Brief Definitive Report

Lymphocyte CC Chemokine Receptor 9 and Epithelial Thymus-expressed Chemokine (TECK) Expression Distinguish the Small Intestinal Immune Compartment: Epithelial Expression of Tissue-specific Chemokines as an Organizing Principle in Regional Immunity

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Abstract

The immune system has evolved specialized cellular and molecular mechanisms for targeting and regulating immune responses at epithelial surfaces. Here we show that small intestinal intraepithelial lymphocytes and lamina propria lymphocytes migrate to thymus-expressed chemokine (TECK). This attraction is mediated by CC chemokine receptor (CCR)9, a chemoattractant receptor expressed at high levels by essentially all $CD4^+$ and $CD8^+$ T lymphocytes in the small intestine. Only a small subset of lymphocytes in the colon are $CCR9^+$, and lymphocytes from other tissues including tonsils, lung, inflamed liver, normal or inflamed skin, inflamed synovium and synovial fluid, breast milk, and seminal fluid are universally $CCR9^-$. TECK expression is also restricted to the small intestine: immunohistochemistry reveals that intense anti-TECK reactivity characterizes crypt epithelium in the jejunum and ileum, but not in other epithelia of the digestive tract (including stomach and colon), skin, lung, or salivary gland. These results imply a restricted role for lymphocyte CCR9 and its ligand TECK in the small intestine, and provide the first evidence for distinctive mechanisms of lymphocyte recruitment that may permit functional specialization of immune responses in different segments of the gastrointestinal tract. Selective expression of chemokines by differentiated epithelium may represent an important mechanism for targeting and specialization of immune responses.

Key words: leukocyte • gastrointestinal tract • trafficking • epithelium • lamina propria lymphocytes

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Introduction

Recent studies have shown that certain constitutively expressed chemokines play an important role in regulating homeostatic lymphocyte recirculation through secondary lymphoid organs, whereas others may help control tissue-specific targeting of lymphocytes to extralymphoid organs (1, 2). The first examples of chemokines that participate in nonlymphoid tissue–selective lymphocyte targeting have recently been identified: thymus and activation-regulated chemokine (TARC; reference 3) and cutaneous T cell–attracting chemokine (CTACK; reference 4). TARC is constitutively expressed by venular endothelium in the skin and can trigger rapid adhesion of circulating skin-homing memory lymphocytes expressing CC chemokine receptor (CCR)4 (3). CTACK, expressed by skin epidermal keratinocytes, is chemotactic for a similar subset of skin-homing memory lymphocytes (4). Together, these two chemokines and their receptors may regulate memory lymphocyte adhesion to cutaneous vascular endothelium and subsequent entry into and localization within the skin.

Like the skin, mucosal sites such as the gastrointestinal tract are constantly exposed to potential pathogens, and thus present highly specialized challenges to the immune system. Here we have explored the potential for specialized chemokine involvement in lymphocyte localization in the gastrointestinal tract. The chemokine thymus-expressed chemokine (TECK) is highly expressed at the message level in the small intestine (5–7), and has recently been localized by in situ hybridization to the intestinal epithelium in the mouse. Interestingly, CCR9, the only known receptor for TECK, is expressed by discrete subsets of circulating memory $CD4^+$ and $CD8^+$ lymphocytes expressing the intestinal homing receptor $\alpha_4\beta_7$, but not by other systemic memory lymphocyte subsets (5), suggesting that CCR9 may be a receptor used preferentially by lymphocytes involved in aspects of gut immunity. Consistent with this, CCR9 is expressed on lymphocytes isolated from human small intestine (5). Together, these studies suggest a role for TECK and CCR9 in the intestinal immune compartment, but the extent to which this chemokine receptor–ligand pair is specifically associated with the intestinal compartment versus other mucosal and epithelial sites remains unclear.

Here, we report an extraordinarily selective association of both epithelia expressing TECK and lymphocytes expressing CCR9 with the immune compartment of the small intestine. The results support a novel paradigm in which epithelial cell–expressed chemokines can provide specific "addressin" signals controlling cellular recruitment and thus the character of immune responses at different epithelial surfaces.

