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

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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, cytototoxic effector CD8+ T cells, and CD14lowCD16high monocytes. Albeit at relatively high concentrations, CCL26 induced calcium flux and chemotaxis in mouse L1.2 cells expressing human CX3CR1 but not mouse CX3CR1 and competed with CX3CL1 for binding to CX3CR1. In chemotaxis assays using human PBMCs, CCL26 attracted not only eosinophils but also CD16+ NK cells, CD45RA+CD27−CD8+ T cells, and CD14lowCD16high monocytes. Intrapерitoneal injection of CCL26 into mice rapidly recruited mouse eosinophils and intra-peritoneal cavity. IL-4–/IL-13–stimulated HUVECs produced CCL26 and efficiently induced adhesion of cells expressing CX3CR1. Real-time PCR showed that skin lesions of psoriasis consistently contained CX3CL1 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 CX3CR1 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: 6472–6479.

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–/IL-13–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 level of surface CX3CR1 subdivides blood monocytes into two major functional subsets; namely, CD14+CD16+ CX3CR1low inflammatory monocytes and CD14−CD16+ CX3CR1high homeostatic monocytes. Taken together, CCL26 could be a particularly versatile chemokine, being both a less-fit 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-IRES-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 (BD Biosciences, Mountain View, CA). HUVECs were purchased from Sankyo Junyaku (Tokyo, Japan). Human PBMCs were isolated from heparinized venous blood by dextran T500 sedimentation 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 (BD Biosciences, Mountain View, CA). HUVECs were purchased from HUVECs (BD Biosciences, Mountain View, CA). Pertussis toxin (PTX) was purchased from Wako Pure Chemical Industries, Ltd. (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 List Biological Laboratories (Campbell, CA). 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 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).

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. Allopoxycyanin-labeled anti-CD4 (13B8.2), PerCP-Cy5.5-labeled anti-CD16 (3G8), FITC-labeled control mouse IgG2a, and allopoxycyanin-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 (H100), PerCP-Cy5.5-labeled anti-CD19 (SJ25C1), and FITC-labeled anti-CD14 (M5E2) 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 with 10^5 cells/ml with the mixture of FITC-labeled mAb, PE-labeled mAb, and allopoxycyanin-labeled mAb. In some experiments, cells were stained first with a nonlabeled primary mAb and then with FITC-, PE-, or isotype-matched control Abs (Cedarlane, Ontario, Canada). After washing, cells were immediately analyzed on FACS Calibur (BD Biosciences) using appropriate settings.

CX3CR1 internalization

Cells were suspended at 10^6 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 reagent (Invitrogen Life Technologies, Carlsbad, CA). Total RNAs were amplified in a final volume of 20 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).

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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 $1 \times 10^5$ 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 $1 \times 10^5$ 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 using 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 $1 \times 10^6$ 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). Smears were made on glass slides and stained with May-Grunwald and Giemsa solutions. Eosinophil numbers were obtained by counting at least 500 cells per slide. All animal experiments were performed in accordance with the guideline of the Center of Animal Experiments, Kinki University Faculty of Medicine (Osaka, Japan).

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 Biosystem).
Biosystems). Quantification of the gene expression was performed using the Sequence Detector System Software (Applied Biosystems).

Statistical analysis
Statistical significance was determined using Student 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). Further...
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 CD14lowCD16homeostatic 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 CD14lowCD16+ 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 CD14lowCD16+ 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.

FIGURE 7. In vivo recruitment of cells expressing CCR3 and CX3CR1 by CCL26. Male BALB/c mice were i.v. injected with CFSE-labeled human CD16+ NK cells (purity, >95%). After 14 h, mice were i.p. injected with recombinant human CX3CL1 or CCL26. After 6 h, mice were sacrificed, and peritoneal lavage fluids were collected. CFSE-labeled human NK cells were counted by flow cytometry, and mouse eosinophils were counted by staining with May–Grünwald and Giemsa solutions on the slides. Results from three separate experiments are shown as mean ± SEM.

FIGURE 8. Coexpression of CCL26 and CX3CR1 in AD. A, Quantitative real-time PCR analysis for the expression of CCL26, CCR3, CX3CL1, and CX3CR1 mRNAs in various skin tissues. Total RNAs were prepared from formalin-fixed, paraffin-embedded sections from normal skin tissues (n = 5), skin tissues from psoriasis patients (n = 5), and skin tissues from AD patients (n = 13). Real-time PCR was performed to quantify mRNA expression of CCL26, CCR3, CX3CL1, and CX3CR1. The results are normalized by the expression of the β2-microglobulin gene (B2M). Representative results from two separate experiments are shown. B, Correlation analysis between the level of mRNA expression of CCL26, CCR3, CX3CL1, and CX3CR1 and the severity of AD. The AD patients were grouped into two groups according to the criteria of Hanifin and Rajka. *p < 0.05.
eosinophils 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. In contrast, 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+CD27+CD8+ T cells (terminally differentiated effector CD8+ T cells), and CD14low/CD16high 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. CX3CR1 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. CX3CL1 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**

The authors have no financial conflicts of interest.

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