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Naive T Cell Recruitment to Nonlymphoid Tissues: A Role for Endothelium-Expressed CC Chemokine Ligand 21 in Autoimmune Disease and Lymphoid Neogenesis

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Naive T cells are usually excluded from nonlymphoid tissues. Only when such tertiary tissues are subjected to chronic inflammation, such as in some (but not all) autoimmune diseases, are naive T cells recruited to these sites. We show that the CCR7 ligand CC chemokine ligand (CCL)21 is sufficient for attracting naive T cells into tertiary organs. We performed intravital microscopy of cremaster muscle venules in T-GFP mice, in which naive T cells express green fluorescent protein (GFP). GFP+ cells underwent selectin-dependent rolling, but no firm adherence (sticking). Superfusion with CCL21, but not CXC chemokine ligand 12, induced integrin-dependent sticking of GFP+ cells. Moreover, CCL21 rapidly elicited accumulation of naive T cells into sterile s.c. air pouches. Interestingly, a second CCR7 ligand, CCL19, triggered T cell sticking in cremaster muscle venules, but failed to induce extravasation in air pouches. Immunohistochemistry studies implicate ectopic expression of CCL21 as a mechanism for naive T cell traffic in human autoimmune diseases. Most blood vessels in tissue samples from patients with rheumatoid arthritis (85 ± 10%) and ulcerative colitis (66 ± 1%) expressed CCL21, and many perivascular CD45RA+ naive T cells were found in these tissues, but not in psoriasis, where CCL21+ vessels were rare (17 ± 1%). These results identify endothelial CCL21 expression as an important determinant for naive T cell migration to tertiary tissues, and suggest the CCL21/CCR7 pathway as a therapeutic target in diseases that are associated with naive T cell recruitment.


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3 Abbreviations used in this paper: PP, Peyer’s patches; LN, lymph node; PLN, peripheral LN; HEV, high endothelial venules; PNAd, peripheral node addressin; CCL, CC chemokine ligand; MadCAM, mucosal addressin cell adhesion molecule; CXCL, CXC chemokine ligand; GFP, green fluorescent protein; vWF, von Willebrand factor; WSR, wall shear rate; RA, rheumatoid arthritis; UC, ulcerative colitis; DIIg, digoxigenin; RAST, RA synovial tissue; EC, endothelial cell; PSGL, P-selectin glycoprotein ligand; EGFP, enhanced GFP; DC, dendritic cell.

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Recent studies have implicated chemokines, such as CCR7 ligands, in lymphoid neogenesis; transgenic expression of CCL21 or, to a lesser degree CCL19, led to the formation of LN-like structures in pancreatic islets (10–12). Interestingly, lymphoid neogenesis was absent in CCL21-transgenic mice that were lymphocyte-deficient, but was still evident in animals that were deficient in B but not T cells, indicating that the effect of ectopically expressed CCL21 is T cell-dependent (10, 11). These studies elegantly demonstrate that certain transgenically expressed chemokines are sufficient to mediate naive T cell accumulation within nonlymphoid tissues in vivo. However, a drawback of these reports is that the transgenic chemokines were continuously present during development and adult life, and therefore could have had indirect effects on lymphocyte migration. Thus, conclusions about their effects on naive T cell recruitment during homeostatic and acute and chronic inflammatory conditions in adult animals cannot be drawn. These questions are of importance, as recent evidence suggests that Ag can be presented to naive T cells in tertiary lymphoid tissues (13). Such priming of T cells in nonlymphoid sites might be of benefit for tumor therapy (13), but could also be involved in the pathogenesis or progression of autoimmune diseases, because locally produced autoantigens may be presented to naive T cells directly at the site of inflammation. Thus, a better understanding of the mechanisms of lymphoid neogenesis/naive T cell trafficking to nonlymphoid tissues could facilitate the development of new therapies for human disease.

