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The Membrane-Bound Chemokine CXCL16 Expressed on Follicle-Associated Epithelium and M Cells Mediates Lympho-Epithelial Interaction in GALT

Koji Hase,* Takaya Murakami,* Hiroyuki Takatsu,*† Takeshi Shimaoa,‡ Mitsutoshi Iimura,§ Kimiyo Hamura,§ Kazuya Kawano,* Sayaka Ohshima,* Risa Chihara,* Kikuji Itoh,¶ Shin Yonehara,‡ and Hiroshi Ohno*†‡

The recently identified CXCL16 has dual functions as a transmembrane adhesion molecule and a soluble chemokine. In this study we found that CXCL16 mRNA and protein were expressed constitutively on the follicle-associated epithelium covering Peyer’s patches (PPs), isolated lymphoid follicles, and cecal patches, but minimally on the villous epithelium in the murine gastrointestinal tract. The CXCL16 receptor CXCR6/Bonzo was constitutively expressed on subpopulations of CD4+ and CD8+ T cells isolated from PPs. The expression of CXCR6/Bonzo on the PP T cells was up-regulated after stimulation with anti-CD3 and anti-CD28 mAbs. The activated PP T cells showed chemotactic migration in response to the soluble N-terminal chemokine domain of CXCL16. Furthermore, the activated PP T cells selectively adhered to cells expressing murine CXCL16. To determine the physiological role of CXCL16 in GALT, we first carefully analyzed T cell distribution in PPs. T cells localized not only in the interfollicular region but also at a lesser frequency in the subepithelial dome (SED) and in the germinal center of lymphoid follicles. Consistently, the majority of the adoptively transferred activated T cells migrated into the SED and the interfollicular region. However, the neutralization of CXCL16 specifically reduced the migration of the adoptive, transferred, activated T cells into the SED of PPs. These data suggest that CXCL16 expressed on the follicle-associated epithelium plays an important role in the recruitment and retention of activated T cells in the SED and should, at least partially, be responsible for lymphocyte compartmentalization in GALT. The Journal of Immunology, 2006, 176: 43–51.

Peyer’s patches (PPs)† and isolated lymphoid follicles (ILFs) are important inductive sites for mucosal immune response against the vast majority of intestinal foreign Ags (1, 2). The luminal side of PPs and ILFs is covered by the follicle-associated epithelium (FAE), which contains limited numbers of goblet cells and enteroendocrine cells but instead harbors M (membranous or microfold) cells (3, 4). M cells play a pivotal role in immunosurveillance on the mucosal surface by recognizing and taking up foreign macromolecules through active transepithelial vesicular transport (5). The transcytosed Ags are transferred to CD11b+ myeloid dendritic cells (DCs) accumulating in the subepithelial dome (SED) beneath the FAE (6). The Ag-primed DCs undergo maturation and migration to the T cell-rich interfollicular region (IFR) for Ag presentation, leading to the onset of T-B collaboration and the formation of IgA-producing plasma cells (7).

The spatial distribution of immune cells in PPs is most likely controlled by chemokine-driven processes. For instance, CCL20 is constitutively expressed by the FAE in both mice and human (8–10). Similarly, murine CCL9 and its potential human counterpart, CCL23, are selectively produced by the FAE but not by intestinal epithelial cells (IECs) (6, 10). The receptors for these chemokines, CCR1 and CCR6, are expressed by myeloid DCs. The blockade of CCL9 with a neutralizing Ab results in the failure to recruit myeloid DCs into the subepithelial dome (SED) of PPs (6). CCR6-deficient mice also manifest altered localization of myeloid DCs in certain circumstances and the reduced size of PPs (11–13). Collectively, these data clearly indicate that FAE-derived chemokines contribute to the migration of myeloid cells, which may be important for the maintenance of microarchitecture in GALT. However, it remains unknown whether lymphocytes are also attracted to FAE-derived chemokines in the context of lympho-epithelial interaction.

