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Binding of Lymphoid Chemokines to Collagen IV That Accumulates in the Basal Lamina of High Endothelial Venules: Its Implications in Lymphocyte Trafficking

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Certain lymphoid chemokines are selectively and constitutively expressed in the high endothelial venules (HEV) of lymph nodes and Peyer’s patches, where they play critical roles in the directional migration of extravasating lymphocytes into the lymphoid tissue parenchyma. How these chemokines are selectively localized and act in situ, however, remains unclear. In the present study, we examined the possibility that basal lamina-associated extracellular matrix proteins in the HEVs are responsible for retaining the lymphoid chemokines locally. Here we show that collagen IV (Col IV) bound certain lymphoid chemokines, including CCL21, CXCL13, and CXCL12, more potently than did fibronectin or laminin-1, but it bound CCL19 and CCL5 only weakly, if at all. Surface plasmon resonance analysis indicated that Col IV bound CCL21 with a low nanomolar K_a, which required the C-terminal region of CCL21. Col IV can apparently hold these chemokines in their active form upon binding, because the Col IV-bound chemokines induced lymphocyte migration efficiently in vitro. We found by immunohistochemistry that Col IV and CCL21, CXCL13, and CXCL12 were colocalized in the basal lamina of HEVs. When injected s.c. into plt/plt mice, CCL21 colocalized at least partially with Col IV on the basal lamina of HEVs in draining lymph nodes. Collectively, our results suggest that Col IV contributes to the creation of a lymphoid chemokine–rich environment in the basal lamina of HEVs by binding an array of locally produced lymphoid chemokines that promote directional lymphocyte trafficking from HEVs into the lymphoid tissue parenchyma. The Journal of Immunology, 2007, 179: 4376–4382.

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mphocytes continuously recirculate between the blood and the lymph by way of the secondary lymphoid tissues, where Ags and APCs are selectively localized (1–3). To enter lymph nodes (LN) and Peyer’s patches (PP), lymphocytes in the blood selectively adhere to and transmigrate across high endothelial venules (HEV) in which certain lymphoid chemokines are constitutively expressed; these chemokines play a critical role in lymphocyte trafficking to the LNs and PPs (4–7).

Chemokines are a group of structurally related small, soluble proteins that regulate the trafficking of various types of cells by activating specific seven-transmembrane G protein–coupled receptors (8). Although chemokines have long been believed to induce directional cell migration by forming soluble gradients, it is unlikely that such gradients can remain stable in the presence of continuous body fluid flow (9). Thus, it has been hypothesized that, to form a stable immobilized gradient under continuous body fluid flow, chemokines must be immobilized in situ by chemokine-bind- ing molecules, such as glycosaminoglycans (GAG; Refs. 8–10). In agreement with this idea, genetically engineered mutants of CCL5, CCL4, and CCL2 with impaired GAG-binding capacities could not recruit inflammatory cells when they were administered i.p., even though they fully retained their chemotactic activity in vitro (11). These observations suggest that the GAG-binding ability is critical for the activity of particular chemokines in vivo.

In addition to the GAGs, structurally different classes of molecules can also bind chemokines and regulate their spatial distribution and functions (2, 12, 13). We previously showed that Duffy Ag receptor for chemokines (DARC; Ref. 12) and mac25/angiomodulin (AGM; Ref. 13) are expressed in HEVs and bind distinct sets of chemokines: DARC is expressed on the luminal surface of HEVs, selectively binds a set of proinflammatory chemokines, and scavenges them (12); whereas mac25/AGM selectively accumulates in the basal lamina of HEVs, preferentially binds certain chemokines, including CCL21, CXCL10, and CCL5, and presents them to lymphocytes (13). Mac25/AGM can also bind certain extracellular matrix (ECM) proteins, including collagen IV (Col IV), which also accumulates in the basal lamina of HEVs (14). The molecular mechanisms underlying the selective retention of chemokines in the basal lamina, however, remain to be fully elucidated.

