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Eotaxin-3/CC Chemokine Ligand 26 Is a Functional Ligand for CX3CR1

Takashi Nakayama,* Yoshiko Watanabe,† Naoki Oiso,‡ Tomonori Higuchi,* Akiko Shigeta,* Nobuyuki Mizuguchi,§ Fuminori Katou,† Kenji Hashimoto,† Akira Kawada,‡ and Osamu Yoshie*

Eotaxin-3/CCL26 is a functional ligand for CCR3 and abundantly produced by IL-4-/IL-13-stimulated vascular endothelial cells. CCL26 also functions as a natural antagonist for CCR1, CCR2, and CCR5. In this study, we report that CCL26 is yet a functional ligand for CX3CR1, the receptor for fractalkine/CX3CL1, which is expressed by CD16+ NK cells, cytotoxic effector CD8+ T cells, and CD14lowCD16high monocytes. Intraperitoneal injection of CCL26 into mice rapidly recruited mouse eosinophils and intra-abdominal allergic diseases by attracting eosinophils via CCR3 and killer lymphocytes and resident monocytes via CX3CR1. Particularly when the expression of CX3CL1 is low. Collectively, CCL26 is another agonist for CX3CR1 and may play a dual role at high levels. Thus, CCL26 may be partly responsible for the recruitment of cells expressing CX3CR1 in atopic dermatitis mRNA in only about half of the samples. Nevertheless, the skin lesions from both diseases consistently contained CX3CR1 mRNA but not CCL26 mRNA, whereas those of atopic dermatitis contained CCL26 mRNA in all samples but CX3CL1 mRNA in only about half of the samples. Nevertheless, the skin lesions from both diseases consistently contained CX3CR1 mRNA at high levels. Thus, CCL26 may be partly responsible for the recruitment of cells expressing CX3CR1 in atopic dermatitis particularly when the expression of CX3CL1 is low. Collectively, CCL26 is another agonist for CX3CR1 and may play a dual role in allergic diseases by attracting eosinophils via CCR3 and killer lymphocytes and resident monocytes via CX3CR1. The Journal of Immunology, 2010, 185: 000–000.

Chemokines play pivotal roles in health and disease by controlling migration and tissue localization of specific types of cells expressing their cognate receptors (1, 2). Humans have >45 chemokines, which are grouped into four subfamilies (CXC, CC, C, and CX3C) by the structural motif of the N-terminal conserved cysteine residues. Chemokine receptors belong to the seven-transmembrane G protein-coupled receptor family. In humans, there are at least 18 signaling chemokine receptors, which are also grouped into four subfamilies based on the subfamily of their signaling ligands (1, 2). Chemokines and their receptors, especially those involved in inflammatory responses, tend to have highly promiscuous relationships (1, 2). This is partly because the chemokine system has undergone rapid gene expansions through repeated gene duplication events during the vertebrate evolution (1, 2).

CCR3 is a CCR selectively expressed on eosinophils, basophils, and some Th2 cells (3–7). A large number of CC chemokines are known to function as an agonist for CCR3: eotaxin/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26, RANTES/CCL5, MCP-2/CCL8, MCP-3/CCL7, and MCP-4/CCL17 (3, 4, 8–11). Among them, the three eotaxins are regarded as most important because of their high specificity for CCR3. We have originally identified eotaxin-3/CCL26 through a genomic sequence analysis on the vicinity of the gene encoding eotaxin-2/CCL24 at the human chromosome region of 7q11.23 (9). We coined this chemokine as eotaxin-3 because of its close genomic relationship with eotaxin-2/CCL24 and its specificity to CCR3 (9). Independently, Shinkai et al. (12) have also identified CCL26 from IL-4–stimulated HUVECs by a differential display analysis. They showed that CCL26 was abundantly produced by endothelial cells stimulated with IL-4 or IL-13 (12). Furthermore, Cuvelier and Patel (13) showed that CCL26 was associated on the surface of IL-4–stimulated endothelial cells and promoted eosinophil transmigration especially in shear conditions. Interestingly, CCL26 is stored in Weibel–Palade bodies, the endothelial cell-specific storage granules, and is rapidly released upon secretogenic stimuli (14). Subsequent studies have further shown that IL-4 as well as IL-13 induces CCL26 in a wide variety cells such as fibroblasts, bronchial epithelial cells, airway smooth muscle cells, intestinal epithelial cells, and epidermal keratinocytes (15–19). Thus, CCL26 is likely to play a major role in the emigration and migration of eosinophils and other CCR3-expressing cells in Th2-shifted conditions. Indeed, the serum levels of CCL26 but not those of CCL11 or CCL24 were shown to be well correlated with the disease severity of atopic dermatitis (AD) (20). Similarly, CCL26 but not CCL11 or CCL24 was dramatically upregulated in bronchial biopsies of asthmatic patients 24 h after allergen challenge (21). Furthermore, CCL26 but not CCL11 or CCL24 was highly elevated in the blood and affected tissues in Churg–Strauss syndrome, which is charac-
terized by excessive eosinophil accumulation in peripheral blood and affected tissues and development of granulomatous vasculitic organ damage (22). CCL26 has also been shown to be highly expressed in the lesions of eosinophilic esophagitis, an emerging disorder with a poorly understood pathogenesis (23, 24), and a single-nucleotide polymorphism of the CCL26 gene is significantly associated with the disease susceptibility (23). Collectively, CCL26 appears to have a highly important role in allergic and other pathological conditions where tissue infiltration of eosinophils is prominent. Unfortunately, however, the mouse CCL26 gene is a pseudogene (2), making it impossible to study the in vivo functions of CCL26 using mouse models.