We have recently performed transcriptome analysis of FAE and IECs using high-density oligonucleotide microarray (14) and found that the expression level of CXCL16 is remarkably higher in the FAE than in the IECs. CXCL16 is one of the two known chemokines that are associated with the plasma membrane through a mucin-like stalk and a transmembrane domain. The membrane-bound and soluble forms of CXCL16 have completely different physiological functions. Although the membrane-bound form...
functions as a cell adhesion molecule, a scavenger, and a phagocytic receptor for oxidized low-density lipoproteins and bacteria (15, 16), it functions as a soluble chemokine when proteolytically released (17, 18). To date, a disintegrin and metalloproteinase do- main-10 (ADAM10) is thought to be the key regulator of CXCL16 shedding (19, 20). The expression of CXCL16 was first identified on Ag-primed DCs, and its receptor CXCR6/Bonzo, was detected on activated T cells, suggesting the role of CXCL16 as a mediator of DC-T cell interaction. Other studies have shown the expression of CXCL16 on bone marrow stromal cells, macrophages, and aortic smooth muscle cells in atherosclerotic lesions (16, 21, 22).

In this work we demonstrate that CXCL16 mRNA and protein are produced throughout the FAE. CXCR6 is moderately expressed by most CD8+ T cells and a subpopulation of CD4+ T cells in PPs, and its expression is highly up-regulated after in vitro activation. The activated T cells show migration and cell adhesion activities in response to CXCL16. Furthermore, the administration of CXCL16 neutralizing mAb significantly inhibited the migration of activated T cells to the SED. These results suggest that CXCL16 may function in promoting the interaction between the FAE and T cells, which could account for activated T cell trafficking and retention in the SED in GALT.

Materials and Methods

Animals

BALB/c and C57BL/6d mice were purchased from CLEA Japan and maintained under specific pathogen-free conditions in RIKEN animal facilities until use in experiments at 7–10 wk old. In certain experiments, BALB/c mice were maintained under germfree (GF) or conventional (CV) conditions at the animal facility of the University of Tokyo as described (23). All animal experiments were approved by the Animal Research Committee of the RIKEN Yokohama Research Institute and of the University of Tokyo.

Isolation of the FAE and IECs

The FAE and IECs were isolated as described previously (14). Briefly, PPs were dissected from murine small intestine and soaked in HBSS containing 30 mM EDTA (pH 7.2). After incubation at room temperature for 20 min, the FAE was isolated by manipulation with a fine needle under a transillumination stereomicroscope (MZ12.5; Leica Microsystems). IECs were also isolated in the same manner from small pieces of the duodenum, the jejunum and the ileum after excluding PPs. The isolated epithelial sheets were almost exclusively composed of epithelial monolayers with trace amounts of lymphocytes (14).

Detection of chemokine mRNA expression

Total RNA was extracted from the FAE and IECs using a commercial kit (RNaseasy; Qiagen). One microgram of total cellular RNA was reverse-transcribed using SuperScript II (Invitrogen). Chemokine mRNA expression of murine FAE and IECs was assessed with a mouse chemokines and receptors gene array (GE Array Q series; SuperArray) following the manufacturer’s instructions. Real-time PCR analysis was performed to quantify the CXCL16 mRNA expression level using the SYBR® Green PCR assay and an ABI Prism 7000 sequence detection system as described previously (24). The CXCL16 mRNA expression of each sample was calculated by extrapolating to a standard curve obtained with an authentic sample containing large amounts of CXCL16 mRNA, and the values calculated were normalized to the expression level of GAPDH. Amplification of the expected single products was confirmed using 1% agarose gels and ethidium bromide staining. The specific primers for murine chemokines are 5'-GGGGCTTTGGACC-3' (forward) and 5'-TTTGCGTCACGTTT-3' (reverse) for CXCL16 and 5'-GGCTGAGCAGTTGTCGTT-3' (forward) and 5'-TGTAGTCCCGGGTTTT-3' (reverse) for CCL9. Murine CCL20 and GAPDH primers were described elsewhere (24, 25).

