Demonstration of functional role of TECK/CCL25 in T lymphocyte-endothelium interaction in inflamed and uninflamed intestinal mucosa

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GASTROINTESTINAL MUCOSA PRESENTS highly specialized challenges to the immune system because it is constantly exposed to potential antigens and pathogens. The thymus-expressed chemokine (TECK/CCL25), which had initially been reported to be produced by thymic cells (25), is highly expressed at the message level in the small intestine (25, 30, 32) and has recently been localized to the crypt epithelium of the jejunum and ileum (12). TECK/CCL25 is also expressed by endothelial cells and a subset of cells in the intestinal lamina propria of the human small intestine (18). Interestingly, the only known receptor for TECK/CCL25, CCR9, is expressed by discrete subsets of circulating memory CD4+ and CD8+ lymphocytes expressing the intestinal homing receptor α4β7 but not by other systemic memory lymphocyte subsets (32), suggesting that CCR9 may be a receptor used preferentially by lymphocytes involved in gut immunity.

TECK expression is restricted to the small intestine. Immunohistochemistry reveals that, in humans, anti-TECK/CCL25 reactivity characterizes the small intestinal mucosal epithelium but not the other epithelia of the digestive tract (stomach or colon) (12, 17, 18). It has been reported that CCR9 expression in freshly isolated small intestinal lamina propria mononuclear cells is significantly higher than in colonic counterparts or peripheral blood lymphocytes (18). It has been also shown in vitro that TECK/CCL25 is selectively chemotactic for lamina propria mononuclear cells of the small intestine but not for those of the colon (18). The same group of researchers has recently demonstrated that in patients with Crohn’s disease TECK/CCL25 expression is altered in inflamed small intestine but not in inflamed colon, being intensely expressed in patchy distribution in crypt epithelial cells in proximity to lymphocytic infiltrates (17). These results imply that the selective expression of TECK/CCL25 in the small intestine is evidence for distinctive mechanisms of lymphocyte recruitment in different segments of the gastrointestinal tract; that is, regionally different mechanisms underlie the homing of CCR9+ intestinal memory T cells to the small intestine rather than the colon. This regional specialization may represent an important mechanism for segregating the immune responses of the small intestine from those of the colon in healthy and diseased conditions (12, 18), but there has been little direct evidence that the TECK/CCL25-CCR9 pathway is actually involved in organ-specific homing of lymphocytes to the intestinal mucosa in vivo.

CCR9 is expressed not only in lamina propria lymphocytes (LPLs) but also in most small intestinal intraepithelial lymphocytes (IELs) (22, 32). The production of TECK/CCL25 in the epithelium lining the small intestine suggests that TECK may act as a mediator of intercellular communication between the epithelium and IELs (13, 22, 30). It was recently shown that the intraepithelial T cell-to-epithelial cell ratio is decreased in the small intestine of CCR9-deficient mice, an observation that can be accounted for by a marked diminution of the T cell receptor γδ+ compartment (29). These results suggest that the TECK/CCL25-CCR9 pathway is essential for the appearance of IELs in small intestinal mucosa. Although there is little information about the exact route and mechanisms by which IELs reach the intestinal epithelium, the TECK/CCL25-CCR9...
pathway may play some important roles in the development and trafficking of IELs (11, 24).

In the work reported here, we used the intravital microscopic procedure for monitoring the dynamic process of lymphocyte migration (1) to examine how TECK/CCL25 and its receptor CCR9 are involved in T-lymphocyte (LPLs and IELs)-endothelial cell adhesion in small intestinal and colonic mucosa and 2) to determine whether the contribution of the TECK/CCL25-CCR9 system in T-lymphocyte recruitment to the inflamed mucosa differs from that of normal mucosa after both sites are challenged with TNF-α.