Materials and Methods

Abs and Reagents. Anti–human CCR9 mAbs 3C3 (mouse IgG_{2b}) and 96-1 (mouse IgG_1) have been described (5). Anti–human TECK mAb 52513.111 (mouse IgG_{2b}) and isotype control mAb 20116.11 (mouse IgG_{2b}) were from R&D Systems. Directly conjugated mouse anti–human CD3-FITC (IgG_1, clone UCHT1), TCRαβ-FITC (IgM, clone T10B9.1A-31), CD4-allophycocyanin (IgG_1, clone RPA-T4), and CD8-PE (IgG_1, clone RPA-T8) were from BD PharMingen. BSA fraction V, dithiothreitol, sodium azide, EDTA, and olive oil were from Sigma-Aldrich. Crude collagenase (CLS-2) was from Worthington Biochemical Corp. Recombinant human TECK and IFN-inducible T cell α chemoattractant (I-TAC) were from PeproTech.

Tissue Sources and Lymphocyte Isolation. Normal human jejunum, ileum, colon, lung, facial skin, inflamed liver, and inflamed synovial tissue were from patients undergoing various surgical procedures. Synovial fluid was from patients undergoing diagnostic arthroscopy. Breast milk was obtained from nursing volunteers, and seminal fluid was obtained from normal male volunteers. All human subject protocols were approved by the Institutional Review Boards at Stanford University, the University of Oslo, Leicester University, or Robert Wood Johnson Medical School.

Lymphocytes from the epithelium and lamina propria of human intestine were isolated as described previously (8). Lymphocytes were isolated from normal human skin by first using a razor blade to separate the epidermis and a portion of the dermis from the lower dermis and subcutaneous fat. The epidermal pieces were cut into strips and incubated in cold 5 mM EDTA/HBSS for 120 min with vigorous stirring. The supernatant from this step was spun down to obtain released lymphocytes, and the remaining strips were crushed through a 50 mesh strainer to obtain additional lymphocytes. To obtain lymphocytes from inflamed skin, delayed-type hypersensitivity reactions were induced by poison oak application, or by intradermal injection of *Candida* allergen (0.1 ml Candin®; Allermed Laboratories, Inc.) in an allergic volunteer, and epidermal blisters were raised on the affected volar aspect of the forearm as described previously (9), and after 24 h, lymphocyte-containing blister fluid was drained. Lymphocytes were isolated from normal lung by finely mincing the tissue and sieving the resulting suspension through gauze. Lymphocytes were isolated from explant livers by first cutting the liver into small 1-cm^2 pieces, washing in RPMI 1640 supplemented with 10% fetal bovine serum, and then homogenizing into a cell suspension with a Stomacher 400 (Seward). Lymphocytes were isolated from synovial tissue as described (10). Synovial fluid, breast milk, and seminal fluid were diluted with FACS® buffer (PBS supplemented with 0.1% azide and 1% BSA), centrifuged at 250 *g*, and resuspended in FACS® buffer. Cell suspensions from liver, synovial fluid, breast milk, and seminal fluid were layered over Ficoll (Amersham Pharmacia Biotech) and the mononuclear cell layer was used for FACS® analysis. We found that the treatments required to dissociate these tissues did not affect CCR9 expression, and other lymphocyte markers such as LFA-1 were positive on all isolated lymphocyte populations (data not shown).

FACS® Analysis. Tissue lymphocytes were stained and gated for CD3 (or TCRαβ) expression, then further subdivided by CD4 or CD8 expression. Unconjugated anti-CCR9 mAbs (or isotype-matched control mAbs) were detected using a biotinylated horse anti–mouse IgG secondary Ab (Vector Laboratories) and streptavidin–peridinine chlorophyll protein (BD PharMingen). Four-color flow cytometry was done on a FACSCalibur™ (Becton Dickinson) using CELLQuest™ software, v3.1 (Becton Dickinson).