In situ hybridization (ISH)

The full-length sequence of CXCL16 was amplified from FAE-derived cDNA by PCR using the primers 5'-GGGCTTTGGACC-3' (forward) and 5'-GGCTGAGCAGTTGTCGTT-3' (reverse). The PCR product was digested by BamHI and XhoI and was ligated into pcDNA3.0 digested by the same enzymes. Digoxigenin-labeled RNA probe was prepared by in vitro transcription
with T7 or SP6 RNA polymerase (Roche) using the above-mentioned plasmid vector digested by XhoI or BamHI as template, respectively. ISH was performed with a Discovery™ automated ISH system and a RiboMap kit in accordance with the manufacturer’s instructions (Ventana Japan) as described previously (14). Briefly, 4% buffered formalin fixed sections of murine PPs were deparaffinized, treated with protease, and hybridized with 50 ng of murine CXCL16 (mCXCL16)-specific antisense riboprobe or the murine PPs were deparaffinized, treated with protease, and hybridized with 50 ng of murine CXCL16 (mCXCL16)-specific antisense riboprobe or the control sense probe for 6 h at 65°C. The sections were then incubated with biotin-labeled anti-digoxigenin Ab (Jackson ImmunoResearch Laboratories) for 20 min at 37°C, followed by incubation with alkaline phosphatase-biotin-labeled anti-digoxigenin Ab (Jackson ImmunoResearch) followed by streptavidin for 16 min at 37°C. The signal was detected with a BlueMap NBT/BCIP substrate kit (Ventana Japan), and the sections were counterstained with Nuclear Fast Red.

**Immunohistochemistry**

For CXCL16 immunostaining, 1% zinc sulfate/4% formalin (Richard-Allan Scientific)-fixed sections of murine PPs (5 μm) were deparaffinized, rehydrated, and treated with 0.3% H2O2 in PBS for 20 min at room temperature to block endogenous peroxidase activity. The sections were incubated with 0.5% blocking buffer (Roche) in PBS for 30 min at room temperature and then with 2 μg/ml goat anti-mouse CXCL16 polyclonal Ab (R&D Systems) or an identical concentration of control goat IgG overnight at 4°C. The binding of primary Ab was detected with 4 μg/ml biotinylated donkey anti-goat IgG (Jackson Immunoresearch) followed by streptavidin-HRP (ABC Elite; Vector Laboratories), visualized with 3,3'-diaminobenzidine (DakoCytomation), and counterstained with hematoxylin (DakoCytomation). For immunofluorescence staining, CXCL16-specific binding was visualized with tyramide-FITC (PerkinElmer) and counterstained with 10 μg/ml rhodamine-labeled Ulex europaeus agglutinin-I (UEA-1) (Vector Laboratories) followed by DAPI. The specimens were analyzed with a DM-IRE2 confocal laser scanning microscope and Leica confocal software (Leica Microsystems).

For immunohistochemical analysis of human CXCL16, endoscopic biopsies were obtained from the noninflamed terminal ileum containing lymphoid follicles of human subjects. The biopsy samples were fixed in 1% zinc sulfate/4% formalin (Richard-Allan Scientific) and immunostained using goat anti-human CXCL16 polyclonal Ab (R&D Systems) as mentioned above for murine samples. The studies were approved by the Committee on Human Subjects in RIKEN and Tokyo Women’s Medical University.

The distribution of T cells in PPs was determined by immunofluorescence staining. Frozen sections (5 μm) of PPs were fixed with paraformaldehyde (BD Pharmingen) and incubated with anti-CD16/CD32 mAb (clone 93; eBioscience) for 30 min at room temperature to block nonspecific Fc binding, followed by 4 μg/ml biotinylated anti-CD3ε mAb (145–2C11; BD Pharmingen) or control IgG and finally by Alexa 488-conjugated streptavidin (Invitrogen) and DAPI.