MATERIALS AND METHODS

Separation of LPLs and IELs from mouse intestine. Female BALB/c mice at 8 wk of age were used. They were maintained on standard laboratory chow diet. The care and use of laboratory animals was in accordance with the guidelines of Keio University, Animal Research Committee. LPLs were isolated from mice by the modified procedures as described by Davies and Parrot (5). Briefly, inverted small intestine was cut into four segments and all Peyer’s patches were removed. These segments were transferred to a 50-ml conical tube tube of 54 ml of 5% fetal calf serum in Ca2+/Mg2+-free HBSS (GIBCO, Grand Island, NY) and shaken in 150 rpm in the horizontal position in an orbital shaker for 45 min at 37°C. Cell suspensions were removed, and the remaining fragments were then transferred to flasks containing HBSS with 90 U/ml of collagenase type 1 (Sigma, St. Louis, MO) and stirred gently for 25 min in 37°C. Cell suspensions containing LPLs were filtered through nylon mesh and then centrifuged. LPLs were purified by using a 44–70% discontinuous Percoll (Pharmacia Biotech, Uppsala, Sweden) gradient. After centrifugation at 600 g for 20 min at 20°C, cells at the interface were collected, washed, and resuspended in RPMI 1640 medium (pH 7.4; GIBCO) with 5% fetal calf serum on ice until used.

IELs were isolated by using procedures slightly modified from those described previously (9). Briefly, inverted intestine was cut into four segments that were transferred to a 50-ml conical tube containing 45 ml of 5% fetal calf serum in Ca2+/Mg2+-free HBSS. After the tube was shaken in the horizontal position in an orbital shaker for 45 min at 37°C, cell suspensions were collected and passed through a glass wool column to remove cell debris and adherent cells. Cells were then resuspended in 30% (wt/vol) Percoll and centrifuged for 20 min at 1,800 rpm. After this centrifugation, the cells at the bottom of the resulting tube containing HBSS with 90 U/ml of collagenase type 1 (Sigma, St. Louis, MO) and stirred gently for 25 min in 37°C. Cell suspensions containing LPLs were filtered through nylon mesh and then centrifuged. LPLs were purified by using a 44–70% discontinuous Percoll (Pharmacia Biotech, Uppsala, Sweden) gradient. After centrifugation at 600 g for 20 min at 20°C, cells at the interface were collected, washed, and resuspended in RPMI 1640 medium (pH 7.4; GIBCO) with 5% fetal calf serum on ice until used.

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Flow cytometric analysis on cell surface molecules. Expression of various markers (CD3, CD4, CD8, and adhesion molecules (α4β1, α5β1, and β2-integrin, L-selectin, and CD11a) on the surface of LPLs and IELs was determined by flow cytometric analysis by using FACSort (Becton Dickinson, Mountain View, CA). Dead cells were excluded from analysis on the basis of performed iodide dye exclusion. The purity of CD3-positive cells was shown to be at least 95% in both LPLs and IELs. As we previously reported (7), LPLs consisted of 54% of CD4 cells and 34% of CD8 cells. There was a slight expression of α4β1-integrin among LPLs and IELs. There was a strong surface expression of α5β1 and β2-integrin in CD11a and CD11b, but L-selectin was only slightly expressed. IELs used in the migration studies comprised four subpopulations (65% CD4-negative/CD8-positive cells, 8% CD4-positive/CD8-negative cells, 22% double-negative cells, and 5% double-positive cells) (11). The IELs always showed a strong expression of α4β1 and β2-integrin molecules on their surface. There was also an expression of CD11a and a weak expression of α5β1-integrin but no expression of L-selectin molecules.

Analysis of CCR9 expression by RT-PCR. To determine whether CCR9 is actually expressed in isolated LPLs and IELs, the RT-PCR method was applied. Total RNA was extracted from LPLs and IELs by using the RNeasy Mini Kit (Qiagen, Tokyo, Japan). RT-PCR was performed using an RNA PCR kit (Takara Biomedical, Inc., Tokyo, Japan) version 2.1 (Takara Biomedicals, Osaka, Japan), 1 μg RNA, and 0.2 μM primers, according to the manufacturer’s instructions. Total RNA was converted to cDNA by oligo(dT) adapter primer (Takara Biomedical). Reverse transcription mixture was subjected to PCR with 0.2 μM specific primer in a reaction containing 0.2 mM 2-deoxy-ribonucleotide 5’-triphosphate, 2.5 units Taq polymerase (Takara Biomedicals), and 2.5 mM MgCl2. Amplification consisted of an initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1.5 min; and a final extension at 72°C for 7 min in the iCycler thermal cycler (Bio-Rad Laboratories, Tokyo, Japan). The primers for β-actin were 5’-ATGTTTGGAGACCTTCAACACC-3’ and 5’-TCTCCAGG CAGGGCAGAGGAT-3’. The primers for CCR9 were 5’-GAAGATGTTTGAGACCTTCAACACC-3’ and 5’-CCCTCAGCAATCTCTGCGCCA-3’. The PCR products were fractionated on 3.0% agarose gel and visualized by ethidium bromide staining.