We have determined the minimal requirements for naive T cell entry into nonlymphoid tissues in adult animals. We found that these cells underwent selectin-mediated rolling, but were incapable of firm adherence in acutely inflamed peripheral tissue. Exogenous application of CCL21, but not CXCL12, rapidly induced accumulation of naive T cells, even in the absence of HEV or vascular adhesion molecules. Consistent with this finding, we found that CCL21 is expressed on PNAd⁺ and PNAd⁻ blood vessels in human diseases accompanied by lymphoid neogenesis. Importantly, the presence of naive T cells in perivascular areas correlated with CCL21 expression in blood vessels. These results suggest that de novo expression of CCL21 on endothelial cells could represent a key step for naive T cell recruitment in the periphery. In addition, these findings implicate CCL21 in the pathogenesis of those autoimmune diseases associated with lymphoid neogenesis, and suggest the CCL21/CCR7 pathway as a potential therapeutic target in these diseases.

Materials and Methods

Mice

T-GFP mice, in which green fluorescent protein (GFP) is expressed under the control of the murine CD4 proximal enhancer and promoter without the intronic silencer, have been described previously (14). Mice were housed and bred in a specific pathogen-free/virus Ab-free animal facility. All experiments were in accordance with National Institutes of Health guidelines and approved by the Committees on Animals of both Harvard Medical School and The Center for Blood Research (Boston, MA).

Abs and chemokines

Hybridoma cells for mAb 9A9 (rat IgG2b) and mAb 5H1 (rat IgG1), which neutralize mouse E-selectin and P-selectin, respectively, and the nonbinding control mAb 2A1 (rat IgG2b) were kindly provided by Dr. B. Wolitzky (Hoffman LaRoche, Nutley, NJ). Hybridoma cells for anti-L-selectin mAb Mel-14 (rat IgG2a), anti-PNA/Ad mAb MECA-79 (rat IgM), and anti-LFA-1 mAb Thb 213 (rat IgG2b) were a kind gift of Dr. E. Butcher (Stanford University, Stanford, CA). The following Abs were used: anti-CD3, anti-CD44, and anti-CD62L (BD Pharmingen, San Diego, CA); anti-human CD45RA (clone L48), anti-human HLA-DR (L243), anti-human CD11c (SHCL-3), and anti-human CD45RA (HL100; BD Biosciences, Mountain View, CA); anti-human CD3, anti-human vWillebrand factor (vWF), and anti-human CD5 (DR25; DAKO, Carpinteria, CA); anti-human CD4 (MAT-4; Medscand Diagnostics, Malmoe, Sweden); anti-human CCL21 (R&D Systems, Minneapolis, MN); and anti-human mAb PAL-E (Research Diagnostics, Flanders, NJ). Mouse mAb R19 against human CD3 (IgG3) and FITC-conjugated goat anti-human vWF were gifts from J. Hilgers (Bioprobe, Amsterdam, The Netherlands) and Dr. E. Butcher, respectively. Recombinant murine TNF, CXCL12, CCL19, and CCL21 were purchased from R&D Systems. For some experiments, human recombinant CCL21 (kindly provided by T. Springer, Center for Blood Research, Boston, MA) was used. No difference in chemotactic response of naive murine T cells to murine or human CCL21 was observed (data not shown). The use of the CCL19 (EBI1 ligand chemokine/macroage-inflammatory protein-3β)-Ig chimera to detect CCR7 on murine T cells was described previously (15).

