latter would be expected to block extravasation of CT-specific IgA ASCs in both small intestine and colon. This was probably due in part to the vast numerical dominance (~10 times) of total CT-specific IgA ASCs homing into small intestine over those homing into colon (data not shown). Collectively, these data supported that CCL25 and CCL28 play a direct role in the extravasation of IgA ASCs in small intestine (CCL25 and CCL28) and colon (CCL28).

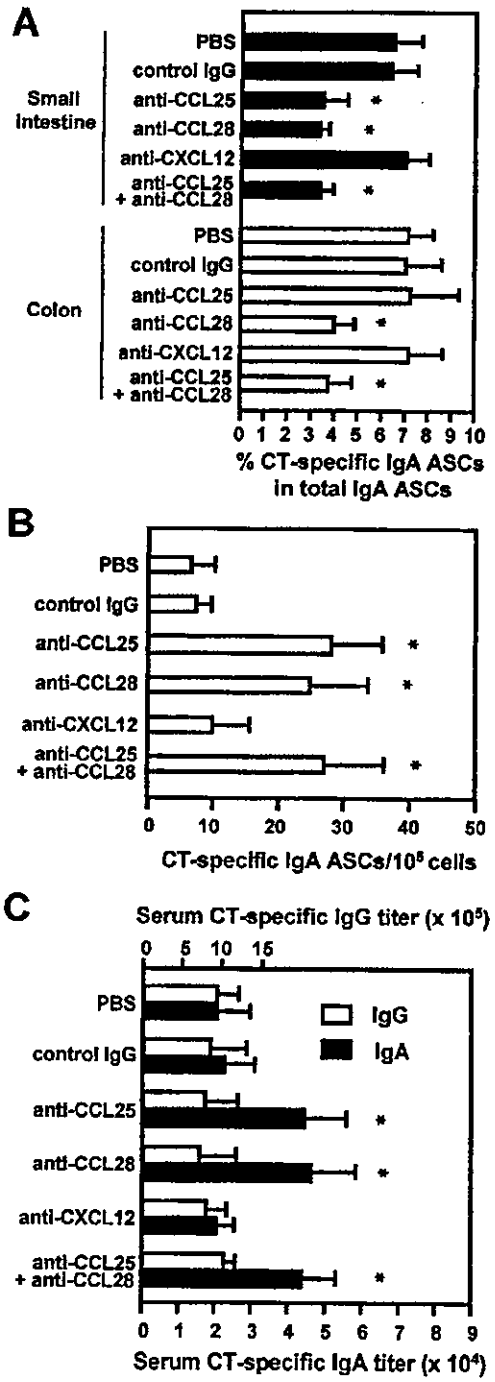

FIGURE 5. Effects of blocking CCL25, CCL28, and CXCL12 on intestinal homing of Ag-specific IgA ASCs. BALB/c mice primed with CT were immunized orally and per rectum with CT and also injected i.p. with PBS, normal goat IgG, goat anti-mouse CCL25, goat anti-mouse CCL28, or goat anti-human/mouse CXCL12, as indicated. After 6 days, lymphocytes were prepared from lamina propria of small intestine and colon. Serum samples and blood leukocytes were also prepared. Each group consisted of at least five mice. Statistical analysis was done with Student's *t* test. *A*, Intestinal homing of CT-specific IgA ASCs. Cells were prepared from lamina propria of small intestine and colon. CT-specific IgA ASCs and total IgA ASCs were enumerated by ELISPOT. For details, see *Materials and Methods*. Data represent mean ± SD from four separate experiments. *, $p < 0.05$. *B*, CT-specific IgA ASCs in peripheral blood. CT-specific IgA ASCs in peripheral blood leukocytes were enumerated by ELISPOT. For details, see *Materials and Methods*. Data represent mean ± SD from three separate experiments. *, $p < 0.05$. *C*, Serum CT-specific IgG and IgA titers. CT-specific IgG titers (□) and IgA titers (■) were determined with ELISA. For details, see *Materials and Methods*. Data represent mean ± SD from four separate experiments. *, $p < 0.05$.

CCL25 and CCL28 induce α_4 integrin-dependent adhesion of IgA ASCs to MAdCAM-1 and VCAM-1

Plasma cells homing to intestine are known to express the mucosal homing receptor $\alpha_4\beta_7$ integrin (29), whereas its ligand MAdCAM-1 is predominantly expressed by postcapillary venules in intestinal mucosal tissues (30). In mice lacking β_7, plasma cell numbers in the intestinal lamina propria were greatly reduced (31). Furthermore, VCAM-1, which is another ligand of $\alpha_4\beta_7$ integrin, is expressed on the endothelium of nonintestinal mucosal tissues that also express CCL28 (5). Given the presumed roles of CCL25 and CCL28 in extravasation of IgA ASCs in intestinal tissues, we next asked whether these chemokines were capable of inducing firm adhesion of IgA ASCs to MAdCAM-1 and VCAM-1 in vitro. We tested IgA ASCs in MLN as responding cells because MLN contained IgA ASCs vigorously responding to CCL25 and CCL28 in chemotaxis assays (Fig. 3). Cell adhesion assays were performed under static conditions, and total and bound IgA ASCs were enumerated with IgA-specific ELISPOT. As shown in Fig. 6, both CCL25 and CCL28 significantly enhanced adhesion of IgA ASCs to MAdCAM-1 and VCAM-1, with CCL28 being more efficient than CCL25. Furthermore, anti-α_4 (CD49d), but not control IgG, effectively blocked CCL25- and CCL28-induced adhesion of IgA ASCs to MAdCAM-1 and VCAM-1, confirming activation of $\alpha_4\beta_7$ integrin expressed on IgA ASCs by CCL25 and CCL28.

Discussion

IgA plasma cells play pivotal roles in mucosal immunity against invading microorganisms. Although IgA plasma cells generated in small intestine and colon preferentially home into small intestine and colon, respectively (32), those generated by immunization via nasal cavity preferentially home into nasopharyngeal and respiratory mucosal tissues, but not into intestinal tissues (33, 34). Such differential migratory properties of IgA plasma cells in accordance with their tissue origin are likely to be explained in part by their expression of a different set of chemokine receptors (5). Recently, Butcher and his coworkers (10, 17, 18) have elegantly demonstrated the differential roles of CCL25 and CCL28 in the mucosal immunity. Although CCL25 is selectively expressed in the small intestine, its receptor CCR9 is expressed by almost all T cells in the small intestine and a fraction of IgA ASCs. In contrast, CCL28 is widely expressed in intestinal and nonintestinal mucosal tissues, and its receptor CCR10 is expressed by almost all IgA ASCs. Notably, however, T cells homing to intestinal tissues hardly express CCR10. In fact, T cells expressing CCR10 are CLA^+ memory/effector T cells, which preferentially migrate to skin where epidermal keratinocytes produce CCL27, another ligand of CCR10 (18, 24, 35). Thus, the CCL25/CCR9 axis may play a specific role in small intestine by attracting memory/effector T cells and a fraction of IgA ASCs directed to small intestine, whereas the CCL28/CCR10 axis may play a more unifying role in the common mucosal immune system by recruiting IgA ASCs to a wide variety of mucosal tissues (10, 17, 18).

In the present work, we have further explored the roles of chemokines in intestinal homing of IgA plasma cells. First, we have demonstrated highly differential expression of CCL25, CCL27, CCL28, and CXCL12 mRNA in various mouse tissues, including

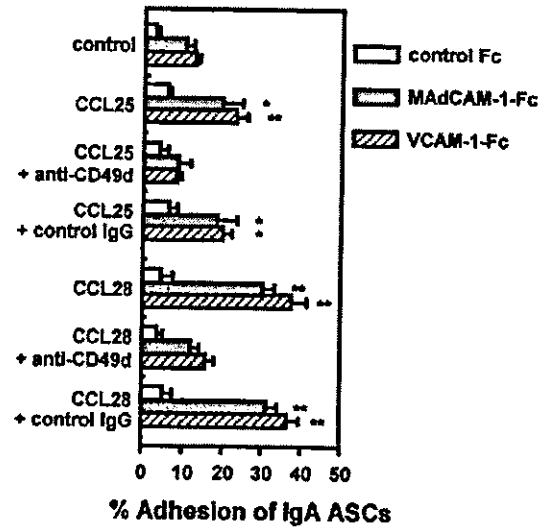

FIGURE 6. Induction of α_4 integrin-dependent adhesion of IgA ASCs to MAdCAM-1 and VCAM-1 by CCL25 and CCL28. Single cells were prepared from MLNs. Cells were treated without or with CCL25 or CCL28 at 300 nM and immediately added to microtest plates coated with MAdCAM-1-Fc, VCAM-1-Fc, or control Fc. After incubation at 37°C for 5 min, nonadherent cells were gently washed away. IgA ASCs in input and adherent cell populations were enumerated by IgA-specific ELISPOT. For details, see *Materials and Methods*. Data represent mean ± SD from three separate experiments. *, $p < 0.05$; **, $p < 0.01$ (Student's t test).