In this study, we examined the chemokine-binding abilities of various ECM proteins using two different solid-phase binding assays and found that Col IV is colocalized with certain lymphoid chemokines in the basal lamina of HEVs and can bind these chemokines and present them to lymphocytes to induce cell migration. We also found that exogenously administered CCL21 binds to the
HEV basal lamina where Col IV is abundantly expressed. Together, these observations suggest that Col IV contributes to the retention of certain chemokines in the basal lamina of HEVs, generating a chemokine-rich microenvironment to support rapid and directional lymphocyte trafficking across the HEVs.

Materials and Methods

Animals

All animal experiments were performed using protocols approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine. Female C57BL/6 mice were purchased from Japan SLC. The platelet mice (15) on a C57BL/6 background were provided by Dr. Hideki Nakano (Duke University, Durham, NC). The mice were reared under specific pathogen-free conditions and used at 7–10 wk of age.

Chemokines and ECM proteins

Unless otherwise noted, the chemokines and ECM proteins used in this study were of mouse origin. CCL5, CCL19, CCL21, CXCL12, and human CCL21 were purchased from R&D Systems, and CXCL13 was from DAKO. Human CCL21 lacking 3 C-terminal amino acids (CCL21-T Ref. 16) was a gift from Dr. T. Springer (Harvard Medical School, Cambridge, MA). Col IV (Sigma-Aldrich) and laminin-1 (LN-1; Invitrogen Life Technologies) were both derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma cells. Col IV was purified as reported previously (17, 18) and was judged to be >95% pure based on the pattern of proteins apparent after gel electrophoresis on 5% polyacrylamide gels. Western blot analysis showed that this preparation predominantly consisted of 185 and 170 kDa bands that react with anti-Col IV Abs. Decoration with GAGs appears to be minimal, because neither the anti-chondroitin sulfate mAb CS56 nor the anti-heparan sulfate mAb 10E4 bound to the Col IV at significant levels, as assessed by ELISA. Fibronectin (FN) was isolated from mouse plasma as reported previously (19).

ELISAs

Wells of 96-well plates were coated with Col IV, FN, LN-1, or BSA (20 μg/ml in PBS) at 4°C overnight and blocked with 3% BSA-PBS. The plates were incubated with or without CCL21, CCL19, CXCL13, CXCL12, or CCL5 (50 nM) at room temperature for 1 h. To detect chemokine binding, the plates were incubated with biotinylated rabbit anti-CCL21 Ab (PeproTech), biotinylated goat anti-CCL19 Ab (DAKO), goat anti-CCL5 Ab (R&D Systems), goat anti-CXCL12 Ab (PeproTech), or rabbit anti-CXCL5 Ab (PeproTech) for 1 h, followed by HRP-conjugated streptavidin (Zymed) or HRP-conjugated secondary Abs to goat IgG (American Qualex) or rabbit IgG (American Qualex). After the plates were washed with PBS containing 0.1% BSA and 0.05% Tween 20, o-phenylenediamine solution containing H₂O₂ was added, and the OD₄₅₄ was read in a microtiter plate reader. In some experiments, ELISA plates were coated with CCL21, CCL19, CXCL13, CXCL12, or CCL5 (400 nM) overnight at 4°C and blocked with 3% BSA-PBS. The plates were incubated with or without Col IV (10 μg/ml) at room temperature for 1 h, followed by rabbit anti-Col IV Ab (Chemicon). For competitive binding-inhibition studies, ELISA plates coated with Col IV (20 μg/ml) were incubated with CXCL13 or CXCL12 (50 nM) in the presence or absence of various concentrations of CCL21 or CCL5 at room temperature for 1 h. The nonspecific signals in each experiment were subtracted from the specific binding signals, and the data are represented as the mean ± SD of triplicate determinations.