CCL26 is a relatively low-affinity agonist for CCR3 with a potency ~10-fold less than that of eotaxin/CCL11 (9). Furthermore, CCL26 has been shown to act as a natural antagonist for CCR1, CCR2, and CCR5 (25, 26). These findings indicate that CCL26 is a less well fitting but widely interacting ligand for multiple chemokine receptors. During a thorough reexamination of various human chemokines on their reactivity on the panel of murine L1.2 cell lines stably expressing human chemokine receptors (CCR1 to 10, CXCR1 to 7, XCR1, and CX3CR1), we unexpectedly found that CCL26 functions as an agonist for CX3CR1, the receptor for fractalkine/CX3CL1 (27), and thus efficiently attracts cells expressing CX3CR1 in vitro and in vivo. CX3CR1 is known to be mainly expressed by CD16high NK cells, terminally differentiated cytotoxic CD8+ T cells, and monocytes (27, 28). Furthermore, Geissmann et al. (29) have recently shown that the expression of surface CX3CR1 subdivides blood monocytes into two major functional subsets; namely, CD14+CD16 CX3CR1imm inflammatory monocytes and CD14−CD16+CX3CR1high homeostatic monocytes. Taken together, CCL26 could be a particularly versatile chemokine, being a dual agonist for CCR3 and CX3CR1 and a triple antagonist for CCR1, CCR2, and CCR5.

### Materials and Methods

#### Cells and tissues

A mouse pre-B cell line L1.2 was a kind gift of E. Butcher (Stanford University School of Medicine, Stanford, CA). The panels of L1.2 transfectants stably expressing the whole set of human chemokine receptors were generated by using a retroviral vector pMX-IRESC-EGFP as described previously (30). L1.2 cells were infected with the recombinant viruses, and cells expressing the EGFP marker were sorted using FACSVantage SE (Becton Dickinson, Mountain View, CA). HUVECs were purchased from Sanko Junyaku (Tokyo, Japan). Human PBMCs were isolated from heparinized venous blood obtained from healthy adult donors by using the density gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Piscataway, NJ). Negative selection with the IMagnet System (BD Biosciences) was used to prepare CD8+ T cells (purity, >96%) and CD16+ CD56− NK cells (purity, >95%) from PBMCs. Human eosinophils (purity, >95%) were isolated as described previously (31). In brief, buffy coat was prepared from heparinized venous blood by dextran T500 sedimentation and subjected to a density centrifugation on 1.088 g/ml Percoll (Pharmacia Biotech, Uppsala, Sweden). Eosinophils were further purified by negative selection using anti-CD16–bound micromagnetic beads and the IMagnet System. Skin tissues were obtained from patients with dermatological diseases with written informed consent. The severity of AD was assessed by the criteria of Hanifin and Rajka. This study was approved by the ethical committee of Kinki University Faculty of Medicine (Osaka, Japan).