small intestine and colon (Fig. 1). Second, we have demonstrated that IgA ASCs in PP, MLN, small intestine, and colon mostly coexpress CCR9, CCR10, and CXCR4 on their cell surface (Fig. 2) and are mostly capable of responding to CCL25, CCL28, and CXCL12 in chemotaxis assays (Fig. 3). Thus, IgA ASCs in these tissues can be guided by any of these chemokines alone or in combination. However, given the highly differential expression of CCL25, CCL28, and CXCL12 in tissues such as PP, MLN, small intestine, and colon (Fig. 1), it is likely that IgA ASCs entering into MLN are mainly guided by CXCL12, whereas those homing into small intestine are guided by CCL25 and CCL28, and yet those homing into colon are guided by CCL28 and CXCL12. Consistently, IgA ASCs in the colon expressed CCR9 at low levels (Fig. 2) and poorly responded to CCL25 in chemotaxis assays (Fig. 3) (10, 17, 18). Thus, a subset of IgA ASCs, possibly generated in the colon, may express CCR9 only at low levels. Third, we have immunologically demonstrated the presence of CCL25 in the epithelial cells of small intestine, CCL28 in the epithelial cells of both small intestine and colon, and CXCL12 in the epithelial cells of colon (Fig. 4*A*). CCL25 was reported to be localized in the crypts of Lieberkühn in human small intestine (12). In mouse small intestine, however, we detected CCL25 more widely in villus epithelial cells and within the villi. Thus, in mice, CCL25 may be produced by most villus epithelial cells and secreted into subepithelial tissue spaces. As noted previously in human small intestine (12), we have also demonstrated the presence of CCL25 on the venular endothelium of mouse small intestine. Furthermore, we have demonstrated for the first time the presence of CCL28 on the venular endothelium of both small intestine and colon in mice (Fig. 4*B*).

The above mentioned results strongly suggested that CCL25 plays a direct role in extravasation of IgA ASCs in small intestine, while CCL28 does so in both small intestine and colon. We have indeed demonstrated that goat anti-CCL25 dramatically reduced homing of Ag-specific IgA ASCs into small intestine, whereas goat anti-CCL28 reduced homing of Ag-specific IgA ASCs into both small intestine and colon (Fig. 5*A*). In contrast, no such reduction in homing of Ag-specific IgA ASCs was seen in mice treated with goat anti-CXCL12. Reciprocally, there were selective increases in Ag-specific IgA ASCs and Ag-specific IgA titers in the blood of mice treated with anti-CCL25 or anti-CCL28 (Fig. 5, *B* and *C*). Furthermore, we confirmed that both CCL25 and CCL28 were capable of inducing adhesion of freshly isolated IgA ASCs to MAdCAM-1 and VCAM-1 via activation of $\alpha_4\beta_7$ integrin (Fig. 6). These results strongly support the notion that the extravasation of IgA ASCs into small intestine is mediated by both CCL25 and CCL28, whereas the extravasation of IgA ASCs into colon by CCL28. Collectively, CCL25 and CCL28 are likely to play essential roles in intestinal homing of IgA ASCs primarily by promoting their extravasation from small venules into intestinal lamina propria. After that, CCL25 and CCL28 produced by epithelial cells of small intestine may further navigate IgA ASCs within lamina propria of small intestine, whereas CCL28 and CXCL12 produced by epithelial cells of colon may guide IgA ASCs within lamina propria of colon (Fig. 4*A*). Because of a relatively low neutralizing potency of anti-CXCL12 in comparison with those of anti-CCL25 and anti-CCL28 (see *Materials and Methods*), however, we could not formally exclude a role of CXCL12 in extravasation of IgA ASCs, especially in the colon. Furthermore, we could not formally exclude some nonspecific effects of injected goat Abs on the homeostatic regulation of IgA ASCs via Fc receptors and/or complement receptors. Thus, generation of mice with targeted disruption of CCL25 and CCL28 genes would be necessary to further define their roles in the intestinal homing of IgA ASCs.

By using 24-h in vivo homing assays, Reiss et al. (36) demonstrated that either CCL27 (a CCR10 ligand selectively expressed by epidermal keratinocytes) or CCR4 (probably via its ligand CCL17 selectively expressed by cutaneous microvascular endothelial cells) was sufficient for recruitment of skin-homing memory T cells into inflamed skin. Thus, CCL27 and CCR4 apparently play a redundant role in recruitment of activated memory T cells in inflamed skin. In this study, in contrast, blocking of either CCL25 or CCL28 similarly reduced homing of Ag-specific IgA ASCs in small intestine. Thus, CCL25 and CCL28 appear to play a nonredundant role in recruitment of IgA ASCs into small intestine. This may be in part due to their relatively low levels of expression in small intestine (Fig. 1).

Recently, by using CCR9-deficient mice, Pabst et al. (37) have also demonstrated that CCR9 contributes to the localization of plasma cells to the small intestine. In these mutant mice, IgA plasma cells in lamina propria were reduced by ~50% in small intestine, but not in colon. Thus, their observations were highly consistent with our findings and also support that CCL28/CCR10 signals do not completely compensate for CCL25/CCR9 signals in homing of IgA ASCs in small intestine. In CCR9-deficient mice, however, serum OVA-specific IgA titers were barely elevated even after repeated oral immunization with OVA (37). This suggests that not only homing of IgA plasma cells, but also T cell-dependent immune responses in small intestine were severely impaired in CCR9-deficient mice. In this study, however, we demonstrated increases in CT-specific IgA titers and IgA ASCs in the blood of mice treated with anti-CCL25 or anti-CCL28 (Fig. 5, *B* and *C*). This suggests that immune responses to orally administered CT were mostly intact, but homing of CT-specific IgA ASCs into small intestine and/or colon was acutely impaired in these mice. Unexpectedly, the combined treatment with anti-CCL25 and anti-CCL28 did not further reduce homing of Ag-specific IgA ASCs in small intestine (Fig. 5*A*). Homing of IgA ASCs was also only partially blocked by anti-CCL28 in colon (Fig. 5*A*). Even though

we could not exclude incomplete neutralization of CCL25 or CCL28 in mice treated with anti-CCL25 or anti-CCL28, these results might indicate the presence of long-lived CT-specific IgA ASCs in these mice that were systematically preimmunized with CT. Another possibility may be that some IgA ASCs are still capable of entering into intestinal tissues by responding to other chemotactic factors. These possibilities remain to be seen.

Finally, we observed some mononuclear cells strongly positive for CCL28 in PPs and lamina propria of small intestine (Fig. 4*B*). The identity of these cells remains to be seen. It also remains to be seen whether CCL25 and/or CCL28 are directly produced by endothelial cells in the small intestine and colon or are produced by epithelial cells, transported to endothelial cells in the lamina propria, and presented on their cell surface. We have also demonstrated a highly restricted expression of CCR9 and CCR10 in postfollicular B cells in PP and MLN (Fig. 2). Thus, CCR9 and CCR10 are expressed only at the terminal stages of B cell differentiation. The molecular mechanisms of restricted expression of CCR9 and CCR10 in the plasma cell stage remain to be seen. It also remains to be seen how and to what extent CCR9 and CCR10 are restricted to IgA plasma cells than those producing other classes of Ig. Furthermore, the signaling functions of CCR9, CCR10, and CXCR4 may also be dynamically regulated along the process of plasma cell differentiation and homing (Fig. 3) (8). These possibilities remain to be addressed.