Surface plasmon resonance assay

All surface plasmon resonance assays were performed using a BIAcore 2000 instrument (BIACORE). ECM proteins were covalently immobilized on the CM-5 sensor chip by the amine-coupling method, according to the manufacturer’s instructions. Briefly, the CM-5 chip was activated with a 1:1 mixture of 75 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 11.5 mg/ml N-hydroxysuccinimide for 7 min. FN (10 μg/ml in 10 mM sodium citrate buffer, pH 4.0), Col IV (20 μg/ml in 10 mM sodium citrate buffer, pH 4.0), or LN-1 (20 μg/ml in 10 mM sodium citrate buffer, pH 4.0) was injected over the CM-5 sensor chip. The remaining active groups on the matrix were blocked with 1 M ethanolamine-HCl, pH 8.5. The amounts of FN, Col IV, and LN-1 immobilized on the sensor chip were 6400, 6700, and 6200 resonance units (RU), respectively. All subsequent binding experiments were performed at 25°C in 10 mM HEPES buffer, pH 7.4, containing 0.15 M NaCl. Various concentrations of chemokines were injected at a flow rate of 50 μl/min for 1 min, and then the sensor chip was regenerated for 1 min using 1 M NaCl. After subtraction of the blank cell from each response value, the kinetic parameters (kₐ, association rate constant; kₐd, dissociation rate constant; Kᵦ, equilibrium dissociation constant) were determined by collectively fitting the overlaid sensograms locally using the BIAscan software to the 1:1 Langmuir binding model with mass transfer. In some experiments, chemokines (100 nM) were injected and the maximum binding during the association phase was scored.

Transwell migration assay

All migration assays were performed in 24-well plate inserts with 5-μm pore size polycarbonate membranes (Costar) as described previously (20). In brief, to coat the bottom side of the transwell insert membranes, the inserts were placed in the wells of 24-well plates that each contained a 75-μl drop of Col IV or BSA (20 μg/ml in PBS). After an overnight incubation at 4°C, the inserts were incubated with a chemokine as indicated or PBS (60 μl) at room temperature for 1 h. To remove unbound chemokines, the transwell membranes were washed extensively with PBS. Plastic-nonadherent spleen cells (5 × 10⁵ cells/100 μl) resuspended in the assay medium (0.5% BSA-RPMI 1640) were added to the upper chamber, and assay chamber medium (600 μl) was added to the lower chamber. The number of migrated cells was counted by a FACScan (BD Biosciences) using a predetermined number of 0.6-μm-diameter beads (Polyscience) as an internal standard.

Immunofluorescence analysis

Frozen sections (8 μm) of mouse LNs were fixed in cold acetone for 10 min. For two-color immunohistochemical analyses of chemokines and Col IV, the following combinations of anti-chemokine and anti-Col IV Abs were used: 1) rabbit anti-CCL21 Ab (PeproTech) and goat anti-Col IV Ab (Chemicon); 2) goat anti-CXCL12 Ab (Santa Cruz Biotechnologies) or goat anti-CXCL13 Ab (Santa Cruz Biotechnologies) and rabbit anti-Col IV Ab (Chemicon). Next, the tissue sections were incubated with either a mixture of biotin-conjugated donkey anti-rabbit IgG (Chemicon) and Alexa 488-conjugated chicken anti-goat IgG (Molecular Probes), or biotin-conjugated donkey anti-goat IgG (Chemicon) and Alexa 488-conjugated chicken anti-rabbit IgG (Molecular Probes), followed by incubation with alkaline phosphatase-conjugated avidin-biotin complex reagent (Vector Laboratories). Labeling by the enzyme conjugates was developed with Vector Red (Vector Laboratories). In some experiments, frozen sections (5 μm) of mouse LNs were treated with collagenase D (Roche; 500 μU/ml) without fixation in the presence of a protease inhibitor mixture (Roche). After blocking, the sections were incubated with rabbit anti-CCL21 Ab (PeproTech) and rat anti-ER-TR7 Ab (BMA), followed by incubation with biotin-conjugated donkey anti-rabbit IgG and fluorescein-conjugated goat anti-rat IgG (Cappel). As a negative control, collagenase added with EDTA (10 μM) was used.

s.c. chemokine injection

Mice were anesthetized by the i.p. injection of 100 mg/kg ketamine (10 mg/ml in saline). From 10 to 20 μl of CCL21 solution (250 μg/ml) were injected into the right hind footpad, and PBS was injected into the left hind footpad. After 90 min, the mice were sacrificed, and the popliteal LNs were removed (5).

Statistical analysis

The data were analyzed using Student’s t test.