Intravital analysis of lymphocyte migration in murine intestinal mucosa. Lymphocytes (1 × 107) were incubated in a carboxyfluorescein diacetate succinimidyl ester solution (CFDSE; Molecular Probes, Eugene, OR) (20 μl of 15.6 mM stock solution was diluted with 20 ml of RPMI 1640 medium) for 30 min at 37°C. The labeled lymphocytes were immediately centrifuged through a cushion of heat-inactivated fetal bovine serum and washed twice with a cold suspension medium. The cells were resuspended in 0.2 ml of the medium and used within 30 min.

After an intraperitoneal injection of pentobarbital sodium (50 mg/kg), the abdomen was opened via a midline incision. For the observation of small intestinal mucosa, a 5-cm ileal segment was gently placed onto the plate. A longitudinal incision was made with microcautery along its antimesenteric border. The intestine was kept warm and moist by continuous superfusion with physiological saline warmed to 37°C. The adjacent intestinal segment and mesentry were covered with absorbent cotton soaked with Kreb-Ringer solution. Suitable area of the microcirculation in small intestinal mucosa was observed from the mucosal surface with an inverted-type fluorescence microscope (Diaphot TMD-2S; Nikon, Tokyo, Japan) and was recorded on a videotape. Behavior of fluorescently labeled lymphocytes was visualized on the television monitor through the fluorescence microscope equipped with a silicon intensified target image tube camera with a contrast-enhancing unit (model C-2400-08; Hamamatsu Photonics, Shizuoka, Japan) according to the method described previously (7, 11, 16). Epilumination was achieved with filters of excitation at 470–490 nm and emission at 520 nm. Lymphocytes (1 × 107 dissolved in 0.3 ml) were injected into the jugular veins of recipient mice for 3 min. Cell kinetics of lymphocyte after infusion and the interaction with microvascular beds of small intestinal mucosa were monitored and continuously recorded on S-VHS videotapes for the first 20 min and then 40 min with 10-min intervals. The lymphocytes adherent to the same position of villus microvessels without movement for >30 s were defined as sticking lymphocytes.

In another set of experiments, colonic mucosa, a 3-cm colonic segment 1 cm distal to the cecal valve, was chosen for the observation. The microcirculation of colonic mucosa was also observed from the mucosal surface, and lymphocyte migration was observed through a fluorescence microscope as previously described (28). The lymphocytes staying >30 s in mucosal microvessels were defined as sticking lymphocytes.

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Administration of TNF-α and blocking of TECK/CCL25-CCR9 interaction. TNF-α (cat. no. 410-MT; R&D Systems, Minneapolis, MN) was administered (25 μg/kg) intraperitoneally to mice 5 h before intravital observation of lymphocyte migration (8, 28). Lymphocytes exposed to a high concentration of a chemokine become refractory to the subsequent stimulation with the same chemokine, or other agonists through the same receptor, as a described phenomenon of ligand-induced homologous receptor desensitization (2, 4, 31). Desensitization of lymphocytes with TECK/CCL25 was examined as to whether it could inhibit in situ signals that trigger integrin-mediated adhesion in normal and TNF-α-stimulated small intestinal and colonic microvessels. Chemokine mouse TECK/CCL25 (481-TK) was purchased from R&D Systems, was prepared as a 0.5 mg/ml stock solution in saline, and was used immediately or stored in aliquots at −80°C. After being labeled with CFDSE, T lymphocytes (LPLs and IELs; 1.5 × 10^7 cells/ml) in medium were desensitized by incubation for 45 min with 1 μM TECK/CCL25 (agonist for CCR9) before infusion. Meanwhile, control cells were incubated with saline for 45 min. After being washed in serum, the cells were resuspended at 0.2 ml in RPMI 1640 medium and used immediately. In some experiments, desensitized T lymphocytes (1 × 10^7) were labeled with tetramethylrhodamine-5-(and-6)-isothiocyanate (Molecular Probes) and mixed with an equal number of nontreated cells labeled with CFSE. They were simultaneously exposed to Tris-buffered saline (pH 7.4) at 4°C, washed, filtered at 520–550 nm after infusion from the jugular vein (27). For the experiment of in vivo inhibition, mice received anti-TECK/CCL25 antibody (cat. no. 89818; R&D Systems) or rat IgG2b control antibody (cat. no. A95–1; BD Pharmingen, San Diego, CA) intravenously from the jugular vein 25 min (25 μg) before the cell injection and intraperitoneally 1 day (75 μg) and 2 days (50 μg) before (13).