Tissue Northern and Dot Blots. $PolyA^+$ mRNA from various human tissues was purchased from CLONTECH Laboratories, Inc. or Clemente Associates, Inc., separated on a 1.5% formaldehyde-agarose gel, blotted, and probed with ^{32}P-labeled cDNA using standard protocols. A human MTE™ array of multiple human tissue $polyA^+$ mRNAs (CLONTECH Laboratories, Inc.) was hybridized according to the manufacturer's instructions.

In Situ Hybridization. A 348-bp digoxigenin (DIG)-labeled riboprobe was generated from the coding region of human TECK using the DIG RNA labeling kit according to the manufacturer's directions (Boehringer). All further steps were performed as described (11). In brief, frozen tissue sections were fixed in 4% paraformaldehyde and washed in 0.1% active diethylpyrocarbonate. Hybridization was performed in a solution of 50% formamide, 5× SSC, 50 μg/ml yeast tRNA, 100 μg/ml heparin, 1× Denhardt solution, 0.1% Tween 20, 0.1% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate, and 5 mM EDTA overnight at 59°C with 500 ng/ml of riboprobe. A high stringency wash was performed in the following sequence: 2× SSC (30 min), 2× SSC (1 h, 65°C), and 0.1× SSC (1 h, 65°C). DIG was visualized by means of an alkaline phosphatase–conjugated sheep anti-DIG and nitro blue tetrazolium chloride/5-bromo-4 chloro-3 indolyl phosphate, disodium salt substrate according to the manufacturer (Boehringer).

Immunohistology. Frozen sections of various optimal cutting temperature–embedded tissues were fixed for 10 min at room temperature in 4% paraformaldehyde/PBS, washed for 10 min in PBS, blocked with 100% goat serum for 10 min, then incubated with 2.5 μg/ml mouse anti–human TECK Ab (R&D Systems) or an isotype control mAb (R&D Systems) in 25% goat serum/PBS (GIBCO BRL), washed once in PBS, incubated with a 1:50 dilution of a goat anti–mouse-PE $F(ab')_2$ secondary Ab (BD PharMingen), washed, and visualized by confocal microscopy. Serial tissue sections were stained with a mouse anti–human "pan" cytokeratin Ab (IgG_1, clone C-11; Sigma-Aldrich) to highlight the epithelial layers.

Chemotaxis of Intestinal Lymphocytes. Chemotaxis assays were performed essentially as described (3) in RPMI 1640 with 0.5% BSA for 3 h. 10^6 intraepithelial lymphocytes (IELs) or 5×10^5 lamina propria lymphocytes (LPLs), isolated from jejunal sections removed during gastric bypass surgeries as described previously (5), were placed in the top well of each insert. For Ab blockade experiments, IELs or LPLs were incubated with 40 μg/ml of anti-CCR9 mAb 3C3, control mouse IgG_{2b} (clone 49.2; BD PharMingen), or medium alone for 10 min at 4°C before addition to the insert.

Results and Discussion

We initially asked whether small intestinal lymphocytes could chemotax to TECK, as predicted by their expression of the known TECK receptor CCR9 (5). In a standard transwell assay, both IELs (data not shown) and LPLs migrated to TECK with the typical bell-shaped dose–response curve characteristic of chemotaxis, with an optimal TECK concentration of ~300 nM (Fig. 1 A). Importantly, anti-CCR9 mAb 3C3 reduced migration almost to background levels (Fig. 1 B). In contrast, migration to I-TAC, a chemokine that signals through the independent receptor CXCR3 (Fig. 1 C), was not inhibited by anti-CCR9. We conclude that small intestinal lymphocytes migrate to TECK, and that this response is predominantly, or exclusively, mediated through CCR9.