Results

Col IV preferentially binds lymphoid chemokines such as CCL21, CXCL13, and CXCL12

The endothelial cells of HEVs are surrounded by a well-developed basal lamina, across which large-scale lymphocyte trafficking takes place continuously. Although certain lymphoid chemokines are abundant on the basal lamina of HEVs, the molecular mechanisms underlying their selective retention are largely unknown. We previously showed that a basal lamina-associated protein, mac25/AGM, binds and presents certain chemokines, including CCL21 and CXCL10, to lymphocytes (13). This observation prompted us to investigate whether other HEV ECM components can also bind chemokines. To investigate this issue, we immobilized FN, Col IV, LN-1, or BSA on plastic supports and examined the binding of lymphoid (CCL21, CCL19, CXCL13, or CXCL12)

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or inflammatory (CCL5) chemokines using chemokine-specific Abs. As shown in Fig. 1A, Col IV appeared to bind lymphoid chemokines CCL21, CCL19, CCL5, CXCL13, or CXCL12 (50 nM) at room temperature for 1 h. Chemokine binding was detected with specific Abs. *, p < 0.001, and **, p < 0.05, vs BSA. B, CCL21, CCL19, CCL5, CXCL13, or CXCL12 (400 nM) were immobilized on a 96-well plate at 4°C overnight. After being blocked with BSA, the wells were incubated with Col IV (10 μg/ml) at room temperature for 1 h. The bound Col IV was detected with a specific Ab to Col IV. The nonspecific signals in each experiment were subtracted from specific binding signals, and data represent the mean ± SD of triplicate determinations. *, p < 0.001, and **, p < 0.05, vs BSA.

**FIGURE 1.** Interactions of various ECM proteins with chemokines. A. ECM proteins (20 μg/ml) were immobilized on a 96-well plate at 4°C overnight. The wells were blocked with BSA and incubated with CCL21, CCL19, CCL5, CXCL13, or CXCL12 (50 nM) at room temperature for 1 h. Chemokine binding was detected with specific Abs. *, p < 0.001, and **, p < 0.05, vs BSA. B. CCL21, CCL19, CCL5, CXCL13, or CXCL12 were perfused onto a Col IV-immobilized sensor chip at a flow rate of 50 μl/min at 25°C for 1 min, and the binding constants during the association and dissociation phases were determined.

or inflammatory (CCL5) chemokines using chemokine-specific Abs. As shown in Fig. 1A, Col IV appeared to bind lymphoid chemokines CCL21, CCL19, and CXCL12 more potently than did FN or LN-1. Col IV also bound CCL19, but only weakly. Col IV, FN, and LN-1 bound little or no CCL5 (Fig. 1A) (13). BSA bound CXCL13 at low levels but failed to bind CCL21, CXCL12, or CCL5. Although heparan sulfate has been reported to bind lymphoid chemokines avidly (16, 21), pretreatment of immobilized Col IV with heparitinase (22) did not affect the chemokine binding significantly (data not shown); in agreement with this observation, we found the Col IV to be little decorated with GAGs as described in **Materials and Methods.** These results indicate that a distinct ECM component of the HEV basal lamina, Col IV, binds certain lymphoid chemokines better than FN or LN-1 does.

Essentially the same results were obtained in a reverse type of binding assay in which we examined the binding of Col IV to immobilized chemokines (Fig. 1B). In this analysis, Col IV bound CCL21 and CXCL13 strongly, CXCL12 moderately, and CCL19 weakly, and this result was subsequently confirmed by the surface plasmon resonance assay, as shown below. These results collectively indicate that Col IV has a unique chemokine-binding profile with a clear preference for CCL21, CXCL13, and CXCL12, which promote lymphocyte trafficking across HEVs.

**FIGURE 2.** Analysis of molecular interactions between Col IV and lymphoid chemokines by surface plasmon resonance measurement. A. Chemokines (100 nM) were injected at a flow rate of 50 μl/min at 25°C for 1 min and allowed to interact with immobilized ECM proteins in the BIAcore system. Bars represent the maximum RU values of the association phase. B. Various concentrations of CCL21, CCL19, CXCL13, or CXCL12 were perfused onto a Col IV-immobilized sensor chip at a flow rate of 50 μl/min at 25°C for 1 min, and the binding constants during the association and dissociation phases were determined.