#### Calcium mobilization assay

Recombinant human chemokines were all purchased from R&D Systems (Minneapolis, MN). Human CCL26 and CX3CL1 were also purchased from PeproTech (Rocky Hill, NJ). Pertussis toxin (PTX) was purchased from Sigma-Aldrich (St. Louis, MO). Cells were suspended at 104 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 on an F2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) and stimulated with each recombinant human chemokine. Emission fluorescence at 510 nm was measured upon excitation at 340 and 380 nm, and the fluorescence intensity ratio (R340/380) was obtained.

#### Chemotaxis assay

The chemotaxis assay was performed using Transwell plates with 5-μm pore polycarbonate membrane filters (Corning, Corning, NY) as described previously (31). Cells migrated into the lower wells were lysed with 0.1% Triton X-100 (Wako, Osaka, Japan) and quantified using PicoGreen dsDNA reagent (Molecular Probes).

### Binding assay

Soluble human CX3CL1 fused with the secreted form of placental alkaline phosphatase (CX3CL1–SEAP) was produced as described previously (32). For binding experiments, 2 × 105 cells were incubated at 16°C for 1 h with 1 nM CX3CL1–SEAP without or with increasing concentrations of competitors in 200 μl RPMI 1640 + 20 mM HEPES (pH 7.4) + 0.1% BSA. Cells were washed and lysed in 50 μl 1% Triton X-100 in 10 mM Tris-HCl (pH 8). Cell lysates were heated at 65°C for 10 min to inactivate cellular phosphatases and centrifuged to remove cell debris. Alkaline phosphatase activity in 10 μl lysate was determined by chemiluminescence using the AURORA AP kit (ICN Biomedicals, Costa Mesa, CA). The binding data were analyzed with GraphPad PRISM (GraphPad Software, San Diego, CA).

#### Flow cytometry

Anti-CX3CR1 (clone 2A9-1) labeled with FITC or PE was purchased from MBL International (Woburn, MA). Unlabeled anti-CCR3 (61828) was purchased from R&D Systems. Allophycocyanin-labeled anti-CD4 (13B8.2), PerCP-Cy5.5–labeled anti-CD16 (3G8), FITC-labeled control mouse IgG2a, and allophycocyanin-labeled control mouse IgG1 were purchased from Beckman Coulter (Marseille, France). FITC-labeled anti-CD27 (LT27) was purchased from Serotec (Hokkaido, Japan). PE-labeled anti-CD45RA (HI100), PerCP-Cy5.5–labeled anti-CD19 (SJ25C1), and FITC-labeled anti-CD14 (M2E2) were purchased from BD Biosciences. Isotype-matched control Abs were also purchased from BD Biosciences. Cells were suspended in ice-cold PBS containing 2% FCS and 0.05% sodium azide (staining buffer) and treated with normal human serum for 20 min to block the Fc receptors. After washing, cells were incubated for 30 min with the mixture of FITC-labeled mAb, PE-labeled mAb, and allophycocyanin-labeled mAb. In some experiments, cells were stained first with a nonlabeled primary mAb and then with FITC-, PE-, or allophycocyanin-labeled secondary Abs (Cedarlane, Ontario, Canada). After washing, cells were immediately analyzed on FACSCalibur (BD Biosciences) using appropriate gatings.

#### CX3CR1 internalization

Cells were suspended at 105 cells/ml in RPMI 1640 + 20 mM HEPES (pH 7.4) + 0.1% BSA and incubated at 37°C for 30 min with CCL26 or CX3CL1. After washing with PBS, the surface expression of CX3CR1 was determined by flow cytometry using FACSCalibur (BD Biosciences).

#### RT-PCR

Total RNAs were prepared from cells and tissue sections using TRIzol (Invitrogen Life Technologies) and reverse transcribed using RT-PCR Kit (Qiagen, Hilden, Germany) and RNeasy FFPE Kit (Qiagen), respectively. Total RNAs (1 μg) were reverse transcribed using oligo(dT)14 primer and SuperScript II reverse transcriptase (Invitrogen Life Technologies). Resulting first-strand DNAs (equivalent to 20 ng total RNAs) and original total RNAs (20 ng) were amplified in a final volume of 20 μl containing 10 pmol of each primer and 1 U Ex-Taq polymerase (Takara Bio, Kyoto, Japan). The amplification conditions consisted of initial 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 33 cycles for CCL26 and CX3CL1 and 27 cycles for GAPDH. The amplification products (10 μl each) were subjected to electrophoresis on 2% agarose and stained with ethidium bromide. The primers used were +5′-CTCCTGAG-TTCCTCACCTTTGGA-3′ and −5′-TCACAAATTGTTTGCGAGTTTCA-3′ for CCL26 (Forward: 5′-GCCTTCCTACCTAAGATGCT-3′ and −5′-TG- GAGGTGGAGAATGGTCAAGGCTG-3′; Reverse).
ELISA
CCL26 and CX3CL1 in culture supernatants were measured using ELISA kits (R&D Systems). For standardization of the assays, serially diluted recombinant CCL26 or CX3CL1 were always included in each assay.