The intestines have historically been considered part of a "common mucosal immune system" (12), so we were interested in the potential association of infiltrating $CCR9^+$ lymphocytes, and of TECK, with other regions of the gastrointestinal tract, with other mucosal epithelia, and with

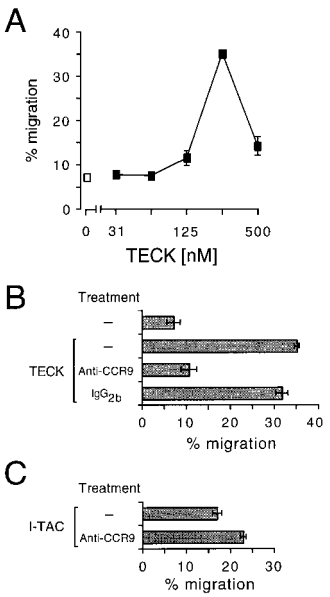


Figure 1. Human LPL chemotaxis to human TECK is mediated by CCR9. (A) Whole populations of LPLs isolated from the human small intestine chemotax efficiently to hTECK. TECK-induced chemotaxis is mediated exclusively by the chemokine receptor CCR9 since (B) the anti-CCR9 Ab 3C3 (40 μg/ml) blocks chemotaxis to hTECK whereas (C) the same Ab does not block chemotaxis to hI-TAC, which signals through CXCR3 expressed on virtually all LPLs. Data in A are the mean ± SD of duplicate wells ($n = 3$) and data in B and C are the mean ± SD of triplicate wells ($n = 2$). 250 nM TECK and 100 nM I-TAC were used in B and C.

nonmucosal epithelial tissues. Essentially all $CD4^+$ and $CD8^+$ T cells in both the intraepithelial and lamina propria compartments of the jejunum displayed high levels of CCR9 (Fig. 2, A and B). Lymphocytes isolated from an ileal specimen displayed an intermediate phenotype, with ~50% of $CD4^+$ and $CD8^+$ lymphocytes bearing CCR9 (data not shown). Interestingly, a smaller percentage of the lymphocytes isolated from the colon expressed CCR9 compared with jejunal lymphocytes (Fig. 2 J). These results suggest an unexpected level of differentiation between, and specialization of, lymphocytes in different segments of the gastrointestinal tract.

Even more surprising was the complete lack of CCR9 expression on $CD4^+$ and $CD8^+$ lymphocytes infiltrating a variety of other normal and inflamed mucosal and other epithelia-associated tissues. Lymphocytes from normal lung tissue (Fig. 2 C) were uniformly $CCR9^-$, as were lymphocytes from hepatitis C–infected liver (Fig. 2 D). Lymphocytes in breast milk (Fig. 2 E) and seminal fluid (data not shown), secretions of two epithelial glands, were also $CCR9^-$. Lymphocytes infiltrating normal (Fig. 2 F) and inflamed (delayed-type hypersensitivity to *Candida* or contact hypersensitivity to poison oak; Fig. 2 G) skin were uniformly negative as well, as were lymphocytes infiltrating inflamed joints (in the synovial tissue or the synovial fluid) in rheumatoid arthritis (Fig. 2, H and I) and other arthropities including psoriatic arthritis (synovial fluid), osteoarthritis (synovium and synovial fluid), ankylosing spondylitis (synovium), and acetabular osteolysis (synovium; data not shown). All lymphocytes analyzed by flow cytometry were isolated by mechanical means, obviating the need for enzymatic digestion that might alter cell surface antigen levels. (The sole exception were the lymphocytes isolated from synovium; in this case, parallel treatment of peripheral blood lymphocytes with collagenase confirmed lack of alteration in detectable CCR9 levels by the isolation procedure used.) Similar lack of CCR9 expression was found