Chemotaxis assay for lymphocytes. To examine whether there is heterologous cross-desensitization between CCR9 and other chemokine receptors, migration of cells (LPLs and IELs) was assessed by using Transwell tissue culture inserts with a 5-μm pore size polycarbonate filter (Kurabo Biomedical, Neyagawa, Japan) as described previously (10, 12) with modifications. In brief, untreated lymphocytes or desensitized lymphocytes with TECK/CCL25 were suspended at 1 × 10^7/ml in RPMI 1640 medium supplemented with 0.5% BSA, and 100-μl aliquots were loaded into the upper inserts. Chemokine or control buffer aliquots of 250 μl, prepared in the same medium, were placed in the lower wells. Chambers were incubated for 2 h, and cell migration was quantified by counting the number of cells through a light microscope. All assays were conducted in triplicate. For the migration assay, the following chemokines were obtained from R&D Systems and were placed in the lower chamber at the concentration (in nm) of 250 TECK/CCL25 (481-TK), 100 MCP-1/CCL2 (479-JE), 100 RANTES/CCL5 (478-MR), 50 SDF-1α/CXCL12 (460-SD), 100 IP-10/CXCL10 (466-CR), 100 MEC/CCL28 (533-VL), 100 6C-cine/CCL1 (457–6C), and 100 MIP-1α/CCL3 (450-MA).

In situ visualization of immunolocalization of TECK/CCL25. Alexa 546 protein labeling kits were used as directed by the manufacturer (Molecular Probes). Monoclonal anti-mouse antibody TECK/CCL25 (89818) was obtained from R&D Systems, and Alexa 546-conjugated monoclonal antibodies to mouse TECK/CCL25 were made in our laboratories. For in situ staining of immunolocalization of TECK/CCL25, 50 μg of antibody was injected via jugular vein in a single bolus under pentobarbital anesthesia (27). Intestinal mucosa was observed from the mucosal surface after opening the intestinal lumen along its anti-mesenteric side. Behavior of fluorescence-labeled lymphocytes (1 × 10^7) was also simultaneously visualized after the infusion on the television monitor with the fluorescence microscopy (488-nm excitation for CFDSE and 543-nm excitation for Alexa 546). Lymphocytes were simultaneously observed through filters of excitation of infused labeled lymphocytes was visualized on the monitor, with a fluorescence microscope (model BX51WI; Olympus) with a contrast-enhancing unit (model M3204C; Olympus). After 20 min and 1 h, animals were killed and the small intestine and colon were immediately removed for further examination with the microscope. There was no significant difference between the mice killed at the two different time points. For the control experiment, isotype-matched nonbinding antibody (cat. no. A95-1; BD Pharmingen), with the similar fluorescence/protein ratio, was administered. Moreover, in some experiments, an excess amount (100 μg) of unlabeled anti-TECK/CCL25 (cat. no. 89818; R&D Systems) was administered from the jugular vein 20 min before to block specific binding of fluorescent anti-TECK/CCL25.