Cell adhesion assay
Recombinant human cytokines were purchased from R&D Systems. HUVECs were plated at 1 x 10^4 cells/well in a 96-well plate and treated with IL-4 (20 ng/ml) or IFN-γ (100 ng/ml) for 24 h. After replacing the medium, HUVECs in each well were incubated with 1 x 10^5 cells labeled with PKH26 (Sigma-Aldrich, St. Louis, MO) for 20 min at 37˚C. After gentle washing, cells adhering to HUVECs were released by trypsin-EDTA and counted on FACSCalibur (BD Biosciences).

Cell mobilization in mice
BALB/c mice, male and 9 wk old, were purchased from CLEA (Tokyo, Japan) and kept in specific pathogen-free conditions for at least 1 wk before experiments. Human CD16+ NK cells (purity, >95%) were labeled with 2 μM CFSE (Molecular Probes) for 15 min. Mice were injected i.v. with 1 x 10^6 CFSE-labeled NK cells suspended in 200 μl PBS. After 14 h, mice were injected i.p. with 400 μl PBS or PBS containing 5 μg human CX3CL1 or CCL26. After 6 h, mice were sacrificed by cervical dislocation, and 6 ml PBS containing 10% FCS and 10 mM EDTA was injected into the peritoneal cavity. After gentle massage, PBS was recovered from each mouse. CFSE-labeled human NK cells were counted using FACSCalibur (BD Biosciences).

Quantitative PCR
Quantitative real-time PCR was carried out using TaqMan kit and the 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The conditions for PCR were 50˚C for 2 min, 95˚C for 10 min, and then 50 cycles of 95˚C for 15 s (denaturation) and 60˚C for 1 min (annealing extension). The primers and fluorogenic probes for CCL26, CX3CL1, CX3CR1, and β2-microglobulin were obtained from TaqMan kits (Applied Biosystems).

FIGURE 1. CCL26 induces calcium mobilization via CX3CR1. A, CCL26 induces calcium mobilization in L1.2 cells expressing CCR3 (L1.2-CCR3) and those expressing CX3CR1 (L1.2-CX3CR1). Cells were loaded with fura 2-AM and stimulated with CCL11, CX3CL1, and CCL26 at indicated concentrations (5 x 10^5 cells per assay). Intracellular calcium mobilization was measured on a fluorescence spectrophotometer. Pretreatment of L1.2-CX3CR1 with PTX at 500 ng/ml was performed at 37˚C for 30 min. Representative results from three separate experiments are shown. B, Desensitization experiments. L1.2-CX3CR1 cells were loaded with fura 2-AM and sequentially stimulated with CCL26 and CX3CL1 as indicated. Intracellular calcium mobilization was measured on a fluorescence spectrophotometer. Representative results from three separate experiments are shown.

FIGURE 2. CCL26 induces chemotaxis via CX3CR1. A, Dose-response experiments. L1.2 cells transfected with the vector only (L1.2-vector), stably expressing CCR3 (L1.2-CCR3) or stably expressing CX3CR1 (L1.2-CX3CR1), were examined for cell migration to indicated concentrations of CCL26 and CX3CL1. B, A checkerboard-type analysis. L1.2-CX3CR1 cells were examined for cell migration with CCL26 added to upper and/or lower wells at 100 nM as indicated. C, Inhibition by PTX. L1.2-CX3CR1 cells were pretreated at 37˚C for 30 min without or with PTX at 500 ng/ml and examined for cell migration to CCL26 at 100 nM. D, Additive effects of CCL26 and CX3CL1. L1.2-CX3CR1 cells were examined for cell migration to indicated concentrations of CCL26 without (open columns) or with 1 nM of CX3CL1 (closed columns). Cell migration was expressed as percentage input cells. Results from three separate experiments are shown as mean ± SEM. *p < 0.05.
Biosystems). Quantification of the gene expression was performed using the Sequence Detector System Software (Applied Biosystems).