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References

1. Yoshie, O., H. Nomiyama, and T. Imai. 2001. Chemokines in immunity. *Adv. Immunol. 78:57.*
2. Butcher, E. C., M. Williams, K. Youngman, L. Rott, and M. Briskin. 1999. Lymphocyte trafficking and regional immunity. *Adv. Immunol. 72:209.*
3. Sallusto, F., C. R. Mackay, and A. Lanzavecchia. 2000. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu. Rev. Immunol. 18:593.*
4. Calame, K. L. 2001. Plasma cells: finding new light at the end of B cell development. *Nat. Immunol. 2:1103.*
5. Kunkel, E. J., and E. C. Butcher. 2003. Plasma-cell homing. *Nat. Rev. Immunol. 3:822.*
6. Cyster, J. G. 2003. Homing of antibody secreting cells. *Immunol. Rev. 194:48.*
7. Hargreaves, D. C., P. L. Hyman, T. T. Lu, V. N. Ngo, A. Bidgol, G. Suzuki, Y. R. Zou, D. R. Littman, and J. G. Cyster. 2001. A coordinated change in chemokine responsiveness guides plasma cell movements. *J. Exp. Med. 194:45.*
8. Wehrli, N., D. F. Legler, D. Finke, K. M. Toellner, P. Loetscher, M. Baggiolini, I. C. MacLennan, and H. Acha-Orbea. 2001. Changing responsiveness to chemokines allows medullary plasmablasts to leave lymph nodes. *Eur. J. Immunol. 31:609.*
9. Hauser, A. E., G. F. Debes, S. Arce, G. Cassese, A. Hamann, A. Radbruch, and R. A. Manz. 2002. Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response. *J. Immunol. 169:1277.*
10. Bowman, E. P., N. A. Kuklin, K. R. Youngman, N. H. Lazarus, E. J. Kunkel, J. Pan, H. B. Greenberg, and E. C. Butcher. 2002. The intestinal chemokine thymus-expressed chemokine (CCL25) attracts IgA antibody-secreting cells. *J. Exp. Med. 195:269.*
11. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman, P. Wooding, A. Miazek, M. G. Mattei, M. Malissen, B. R. Jordan, et al. 2000. The chemokine TECK is expressed by thymic and intestinal epithelial cells and attracts double- and single-positive thymocytes expressing the TECK receptor CCR9. *Eur. J. Immunol. 30:262.*
12. Papadakis, K. A., J. Prehn, V. Nelson, L. Cheng, S. W. Binder, P. D. Ponath, D. P. Andrew, and S. R. Targan. 2000. The role of thymus-expressed chemokine and its receptor CCR9 on lymphocytes in the regional specialization of the mucosal immune system. *J. Immunol. 165:5069.*
13. Hieshima, K., H. Ohtani, M. Shibano, D. Izawa, T. Nakayama, Y. Kawasaki, F. Shiba, M. Shiota, F. Katou, T. Saito, and O. Yoshie. 2003. CCL28 has dual roles in mucosal immunity as a chemokine with broad-spectrum antimicrobial activity. *J. Immunol. 170:1452.*
14. Nakayama, T., K. Hieshima, D. Izawa, Y. Tatsumi, A. Kanamaru, and O. Yoshie. 2003. Cutting edge: profile of chemokine receptor expression on human plasma cells accounts for their efficient recruitment to target tissues. *J. Immunol. 170:1136.*
15. Wang, W., H. Soto, E. R. Oldham, M. E. Buchanan, B. Homey, D. Catron, N. Jenkins, N. G. Copeland, D. J. Gilbert, N. Nguyen, et al. 2000. Identification of a novel chemokine (CCL28), which binds CCR10 (GPR2). *J. Biol. Chem. 275:22313.*
16. Pan, J., E. J. Kunkel, U. Gosslar, N. Lazarus, P. Langdon, K. Broadwell, M. A. Vierra, M. C. Genovese, E. C. Butcher, and D. Soler. 2000. A novel chemokine ligand for CCR10 and CCR3 expressed by epithelial cells in mucosal tissues. *J. Immunol. 165:2943.*
17. Lazarus, N. H., E. J. Kunkel, B. Johnston, E. Wilson, K. R. Youngman, and E. C. Butcher. 2003. A common mucosal chemokine (mucosae-associated epithelial chemokine/CCL28) selectively attracts IgA plasmablasts. *J. Immunol. 170:3799.*
18. Kunkel, E. J., C. H. Kim, N. H. Lazarus, M. A. Vierra, D. Soler, E. P. Bowman, and E. C. Butcher. 2003. CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells. *J. Clin. Invest. 111:1001.*
19. Nakayama, T., R. Fujisawa, H. Yamada, T. Horikawa, H. Kawasaki, K. Hieshima, D. Izawa, S. Fujiie, T. Tezuka, and O. Yoshie. 2001. Inducible expression of a CC chemokine liver- and activation-regulated chemokine (LARC)/macrophage inflammatory protein (MIP)-3α/CCL20 by epidermal keratinocytes and its role in atopic dermatitis. *Int. Immunol. 13:95.*
20. Pierce, N. F., and J. L. Gowans. 1975. Cellular kinetics of the intestinal immune response to cholera toxoid in rats. *J. Exp. Med. 142:1550.*
21. Vicari, A. P., D. J. Figueroa, J. A. Hedrick, J. S. Foster, K. P. Singh, S. Menon, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, K. B. Bacon, and A. Zlotnik. 1997. TECK: a novel CC chemokine specifically expressed by thymic dendritic cells and potentially involved in T cell development. *Immunity 7:291.*
22. Homey, B., W. Wang, H. Soto, M. E. Buchanan, A. Wiesenborn, D. Catron, A. Muller, T. K. McClanahan, M. C. Dieu-Nosjean, R. Orozco, et al. 2000. Cutting edge: the orphan chemokine receptor G protein-coupled receptor-2 (GPR-2, CCR10) binds the skin-associated chemokine CCL27 (CTACK/ALP/ILC). *J. Immunol. 164:3465.*
23. Jarmin, D. I., M. Rits, D. Bota, N. P. Gerard, G. J. Graham, I. Clark-Lewis, and C. Gerard. 2000. Cutting edge: identification of the orphan receptor G-protein-coupled receptor 2 as CCR10, a specific receptor for the chemokine ESkine. *J. Immunol. 164:3460.*
24. Morales, J., B. Homey, A. P. Vicari, S. Hudak, E. Oldham, J. Hedrick, R. Orozco, N. G. Copeland, N. A. Jenkins, L. M. McEvoy, and A. Zlotnik. 1999. CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. *Proc. Natl. Acad. Sci. USA 96:14470.*
25. Ishikawa-Mochizuki, I., M. Kitaura, M. Baba, T. Nakayama, D. Izawa, T. Imai, H. Yamada, K. Hieshima, R. Suzuki, H. Nomiyama, and O. Yoshie. 1999. Molecular cloning of a novel CC chemokine, interleukin-11 receptor α-locus chemokine (ILC), which is located on chromosome 9p13 and a potential homologue of a CC chemokine encoded by molluscum contagiosum virus. *FEBS Lett. 460:544.*
26. Oliver, A. M., F. Martin, and J. F. Kearney. 1997. Mouse CD38 is down-regulated on germinal center B cells and mature plasma cells. *J. Immunol. 158:1108.*
27. Ardavin, C., P. Martin, I. Ferrero, I. Azcoitia, F. Anjuere, H. Diggelmann, F. Luthi, S. Luther, and H. Acha-Orbea. 1999. B cell response after MMTV infection: extrafollicular plasmablasts represent the main infected population and can transmit viral infection. *J. Immunol. 162:2538.*
28. Youngman, K. R., M. A. Franco, N. A. Kuklin, L. S. Rott, E. C. Butcher, and H. B. Greenberg. 2002. Correlation of tissue distribution, developmental phenotype, and intestinal homing receptor expression of antigen-specific B cells during the murine anti-rotavirus immune response. *J. Immunol. 168:2173.*
29. Farstad, I. N., T. S. Halstensen, A. I. Lazarovits, J. Norstein, O. Fausa, and P. Brandtzaeg. 1995. Human intestinal B-cell blasts and plasma cells express the mucosal homing receptor integrin $\alpha_4\beta_7$. *Scand. J. Immunol. 42:662.*
30. Briskin, M., D. Winsor-Hines, A. Shyjan, N. Cochran, S. Bloom, J. Wilson, L. M. McEvoy, E. C. Butcher, N. Kassam, C. R. Mackay, et al. 1997. Human mucosal addressin cell adhesion molecule-1 is preferentially expressed in intestinal tract and associated lymphoid tissue. *Am. J. Pathol. 151:97.*
31. Wagner, N., J. Lohler, E. J. Kunkel, K. Ley, E. Leung, G. Krissansen, K. Rajewsky, and W. Muller. 1996. Critical role for β_7 integrins in formation of the gut-associated lymphoid tissue. *Nature 382:366.*
32. Pierce, N. F., and W. C. Cray, Jr. 1982. Determinants of the localization, magnitude, and duration of a specific mucosal IgA plasma cell response in enterically immunized rats. *J. Immunol. 128:1311.*
33. Johansson, E. L., L. Wassen, J. Holmgren, M. Jertborn, and A. Rudin. 2001. Nasal and vaginal vaccinations have differential effects on antibody responses in vaginal and cervical secretions in humans. *Infect. Immun. 69:7481.*
34. Brandtzaeg, P., E. S. Baekkevold, I. N. Farstad, F. L. Jahnsen, F. E. Johansen, E. M. Nilsen, and T. Yamanaka. 1999. Regional specialization in the mucosal immune system: what happens in the microcompartments? *Immunol. Today 20:141.*
35. Hudak, S., M. Hagen, Y. Liu, D. Catron, E. Oldham, L. M. McEvoy, and E. P. Bowman. 2002. Immune surveillance and effector functions of CCR10$^+$ skin homing T cells. *J. Immunol. 169:1189.*
36. Reiss, Y., A. E. Proudfoot, C. A. Power, J. J. Campbell, and E. C. Butcher. 2001. CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. *J. Exp. Med. 194:1541.*
37. Pabst, O., L. Ohl, M. Wendland, M. A. Wurbel, E. Kremmer, B. Malissen, and R. Forster. 2004. Chemokine receptor CCR9 contributes to the localization of plasma cells to the small intestine. *J. Exp. Med. 199:411.*

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Human P-selectin Glycoprotein Ligand-1 (PSGL-1) Interacts with the Skin-associated Chemokine CCL27 via Sulfated Tyrosines at the PSGL-1 Amino Terminus*

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P-selectin glycoprotein ligand-1 (PSGL-1), a sialomucin expressed on leukocytes, is a major ligand for P-selectin and mediates leukocyte rolling on the endothelium. Here we show that human PSGL-1 interacts with CCL27 (CTACK/ILC/ESkine), a skin-associated chemokine that attracts skin-homing T lymphocytes. A recombinant soluble form of PSGL-1 (rPSGL-Ig) preferentially bound CCL27 among several chemokines tested. This interaction was abrogated by arylsulfatase treatment of rPSGL-Ig, suggesting that sulfated tyrosines play a critical role. In contrast, removal of either *N*-glycans or *O*-glycans by glycosidase treatment of rPSGL-Ig did not affect the interaction. The binding of CCL27 to a recombinant PSGL-1 synthesized in the presence of a sulfation inhibitor was lower than that produced in normal medium. Moreover, mutation of the tyrosines at the amino terminus of PSGL-1 to phenylalanine abolished the binding, further supporting the role of sulfated tyrosines in the CCL27-PSGL-1 interaction. Functionally, rPSGL-Ig reduced the chemotaxis of L1.2 cells expressing CCR10, the receptor for CCL27. In addition, the expression of human PSGL-1 on CCR10-expressing L1.2 cells resulted in reduced chemotaxis to CCL27. These findings suggest a role for PSGL-1 in regulating chemokine-mediated responses, in addition to its role as a selectin ligand.