Intravital microscopy and image analysis

Cremaster muscles were prepared as described previously (16). Briefly, adult male T-GFP mice were anesthetized by i.p. injection of 0.25 ml of a combination of ketamine HCl (5 mg/ml) and xylazine (1 mg/ml). The right cremaster muscle was prepared and covered with sterile, bicarbonate-buffered Ringer’s injection solution (pH 7.4). The surgical procedure as described in this study takes ~30 min. GFP⁺ leukocytes were visualized through a ×40 water-immersion objective (Zeiss Achroplan NA 0.75; Oberkochen, Germany) by video-triggered stroboscopic epi-illumination on an intravital microscope (IV-500; Mikron Instruments, San Marcos, CA). One to three venular trees were chosen and 1-min recordings were made of individual segments of several postcapillary and small collecting venules at 5-min intervals to assess baseline rolling. Subsequently, 50 μg of mAb (9A9, 5H1, Mel-14, Thb-213, or 2-A41) were injected i.v. Fifteen minutes later, the same venules were repeatedly recorded for 1–3 min intervals until ~45 min after mAb injection. To study the effects of chemokines on T cell adhesion, one to two vessel trees in a preparation were chosen. To identify sticking cells, individual venular branches were recorded for 1 min each throughout an entire vessel tree. The superfusion buffer was then replaced with prewarmed buffer alone or buffer containing chemokines (100 nM). The same vessel segments were recorded again 5–25 min and 45–60 min after buffer replacement. For some experiments, TNF-α (500 ng) was injected intracostal 2–3 h before the preparation.

The rolling fraction for each individual venule was determined as the percentage of leukocytes that interacted detectably with the vascular wall within the total number of fluorescent cells that passed a vessel during the observation period. Interactions were defined as any transient or continuous slowing of a GFP⁺ cell below the velocity of noninteracting blood cells in the same vessel. Firm adherence (sticking) was defined as a cell that remained stationary for ≥30 s. Vessel cross-sectional diameters (D) and velocities of individual rolling (Vrollo, at least 10 consecutive per- venule) and noninteracting (Vnon, at least 20 consecutive cells per venule) leukocytes, as well as wall shear rate (WSR) and wall shear stress, were determined off-line as described previously using a PC-based interactive image analysis system (17, 18).

Induction of air pouches

Air pouches were generated by s.c. injection of sterile air (3–5 ml initially, 3 ml after 72 h) in the back of each mouse as described (19). After 6 days, PBS alone or PBS containing TNF-α (500 ng), CXCL12, CCL19 (1 or 2 μg), or CCL21 (2 μg) were injected into the air pouch at time points 0 and 4 h. Accumulated leukocytes were harvested from the pouch 8 h after the first injection by PBS lavage.

Immunofluorescence staining and quantification of naive T cells and CCL21⁺ blood vessels

Serial cryosections (8 μm) of inflamed human tissue from various inflammatory lesions including rheumatoid arthritis (RA) (n = 4), ulcerative colitis (UC) (n = 5), and psoriasis (n = 2) were used for analysis. Control material consisted of palatine tonsils (n = 3), normal colon (n = 3), and normal skin (n = 2) obtained from individuals undergoing tonsillectomy, colectomy (due to long-lasting chronic obstruction), or mastectomy, respectively. All procedures at Rikshospitalet involving patient material were performed in agreement with the Helsinki Declaration and were approved by the Regional Ethical Committees for Medical Research Ethics (Health Region South, Oslo, Norway). Patient data, pathohistological grading, and treatment schedules are provided in Table I. Three-color immunostaining with primary Abs and appropriate secondary- and third-step reagents was performed as described previously (20).

For quantification of naive T cells and CCL21⁺ vessels, 10 microscopic fields from each sample of diseased tissue were selected in areas where CD3⁺ cells were juxtaposed to vWFP⁺ (or PAL-E⁺) blood vessels (×400)
the peripheral blood of T-GFP mice.

In the T-GFP strain, transgenic GFP is strongly and selectively expressed by T cells, making them easily detectable by flow cytometry and intravital fluorescence microscopy (Fig. 1A; Refs. 14, 15, 21). To specifically assess the adhesive behavior of naive T cells in vivo, it is necessary to distinguish them from Ag-experienced T cells, which migrate readily to nonlymphoid sites (22, 23). T-GFP mice allow this distinction to some extent, because prolonged antigenic stimulation (for 0.90%) of effector memory T cells. A similar procedure was performed in parallel sections for enumeration of CCL21 vessels in the total population of vWF blood vessels (median number of counted blood vessels/sample of diseased tissue: 38, range 23–48).