**Preparation of PP lymphocytes**

Murine PP lymphocytes were prepared according to a method described previously (14). Briefly, PPs were excised from the intestinal wall and dissociated with collagenase solution containing 0.5 mg/ml collagenase (Nitta gelatin), 0.5 mg/ml DNase I (Roche), 2% FBS, 100 μg/ml streptomycin, and 20 mM HEPES (pH 7.2) in RPMI 1640 at 37°C for 20 min to obtain single-cell suspensions. The cell dissociation was repeated again in fresh collagenase solution. The single-cell suspensions were pooled and washed with RPMI 1640 twice and subjected to Percoll gradient separation to remove epithelial cells. For the in vitro activation of T cells, PP lymphocytes were cultured in a plastic dish coated with anti-CD3ε (145–2C11) and anti-CD28 (37.5) mAbs (BD Pharmingen) in RPMI 1640 medium supplemented with 10% FBS and 4 ng/ml IL-2 (R&D systems) for 3–4 days and further cultured for 2–3 days without stimulation.

**FIGURE 3.** CXCL16 mRNA expression on the FAE. In situ hybridization was performed with CXCL16-specific antisense (A and B) or control sense (C and D) probe. A positive signal for CXCL16 mRNA was observed in the FAE (arrowheads) of murine PPs but not in the villous epithelium (arrows). Scale bars, 200 (A and C) or 50 (B and D) μm.

**FIGURE 4.** CXCL16 immunostaining of murine small intestine. A–F. Sections were stained with anti-CXCL16 polyclonal Ab (A–C) or control goat IgG (D–F). Positive staining was observed throughout the FAE (A and B) but not in the villous epithelium (C). G–I, confocal images of CXCL16 (green) and UEA-1 (red) staining, with DAPI (white) as counter nuclei staining. The merged image (I) clearly shows CXCL16 expression on the basolateral plasma membrane of M cells (arrowheads) and the other FAE cells. Scale bars, 100 (A, C, D, and F), 50 (E), or 20 (G–I) μm.
Flow cytometric analysis

CXCR6-expressing cells were detected with mCXCL16-human Fcγ fusion protein as described (18, 26). Briefly, PP lymphocytes were incubated with CXCL16-Fcγ fusion protein or control human Fcγ, and specific binding was detected with PE-conjugated anti-human Fcγ (Jackson ImmunoResearch). To characterize cell populations, PP lymphocytes were further stained with the following: FITC-conjugated mAbs against CD3ε (145–2C11) and CD62L (MEL14); PE-conjugated mAbs against CD4 (GK1.5) and CD11c (HL3); PerCP-conjugated mAbs against CD8α (53-6.7) and B220 (RA3–6B2); and biotinylated mAbs against CD11b (M1/70), CD25 (7D4), and CD44 (IM7) in combination with streptavidin-PerCP and streptavidin-PE-Cy7 (all from BD Pharmingen). The stained cells were analyzed using FACSCalibur with CellQuest software (BD Biosciences).

Chemotaxis and cell adhesion assay

Chemotaxis assays using a 3-μm Transwell chamber (Corning Costar) were performed as described previously (27). Cell adhesion assay was performed essentially as described (26). Briefly, activated CD4⁺ or CD8⁺ T cells derived from PPs were suspended at 5 × 10⁵/ml in RPMI 1640 medium containing 0.5% BSA and 20 mM HEPES (pH 7.4). The cell suspension was transferred to 24-well plates (0.2 ml/well), where CHO cells stably transfected with mCXCL16 or wild-type CHO cells were pre-seeded. After incubation for 60 min at 37°C, nonadherent cells were removed by extensive washing with PBS three times. Adherent T cells were recovered with cell dissociation buffer (Sigma) and counted by FACSCalibur (BD Bioscience).

In vivo neutralization of CXCL16

Activated PP T cells were labeled with a PKH26 red fluorescent cell linker kit (Sigma) following the manufacturer’s instructions. To neutralize the biological activity of CXCL16, female C57BL/6J mice received i.v. injections of 200 μg of anti-CXCL16 blocking mAb (clone 12–81) (26, 28) or control rat IgG at 16 h before and 4 h after transferring PKH26-labeled cells.