Western blot analysis of TECK/CCL25 expression. Small intestinal and colonic tissues were homogenized in lysis buffer containing 1% Triton X-100 and a mixture of protease inhibitors. The protein content was measured, and equal amounts of protein were separated. Samples were diluted in NuPage sample buffer (Invitrogen, Carlsbad, CA) and heated to 70°C for 10 min. Soluble supernatants were then resolved by SDS-PAGE on a 10% NuPage bis-tris gel (Invitrogen) and electrophoretically transferred onto polyvinylidene difluoride membranes (model IPVH0010; Millipore, Bedford, MA). Membranes were blocked with Block Ace (UK-B25; Dainippon Pharmaceutical, Osaka, Japan) for 1 h at room temperature. The blots were then incubated with the primary antibody anti-mouse TECK/CCL25 and antibody AF-481-NA (R&D Systems) and anti-actin antibody A-3937 (Sigma) overnight at 4°C. Washed in Tris-buffered saline-0.05% Tween 20 (TBST), and further incubated with the secondary antibody linked to alkaline phosphatase. Bound alkaline phosphatase was visualized with an alkaline phosphatase substrate kit (model SK-5300; Vector Laboratories, Burlingame, CA). Developed films were scanned and quantified by using NIH Image software, version 1.60 (National Institutes of Health, Bethesda, MD).

Statistics. All results are expressed as means ± SE. Differences among groups were evaluated by one-way ANOVA and Fisher’s post hoc test. Statistical significance was set at P < 0.05.

RESULTS

Desensitization of CCR9 with TECK/CCL25. The specificity of CCR9 blocking with TECK/CCL25 was examined by using in vitro lymphocyte chemotaxis assay. Figure 1 shows the chemotaxis of control and TECK/CCL25-treated cells toward different kinds of chemokines. Desensitization of lymphocytes with TECK/CCL25 showed a drastic inhibition of chemotactic ability of the cells toward the chemokine in case of either LPLs and IELs. There was no significant difference in chemotaxis ability toward TECK/CCL25 between LPLs and IELs. This remarkable inhibition of chemotaxis by TECK/CCL25 desensitization was shown to continue for at least 60 min (data not shown). As shown in Fig. 1, in either the LPL (A) or IEL (B) population, the chemotactic response toward other chemokines was not different between control cells and cells desensitized with TECK/CCL25, indicating that there was no heterologous cross-desensitization, at least in the case of CCR9 desensitization. When comparing control lymphocytes with lymphocytes whose CCR9 had been desensitized with TECK/CCL25, we found that this desensitization affected neither cell viability nor surface levels of adhesion receptors CD11a, α4- and αE-integrin, and β7-integrin (data not shown).

Effect of inhibition of TECK/CCL25-CCR9 system on LPL and IEL adherence to microvessels in normal small intestinal and colonic mucosa. We monitored the migration of LPLs and IELs to the normal small intestinal and colonic mucosal microvessels by observation from the mucosal side. We then investigated the effect of inhibition of TECK/CCL25-CCR9 system by either desensitization of CCR9 or anti-TECK/
increased. IELs showed an accumulation profile similar to that of LPLs, and the number adhering at 60 min was significantly greater than the number adhering at 20 min (data not shown). Desensitization with the CCR9 ligand TECK/CCL25 inhibited the accumulation of both LPLs and IELs in the small intestinal mucosal microvessels (Figs. 2B and 3). The degree of inhibition by desensitization did not differ significantly between LPLs and IELs, and at 60 min the number of these cells sticking was decreased to 40.2 and 49.4% of the number of control cells sticking, respectively. When the equal number of control cells and desensitized cells were simultaneously infused, dominant accumulation of control cells was confirmed by two-color analysis (Fig. 2C). Administration of anti-TECK/CCL25 to the mice also significantly decreased the LPLs and IELs sticking in small intestinal microvessels, which was comparable with the results of desensitization (Fig. 3). The total flux of TECK/CCL25-treated lymphocytes through small intestinal microvessels was, if anything, slightly increased over that of control lymphocytes, and there was no change in the case of anti-TECK/CCL25 antibody treatment. On the other hand, as shown in Figs. 2D and 3, only a few LPLs and IELs had adhered to the colonic microvessels after infusion under normal conditions. Moreover, it should be noted that desensitization with TECK/CCL25 did not inhibit the accumulation of injected LPLs or IELs in the colonic mucosal microvessels (Figs. 2E and 3). This was further confirmed by the two-color analysis, in that control and desensitized cells were equally found in the colonic mucosal microvessels (Fig. 2F). Similarly, anti-TECK/CCL25 antibody did not produce any significant effect on lymphocytes sticking to the colonic mucosal microvessels (Fig. 3).