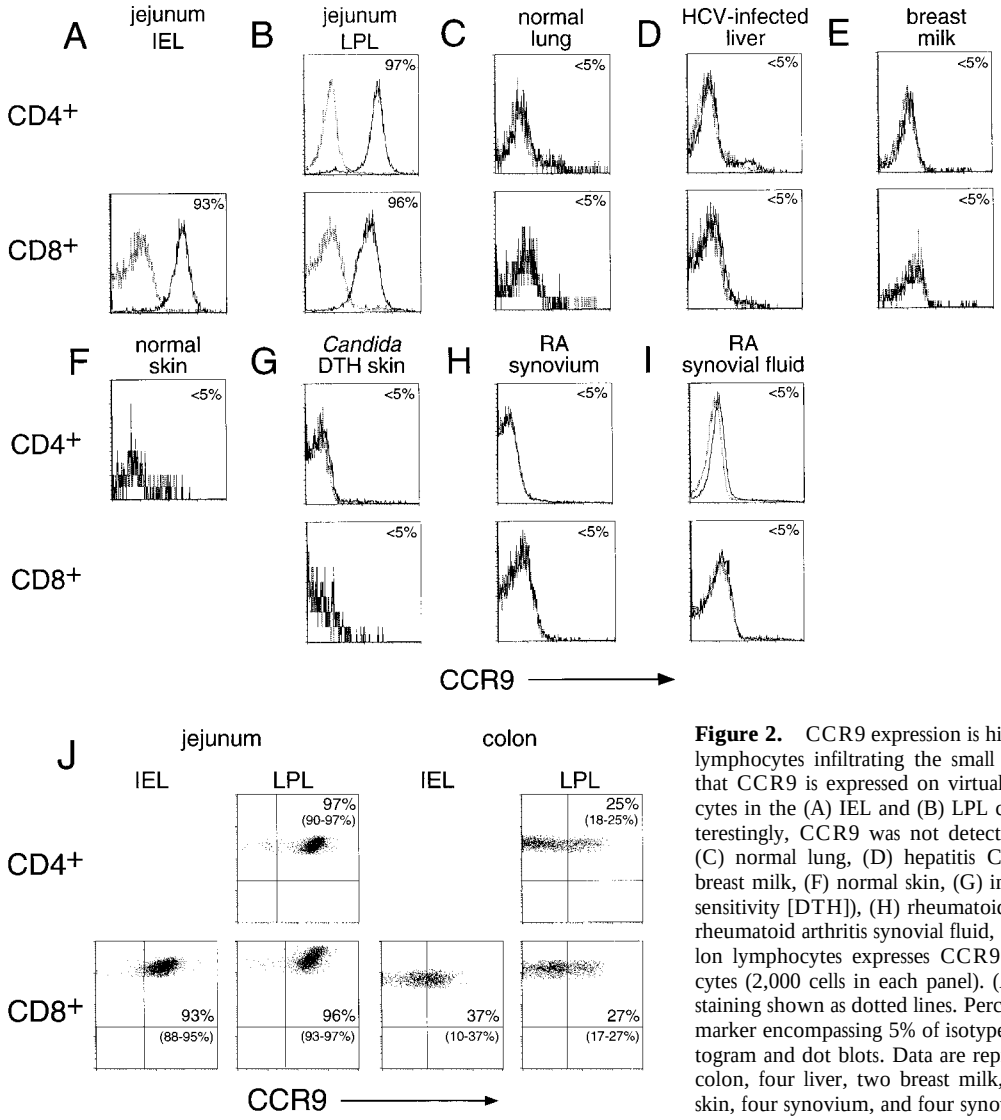
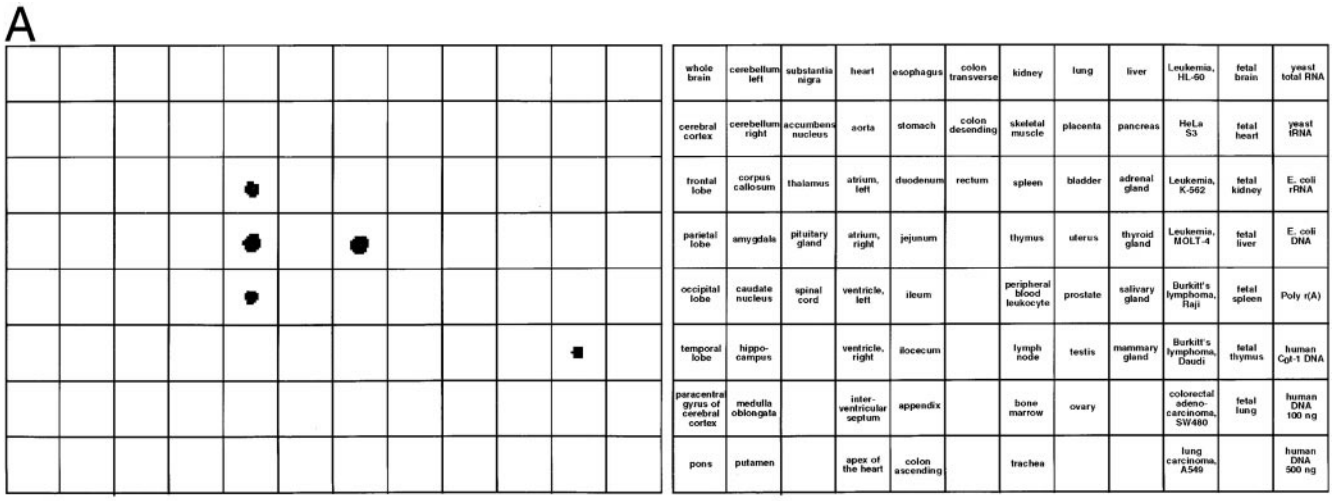