**Col IV binds CCL21, CCL19, CXCL13, and CXCL12 with different affinities**

We next performed a surface plasmon resonance assay using the BIAcore system. We immobilized approximately equal amounts of FN, Col IV, or LN-1 onto the CM-5 sensor chip (measured in RU), and injected 100 nM chemokines over the chip, to test their binding. Binding was scored as the maximum binding during the association phase. The results were consistent with those obtained in the chemokine-binding assays described above: immobilized FN, Col IV, and LN-1 bound CCL21, CCL19, CXCL13, and CXCL12 at variable levels; and Col IV bound CCL21, CXCL13, and CXCL12 more potently than did FN or LN-1 (Fig. 2A). Next, to determine the $k_{on}$, $k_{off}$, and $K_D$ for chemokine binding to Col IV, we used varying concentrations of CCL21, CCL19, CXCL13, and CXCL12 for perfusion and analyzed the kinetics by fitting the overlaid sensorgrams to the 1:1 Langmuir binding model with mass transfer (Fig. 2B). As summarized in Table I, Col IV bound CCL21 and CXCL13 with high affinity ($K_D = 4.59$ nM and 5.86 nM, respectively). Col IV also bound CXCL12 with a modest affinity ($K_D = 43.1$ nM). The binding kinetics showed that Col IV bound CXCL12 with a similar association rate and a higher dissociation rate than for
CCL21 and CXCL13. Col IV bound CCL19 with a low $K_D$ (421 nM), which was consistent with the results obtained in the chemokine-binding assay described above. In the subsequent experiments, we used CCL21, CXCL13, and CXCL12 to further characterize the chemokine binding to Col IV.

**CCL21 binding to Col IV requires the C-terminal region of CCL21**

We next examined some characteristics of the chemokine binding to Col IV. Competitive binding studies showed that the addition of CCL21 inhibited the binding of CXCL13 and CXCL12 to Col IV moderately, whereas the addition of CCL5 did not inhibit their binding at all (Fig. 3A), suggesting that the CCL21-, CXCL13-, and CXCL12-binding regions of Col IV overlapped at least partially or were geographically related to each other. To gain further insight into the mode of chemokine binding to Col IV, we next examined the binding of C-terminally truncated CCL21 (CCL21-T) to Col IV; CCL21-T lacks its C-terminal tail, which contains clusters of basic amino acid residues that are implicated in the binding of CCL21 to certain GAGs (16, 23). As expected, CCL21-T failed to bind to immobilized Col IV, whereas intact CCL21 bound to it strongly, as assessed by a modified ELISA-type binding assay (Fig. 3B) and the BIAcore assay (Fig. 3C). These observations suggest that the extended C terminus of CCL21 is critically important for its binding to Col IV.

**Col IV can bind certain chemokines and present them to lymphocytes to promote lymphocyte migration**

We next investigated whether Col IV binding affected the chemotactic activities of chemokines in the transwell cell migration assay. To this end, we coated the lower side of transwell membranes with Col IV and then added intact CCL21 or C-terminally truncated CCL21-T to the Col IV-coated side of the filter. Unbound chemokine was removed by extensive washing, and lymphocyte migration across the filter was measured. As shown in Fig. 4A, intact CCL21 added to the Col IV-coated filters induced lymphocyte migration in a dose-dependent manner, which is consistent with the idea that ECM-bound chemokines are functional in inducing chemotaxis and that Col IV is an efficient chemokine-binding substrate. In contrast, CCL21-T, which showed poor binding to Col IV, was also quite poor at inducing chemotaxis under these conditions (Fig. 4A, left), although intact CCL21 and CCL21-T in the soluble phase were equally potent in inducing lymphocyte chemotaxis in a conventional transwell filter assay (Fig. 4A, right). In addition, as shown in Fig. 4B, CCL1, CXCL13, and CXCL12 added to Col IV-coated filters induced lymphocyte chemotaxis 2 to 3 times more efficiently than they did when added to BSA-coated filters.