Results

CXCL16 is constitutively expressed by the FAE

To define FAE-specific chemokines, we first prepared total RNAs from the FAE and IECs (14) of the murine small intestine and compared their chemokine mRNA expression profiles. Among the 32 chemokines tested, 13 chemokine mRNAs were detected in either the FAE or IECs (Fig. 1). The expression profiles of the chemokines in the FAE were characterized by the increase in CCL9/MIP-3β, CCL20/MIP-3α, and CXCL16 and by the decrease in CCL5/RANTES, CCL25/thymus-expressed chemokine, and CX3CL1/fractalkine, compared with those in the IECs. The FAE-specific expression of CXCL16 mRNA was further confirmed by quantitative real-time PCR and ISH analysis. In analogy with other
FAE-specific chemokines such as CCL9 and CCL20 (6, 8, 9), the CXCL16 mRNA expression level was 5- or 15-fold higher in the FAE than in IECs from the small intestine or the colon, respectively (Fig. 2). ISH analysis of murine PPs showed that the transcripts of CXCL16 were distributed throughout the FAE (Fig. 3, A and B; arrowheads) but not in the villous epithelium (arrows).

CXCL16 protein expression was examined by immunohistochemistry using an anti-CXCL16 polyclonal Ab. In agreement with the results of the ISH analysis, the CXCL16 protein was predominantly expressed on the FAE but not on IECs (Fig. 4A). The figure at a higher magnification indicated that CXCL16 was localized on the basolateral side of plasma membrane of the FAE with little if any expression on the apical side (Fig. 4B). Similar results were obtained in other PPs prepared from at least five different mice. There was no difference in the CXCL16 expression on the FAE between BALB/c and C57BL/6 strains (our unpublished observations). We next tested CXCL16 expression on M cells by immunofluorescence costaining with UEA-1 lectin. The expression of CXCL16 was observed on the basolateral plasma membrane of UEA-1⁺ M cells, suggesting a potential role of CXCL16 in lymphocyte recruitment into the M cell pockets (Fig. 4, G–I).

Further study was performed to test whether CXCL16 is generally expressed on the FAE of GALTs other than PPs. To this end, tissue samples, including ILFs and cecal patches, were subjected to immunohistochemical analysis. CXCL16 protein was detected in the FAE of both ILFs and cecal patches (Fig. 5, A and C). Therefore, the constitutive expression of CXCL16 on the FAE is a common feature in the Ag sampling site of GALTs.

To examine whether this feature is the case in human as well, we obtained human biopsy samples from the terminal ileum containing large lymphoid follicles that correspond to murine PPs and performed immunohistochemical analysis. CXCL16 protein expression was detected throughout the human FAE (Fig. 5E). Interestingly, the higher magnification image clearly showed that CXCL16 was localized on the basolateral side of plasma membrane of human FAE (Fig. 5G); the expression pattern was quite similar to that of mCXCL16 (Fig. 4B). These data suggest that CXCL16 expression as well as its intracellular localization was regulated similarly between mice and human.

**CXCL16 expression under GF condition**

It is well known that the FAE contains a limited number of mucin-producing goblet cells that lead to reduced mucin protection in situ (4). The expression of brush border hydrolases in the FAE is also down-regulated as compared with that in IECs (29). This allows external Ags, including commensal bacteria, to easily access the FAE, which might be the reason why CXCL16 is up-regulated in the FAE. To confirm this hypothesis, we compared CXCL16 mRNA and protein expressions on the FAE of BALB/c mice maintained under GF and CV conditions. However, no differences in CXCL16 mRNA and protein expressions between those two conditions were observed (Fig. 6, A and B). This observation suggests that the expression of CXCL16 on the FAE is regulated autonomously rather than induced by the exposure to luminal bacteria.