Leukocyte migration from the blood to tissues is initiated by transient and reversible interactions that capture leukocytes from flowing blood and allow them to roll on the surface of endothelial cells under blood flow. These interactions are primarily mediated by selectins, a family consisting of three cell adhesion molecules: L-selectin (CD62L), which is expressed on most leukocytes, and E-selectin (CD62E) and P-selectin (CD62P), which are expressed on activated vascular endothelium (1, 2). The rolling cells sense activating factors such as chemokines presented on the endothelium, which induce the activation of leukocyte integrins, leading to stable cell attachment and the subsequent transmigration of leukocytes into tissues (3).

P-selectin glycoprotein-1 (PSGL-1[1]; CD162) has been identified as the major ligand for P-selectin on myeloid cells and subsets of activated T cells (4). PSGL-1 mediates P-selectin-mediated neutrophil rolling and migration *in vivo* (5) as well as T cell migration into inflamed skin (6, 7). Besides being a P-selectin ligand, PSGL-1 functions as a ligand for E-selectin and L-selectin *in vivo* (7–9). To bind selectins, PSGL-1 must be modified by specific core-2-type *O*-glycans containing the sialyl Lewis^x (sLe^x) moiety, which is synthesized by multiple glycosyltransferases, including core 2 β-1,6-*N*-acetylglucosaminyltransferase I (C2GlcNAcT-I) and α-1,3-fucosyltransferase VII (FucT-VII) (10). In human skin-homing T cells, the PSGL-1 glycans carry the cutaneous lymphocyte-associated antigen, a sLe^x-related carbohydrate epitope defined by the monoclonal antibody (mAb) HECA-452 (11). In addition to *O*-glycans, PSGL-1 also requires sulfated tyrosines at its amino terminus to bind P-selectin and L-selectin (12–15).

A recombinant PSGL-1 in a selectin-binding glycoform (rPSGL-Ig), consisting of the amino-terminal region of human PSGL-1 fused to the Fc portion of human IgG1, has been developed (12), and it has been shown to have anti-inflammatory effects in various models of inflammation (16–19). Although the effects of rPSGL-Ig have been attributed to its ability to bind selectins, a recent report suggests that it has additional functions, including the ability to bind the murine chemokine KC (20).

Chemokines regulate leukocyte traffic throughout the body during immune surveillance and inflammation. Chemokines presented on the endothelium induce the firm adhesion of rolling leukocytes through the activation of integrins. Chemokines also direct the migration of leukocytes into specific microenvironments within tissues. Recent data reveal that chemokines and their receptors play important roles in controlling the specificity of lymphocyte subsets for certain sites (21). In

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[1] The abbreviations used are: PSGL-1, P-selectin glycoprotein ligand-1; sLe^x, sialyl Lewis^x; C2GlcNAcT-I, core 2 β-1,6-*N*-acetylglucosaminyltransferase I; FucT-VII, α-1,3-fucosyltransferase VII; mAb, monoclonal antibody; OSGE, *O*-sialoglycoprotein endopeptidase; FCS, fetal calf serum; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay.

inflamed skin, the expression of several chemokines, including CCL17 (TARC) and CCL27 (CTACK/ILC/ESkine), is up-regulated (22, 23). CCL27 is expressed exclusively in the skin and attracts cutaneous lymphocyte-associated antigen-positive T cells (24), which express CCR10, the receptor for CCL27 (25, 26), indicating that CCL27 is a skin-associated chemokine that mediates T cell migration into the skin.

Several chemokine receptors, such as CCR5, CCR2b, CX3CR1, and CXCR4, are modified in the amino-terminal region by tyrosine sulfation (27–30). In addition, CCR5 is modified by *O*-glycosylation in the amino-terminal region, which, together with tyrosine sulfation, contributes to its high affinity binding to chemokines (31). Chemokines also bind sulfated glycosaminoglycans, such as heparan sulfate, that are thought to anchor the chemokine for recognition by a receptor-bearing cell, further suggesting the role of sulfation in chemokine binding (32, 33). It is well established that tyrosine sulfation and *O*-glycosylation are the post-translational modifications of PSGL-1 required for selectin binding.

Based on the similar structural requirements of PSGL-1 and some chemokine receptors to bind their ligands with high affinity, we investigated the interaction of rPSGL-Ig with chemokines. Here we show that rPSGL-Ig preferentially bound CCL27, among the various chemokines examined. Our results show that sulfated tyrosines were critical for PSGL-1 binding to CCL27, whereas neither *O*-glycans nor *N*-glycans contributed to the binding. Moreover, rPSGL-Ig and cell surface-expressed PSGL-1 partially inhibited the chemotaxis of L1.2 cells expressing CCR10. Thus, in addition to its well characterized role as a selectin ligand, these results support a role for PSGL-1 in binding certain chemokines and thereby regulating cell responses to those chemokines.

EXPERIMENTAL PROCEDURES

Reagents—The rPSGL-Ig was a kind gift from Wyeth Research (Cambridge, MA). It was developed by linking a truncated human PSGL-1 to the Fc portion of human IgG1 and produced in Chinese hamster ovary cells that had been engineered to express FucT-VII and C2GlcNAcT-I (19). Two hinge-proximal amino acids at positions 234 and 237 within the IgG1 Fc portion are mutated to alanine to reduce complement activation and Fc receptor binding. The chemokine-Fc chimeric protein CCL27-Fc and control Fc were prepared as described previously (34). Human chemokines CCL1 (I-309), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CXCL1 (GRO-α), CXCL4 (PF-4), CXCL5 (ENA-78), CXCL8 (IL-8), CXCL10 (IP-10), and CXCL12 (SDF-1β) were purchased from Peprotech; CCL21 (SLC) was from DakoCytomation; and CCL27 and CCL28 (MEC) were from R & D Systems. CCL17, CCL18 (PARC), CCL19 (ELC), CCL20 (LARC), XCL1 (SCM-1α), and XCL2 (SCM-1β) were kindly provided by Shionogi (Osaka, Japan). Recombinant human P-selectin, E-selectin, biotinylated anti-P-selectin, biotinylated anti-CCL27, and biotinylated anti-CCL28 were purchased from R & D Systems; biotinylated anti-CCL21 was from DakoCytomation; peroxidase-conjugated anti-human IgG, peroxidase-conjugated anti-rabbit IgG, and peroxidase-conjugated anti-mouse IgG+M were from American Qualex; and peroxidase-conjugated streptavidin was from Zymed Laboratories Inc.. Polyclonal anti-PSGL-1 antibodies prepared using a synthetic peptide (QATEYEYLDYDFLPETEPP) based on residues 42–60 of human PSGL-1 were kindly provided by Dr. Bruce Furie (Harvard Medical School, Boston, MA). The anti-PSGL-1 mAbs PL1 and KPL1 were purchased from Immunotech and BD Biosciences, respectively.

Cells—COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS). A mouse pre-B cell line L1.2 was kindly provided by Dr. Eugene Butcher (Stanford University, Stanford, CA) and maintained in RPMI 1640 containing 10% FCS. L1.2 cells expressing human CCR10 were maintained in RPMI 1640 containing 10% FCS and 0.8 mg/ml G418 (Sigma) (34). L1.2 cells coexpressing chemokine receptor and human PSGL-1 were maintained in RPMI 1640 containing 10% FCS, 0.8 mg/ml G418, and 20 μg/ml blasticidin (Invitrogen).