In situ hybridization for CCL21

A 345-bp digoxigenin (DIG)-labeled riboprobe was generated from the coding region of cDNA for human CCL21 with the DIG RNA labeling kit according to the manufacturer’s directions (Boehringer Mannheim, Mannheim, Germany). Hybridization and detection of the hybridized probe were performed as previously described (20).

Statistical analysis

Where appropriate, data are presented as mean ± SEM. Rolling fractions after Ab treatment vs baseline were compared using the Wilcoxon signed rank test. Sticking fractions before vs after superfusion with chemokines after Ab treatment vs baseline were compared using the Wilcoxon signed rank test. Accumulation of naive and central memory T cells in air pouches in response to chemokines and TNF-α was analyzed using the Mann-Whitney test. Sticking fractions before vs after superfusion with chemokines was analyzed using the Mann-Whitney test. Significance was assumed at p < 0.05.

Results

Phenotype of circulating T cells in T-GFP mice

In agreement with previous results (14), all GFP+ cells were CD3+, and naive T cells (CD3+CD44low/–) were uniformly GFP++, L-selectin+ (90 ± 4%) and bound CCL19-Ig chimera

FIGURE 1. Phenotype of circulating T cells in T-GFP mice. Peripheral blood leukocytes isolated from (A) young (8 wk) and (B) aged (1.5 years) T-GFP mice were analyzed by three-color flow cytometry. A, T cells were identified by expression of CD3 after gating on live lymphocytes by for-ward side light scatter (left plot). Nearly all CD44low/– T cells were GFP+++, whereas CD44high cells expressed variable levels of GFP. In B, gates were set on live lymphocytes, and the CD44high GFPlow (R1; a subset of effector memory cells), CD44high GFP++ (R2; central memory cells), and CD44high GFP++ (R3; naive T cells) subsets were analyzed for expression of L-selectin and binding of CCL19-chimeric ligand for CCR7. The CD44high GFP++ cells in the left plot were mostly B cells and a small number (3–5%) of effector memory T cells. Similar FACS profiles were found in each of three mice analyzed for each group. Although young animals contained fewer CD44high T cells, the expression pattern of L-selectin and CCR7 was similar irrespective of the animals’ age (not shown). Numbers in plots represent quadrant percentages.
(88 ± 3%), a ligand for CCR7 (15). CD44^high effector/memory cells fell in three separate categories: GFP^−, GFP^low, and GFP^high (Fig. 1A). Because GFP^− cells escape detection by intravital microscopy, we focused on the two GFP^+ subsets. Most GFP^highCD44^high cells (R2 in Fig. 1B) expressed L-selectin (77 ± 10%) and bound CCL19-Ig chimera (80 ± 7%), comparable to naive T cells (R3 in Fig. 1B; n = 3 mice). Therefore, this subset meets the phenotypic criteria proposed for central memory T cells (24). In contrast, only a minority of GFP^lowCD44^high cells (R1 in Fig. 1B) was L-selectin^− (27 ± 8%) and bound CCL19-Ig (44 ± 2%), which suggests that most cells in this gate belong to a subpopulation of effector memory T cells, similar to the GFP^−CD44^high subset, which we have shown previously to be of effector memory phenotype (25). CD44^highGFP^low cells constituted 1.0 ± 0.1% and 4.0 ± 0.6%, and CD44^highGFP^high cells constituted 2.3 ± 0.4% and 8.5 ± 2.9% of circulating lymphocytes in young (~8 wk) and aged (1.5 years) mice, respectively. This difference in frequency notwithstanding, the surface phenotype of each memory population remained remarkably constant irrespective of the animals’ age (not shown). As mice used in this study for intravital microscopy were young (6–10 wk), only ~5% of all GFP^+ cells were CD44^high memory cells.