Table I. Kinetics of the interaction of Col IV with chemokines

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>$k_{on}$ ($\times 10^6$ M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ ($\times 10^{-2}$ s$^{-1}$)</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL21</td>
<td>4.31 ± 1.34</td>
<td>3.57 ± 1.81</td>
<td>4.59 ± 3.02</td>
</tr>
<tr>
<td>CXCL13</td>
<td>5.46 ± 2.47</td>
<td>2.83 ± 0.43</td>
<td>5.86 ± 2.21</td>
</tr>
<tr>
<td>CXCL12</td>
<td>4.81 ± 2.81</td>
<td>17.8 ± 2.49</td>
<td>43.1 ± 16.2</td>
</tr>
<tr>
<td>CCL19</td>
<td>0.12 ± 0.04</td>
<td>5.39 ± 3.67</td>
<td>421 ± 151</td>
</tr>
</tbody>
</table>

* The apparent $k_{on}$, $k_{off}$, and $K_D$ values for the interaction of Col IV with lymphoid chemokines were determined using the 1:1 Langmuir binding model with mass transfer available in the BIAevaluation 3.0 software in the BIAcore system.

**FIGURE 3.** Binding of wild-type chemokines and a CCL-21 mutant lacking its C-terminal region to Col IV. A. The binding of CXCL12 or CXCL13 (50 nM) to Col IV was examined in the presence or absence of various concentrations of competitor chemokines (CCL21 or CCL5). Chemokine binding was determined using Abs specific for CXCL12 or CXCL13. *, $p < 0.001$. B. Col IV (20 μg/ml) was immobilized on a 96-well plate. The wells were blocked with BSA and incubated with CCL21 or CCL21-T (50 nM) at room temperature for 1 h. The chemokine binding was detected with the anti-CCL21 Ab. C, Col IV was immobilized on a CM-5 sensor chip by an amine-coupling method. CCL21 or CCL21-T (300 nM) was injected over the chip at a flow rate of 50 μl/min at 25°C for 1 min.

**FIGURE 4.** Lymphocyte migration toward chemokines immobilized on Col IV. A. Transwell membranes were coated with Col IV and then incubated with various concentrations of CCL21 or CCL21-T at room temperature for 1 h. After extensive washing, lymphocyte migration was tested (left). *, $p < 0.05$. CCL21 or CCL21-T (100 nM) was added to the lower chamber of transwell plates, and their chemotactic effects were examined (right). B, transwell membranes were coated with Col IV or BSA (20 μg/ml) and then incubated with CCL21 (100 nM), CXCL13 (300 nM), or CXCL12 (100 nM) for 1 h at room temperature. After extensive washing, the chemotactic effects of the immobilized chemokines were examined. Relative cell migration was calculated as the ratio of the number of migrated cells induced by the chemokines added to the Col IV-coated filter to the number induced by the chemokines added to the BSA-coated filter. Values are the mean ± SD of triplicate determinations.
These observations collectively indicate that Col IV can retain lymphoid chemokines, including CCL21, CXCL13, and CXCL12, and serve as a chemokine-presenting substrate to lymphocytes, promoting their chemokine-dependent migration.

CCL21, CXCL13, and CXCL12 colocalize with Col IV in the abluminal aspect of HEVs under physiological conditions and exogenously administered CCL21 accumulates in this area.

HEVs express multiple lymphoid chemokines, including CCL21, CXCL13, and CXCL12 (2, 6, 7, 13). To investigate the possibility that Col IV is involved in retaining such chemokines on the basal lamina of HEVs in situ, we performed a two-color immunohistochemical analysis of mouse LNs using specific Abs to chemokines and Col IV, respectively. As shown in Fig. 5A, CCL21, CXCL13, and CXCL12 were all detected on the luminal aspect of HEVs, as reported previously (6, 7, 24). These chemokines were also detected on the abluminal aspect of the HEVs and on the reticular structures surrounding the HEVs, where Col IV was also abundant, and the chemokine signal was stronger on the abluminal than on the luminal side of the HEVs. Merged pictures of sections stained with anti-chemokine and anti-Col IV Abs showed that the lymphoid chemokines colocalized with Col IV in the HEV basal lamina and the surrounding reticular structures.