Generation of Wild-type and Mutant hPSGL-Fc Proteins—To generate the hPSGL-Fc and hPSGL-long-Fc constructs, a fragment corresponding to the first 47 amino acids or the entire extracellular domain of mature human PSGL-1 was amplified by PCR using the 5′ primer CGGGAGCTAGCACAGGCCACCGAATATGAG and the 3′ primer GTAGGATCCCTTGCAGCAGGCTCCACAGTG or CAGCGGATCCTGCTTCACAGAGATGTGGTC. The amplified fragment was digested with NheI and BamHI and ligated into the CD5 leader-IgG1 vector, provided by Dr. Brian Seed (Massachusetts General Hospital, Boston, MA), at the NheI and BamHI sites. To generate the hPSGL-Fc constructs with mutations in tyrosines, the following 5′ primers (with the mutated nucleotides underlined) were used: FFF mutant, CGGGAGCTAGCACAGGCCACCGAATTTGAGTTCCTAGATTTTGATTTCCTG; FYY mutant, CGGGAGCTAGCACAGGCCACCGAATTTGAGTACCTAGATTATGATTTCCTG; YFY mutant, CGGGAGCTAGCACAGGCCACCGAATATGAGTTCCTAGATTATGATTTCCTG; YYF mutant, CGGGAGCTAGCACAGGCCACCGAATATGAGTACCTAGATTTTGATTTCCTG; FFY mutant, CGGGAGCTAGCACAGGCCACCGAATTTGAGTTCCTAGATTATGATTTCCTG; FYF mutant, CGGGAGCTAGCACAGGCCACCGAATTTGAGTACCTAGATTTTGATTTCCTG; and YFF mutant, CGGGAGCTAGCACAGGCCACCGAATATGAGTTCCTAGATTTTGATTTCCTG. COS-7 cells were transfected with the constructs using DEAE dextran. In some experiments, COS-7 cells were cotransfected with hPSGL-Fc constructs and expression plasmids for FucT-VII and *N*-acetylglucosamine-6-*O*-sulfotransferase GST-3 (also called LSST or HEC-GlcNAc6ST) (35, 36). The culture supernatants were harvested 6 days after transfection and applied to a protein A-Sepharose column. The chimeric proteins were eluted with 4 M imidazole (pH 8.0) and dialyzed against phosphate-buffered saline (37). The protein concentration was measured by a BCA protein assay kit (Pierce).

To generate unsulfated hPSGL-Fc protein, COS-7 cells transfected with the hPSGL-Fc construct were cultured for 5 days in sulfate-free medium (Invitrogen) containing 10 mM sodium chlorate. The chimeric protein was purified as described above.

Enzyme Treatment of rPSGL-Ig—To remove sialic acids, rPSGL-Ig was incubated with 1 unit/ml neuraminidase from *Clostridium perfringens* (Sigma) in 50 mM sodium phosphate (pH 5.0) at 37 °C for 1 h. To remove *O*-glycan chains, the rPSGL-Ig was treated with a mixture of enzymes consisting of 1 unit/ml neuraminidase, 0.15 unit/ml β-1,4-galactosidase (Prozyme), 2 units/ml β-*N*-acetylglucosaminidase (Prozyme), and 50 milliunits/ml endo-α-*N*-acetylgalactosaminidase (*O*-Glycanase; Glyko) in 50 mM sodium phosphate (pH 7.0) at 37 °C overnight. To remove *N*-glycan chains, the rPSGL-Ig was treated with 250 units/ml *N*-Glycosidase F (Calbiochem) in 50 mM sodium phosphate (pH 7.5) at 37 °C overnight. For sulfatase treatment, the rPSGL-Ig was incubated with 1 unit/ml sulfatase from abalone entrails (Sigma) in 100 mM sodium acetate (pH 5.0) at 37 °C for 1 h. Control samples were incubated in the same buffer under the same conditions without enzyme.

Dot Blot Assays—To study the binding of PSGL-1 to chemokines, various chemokines and selectins were spotted onto nitrocellulose membranes (Hybond-C; Amersham Biosciences). The membrane was blocked in phosphate-buffered saline containing 3% bovine serum albumin (BSA) and incubated with 4 μg/ml rPSGL-Ig or control human IgG (Sigma) for 1 h. The membrane was then washed and incubated with a 1: 50,000 dilution of peroxidase-conjugated anti-human IgG. The membrane was washed and exposed to ECL reagents (Amersham Biosciences). In some experiments, rPSGL-Ig and control human IgG, untreated or treated with glycosidases or sulfatases, and various preparations of hPSGL-Fc were spotted onto a membrane. The membrane was blocked as described above and incubated with 0.3 μg/ml CCL27 for 1 h. The bound chemokine was detected using a biotinylated anti-CCL27 antibody followed by peroxidase-conjugated streptavidin.

Enzyme-linked Immunosorbent Assay (ELISA)-like Binding Assays—rPSGL-Ig, control human IgG, or various preparations of hPSGL-Fc (10 μg/ml) were immobilized on Sumilon H plates (10 μg/ml, 50 μl/well) at 37 °C for 2 h, and the plates were blocked with 3% BSA in phosphate-buffered saline at 4 °C overnight. The plates were washed and incubated with CCL27, P-selectin, or a polyclonal anti-PSGL-1 antibody (0–3 μg/ml) for 1 h at room temperature, then washed again, and incubated with 0.1 μg/ml biotinylated anti-CCL27, biotinylated anti-P-selectin, or biotinylated anti-rabbit IgG for 1 h at room temperature. The plates were further incubated with a 1:5000 dilution of peroxidase-labeled streptavidin for 1 h at room temperature. To quantify the reaction, *o*-phenylenediamine was added, and the optical density was read at 490 nm on an Immuno Mini NJ-2300 microplate reader (InterMed).

ELISA—Various preparations of hPSGL-Fc (10 μg/ml) were immobilized on Costar 3690 plates (10 μg/ml, 25 μl/well) at 37 °C for 2 h, and the plates were blocked with 3% BSA in phosphate-buffered saline at 4 °C overnight. The plates were washed and incubated with a polyclonal

anti-PSGL-1 antibody, CSLEX-1 (BD Biosciences), G72 (38), or 2H5 (39) for 1 h at room temperature. The plates were washed and then incubated with peroxidase-labeled anti-rabbit IgG or anti-mouse IgG+M for 1 h at room temperature.

Flow Cytometry—To examine the PSGL-1 expression on transfected L1.2 cells, the cells were stained with phycoerythrin-labeled anti-PSGL-1 KPL-1 (BD Biosciences) or nonlabeled PL-2 (Immunotech) followed by fluorescein isothiocyanate-labeled anti-mouse IgG. For CCR10 staining, the cells were incubated with goat anti-CCR10 (ImmunoDetect) followed by Alexa Fluor 488-labeled anti-goat IgG (Molecular Probes). Stained cells were analyzed using an EPICS XL flow cytometer (Beckman Coulter). To assess the chemokine binding by flow cytometry, the cells were incubated with CCL27-Fc or control Fc for 30 min at 4 °C, then washed, incubated with biotinylated goat anti-human IgG (Cappel), and stained with streptavidin-phycoerythrin (BD Biosciences). In some experiments, the cells were treated with 120 μg/ml *O*-sialoglycoprotein endopeptidase (OSGE; Cedarlane) at 37 °C for 1 h and washed before staining. To examine whether anti-PSGL-1 mAbs affect CCL27 binding, the cells were preincubated with 20 μg/ml PL1, KPL1, or control mouse IgG before staining. CCL27-Fc binding was detected using biotinylated goat anti-human IgG adsorbed against mouse IgG using mouse IgG-agarose (Sigma) and streptavidin-phycoerythrin.

Generation of L1.2 or L1.2-CCR10 Cells Expressing Human PSGL-1—To generate the human PSGL-1 expression plasmid, the entire coding region was amplified by PCR from HL-60 cDNA using the following primers: GCTGGATCCGGTGGTGCCATGCCTCTGCAAC and AGTGAATTCAGGGAGGAAGCTGTGCAGGGTG. The amplified product was digested with BamHI and EcoRI and ligated into pcDNA/Myc-His (Invitrogen) at the BamHI and EcoRI sites. Stable L1.2 or L1.2-CCR10 cell lines expressing human PSGL-1 were made by the transfection of L1.2 or L1.2-CCR10 cells with the PSGL-1 plasmid and selection with 20 μg/ml blasticidin.

Chemotaxis Assay—Chemotaxis assays were performed using the ChemoTx system (NeuroProbe). The cells were suspended at 5×10^6 cells/ml in RPMI 1640 containing 0.5% BSA and 10 mM HEPES. Chemokine at the indicated concentrations were placed in the lower chamber, and a filter with a 5-μm pore size was placed on top. In some experiments, rPSGL-Ig was also added to the lower chamber. Aliquots of 1.25×10^5 cells/well were applied to the top surface of the filter, and the plates were incubated at 37 °C in 5% CO_2 for 4 h. The cells that migrated to the bottom were collected and counted on a FACSCalibur (Beckton Dickenson).