**Effect of inhibition of TECK/CCL25-CCR9 system on TNF-α-induced LPL and IEL sticking to microvessels in small intestine and colon.** We then examined the migration of LPLs and IELs to the TNF-α-stimulated small intestinal and colonic mucosal microvessels and investigated the effect of inhibition of TECK/CCL25-CCR9 system to see the role of this system in lymphocytes sticking under inflammatory conditions. The numbers of LPLs (Fig. 4A) and IELs (Fig. 4B) sticking to inflamed small intestinal and colonic mucosa 60 min after infusion are shown for samples in which inflammation had been induced by TNF-α administration. TNF-α treatment substantially increased the number of LPLs sticking to the microvessels of the villus tip in small intestinal mucosa at 60 min. On the other hand, the number of IELs sticking to the small intestinal mucosa increased only slightly after TNF-α treatment. At 10 min, the total number of lymphocytes that had entered small intestinal microvessels did not differ between the TNF-α-treated mice and the control mice (LPLs: control mice, 16.2 ± 3.6/min; TNF-α treated mice, 15.0 ± 2.6/min. IELs: control mice, 12.3 ± 1.1/min; TNF-α treated mice, 13.3 ± 1.7/min). Desensitization with TECK/CCL25 remarkably attenuated the enhanced accumulation of injected lymphocytes (LPLs or IELs) in the small intestinal mucosa of mice after inflammation induced by TNF-α (Fig. 4). It should be noted that the desensitization procedure significantly suppressed the TNF-α-induced lymphocytes sticking to levels similar to those in normal small intestine after CCR9 blocking. There was no significant difference in the degree of inhibition between LPLs and IELs after desensitization, and sticking cells at 60 min were decreased to 28.0 and 35.8%, respectively.
CCL25 was functionally blocked by anti-TECK/CCL25 antibody, we observed the almost similar inhibitory effect to that of CCR9 desensitization on lymphocytes sticking in the small intestine in either LPLs or IELs. The sticking cells at 60 min were decreased to 32.1 and 46.2%, respectively (Fig. 4). TNF-α treatment significantly increased LPLs sticking in the microvessels of the colonic mucosa but not IELs sticking at 60 min. As seen in the uninfamed condition, desensitization with TECK/CCL25 did not significantly attenuate the LPL accumulation induced by TNF-α in the colonic microvessels (Fig. 4). Treatment with anti-TECK/CCL25 antibody did not significantly affect the TNF-α-induced LPL sticking in the colonic mucosa either (Fig. 4). In these experiments, the number of lymphocytes that had entered colonic microvessels at 10 min after infusion did not differ significantly between the different treatment groups.

RT-PCR analysis of CCR9 expression and Western blot analysis of TECK/CCL25 protein expression. To further address the role of TECK/CCL25 in lymphocyte adhesion in small intestinal microvessels, we investigated whether LPLs and IELs express CCR9 and whether TECK/CCL25 protein is expressed in the intestine. The expression of CCR9, a receptor for TECK/CCL25 on LPLs and IELs, was determined at mRNA levels by using RT-PCR, and we could confirm CCR9 mRNA expression in both LPLs and IELs (Fig. 5A).

Western blot analysis of intestinal tissue demonstrated that TECK/CCL25 protein was abundantly expressed in the normal ileum but not in the colon. After TNF-α treatment there was a significant increase in TECK/CCL25 in the ileum, but even under TNF-α stimulation TECK/CCL25 was not detected in the colon (Fig. 5, B and C).

In situ immunovisualization of TECK/CCL25 in normal and TNF-α-stimulated small intestine and colon. Fluorescently labeled anti-mouse TECK/CCL25 monoclonal antibody or control monoclonal antibody was intravenously injected into control and TNF-α-treated mice for intravital staining of TECK/CCL25 at the small intestinal mucosa (Fig. 6). Under normal conditions anti-TECK/CCL25 reacted slightly with lamina propria of the small intestine, but the reacted villi were scattered and not all regions were positively stained (Fig. 6A). In TNF-α-treated animals, on the other hand, strong and diffuse anti-TECK/CCL25 reactivity appeared in almost all of the lamina propria of some intestinal villi, well corresponding to the sites of TECK/CCL25 expression (Fig. 6B). On the other hand,
control monoclonal antibody did not significantly react with the intestinal villi of TNF-α-treated animals (Fig. 6C), and when animals were pretreated with excess amounts of unla-
beled anti-TECK/CCL25, the strong anti-TECK/CCL25 reac-
tivity successfully disappeared (Fig. 6D), suggesting the specific-
ity of intravital staining of TECK/CCL25. Anti-TECK/
CCL25 did not positively react with colonic mucosa in either control or TNF-α-treated animals (data not shown).