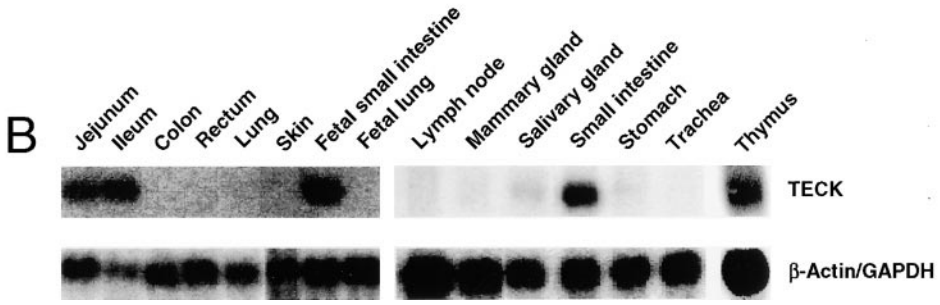
Figure 2. CCR9 expression is highly specific for $CD4^+$ and $CD8^+$ lymphocytes infiltrating the small intestine. FACS® analysis reveals that CCR9 is expressed on virtually all $CD4^+$ and $CD8^+$ lymphocytes in the (A) IEL and (B) LPL compartments of the jejunum. Interestingly, CCR9 was not detected on lymphocytes isolated from (C) normal lung, (D) hepatitis C virus (HCV)-infected liver, (E) breast milk, (F) normal skin, (G) inflamed skin (delayed-type hypersensitivity [DTH]), (H) rheumatoid arthritis (RA) synovium, and (I) rheumatoid arthritis synovial fluid, and (J) a smaller percentage of colon lymphocytes expresses CCR9 compared with jejunal lymphocytes (2,000 cells in each panel). (A–I) Isotype-matched control Ab staining shown as dotted lines. Percentage of $CCR9^+$ cells based on a marker encompassing 5% of isotype control–stained cells in both histogram and dot blots. Data are representative of three jejunum, four colon, four liver, two breast milk, four normal skin, four inflamed skin, four synovium, and four synovial fluid samples.

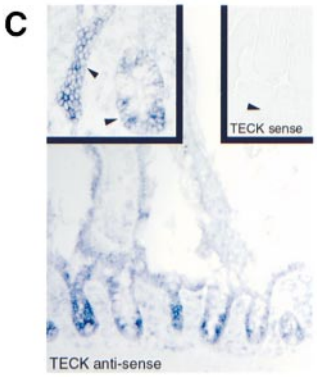
with two independent anti-CCR9 mAbs. Thus, although not all tissues in the body were examined, the results suggest a unique association of the CCR9 with T lymphocytes involved in small intestinal immunity.