RESULTS

PSGL-1 Binds CCL27—To investigate the interaction between chemokines and PSGL-1, we first examined the binding of rPSGL-Ig, a recombinant soluble form of PSGL-1, to chemokines immobilized on a nitrocellulose membrane. rPSGL-Ig consists of the 47 amino-terminal amino acids of mature human PSGL-1, fused to a mutated hinge region of human IgG1 that has reduced complement binding and Fc receptor binding (19). rPSGL-Ig is produced in Chinese hamster ovary cells that have been engineered to express FucT-VII and C2GlcNAcT-I, so it carries the glycans required for binding to P-selectin. In agreement with previous results (12), rPSGL-Ig, which contains only the first 47 amino acids of PSGL-1, bound to P-selectin but not detectably to E-selectin immobilized on the membrane (Fig. 1*A*). Among the human chemokines tested (CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL17, CCL18, CCL19, CCL20, CCL21, CCL27, CCL28, CXCL1, CXCL4, CXCL5, CXCL8, CXCL10, CXCL12, XCL1, and XCL2), rPSGL-Ig preferentially bound to CCL21, CCL27, and CCL28, whereas control human IgG did not bind to these chemokines (Fig. 1*A*). The binding of rPSGL-Ig to the other chemokines was either undetectable or very weak. We next examined the binding of chemokines to rPSGL-Ig and control human IgG immobilized on the membrane. CCL27 bound to rPSGL-Ig but not to control IgG (Fig. 1*B*). Incubation of the membrane with secondary reagents without prior incubation with CCL27 did not result in any binding (data not shown), confirming that the signals represented CCL27 binding. Although rPSGL-Ig had bound to CCL21 and CCL28 when these chemokines were immobilized on the membrane, specific binding of CCL21 and

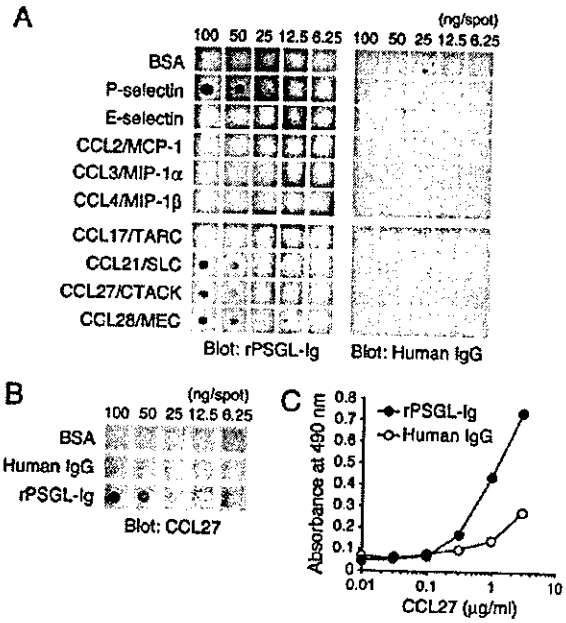

FIG. 1. **PSGL-1 binds to CCL27.** *A*, binding of rPSGL-Ig to chemokines immobilized on a membrane. Selectins, chemokines, and BSA were spotted onto a nitrocellulose membrane. The membrane was incubated with rPSGL-Ig or control human IgG followed by peroxidase-conjugated goat anti-human IgG. Representative blots are shown. *B*, binding of CCL27 to rPSGL-Ig immobilized on a membrane. rPSGL-Ig, control human IgG, and BSA were spotted onto a membrane. The membrane was incubated with CCL27 followed by biotinylated anti-CCL27 and then peroxidase-conjugated streptavidin. A representative blot is shown. *C*, binding of CCL27 to rPSGL-Ig or control human IgG immobilized on a microtiter plate. rPSGL-Ig or human IgG was immobilized on the wells of a microtiter plate. The plate was incubated with the indicated concentrations of CCL27. The bound CCL27 was detected by biotinylated anti-CCL27 and peroxidase-conjugated streptavidin. The data points are the means from duplicate wells. One of three similar experiments is shown.

CCL28 to immobilized rPSGL-Ig was difficult to detect because of high background binding of these chemokines to the membrane, and thus PSGL-1 interaction with these chemokines was not investigated further in this study. CCL27 also bound to rPSGL-Ig immobilized on a microtiter plate in a dose-dependent manner (Fig. 1*C*). These results indicate that the amino-terminal region of PSGL-1 binds CCL27.

PSGL-1 Glycans Are Not Involved in CCL27 Binding—PSGL-1 is modified by sialylated and fucosylated glycans, which are required for its binding to selectins. To study the role of sialylated glycans in PSGL-1 binding to CCL27, we examined the effect of removing the sialic acids from the rPSGL-Ig glycans on the CCL27 binding. Treatment of rPSGL-Ig with sialidase from *C. perfringens*, which hydrolyzes the α-2,3, α-2,6, and α-2,8 glycosidic linkages of terminal sialic residues, did not affect the CCL27 binding, whereas it completely abrogated P-selectin binding (Fig. 2*A*). We next investigated the role of the *O*-glycan chains in rPSGL-Ig binding to CCL27. rPSGL-Ig was treated with a combination of glycosidases that removed *O*-glycan chains. As expected, no binding of P-selectin to rPSGL-Ig was observed after this treatment. In contrast, CCL27 bound to the *O*-deglycosylated rPSGL-1-Ig, suggesting that *O*-glycans are not required for the CCL27 binding (Fig. 2*A*). Because PSGL-1 is also *N*-glycosylated, the role of *N*-glycan chains was also examined by treating rPSGL-Ig with *N*-glycosidase. P-selectin bound to the *N*-deglycosylated rPSGL-Ig, confirming the previous results that *N*-glycans are

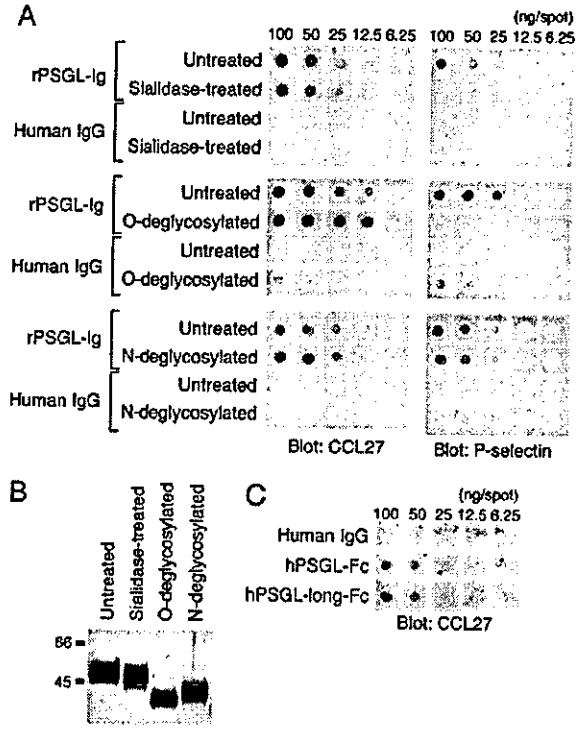

FIG. 2. **Glycosidase treatment of rPSGL-Ig does not abolish CCL27 binding.** *A*, binding of CCL27 and P-selectin to glycosidase-treated rPSGL-Ig. rPSGL-Ig or control human IgG was treated with sialidase, a mixture of enzymes that together cleave *O*-glycans or *N*-glycosidase. Untreated samples were incubated in the same buffer without the enzyme. These samples were spotted onto a membrane, and CCL27 binding and P-selectin binding were examined. *B*, effect of glycosidase treatment on migration on SDS-PAGE. rPSGL-Ig, untreated or treated with the enzymes, was separated by SDS-PAGE, and the gel was subjected to silver staining. *C*, binding of CCL27 to hPSGL-Fc and hPSGL-long-Fc proteins. The samples were spotted onto a membrane, and CCL27 binding was examined.

not required for P-selectin binding (40). CCL27 also bound to the *N*-deglycosylated rPSGL-Ig (Fig. 2*A*). In these experiments, each treatment of rPSGL-Ig led to a shift in its migration on SDS-PAGE (Fig. 2*B*), confirming the effectiveness of the enzymes. The treatment of rPSGL-Ig with a combination of enzymes that removed both the *O*-glycan and *N*-glycan chains did not abrogate the CCL27 binding (data not shown). These results suggest that neither the *O*-glycan nor the *N*-glycan chains on rPSGL-Ig are required for CCL27 binding.

rPSGL-Ig contains only the amino-terminal region of PSGL-1. The remaining extracellular region of PSGL-1 contains a mucin domain with many putative *O*-glycosylation sites. To examine the role of glycans attached to this region, we generated constructs that contained either the same amino-terminal region as rPSGL-Ig, here termed hPSGL-Fc, or the entire extracellular domain of mature human PSGL-1, termed hPSGL-long-Fc; both were fused to the hinge region of human IgG1. These constructs were introduced into COS-7 cells that endogenously express C2GlcNAcT but not FucT-VII. As shown in Fig. 2*C*, CCL27 bound similarly to the hPSGL-long-Fc protein and the hPSGL-Fc protein, suggesting that the mucin domain does not play a role in CCL27 binding to PSGL-1. The cotransfection of COS-7 cells with a plasmid expressing FucT-VII, which enables the chimeric proteins to bind P-selectin, did not significantly affect the CCL27 binding (data not shown). Together, these results suggest that no glycosylation of PSGL-1 is required for its binding to CCL27.