**DISCUSSION**

In the present study, we found evidence that the TECK/
CCL25-CCR9 pathway is involved in organ-specific homing of lymphocytes to the intestinal mucosa in vivo. Blocking CCR9

with an excess amount of TECK/CCL25 or blocking antibody against TECK/CCL25 significantly inhibited accumulation of injected LPLs and IELs in the small intestinal mucosa under normal (uninflamed) conditions, suggesting that the TECK/
CCL25-CCR9 system plays an important role in the physiological recruitment of T lymphocytes from the circulating blood to the small intestine. We also found that under normal conditions, TECK/CCL25 is not expressed in the colonic mucosa and that TECK/CCL25 is not physiologically involved in the lymphocyte recruitment to the colonic mucosa. These observations are in accordance with observations reported previously (12, 18, 30) and suggest that CCR9+ intestinal memory T cells home selectively to the small intestine rather than the colon.

In this study, we demonstrated that TECK/CCL25 can trig-
ger intravascular adhesion of intestinal lymphocytes. Venules
expressing MAdCAM-1, a ligand for intestinal homing recep-

Fig. 3. Effect of inhibition of TECK/CCL25-CCR9 system on LPL (A) and IEL (B) adherence to microvessels in the normal small intestine (ileal) and the colon. The number of T lymphocytes (per mm²) sticking to mucosal microves-
sels at 60 min after infusion of lymphocytes was determined in control (TNF-α-untreated) mice. Lymphocytes (1.5 × 10⁷ cells/ml) were desensitized by incubation for 45 min with 1 μM TECK/CCL25 before infusion. Mean-
while, control cells were incubated with saline for 45 min. The effect of anti-TECK/CCL25 antibody treatment is also shown. Anti-TECK/CCL25 antibody 89818 (R&D Systems) or rat IgG2b control antibody was intrave-
nously administered from the jugular vein 25 min (25 μg) before the cell injection and intraperitoneally 1 day (75 μg) and 2 days (50 μg) before. The treatment using control antibody did not significantly affect the number of lymphocytes sticking (data not shown). Values are means ± SE for 6 animals.

Fig. 4. Effect of inhibition of TECK/CCL25-CCR9 system on TNF-α induced LPL (A) and IEL (B) sticking to microvessels in the small intestine and the colon. The number of T lymphocytes (per square millimeter) sticking to mucosal microvessels 60 min after infusion of lymphocytes was determined in TNF-α-treated mice. TNF-α was administered (25 μg/kg) 5 h before intravital observation of lymphocyte migration. The effects of anti-TECK/CCL25 antibody treatment and CCR9 desensitization with TECK are also shown. Values are means ± SE for 6 animals.
isor α4β7, are prominent near the crypt area in the gut wall (3, 7) and may lead to the selective recruitment of a specialized subset of circulating “intestinal homing” T lymphocytes by the sequential action of α4β7, LFA-1, and TECK/CCL25-CCR9 (12, 18). CCR9 could support the integrin activation required

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**Fig. 5.** A: expression of CCR9 mRNA on IELs and LPLs was determined by RT-PCR. Both LPLs and IELs expressed a 251-bp CCR9 mRNA. RT(-) shows a control without reverse transcriptase. B: Western blot analysis of TECK protein expression in colon and small intestine (ileum). TECK expression was determined under control (normal) and TNF-α-treated (inflamed) conditions. A representative picture from 4 experiments with similar results is shown. C: TECK expression was normalized to control actin and expressed as a relative intensity ratio. *P < 0.05 vs. control ileum.