**CXCR6/Bonzo-expressing cells in PPs**

To identify the cells that respond to CXCL16, we analyzed PP cells for the expression of CXCR6/Bonzo, the receptor of CXCL16, using a fusion protein of mCXCL16 and the human IgG-Fc domain (18, 26). CXCR6/Bonzo was moderately expressed on most of the CD8⁺ T cells of PPs (Fig. 7A). The expression of CXCR6/Bonzo was also observed in a small subpopulation of CD4⁺ T cells. Because the CD4⁺ T cell population is approximately four times larger than the CD8⁺ T cell population in PPs, the total number of CXCR6⁺ cells was comparable between the CD4⁺ and CD8⁺ T cells (2.5 × 10⁵ and 4 × 10⁵ cells in PPs, respectively), despite the lower ratio of CXCR6⁺ expression on the CD4⁺ T cells. The CXCR6⁺ and CXCR6⁻ CD4⁺ populations were further analyzed for CD44 and CD62L expression. The CXCR6⁺ population was mainly composed of CD44highCD62Llow cells (Fig. 7B). In contrast, the CXCR6⁻ population was predominantly CD44highCD62Lhigh, suggesting an activation-dependent expression of CXCR6 by the PP CD4⁺ T cells. Indeed, the CXCR6 expression on the CD4⁺ and CD8⁺ T cells further analyzed for CD44 and CD62L expression.

**FIGURE 7.** Analysis of CXCR6/Bonzo-expressing cells in PP lymphocytes. A, Various cell populations in murine PPs were stained with CXCL16-Fc fusion protein (●) or control IgG (■). Activated T cells were prepared by in vitro stimulation with anti-CD3 and CD28 mAbs. B, CD4⁺ T cells were divided into CXCR6⁺ and CXCR6⁻ populations and further analyzed for CD44 and CD62L expression.
was remarkably up-regulated by in vitro stimulation with anti-CD3/CD28 mAbs (Fig. 7A). This finding is consistent with previous studies that used murine splenic and human peripheral blood T cells (18, 30).

**PP T cells functionally respond to CXCL16**

To examine whether CXCR6 on PP T cells is functional, we first performed an in vitro chemotaxis assay. Both activated PP CD4+ and CD8+ T cells showed migration activity in response to the N-terminal chemokine domain of CXCL16 (Fig. 8A), and the migration was dose-dependent. The migratory response of activated PP T cells was further analyzed for a subset of chemokines that are expressed by the distinct regions of PPs. CCL19 and CXCL13 are expressed by the IFR and the follicular region of PPs, respectively (31, 32). In contrast, CCL20 is predominantly expressed by FAE in PPs (8, 9). Whereas activated CD8+ T cells selectively responded to CXCL16, activated CD4+ T cells retained chemotactic activity to CCL19, especially at a higher concentration (Fig. 8B). Furthermore, the activated PP T cells selectively adhered to mCXCL16-expressing CHO cells, but not to wild-type CHO cells (Fig. 8C). These data suggest that CXCL16 plays a role in the migration and retention of activated T cells in PPs.

**CXCL16 neutralization alters T cell distribution in PPs**

We further examined the physiological role of FAE-derived CXCL16. First, we carefully examined T cell distribution in PPs by immunofluorescence staining with anti-CD3ε mAb. The PP T cells were observed in three different regions of the PPs. Although the majority accumulated in the IFR, a small number of T cells were localized in the peripheral region of the mantle zone and the SED between the FAE and lymphoid follicles (Fig. 9). Some T cells were found in the interepithelial region of FAE, termed M cell pockets. In addition, a third population of T cells was observed inside lymphoid follicles, particularly in the germinal center that is formed at the serosal side of the lymphoid follicles (Fig. 9).

We next administered anti-CXCL16 neutralizing mAb or control rat IgG into host mice before and after transferring fluorescence-labeled activated T cells and examined the distribution of the transferred T cells in the PPs. The transferred T cells retained PP-homing capacity (Fig. 10A), although part of them migrated into the gut lamina propria (our unpublished observation) as demonstrated previously (33, 34). There was no difference in the total number of migrating T cells between the control IgG and anti-CXCL16 mAb-treated groups (480 ± 62 and 489 ± 44 cells/mm², respectively), indicating that CXCL16 does not affect the homing of PP-derived T cells to the gut. Migrating T cells were detected in both the IFR and the SED in the control group (Fig. 10, A and C), where the ratio of T cell frequency in the SED to that in the IFR was ~0.8 (Fig. 10D). In contrast, the SED migration of T cells was markedly inhibited by the CXCL16 mAb treatment, whereas the frequency of T cells in the IFR was increased (Fig. 10, B and C). As a consequence, the SED/IFR ratio of T cell frequency was