Sulfated Tyrosines Are Required for CCL27 Binding to PSGL-1—It has been shown that in addition to *O*-glycosylation, tyrosine sulfation of the amino-terminal region of PSGL-1 is required for its high affinity binding to P-selectin. Treatment of rPSGL-Ig with sulfatase from abalone entrails, which removes sulfates from tyrosines, not only abrogated the P-selectin binding but also the CCL27 binding (Fig. 3*A*). Sulfatase treatment did not alter the efficiency of immobilization of rPSGL-Ig onto the membrane as shown by an anti-IgG blot (Fig. 3*A*). This treatment did not alter the migration pattern of rPSGL-Ig on SDS-PAGE (Fig. 3*B*). In an ELISA-like binding assay where plate-immobilized rPSGL-Ig was treated with sulfatase or left untreated, the binding of both CCL27 and P-selectin to the sulfatase-treated rPSGL-Ig was reduced, whereas a polyclonal antibody that recognizes the amino-terminal region of PSGL-1 bound similarly to the untreated and sulfatase-treated rPSGL-Ig proteins (Fig. 3*C*). These results suggest that the tyrosine sulfation of PSGL-1 plays an important role in its binding to CCL27.

To further define the role of tyrosine sulfation in the CCL27-PSGL-1 interaction, we examined the effect of sodium chlorate, which is an inhibiter of sulfation. COS-7 cells were transfected with the hPSGL-Fc construct and incubated in sulfate-free medium containing sodium chlorate. The unsulfated hPSGL-Fc protein prepared in the presence of sodium chlorate and the hPSGL-Fc prepared from cells grown in regular medium did not differ significantly in the amount secreted into the culture medium or in their migration pattern on SDS-PAGE (data not shown). Although CCL27 bound to hPSGL-Fc prepared in the presence of sulfate, it did not detectably bind to unsulfated hPSGL-Fc in a dot blot assay (Fig. 4*A*). In this experiment, these hPSGL-Fc preparations were similarly immobilized on the membrane (Fig. 4*A*). The reduced binding of CCL27 to unsulfated hPSGL-Fc was also observed in an ELISA-like binding assay (Fig. 4*B*). In contrast, a polyclonal anti-PSGL-1 antibody similarly bound to the sulfated and unsulfated hPSGL-Fc proteins (Fig. 4*B*). These results, together with the effect of sulfatase treatment, strongly indicate that sulfation on tyrosines is critical for PSGL-1 binding to CCL27.

Sulfation of PSGL-1 Glycans Does Not Enhance CCL27 Binding—Sulfation can occur not only on tyrosines but also on glycan chains. To investigate whether sulfation on the PSGL-1 glycans would enhance the CCL27 binding, we transfected COS-7 cells with the hPSGL-Fc construct together with a plasmid for *N*-acetylglucosamine-6-*O*-sulfotransferase GST-3, which can sulfate the *N*-acetylglucosamines in glycans. To compare the CCL27 binding with P-selectin binding, the COS-7 cells were cotransfected with the FucT-VII-expressing plasmid and the hPSGL-Fc and GST-3 constructs. As shown in Fig. 5*A*, CCL27 binding to the hPSGL-Fc protein was not enhanced when GST-3 was cotransfected. Similar results were obtained when the hPSGL-long-Fc construct was used for transfection instead of the hPSGL-Fc construct (data not shown). The hPSGL-Fc protein produced in the presence of GST-3 was shown to react with the mAb G72, which recognizes sialyl 6-sulfo LacNAc, confirming that the hPSGL-Fc glycan was sulfated (Fig. 5*B*). In contrast, P-selectin binding to the hPSGL-Fc protein prepared in the presence of GST-3 and FucT-VII appeared to be slightly enhanced, compared with that produced in the presence of FucT-VII alone (Fig. 5*A*). The generation of the fucosylated glycan was confirmed by reactivity with the mAb CSLEX-1, which recognizes sLe^x, and the mAb 2H5, which recognizes sLe^x as well as 6-sulfo sLe^x (Fig. 5*B*). All of the hPSGL-Fc preparations were similarly recog-

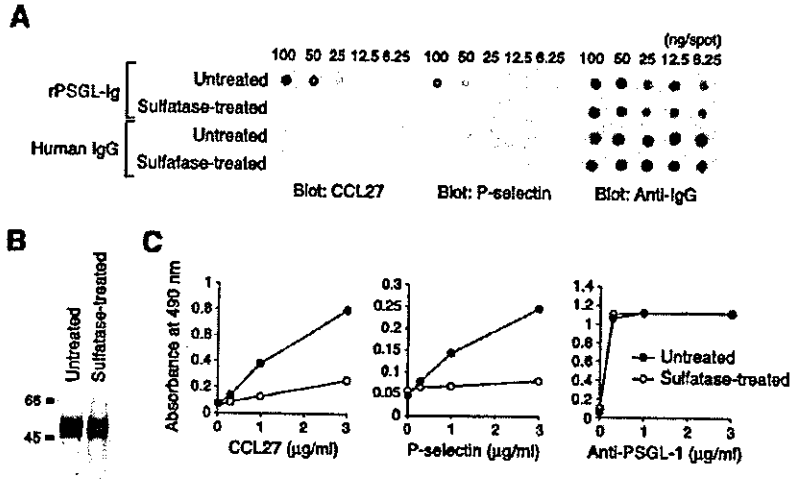

FIG. 3. **Arylsulfatase treatment of rPSGL-Ig abrogates CCL27 binding.** *A*, binding of CCL27 and P-selectin to sulfatase-treated rPSGL-Ig. rPSGL-Ig or control human IgG was treated with arylsulfatase. Untreated samples were incubated in the same buffer without the enzyme. The samples were spotted on a membrane, and CCL27 binding and P-selectin binding were examined. The membrane was also blotted with anti-IgG to show equivalent binding of the unsulfated and sulfatase-treated rPSGL-Ig to the membrane. *B*, migration of sulfatase-treated rPSGL-Ig on SDS-PAGE. rPSGL-Ig, untreated or treated with the enzyme was separated by SDS-PAGE, and the gel was subjected to silver staining. *C*, binding of CCL27, P-selectin, and anti-PSGL-1 antibodies to sulfatase-treated rPSGL-Ig immobilized on a microtiter plate. Microtiter plates were coated with sulfatase-treated or untreated rPSGL-Ig and incubated with CCL27, P-selectin, or polyclonal anti-PSGL-1 antibodies. The values are the means from duplicate wells. The results represent one of three similar experiments.

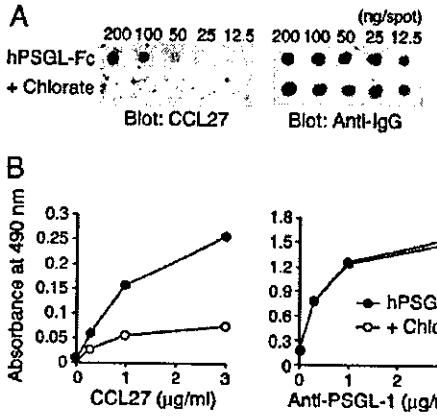

FIG. 4. **Treatment of COS-7 cells with sodium chlorate reduces CCL27 binding to PSGL-1.** *A*, hPSGL-Fc was produced in COS-7 cells in the regular medium or sulfate-free medium containing sodium chlorate. Purified samples were spotted onto a membrane, and CCL27 binding was examined. The membrane was also blotted with anti-IgG to show equivalent binding of the samples. *B*, hPSGL-Fc preparations were immobilized on a microtiter plate, and CCL27 binding and anti-PSGL-1 antibody binding were examined. The values are the means from duplicate wells. The results represent one of three similar experiments.

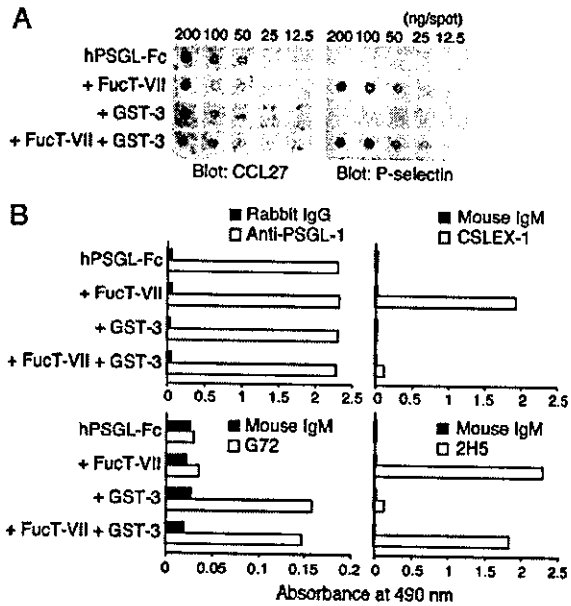

FIG. 5. **Sulfation of the PSGL-1 glycan does not affect CCL27 binding.** *A*, binding of CCL27 and P-selectin to hPSGL-Fc modified by GST-3. COS-7 cells were transfected with the hPSGL-Fc construct with or without the constructs for FucT-VII and GST-3, and the proteins were purified from the culture supernatants. Purified samples were spotted onto a membrane, and CCL27 binding and P-selectin binding were examined. *B*, reactivity of hPSGL-Fc preparations with antibodies against sLe^x-related epitopes. hPSGL-Fc preparations were immobilized on a microtiter plate and assayed for the binding of anti-PSGL-1, G72 (anti-sialyl 6-sulfo LacNAc), CSLEX-1 (anti-sLe^x), or 2H5 (anti-sLe^x/6-sulfo sLe^x). The values are the means from duplicate wells. The results represent one of three similar experiments.

nized by an anti-PSGL-1 antibody (Fig. 5*B*). Thus, the sulfation of glycans on PSGL-1 by GST-3 did not play a significant role in CCL27 binding.