To examine further the possibility that Col IV promotes chemokine binding in vivo, we used plt/plt mice, which show defective expression of CCL21 and CCL19 in HEVs (15) but have an intact basal lamina. CCL21 injected into the footpad of these mice was transported to the HEVs of the draining LNs and accumulated prominently in the abluminal aspect of HEVs and the surrounding reticular structures in the cortex. Col IV staining verified that the injected chemokine was indeed localized to the regions where Col IV was strongly expressed, i.e., the basal lamina of HEVs and the surrounding reticular structures (Fig. 5B). When the frozen sections were treated with collagenase, CCL21 signal was strongly reduced, whereas ER-TR7 staining was unaffected (Fig. 5C); mAb ER-TR7 binds to the basal lamina as well as stromal reticular cells in the lymph node cortex and does not recognize collagens (25). Induction of a loss of enzyme activity by chelation of divalent cations with EDTA abrogated the above-mentioned effect of collagenase, further strengthening the specificity of the observation.

These observations are consistent with the hypothesis that Col IV binds certain lymphoid chemokines in the HEV basal lamina and reticular fibers and presents them to the lymphocytes transmigrating across the HEVs.

Discussion

Col IV is the most abundant constituent of the basal lamina of a variety of tissues (26). Within the LNs, Col IV is largely restricted to the basal lamina of blood vessels and reticular cell networks (14). In particular, Col IV is abundant in the well-developed, thick basal lamina of HEVs, which represents not a single sheet of a uniform thickness but multiple reticular layers that are irregularly organized into regions of loosely associated sheets and thickened masses of tightly associated layers (27). Given that the Col IV-rich HEV basal lamina is in direct contact with the endothelial cells and also with extravasating lymphocytes (28), it is in a strategically suitable location to provide directional cues to the lymphocytes migrating from the HEVs into the parenchymal lymphoid compartment.

In this study, we demonstrated that Col IV binds certain lymphoid chemokines, including CCL21, CXCL12, and CXCL13, that play a critical role in the homeostatic trafficking of lymphocytes across HEVs. We also showed that the Col IV-bound chemokines are functional, because they induced lymphocyte migration efficiently in vitro. The following two pieces of in vivo evidence support the idea that chemokine binding to Col IV is physiologically relevant: 1) CCL21, CXCL12, and CXCL13 colocalized with...
Col IV in the basal lamina of HEVs under physiological conditions; and (2) CCL21 injected s.c. was transported to the HEV regions of draining LNs and prominently colocalized with Col IV on the HEV basal lamina. Although other ECM HEV basal lamina proteins such as FN and LN-1 also bind lymphoid chemokines, our analysis showed clearly that Col IV is a much better binding substrate. These results collectively implicate the unique chemokine-binding ability of Col IV in limiting the diffusion of locally produced lymphoid chemokines and maintaining them in a functionally active state in the vicinity of the HEVs. The requirement for some such supportive environment for chemokine functions in vivo has also been suggested by Lira’s group, who showed that overexpression of CCL21 induces lymphocyte accumulation only in certain tissues (29). The degree of contribution of Col IV to such microenvironment requires further investigation.

Although a variety of chemokines can be produced outside of the vasculature by different parenchymal cells, it is interesting that Col IV preferentially binds CCL21, CXCL13, and CXC12, but not CCL5. Although we do not yet know the precise mechanisms behind selective chemokine binding by Col IV, our competitive binding experiments indicated that the CCL21-, CXCL12-, and CXCL13-binding regions of Col IV overlap at least partially or are geographically related to each other. Electrostatic interactions also appear to play a role in the binding to Col IV by CCL21, because CCL21-T, which lacks clusters of basic amino acids in the C terminus, showed defective binding to Col IV. However, CXCL12 and CXCL13 do not have such basic amino acid clusters in their C-terminal region, indicating that their mode of binding to Col IV might be different from that of CCL21. In addition, the fact that CCL5 has two BBXB motifs that bind to GAGs (30) but fail to bind Col IV indicates that electrostatic interactions alone are not sufficient for chemokines to bind Col IV. Given that Col IV can interact with a variety of structurally different proteins, including ECM components (31, 32), growth factors (33, 34), proteinases (35, 36), and bacterial proteins (37, 38), multiple mechanisms are probably used to bind these molecules. Detailed structural analyses will be needed to understand the precise mechanisms underlying the selective binding by Col IV of lymphoid chemokines.