Statistical analysis
Statistical significance was determined using Student’s t test. We considered $p < 0.05$ as statistically significant.

Results
CCL26 induces calcium mobilization via CX3CR1

To reevaluate the receptor usage of various human chemokines, we tested their ability to induce calcium mobilization using a panel of mouse L1.2 cell lines stably expressing all the known human chemokine receptors (CCR1–10, CXCR1–7, XCR1, and CX3CR1). By testing a total of 32 recombinant human chemokines (CCL1, -2, -3, -4, -5, -7, -8, -11, -13, -14, -15, -16, -17, -18, -19, -20, -21, -22, -24, -25, -26, -27, -28; CXCL4, -8, -9, -10, -12, -13, -16; XCL1; CX3CL1), we found that CCL26 was able to induce calcium mobilization via not only CCR3 but also CX3CR1 in a PTX-sensitive manner (Fig. 1A). This reactivity was specific for CCL26, as other eotaxins (eotaxin/CCL11 and eotaxin-2/CCL24) did not induce such responses in L1.2-CX3CR1 (data not shown).

In desensitization experiments, ~10-fold excess concentrations of CCL26 were required to desensitize CX3CR1 to CX3CL1 effectively, whereas CX3CL1 at equal concentrations completely desensitized CX3CR1 to CCL26 (Fig. 1B). Thus, CCL26 was ~10-fold less in potency than CX3CL1. It was also noted that the calcium mobilization induced by CCL26 was transient compared with that induced by CX3CL1.

CCL26 induces chemotaxis via CX3CR1

The above results prompted us to examine the ability of CCL26 to induce chemotaxis via CX3CR1. As shown in Fig. 2A, CCL26 induced cell migration in both L1.2-CCR3 and L1.2-CX3CR1 with peak responses at ~100 nM, and CX3CL1 induced cell migration in L1.2-CX3CR1 with a peak response at 10 nM. Notably, although the potency of CCL26 was ~10-fold less than that of CX3CL1, its efficacy was comparable with that of CX3CL1. The checkerboard-type analysis confirmed that CCL26 induced chemotaxis, not chemokinesis, in L1.2-CX3CR1 cells (Fig. 2B). Chemotaxis induced in L1.2-CX3CR1 cells by CCL26 was also sensitive to PTX, confirming the Gαi-coupled signaling (Fig. 2C).
HUVECs and induces cell adhesion via CX3CR1.

C

HUVECs were stimulated without or with IL-1

FIGURE 6.

CCL26 is abundantly produced by IL-4–stimulated

A

HUVECs and induces cell adhesion via CX3CR1.

A, RT-PCR analysis. HUVECs were stimulated without or with IL-1β (10 ng/ml), TNF-α (50 ng/ml), IFN-γ (100 ng/ml), IL-4 (20 ng/ml), and their combinations for 24 h, and total RNAs were prepared. PHA-stimulated PBMCs were used as positive control. Reverse transcription-PCR analysis was performed for CCL26, CX3CL1, and GAPDH. Representative results from two separate experiments are shown. B, ELISA assay. HUVECs were seeded in triplicate in 24-well plates and cultured without or with indicated cytokines for 24 h. The contents of CCL26 and CX3CL1 secreted in the culture supernatants were measured by ELISA. Data are shown as mean ± SEM from three separate experiments. C and D, Cell adhesion assay. HUVECs were stimulated without or with IFN-γ (100 ng/ml) or IL-4 (20 ng/ml) for 24 h. Test cells were labeled with PKH26. Adhesion assays were performed as described in Materials and Methods. For blocking experiments, HUVECs were pretreated with 10 μg/ml anti-CX3CL1 or anti-CCL26 for 20 min. L1.2-vector and L1.2-CX3CR1 were pretreated with 10 μg/ml anti-LFA-1 (anti-integrin αL) or anti-VLA-4 (anti-integrin α4) for 20 min. C, L1.2 cells transfected with the vector only (L1.2-vector; open columns)

thermore, CCL26 and CX3CL1 were additive in induction of cell migration in L1.2-CX3CR1 cells (Fig. 2D).