All Three Tyrosines in the Amino-terminal Region of PSGL-1 Are Important for CCL27 Binding—There are three tyrosine residues in the amino-terminal region of human PSGL-1 that can be sulfated. To examine which tyrosine residue is important for CCL27 binding, hPSGL-1-Fc mutants with alterations in one or more of the tyrosines at positions 46, 48, and 51 were generated (Fig. 6*A*). To evaluate the P-selectin binding, wild-type and mutant hPSGL-Fc proteins were also prepared from COS-7 cells cotransfected with the FucT-VII-expressing plasmid. As shown in Fig. 6*B*, CCL27 binding to the hPSGL-Fc protein was dramatically reduced when even one tyrosine was mutated to phenylalanine. In contrast, P-selectin bound to the FYY, YYF, and FYF mutants. Thus, although tyrosine sulfation plays an important role in both the PSGL-1-P-selectin and

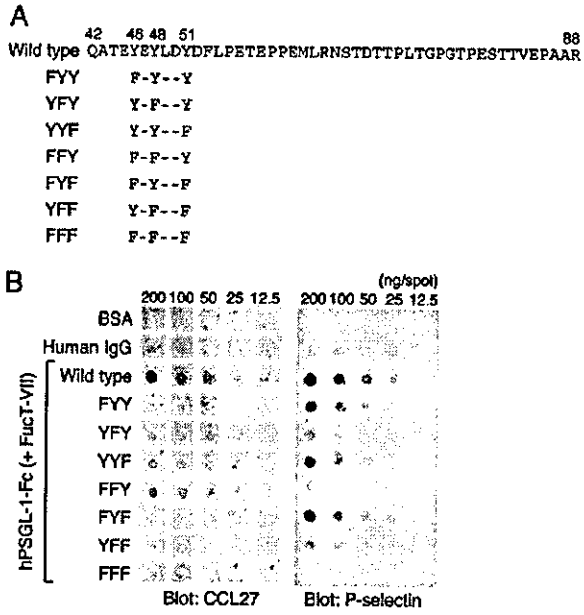

FIG. 6. **CCL27 does not bind to hPSGL-1-Fc with tyrosine mutations.** *A*, sequences of the tyrosine-mutated hPSGL-Fc constructs. Mutant hPSGL-Fc constructs were generated in which one or more tyrosine residues in the amino-terminal region of PSGL-1 were mutated to phenylalanine. *B*, binding of CCL27 and P-selectin to the tyrosine-mutated hPSGL-Fc proteins. Tyrosine-mutated hPSGL-Fc proteins were spotted onto a membrane, and CCL27 binding and P-selectin binding were examined. The blots shown are from one of three similar experiments.

PSGL-1-CCL27 interactions, the relative contribution of each tyrosine to the binding is different in each case. The results suggest that all three tyrosines are required for CCL27 binding, whereas the tyrosine at position 48 plays a dominant role in P-selectin binding.

PSGL-1 Affects CCL27-induced Chemotaxis—To investigate whether PSGL-1 affected CCL27 function, we examined the effect of rPSGL-Ig on the chemotaxis of L1.2 cells expressing human CCR10. rPSGL-Ig reduced the CCL27-induced chemotaxis in a dose-dependent manner, whereas control human IgG1 Fc did not (Fig. 7*A*). The inhibition was observed when rPSGL-Ig was added at 30–100 μg/ml (a molar ratio of 1:3–10). These results suggest that PSGL-1 binding to CCL27 may regulate the chemokine-mediated responses of cells.

We next examined whether cell surface-expressed PSGL-1 would bind CCL27 and affect cell responses to CCL27. The interaction of CCL27 with cell surface-expressed PSGL-1 was studied by examining the binding of a CCL27-Fc chimeric protein to L1.2 cells expressing human PSGL-1. CCL27-Fc bound to L1.2 clones expressing human PSGL-1 more strongly than to the parental L1.2 cells that do not express human PSGL-1 (Fig. 7*B*). The binding of CCL27-Fc to these clones was reduced by treating the cells with OSGE, which cleaves O-sialoglycoproteins such as PSGL-1 (Fig. 7*B*). A decrease in CCL27-Fc binding to parental L1.2 cells by treatment with OSGE was smaller compared with L1.2 clones expressing human PSGL-1. There was no alteration in the control Fc binding to any of these cells by this treatment. These results suggest that human PSGL-1 expressed on the cell surface may bind CCL27. We next tested whether anti-PSGL-1 mAbs that inhibit P-selectin binding would affect CCL27 binding. Neither PL1 nor KPL1 affected the binding of CCL27-Fc to L1.2 clones expressing human PSGL-1 (Fig. 7*C*). PL1 and KPL1 also failed to inhibit the CCL27 binding to rPSGL-Ig immobilized on an ELISA plate (data not shown). These results suggest that the CCL27 binding site on PSGL-1 may not exactly overlap with the epitopes recognized by these antibodies.

We next prepared L1.2 cells expressing both human CCR10 and human PSGL-1 and assayed these cells for the CCL27-induced chemotaxis. The chemotaxis of L1.2 clones expressing both human CCR10 and PSGL-1 was significantly reduced compared with that of L1.2 cells expressing CCR10 alone (Fig. 7*D*). The level of CCR10 expression was similar for all these clones (data not shown). Together, these results support the hypothesis that cell surface-expressed human PSGL-1 binds CCL27 and thereby regulates cell responses to CCL27.

DISCUSSION

In the present study, we investigated the interaction of PSGL-1 with chemokines. We demonstrated that PSGL-1 can bind several chemokines including CCL27. Our data showed that the PSGL-1 interaction with CCL27 is dependent on tyrosine sulfation but not the glycosylation of PSGL-1. All three tyrosines positioned at the amino terminus of PSGL-1 are required for the CCL27 binding. In addition, we showed that PSGL-1 is able to modulate the CCL27-induced response of CCR10-bearing cells.

Tyrosine sulfation is a late post-translational modification that occurs in the trans-Golgi network and is found in a number of secreted and membrane proteins (41). It plays an important role in various protein-protein interactions. Tyrosine sulfation of the amino terminus of PSGL-1 plays an essential role in P-selectin binding (12–14). Recently, sulfation of amino-terminal tyrosine residues of several G protein-coupled receptors, including chemokine and chemoattractant receptors as well as glycoprotein hormone receptors, has been demonstrated (27–30, 42). In these receptors, tyrosine sulfation contributes to the high affinity binding of their ligands. Our data showed that mutation in any one of the tyrosines in the amino terminus of PSGL-1 abrogated the CCL27 binding, indicating that all three tyrosines are required for this binding. Electrostatic interactions are likely to play a role in the binding of the negatively charged region of PSGL-1 generated by sulfated tyrosines to CCL27, which has several basic residues. The requirement of all three tyrosines for CCL27 binding is in contrast to the binding of P-selectin, which was retained to some extent even when tyrosine 46 or 51 was mutated. These findings are in agreement with the results of crystallographic studies showing tyrosine 48 as an important component of the high affinity interaction of PSGL-1 with P-selectin (43). The requirement of all three tyrosines for CCL27 binding might explain why mouse PSGL-1, which has only two tyrosines at its amino terminus, does not bind CCL27 in our assays (data not shown).

Although CCL27 interacts with tyrosine-sulfated PSGL-1, it is unclear whether the binding of CCL27 to its receptor CCR10 requires sulfation of the receptor. Human CCR10 has three tyrosine residues at positions 14, 22, and 32 from the amino terminus, but the Sulfinator, a software tool that predicts tyrosine sulfation sites in protein sequences (44), does not predict the sulfation of any of these tyrosines. The Sulfinator also does not predict tyrosine sulfation in mouse CCR10, which has four tyrosine residues at positions 14, 17, 22, and 32 in the amino-terminal region. It should be experimentally verified whether these tyrosines located in the amino-terminal region of CCR10 are sulfated and contribute to CCL27 binding to CCR10.

Our data also showed that the CCL27 binding to PSGL-1 is not dependent on the glycosylation of PSGL-1. This is in contrast to the selectin binding, which requires sialylated and fucosylated *O*-glycans of PSGL-1. Sialylated *O*-glycans of CCR5