**FIGURE 8.** CXCL16 mediates migration and adhesion of activated PP T cells. A. Dose-dependent effect of CXCL16 on migration of in vitro activated PP CD4+ (○) and CD8+ (●) T cells. B. Chemotactic activity of activated PP CD4+ and CD8+ T cells was performed with various chemokines that are expressed in the distinct regions of PPs at the concentration of 10 (□) or 100 (●) ng/ml. C. Adhesion of activated PP T cells to mCXCL16-expressing (□) or wild-type (●) CHO cells. Chemotaxis and cell adhesion assays were repeated at least three times, and basically similar results were obtained each time.

**FIGURE 9.** T cell distribution in murine PPs. T cells was detected by immunostaining with anti-CD3ε mAb (green). The T cells mainly reside at the IFR, the SED, and the germinal center. A small number of cells were observed in the intraepithelial region of FAE. The sections were counterstained with DAPI (blue). Scale bar represents 100 μm.
CXCL16 is another important molecule for guiding activated T cells from the IFR to the SED in the PP microenvironment.

Discussion

Different from other peripheral lymphoid tissues that filter out Ags in lymph or blood, PPs and ILFs directly take up heterogeneous Ags from the intestinal lumen through the FAE. Therefore, myeloid immature DCs accumulate in the SED beneath the FAE to capture Ags and present them to naive T cells efficiently. Such functional localization of immune cells in the PPs must be tightly controlled by chemokines that are produced by the FAE as well as by stromal cells. Iwasaki and coworkers and other groups (6, 8, 11, 12, 35) have reported that CCL9 and CCL20 are secreted by the FAE, whereas their corresponding receptors, CCR1 and CCR6, respectively, are expressed on CD11b+ DCs that reside in the SED of murine PPs. In this study we have described CXCL16 as being constitutively expressed on the FAE but not on normal absorptive IECs. CXCL16 has been reported to be multifunctional; its membrane-bound form recently turned out to be a phagocytic receptor for bacteria in macrophages (16). Given that the FAE actively takes up mucosal macromolecules through M cells and paradoxically serves as the main portal for bacterial invasion, it is possible to speculate that CXCL16 might mediate bacterial internalization in the gut. However, this does not seem to be the case for CXCL16 expressed by the FAE, because the subcellular localization of CXCL16 was on the basolateral side of the plasma membrane rather than on the apical side. In addition, there was no obvious difference in CXCL16 expression on the FAE between mice maintained under CV and GF conditions. It is therefore plausible to surmise that CXCL16 derived from the FAE functions as a cell adhesion molecule or a soluble chemokine after cleavage.

We demonstrated that a subpopulation of T cells isolated from PPs express CCR6, a CXCL16 receptor. This receptor is functional because CXCL16 mediated the adhesion and migration of CXCR6+ T cells. The fact that the expression level of CXCR6 and migratory response to CXCL16 is higher in CD8+ T cells than in CD4+ T cells could reflect the difference in activation status between the two populations, because CD8+ T cells have been known to respond to Ag stimulation more rapidly than CD4+ T cells (36, 37). CXCR6 is highly up-regulated in PP and splenic T cells after in vitro stimulation (Fig. 7A) (18). CXCR6 has been defined as a marker of effector/memory T cells, because it is selectively expressed on the CD4+ T cells with the activated phenotype (17, 30). We also confirmed that CXCR6+ CD4+ PP T cells mainly possess the memory phenotype (CD44highCD62Llow). In contrast, CCR7 is highly expressed on naive CD4+ and CD8+ T cells and is down-regulated in CXCR6+ effector/memory T cells in human peripheral blood (17, 30), indicating that CXCR6 expression is negatively correlated with CCR7 and CD62L expression. Because CCR7 and CD62L are thought to be lymphoid homing receptors, their down-regulation and the up-regulation of CXCR6 may enable the activated T cells to migrate into inflamed extralymphoid tissue. This possibility coincides with the observation that CXCL16 is induced in the inflamed human liver (38, 39) and the spinal cord in experimental autoimmune encephalomyelitis in mouse (28).