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**Fig. 6.** Simultaneous fluorescence microscopic observation of T lymphocytes (LPLs) and TECK expression in normal (A) and TNF-α-treated small intestinal (ileal) mucosa (B). Monoclonal antibody anti-mouse TECK was conjugated with Alexa 546 and infused into a jugular vein, after which CFDSE-labeled LPLs were infused. Animals were killed 60 min after infusion of anti-mouse TECK monoclonal antibody and CFDSE-labeled lymphocytes, and the small intestinal mucosa was immediately observed through a fluorescence microscope (model BX51WI; Olympus) by using 488-nm excitation for CFDSE and 543-nm excitation for Alexa 546. Under normal conditions, anti-TECK/CCL25 reacted slightly to the lamina propria of small intestine (A). In TNF-α-treated animals, on the other hand, strong and diffuse anti-TECK/CCL25 reactivity appeared, and sticking lymphocytes that had been CFDSE-labeled corresponded well to the sites of TECK/CCL25 expression (B). Control monoclonal antibody did not significantly react to the intestinal villi of TNF-α-treated animals (C), and the strong anti-TECK/CCL25 reactivity disappeared when animals were pretreated with excess amounts of unlabeled anti-TECK/CCL25 (D). In these control experiments (C and D), CFDSE-labeled LPLs were not infused.
for lymphocyte arrest on the lamina propria endothelium. Some chemokines directly direct the migration of leukocytes into and within the extravascular space (26), but this study did not directly confirm that the TECK/CCL25-CCR9 system is also necessary for the subsequent transmigration into the lamina propria of intestine. Differences between desensitized and nondesensitized LPL and IEL accumulation that become significant in the ileum were observed at 60 min but not 20 min after T cell injection (data not shown), suggesting the possibility that CCR9 desensitization not only interfered with intravascular arrest but could also block the emigration of adherent cells. By the in situ visualization of fluorescently labeled anti-TECK monoclonal antibody, we also found that anti-TECK/CCL25 diffusely stained in the lamina propria of villi, suggesting that TECK/CCL25 plays a role in the subsequent migration into the lamina propria. In the small intestine, TECK/CCL25 is mainly produced in the epithelial cells, but another study in humans (18) showed that it is also expressed in some resident lamina proprial stromal cells. The TECK/CCL25 derived from epithelial and stromal cells may diffuse to and be presented by the vascular endothelium (12, 14) to support the intravascular adhesion of intestinal lymphocytes. In this study, we demonstrated that the TECK/CCL25-CCR9 system contributes significantly to the IEL accumulation in the small intestinal mucosa. Our data are in accordance with the observation by Svensson et al. (22) that CCR9 is selectively expressed on murine αEβ7+/naive CD8β+ lymphocytes and is involved in the selective localization of CD8αβ+ lymphocytes to the small intestine after the activation in mesenteric lymph nodes. But in the case of IELs, the exact sequence in which IELs express CCR9-positive IELs should be further investigated.

TNF-α plays a key role in inflammation by triggering chemotaxis and upregulating adhesion molecules (6, 8, 15, 20). TNF-α production in the mucosa has been extensively studied by those interested in inflammatory bowel disease, because anti-TNF-α antibody has recently been shown to ameliorate the symptoms of Crohn’s disease and even to significantly induce a remission of the disease (1, 23). In the present study, we demonstrated that the TECK/CCL25-CCR9 ligand receptor pair plays a significant role in the enhanced lymphocyte recruitment at the small intestinal mucosa even after stimulation with TNF-α. Desensitization of the CCR9 receptor or antibody against TECK/CCL25 reduced the enhanced lymphocyte sticking to levels similar to those seen in normal small intestine, suggesting that the TECK/CCL25-CCR9 system plays a major role under inflamed conditions.

On the other hand, it is interesting to see in our study that in the inflamed colon there was no significant expression of TECK/CCL25 protein and that the TECK/CCL25-CCR9 ligand-receptor pair did not participate in the TNF-α-stimulated T-lymphocyte adhesion to endothelial cells. These results are consistent with the previous reports in which no apparent TECK/CCL25 expression was observed in the inflamed colonic mucosa of humans (17). We speculate that chemokines other than TECK may function in the lymphocyte interaction with microvessels in the inflamed colonic mucosa after the administration of TNF-α.

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