Specific binding of CCL26 to CX3CR1

Previously, we used soluble CX3CL1 tagged with the secreted form of alkaline phosphatase (CX3CL1–SEAP) to quantify its specific binding to CX3CR1 (32). Therefore, we examined specific binding of CCL26 to CX3CR1 through its competition with CX3CL1–SEAP. As shown in Fig. 3A, the specific binding of 1 nM CX3CL1–SEAP to L1.2-CX3CR1 was dose-dependently inhibited by unlabeled CX3CL1 and CCL26 with an IC50 of 2.4 nM and 35 nM, respectively. Furthermore, the specific binding of 1 nM CX3CL1–SEAP to human CD16+ NK cells was also competed by unlabeled CX3CL1 and CCL26 with an IC50 of 1.0 nM and 17 nM, respectively (Fig. 3B). Thus, the binding affinity of CCL26 to CX3CR1 was 10- to 20-fold less than that of CX3CL1, this in keeping with its ~10-fold lower potency in induction of calcium flux and chemotaxis in L1.2-CX3CR1 cells than that of CX3CL1 (Figs. 1 and 2).

CX3CR1 internalization

In general, the binding of a chemokine to its receptor rapidly induces receptor internalization, which is important in the regulation of receptor signaling activity. Therefore, we next examined CX3CR1 internalization by CCL26 and CX3CL1. The surface expression of CX3CR1 was determined before and after treatment with CCL26 or CX3CL1. As shown in Fig. 4, CX3CL1 induced a concentration-dependent internalization of CX3CR1, whereas no internalization was observed in the cells treated with CCL26, even at concentrations that induced the maximum chemotactic responses. This observation may also be in keeping with the low affinity of CCL26 to CX3CR1. Any qualitative differences in the signaling pathway activated by CX3CL1 and CCL26 remain to be seen.

Chemotactic response of cells expressing CX3CR1 in human PBMCs to CCL26

In PBMCs, CCR3 is mainly expressed by eosinophils (3, 4), whereas CX3CR1 is highly expressed by CD16+ NK cells, CD45RA-CD27- CD8+ terminally differentiated cytotoxic T cells, and CD14low CD16high homeostatic monocytes (27–29). We therefore compared chemotactic responses of human PBMCs to CCL26 and CX3CL1 (Fig. 5). As expected, CX3CL1 selectively induced vigorous migration in CD45RA-CD27- CD8+ T cells, CD16+ NK cells, and CD14low CD16high monocytes. Furthermore, although at relatively high concentrations, CCL26 induced vigorous migration not only in eosinophils but also in CD45RA-CD27- CD8+ T cells, CD16+ NK cells, and CD14low CD16high monocytes with an efficacy quite similar to that of CX3CL1.

IL-4–stimulated HUVECs abundantly secrete CCL26 and efficiently induce adhesion of CX3CR1-expressing cells

As reported previously, HUVECs expressed and secreted CCL26 upon IL-4 treatment (12, 13), whereas HUVECs expressed and secreted CX3CL1 upon treatment with proinflammatory cytokines such as IL-1β, TNF-α, and IFN-γ (33, 34) (Fig. 6A, 6B). Furthermore, IFN-γ suppressed the induction of CCL26 by IL-4, and IL-4 suppressed the induction of CX3CL1 by IL-1β, TNF-α, and IFN-γ. Thus, CX3CL1 and CCL26 are produced by HUVECs in

and those stably expressing CX3CR1 (L1.2-CX3CR1; closed columns) were used for the adhesion assay. D, CD16+ NK cells prepared from human PBMCs (purity >95%) were used for the adhesion assay. Cell adhesion was expressed as percentage input cells. Results from three separate experiments are shown as mean ± SEM. *p < 0.01.
a highly reciprocal manner in accordance with the Th1 and Th2 conditions, respectively. It was also notable that the amounts of CCL26 secreted by IL-4–treated HUVECs (∼40 ng/ml) were 10-fold those of CX3CL1 secreted by IFN-γ–treated HUVECs (∼1.5 ng/ml). Thus, the lower potency of CCL26 on CX3CR1 may be easily compensated by its higher production than that of CX3CL1.