The constitutive expression of CXCL16 was observed not only on the FAE of PPs but also on the ILFs and the colonic patches, suggesting its homeostatic role in the inductive site of GALTs. We demonstrated three different regional distributions of T cells in PP microcompartments (Fig. 9). The major population residing in the IFR is most likely composed of CCR7 naive T cells, because there is an abundant expression of CCR7 ligands, CCL19 and CCL21, by stromal and endothelial cells in this area (31, 32). The second population is distributed around the SED and the adjacent peripheral region of lymphoid follicles. Adoptive transfer experiments revealed that CXCR6+ activated T cells migrated into this area at a high frequency (Fig. 10). This migration into the SED was remarkably inhibited by the in vivo neutralization of CXCL16 with blocking mAb, although the total number of migrating T cells into PPs did not change. This finding suggests that CXCL16 guides the SED migration of CXCR6+CCR7intCD62low T cells that is probably activated by mature Ag-primed DCs migrating into the IFR.
However, we do not exclude the possibility that other FAE-derived chemokines such as CCL20 may likewise mediate the interaction between the FAE and T cells, because CCR6, the sole receptor of CCL20, has been reported to be expressed by subsets of memory CD4+ and CD8+ T cells (40–42). It is necessary to confirm whether this is the case in PP T cells, because in vitro activated PP T cells did not respond to CCL20, at least in the condition examined here. The SED zone accumulates external Ags sampled by M cells (43) and may therefore serve as the marginal zone where naïve B cells encounter Ags and the subsequent B-T collaboration occurs. The third population of PP T cells was observed in the germinal center, which is localized beneath the mantle zone of the B cell follicles and near the serosa. This population is surmised to be follicular Th cells or germinal center Th cells observed in the human tonsil (44–48). These cells play a key role in B cell differentiation and Ab production. Because these cells are characterized by CXCR5+CCR7−, they are most likely attracted by BLC/CXCL13 expressed on follicular DCs and stromal cells in the B cell follicles. This hypothesis was confirmed by the observation that CXCR5-deficient T cells failed to migrate into the B cell follicles of peripheral lymph nodes (49). These findings and the results of the present study suggest that the distribution of T cells in GALT could be dependent on the orchestrated expression of three chemokine receptors: CCR7, CCR85, and CXCR6.

Full-length CXCL16 is composed of an N-terminal chemokine domain followed by a mucin-like stalk, a transmembrane domain, and a short cytoplasmic tail (17, 18). Proteolytic cleavage is therefore an important process for CXCL16 to function as a soluble chemoattractant. The membrane-integrated protease, ADAM10, seems to be responsible for this process in fibroblasts and endothelial cells, because CXCL16 release induced by TNF-α and IFN-γ was largely suppressed in ADAM10-deficient cells (19, 20). Although it is unclear whether this is the case for the FAE, at least ADAM10 mRNA is abundantly expressed by the FAE (our unpublished observations). We and others (14, 50) have reported that other membrane-type matrix metalloproteinases, namely, MMP-14, MMP-15, are highly up-regulated in the FAE. These proteases might also participate in CXCL16 processing, although further study is necessary to prove this.

The basolateral plasma membrane of M cells is deeply invaginated, allowing the migration of lymphocytes and mycelial cells into the interepithelial microdomain, called M cell pockets (3). M cell pockets not only effectively shorten the distance from the apical to the basal plasma membranes of M cells but also mediate close contact between M cells and immune cells. However, the detailed mechanism of the M cell pocket formation remains unknown. Half of the lymphocytes observed in the M cell pockets are known. Half of the lymphocytes observed in the M cell pockets are known. Half of the lymphocytes observed in the M cell pockets are known. Half of the lymphocytes observed in the M cell pockets are known. Half of the lymphocytes observed in the M cell pockets are known.