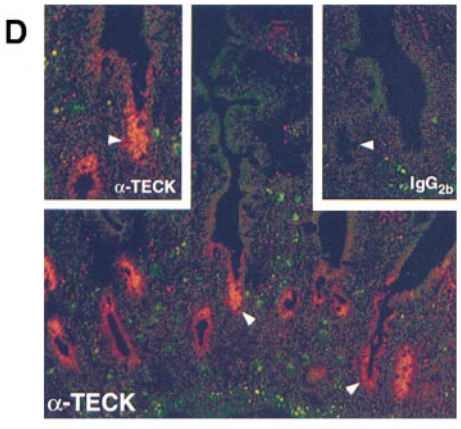
We reasoned that the CCR9 ligand TECK could play an important role in the selective homing and localization of $CCR9^+$ lymphocytes if it were also restricted in expression to the small intestine. Consistent with earlier studies in humans, TECK message was abundant in human thymus and various regions of the small intestine (Fig. 3, A and B), but was not detectable in the colon (Fig. 3, A and B). In addition, we failed to detect TECK message in other epithelial tissues including the skin, kidney, trachea, lung, placenta, bladder, adrenal gland, thyroid gland, salivary gland, prostate gland, and mammary gland (Fig. 3, A and B). These results, together with a recent report on TECK expression in mice (7), are consistent with restricted TECK expression in the small intestine, although in such bulk tissue mRNA preparations, significant TECK expression by a restricted cell population cannot be excluded.

We therefore used morphologic approaches to assess cellular sites of TECK expression more directly. In situ hybridization demonstrated intense expression of TECK message limited to jejunal epithelium, especially the epithelium in the crypts of Lieberkühn, with decreasing levels in epithelial cells moving up the villus (Fig. 3 C). The same expression pattern was observed in the ileum (data not shown). No detectable TECK hybridization was observed in two independent colon, two stomach, and two duodenum specimens. Most important, however, is the pattern of TECK expression at the protein level. Immunofluorescence staining with anti-TECK mAbs revealed strong staining in the small intestinal epithelium, especially in the crypts and lower villus epithelium (consistent with the pattern of in situ hybridization) (Fig. 3 D). Isotype-matched control Abs were negative (Fig. 3 D, inset). Importantly, no specific TECK reactivity was observed in the epithelium of five separate colon samples (Fig. 3 F), in the epithelia of four samples of normal skin (Fig. 3 G), inflamed psoriatic skin (Fig. 3 H), or hives (data not shown), lung