Chemokines are known to induce firm adhesion of cells expressing their cognate receptors to vascular endothelial cells via transient activation of LFA-1 or VLA-4 (1, 35). However, the membrane-anchored form of CX3CL1 is known to induce firm adhesion of CX3CR1-expressing cells even without activation of integrins (27). Therefore, we next examined adhesion of CX3CR1-expressing cells to HUVECs treated with IFN-γ or IL-4. As shown in Fig. 6C, we observed enhanced adhesion of L1.2-CX3CR1 not only to IFN-γ–treated HUVECs but also to those treated with IL-4. The enhanced adhesion of L1.2-CX3CR1 to IFN-γ–treated HUVECs and those treated with IL-4 was significantly inhibited by anti-CX3CL1 and anti-CCL26, respectively. However, anti–LFA-1 or anti–VLA-4 selectively inhibited the adhesion of L1.2-CX3CR1 to IL-4–treated HUVECs but not to IFN-γ–treated HUVECs. Thus, CCL26-mediated adhesion of L1.2-CX3CR1 to HUVECs was dependent on the activation of these integrins. We also confirmed enhanced adhesion of human CD16+ NK cells to both IFN-γ–treated and IL-4–treated HUVECs (Fig. 6D). Again, their adhesion to IFN-γ–treated HUVECs and IL-4–treated HUVECs was effectively suppressed by anti-CX3CL1 and anti-CCL26, respectively.

In vivo recruitment of CCR3- and CX3CR1-expressing cells by CCL26

We next wished to demonstrate attraction of CX3CR1-expressing cells by CCL26 in vivo. Because the mouse homologue of CCL26 is a pseudogene (2, 36), we first examined whether human CCL26 was capable of inducing signals via mouse CCR3 (mCCR3) and mCX3CR1. We found that CCL26 was chemotactic for L1.2 cells expressing mCCR3 but not for those expressing mCX3CR1 (data not shown). Therefore, we first injected CFSE-labeled human CD16+ NK cells into mice via the tail vein. After 14 h, we injected chemokines into the peritoneal cavities of the mice. As shown in Fig. 7, human CCL26 recruited both human NK cells and endogenous mouse eosinophils into the peritoneal cavity, and human CX3CL1 recruited human NK cells. Anti–CCL26 suppressed the recruitment of both human NK cells and mouse eosinophils.
esinophils by CCL26, and anti-CX3CL1 suppressed the recruitment of human NK cells by CX3CL1.

**Dual role of CCL26 in AD**

AD is a Th2-dominant disease, whereas psoriasis a Th1-mediated disease (37, 38). It has been shown that CX3CL1 mRNA is highly elevated in the skin lesion of psoriasis (34). By using quantitative real-time PCR, we compared mRNA levels of CCL26, CCR3, CX3CL1, and CX3CR1 in normal, psoriasis, and AD skin tissues. As shown in Fig. 8A, CCL26 mRNA and CCR3 mRNA were consistently detected at high levels in AD skin samples. On the other hand, CX3CL1 and CX3CR1 transcripts were consistently detected at high levels in psoriasis skin samples. Notably, CX3CR1 mRNA was also consistently detected at high levels in AD skin tissues, although mRNA of its ligand CX3CL1 was detected in only half of the samples. Thus, in AD cases without high CX3CL1 expression, CCL26 might be responsible for the attraction of cells expressing CX3CR1. Unfortunately, we were unable to localize the cells expressing CCL26 or CX3CR1 in situ in AD skin tissues due to the lack of usable Abs.

We also analyzed the correlation of AD disease severity with the mRNA levels of CCL26, CCR3, CX3CL1, and CX3CR1. The disease severity was significantly correlated with the levels of CX3CL1 mRNA but not those of CCL26 mRNA (Fig. 8B). Thus, IL-4-induced CCL26 may play a minor role in AD disease severity, even though it attracts both eosinophils and the cells also attracted by CX3CL1. This may indicate that the shift to Th1 may be responsible for the disease severity of AD (37, 39). However, it should also be reminded that CX3CL1 is inducible not only by the Th1-type cytokine IFN-γ but also by the proinflammatory cytokines such as IL-1β and TNF-α (33, 34), and the disease severity of AD was reported to correlate with the serum levels of TNF-α (40). Thus, the increased levels of CX3CL1 in AD skin tissues may be indirectly correlated with disease severity through the expression levels of TNF-α.