Our transwell cell migration studies clearly showed that Col IV binds chemokines in their active form and promotes lymphocyte migration by presenting these chemokines. We previously reported that various chemokine-binding molecules with distinct chemokine-binding specificities can positively or negatively regulate the biological activities of the chemokines they bind (2, 12, 13). For instance, macrophage migration inhibitory factor (MIF) supports chemokine activities (13), whereas DARC down-regulates them (12); certain GAGs, such as heparan sulfate, potentiate the activity of bound chemokines (21, 39), whereas oversulfated chondroitin sulfates appear to inhibit their activities (16, 23). Selective immobilization of the lymphoid chemokines at the HEV basal lamina may thus provide a regulatory step that determines their function in this specific region as well as a mechanism that imparts directionality to lymphocytes migrating from the inside to the outside of the HEVs.

The function of Col IV may be more complex than just chemokine binding and presentation, because it also serves as an integrin-binding cell-adhesive matrix (26). Therefore, by binding certain lymphoid chemokines, Col IV is likely to provide positional information to migrating lymphocytes by signaling through the cell surface integrins of the lymphocytes as well as their chemokine receptors. Other ECM components, FN and LN-1, that also bind chemokines and serve as integrin ligands may have a similar function. FN is abundant in the HEV basal lamina (14, 40); it binds CXCL12 with a low nanomolar $K_D$ and presents the bound CXCL12 to lymphocytes to induce directed cell migration (20). Our analyses confirmed CXCL12 binding to FN and extended that finding to show that the binding of the lymphoid chemokines to Col IV is more prominent, and that to LN-1 and FN is relatively low. Because these ECM proteins all localize to the HEV basal lamina (14, 40), they may play, more or less, a common role, acting in an overlapping manner to control lymphocyte navigation locally.

Our study also showed that Col IV colocalized with CCL21, CXCL13, and CXCL12 in the reticular cell networks surrounding HEVs. Recent studies by Bajenoff et al. (41, 42) and Katakai et al. (40) indicate that the fibroblastic reticular cell (FRC) network in the LN paracortex serves as a guidance structure for the migration of T and B cells within LNs. Collagens are an important constituent of the FRC network (43) but have been reported to be wrapped by reticular cells, with no more than 10% accessible to migrating lymphocytes (44). However, this finding mostly appears to be the case with collagen type III, a principal component of collagen fibers in the FRC network (45), because Col IV is mainly located on the surface of the network, where it is likely to be exposed to migrating lymphocytes (45). In addition, the association of the reticular fibers and the reticular cells appears to be flexible (44), and direct contacts between the reticular fibers and migrating lymphocytes can be occasionally observed in the LN paracortex (44). Col IV is therefore likely to serve, together with as yet unidentified molecules, as an adhesion substrate on the FRC network that also presents lymphoid chemokines and thus act as a guiding molecule for migrating lymphocytes. On the abluminal aspect of HEVs, haptotaxis (i.e., movement up a chemokine gradient immobilized on a substrate) rather than chemotaxis (i.e., movement up a concentration gradient of soluble-phase chemokine) may be the most important process for migrating lymphocytes, which may recognize chemokines deposited on ECM substrates such as Col IV. Future investigation will shed light on these important regulatory mechanisms for lymphocyte migration.

In conclusion, our study demonstrates that Col IV preferentially binds CCL21, CXCL13, and CXCL12, and colocalizes with these chemokines on the HEV basal lamina and in the reticular cell networks surrounding the HEVs. Exogenous administration of CCL21 to CCL21-deficient plt plt mice restored the chemokine accumulation in the HEV basal lamina. The lymphoid chemokines retained their abilities to induce cell migration after binding to Col IV in vitro, highlighting the possibility that Col IV helps create a lymphoid chemokine-rich microenvironment that promotes rapid and directional lymphocyte trafficking in the HEV regions. Thus, the HEV basal lamina appears to act as a guidance structure for migrating lymphocytes within LNs and PPs that promotes information transfer between migrating lymphocytes and the surrounding stroma, like the paracortical FRC network (40–42). Further investigation into the biological significance of these findings is now warranted, which should lead to a more precise understanding of how lymphocytes determine their positioning in secondary lymphoid tissues in both physiological and pathological settings.

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Disclosures

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References