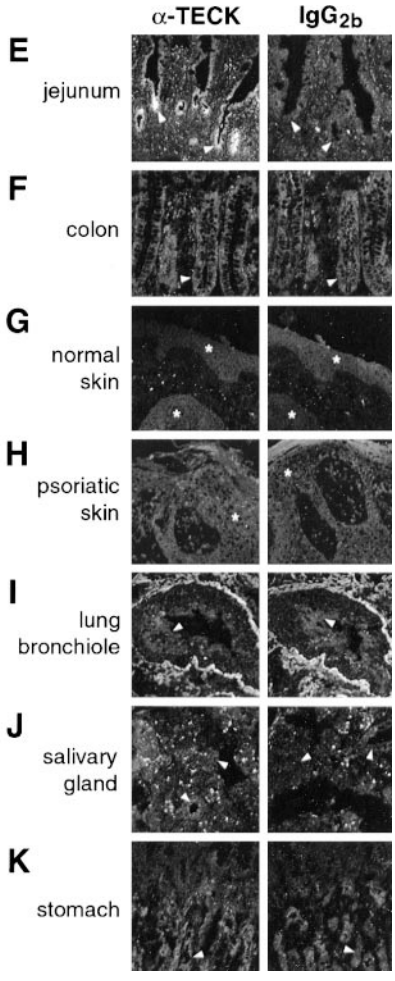


Figure 3. TECK is highly expressed by small intestinal epithelium but not epithelia in other tissues. (A and B) Dot blot and northern blot analysis of TECK mRNA expression in various human mucosal and epithelial tissues demonstrates highly preferential expression of TECK message in the thymus and various segments of the small intestine, but not other epithelial tissues such as the skin, kidney, trachea, lung, placenta, bladder, adrenal gland, thyroid gland, salivary gland, prostate gland, and mammary gland. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. (C) Further analysis by in situ hybridization localizes TECK mRNA expression specifically to the intestinal epithelium in the crypts of Lieberkühn and (D and E) immunohistochemistry localizes TECK protein to the epithelium of the crypts of Lieberkühn and villi in the small intestine. No TECK protein reactivity is detected in the (F) colon or in (G) epithelia from a variety of other human tissues including (G) normal skin, (H) inflamed psoriatic skin, (I) lung bronchioles, (J) salivary gland, or (K) stomach. Ubiquitin staining of the dot blot array revealed that mRNA loading of each tissue was relatively consistent. In situ data are representative of three jejunum samples, and immunohistochemistry data are representative of five jejunum, five colon, four normal skin, three inflamed skin (two psoriatic and one hives), three lung, three salivary gland, and four stomach samples. (D–K) Arrowheads or asterisks highlight epithelia.

bronchioles in three different samples (Fig. 3 I), in three salivary glands (Fig. 3 J), or in four samples of stomach (Fig. 3 K). Although expression below detectable limits cannot be ruled out, we conclude that TECK is produced selectively at high levels by epithelial cells of the small intestine, particularly the crypt epithelium in the jejunum and ileum, consistent with a highly selective role for TECK in the small intestinal immune compartment.

In addition to its ability to attract $CCR9^+$ small intestinal tissue lymphocytes, TECK can attract the small subset of circulating $CCR9^+$ memory cells in human blood (5). These cells constitute a discrete subset of memory cells expressing the intestinal homing receptor $\alpha_4\beta_7$, an integrin receptor for the mucosal vascular addressin MAdCAM-1 (13). Venules expressing MAdCAM-1 in the gut wall are prominent in the basilar lamina propria near the crypts of Lieberkühn (14). Thus, it is attractive to propose that in the jejunum, the juxtaposition of MAdCAM-1–expressing vessels with TECK-expressing epithelium, combined with the expression of $\alpha_4\beta_7$ by circulating $CCR9^+$ memory cells, may lead to the selective recruitment of a specialized subset of circulating "small intestine homing" lymphocytes by sequential action of $\alpha_4\beta_7$, LFA-1, and CCR9/TECK. In this variant of the multistep model of lymphocyte recruitment (15), CCR9 could function primarily in selective transendothelial migration after arrest. Alternatively, epithelium-expressed TECK may also diffuse to and be presented by the vascular endothelium (16) to support the integrin activation required for lymphocyte arrest on lamina propria endothelium. Once lymphocytes have entered the lamina propria of the small intestine, localization within specific compartments such as the epithelium likely occurs by step-by-step migration of lymphocytes through chemokine gradients (17). For instance, $CCR9^+$ lymphocytes selectively recruited into the lamina propria by TECK may then be directed to the villous epithelium by additional chemoattractants, e.g., stromal cell–derived factor 1α, which seems to be differentially expressed by villous epithelial cells (18).

Interestingly, recent studies have revealed selective expression of a distinct chemokine, CTACK, by cutaneous epithelial cells (keratinocytes; reference 4). CTACK selectively attracts circulating cutaneous memory T cells (4). Moreover, like TECK in the small intestinal epithelium, CTACK appears to be constitutively expressed by skin keratinocytes (4). Thus, TECK and CTACK may represent the critical defining components of the small intestinal and cutaneous immune environments, respectively.

In conclusion, our results suggest a highly restricted expression of TECK by small intestinal epithelial cells, and of its receptor CCR9 by lymphocytes infiltrating the small intestine. These results suggest a previously unsuspected specialization of chemokine receptor–ligand expression and hence, of homing mechanisms to different segments of the gastrointestinal tract. Moreover, in conjunction with recent studies revealing restricted epithelial expression of a related CC chemokine, CTACK, by skin keratinocytes, our findings suggest a novel paradigm in which the constitutive expression of chemokine "addressins" by epithelial cells can control the recruitment and localization of specialized lymphocytes, and thus the character of immune responses at different epithelial surfaces.

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