**Discussion**

Eotaxin-3/CCL26 was identified as the third member of the eotaxin family (9, 12), a group of CC chemokines that are regarded to be highly specific for CCR3. Because CCR3 is selectively expressed on eosinophils, basophils, and some Th2 cells (3–7), the eotaxins are considered to play the major roles in allergic diseases. Among the eotaxins, however, CCL26 appears to be particularly important in allergic diseases (20, 21). Furthermore, Ogilvie et al. (25) have demonstrated that CCL26 is a natural antagonist for CCR2 and promotes active movement of monocytes away from a CCL26 gradient especially in synergy with an additional gradient of CCL2, although the mechanism by which CCL26 exerts this repellent effect in a PTX-sensitive manner has not been defined. Moreover, Petkovic et al. (26) have reported that CCL26 acts as a natural antagonist for CCR1 and CCR5 as well. Thus, CCL26 may have multiple roles in allergic inflammation by attracting eosinophils, basophils, and some Th2 cells via CCR3 and concomitantly blocking the recruitment of monocytes and Th1 cells via CCR1, CCR2, and CCR5. Furthermore, recent studies have shown that CCL26 is closely associated in the pathogenesis of diseases such as eosinophilic esophagitis and Churg–Strauss syndrome (22–24). Thus, CCL26 may be an important diagnostic and therapeutic target in the future.

In this study, we have demonstrated that CCL26 is yet another functional ligand for CX3CR1, the fractalkine/CX3CL1 receptor, which is expressed on CD16high NK cells, CD45RA+CD27CD8+T cells (terminally differentiated effector CD8+ T cells), and CD14lowCD16high homeostatic monocytes (27–29). Although the affinity and potency of CCL26 for CX3CR1 are ~10-fold less than those of CX3CL1, the sheer abundance of CCL26 produced by IL-4–or IL-13–stimulated cells may easily compensate for its low affinity and potency as the CX3CR1 agonist. CCR3 is considered to be involved in Th2-mediated diseases including AD and asthma (41, 42), whereas CX3CR1 is reported to be involved in Th1-mediated diseases such as rheumatoid arthritis, diabetes, lichen planus, and psoriasis (34, 43, 44). Furthermore, CCL26 and CX3CL1 are reciprocally induced in vascular endothelial cells by Th1 and Th2 cytokines, respectively (12, 33, 34). Thus, CCL26 and CX3CL1 are likely to have a highly reciprocal role in blood cell–endothelial interactions in Th2 and Th1 conditions, respectively. However, from the current results, CCL26 may also have a role in extravasation of CX3CR1-expressing cells in Th2-shifted conditions. We have indeed demonstrated that although CX3CL1 mRNA was not always detected, the mRNA levels of CX3CR1 were consistently augmented together with those of CCL26 in AD skin lesions (Fig. 8). Thus, the extravasation of CX3CR1-expressing cells into AD skin tissue may be mediated by CCL26 in Th2-shifted conditions.

Recently, it has been suggested that cytotoxic effector lymphocytes are also involved in the pathogenesis of allergic diseases such as AD and asthma (45–47). Yawalkar et al. (45) reported that cytotoxic effector lymphocytes existed in AD and psoriasis skin at the same levels, suggesting that cell-mediated cytotoxic mechanisms could be involved in epidermal cell injury of AD. Furthermore, Echigo et al. (48) reported that infiltration of CX3CR1-expressing lymphocytes was increased in skin tissues of not only psoriasis but also AD. CCL26 was expressed in endothelial cells in chronic skin lesions of AD, and serum CX3CL1 levels correlated with the disease severity. In the current study, we have also shown that, although CX3CR1 mRNA expression is consistently increased in AD skin, CX3CL1 mRNA levels are increased in only a fraction of cases with a significant correlation with the disease severity (Fig. 8). Thus, CCL26 may be responsible for the infiltration of cells expressing CX3CR1 in AD skin where CX3CL1 expression is low. CCL26-induced infiltration of cells expressing CX3CR1 may promote the shift from Th2 to Th1, resulting in production of CX3CL1.

Collectively, CCL26 is another agonist for CX3CR1. Thus, it may play a dual role in the pathogenesis of allergic and other diseases such as eosinophilic esophagitis and Churg–Strauss syndrome by attracting not only CCR3-expressing cells but also CX3CR1-expressing cells (22–24). The role of cells expressing CX3CR1 such as terminally differentiated CD8+ killer T cells and CD16+ NK cells in the pathogenesis of allergic diseases, eosinophilic esophagitis, and Churg–Strauss syndrome remains to be seen.

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**Disclosures**

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