**Naive T cell behavior in cremaster muscle venules**

Having established that the vast majority (≥95%) of detectable (i.e., GFP^high) cells in the bloodstream of healthy young T-GFP mice are naive T cells, we set out to dissect the molecular mechanisms by which these cells interact with microvessels in nonlymphoid tissues. We used intravital microscopy of the cremaster muscle microvasculature, a model of trauma-induced, moderate inflammation (26, 27). Rolling was defined as any transient or continuous interaction of GFP^+ cells below the velocity of noninteracting GFP^+ cells in the same vessel. The baseline rolling fraction of GFP^+ cells in cremaster muscle venules (n = 46

![FIGURE 2.](image-url)
transmitted light revealed that TNF-α 359 cells/24 venules/12 animals) in the absence of cytokine (Fig. 2A). Hemodynamic parameters before and after Ab treatment (50 μg/mouse) revealed no significant differences (Table II). Treatment with nonbinding isotype control mAb 2-4A1 (n = 11/3), with anti-LFA-1 (n = 16/3), or with anti- E-selectin (n = 11/3) did not alter rolling fractions (p > 0.05). A moderate reduction in rolling was induced by anti-P-selectin mAb (41% inhibition; p < 0.01; n = 12/3). In contrast, rolling was nearly abolished by anti-L-selectin mAb (97% inhibition; p < 0.001; n = 10/3).

We also studied the effects of deliberate induction of a strong inflammatory response on naive T cell behavior. Intrascrotal injection of 500 ng of TNF-α 2-3 h before the experiment did not change rolling fractions of GFP+ cells (32 ± 2%; n = 11/4) compared with control conditions (Fig. 2C). However, while the median rolling velocity (Vroll) of GFP+ cells was 170.8 μm/s (n = 359 cells/24 venules/12 animals) in the absence of cytokine (Fig. 2D), TNF-α pretreatment decreased this parameter to 97.5 μm/s (p < 0.001; n = 90/6/3).

CCL21 induces sticking of GFP+ cells in cremaster muscle microvasculature

Having established that naive T cells roll in cremaster muscle venules, we next determined whether they also undergo sticking (defined as stationary arrest for ≥30 s) when exposed to inflammatory stimuli (Fig. 3). Under baseline conditions, firmly adherent GFP+ cells were rare (3.5 ± 1.4 stickers/mm²; n = six venular trees/three animals). Pretreatment of cremaster muscles with TNF-α did not increase sticking of GFP+ cells (2.4 ± 0.8 stickers/mm²; n = 8/4; p > 0.05), even though intravital microscopy under transmitted light revealed that TNF-α induced a marked accumulation of GFP+ (mostly myeloid) leukocytes.

We next asked whether sticking could be induced by supplementing exogenous chemokines that are known to stimulate integrin adhesiveness on naive T cells under flow in vitro. After observing the adhesive behavior of GFP+ cells during a 30-min control period, cremaster muscles were superfused with either CCL21, CCL19, or CXCL12 (each 100 nM) for at least 45 min (Fig. 3A). CCL21 superfusion led to a significant linear increase in the number of sticking GFP+ cells (n = 6 venular trees/3 animals; 77.0 ± 15.2 GFP+ cells/mm² after 45 min superfusion; p < 0.05 vs control period), but did not significantly alter microhemodynamic conditions (Table II). Approximately 0.3% of cells in the total flux of GFP+ cells became firmly adherent, which at a rolling fraction of ~35%, corresponds to a sticking fraction (i.e., sticking cells in percent of rolling cells) of ~1%. CCL19 superfusion of the cremaster muscle induced firm adhesion of GFP+ cells similarly to CCL21 (n = 3/2; 70.0 ± 17.1 GFP+ cells/mm² at 45 min; Fig. 3). In contrast, superfusion with CXCL12 did not significantly induce sticking of GFP+ cells as compared with baseline values in the same vascular bed (n = 6/3; 12.8 ± 3.7 GFP+ cells/mm²; Fig. 3). However, when fluorescently labeled in vitro-generated central memory CD8+ T cells were injected into cremaster muscles, CXCL12 superfusion at equimolar concentration (100 nM) induced firm adhesion of these cells, suggesting that the chemokine

<table>
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<th>Table II. Hemodynamics and leukocyte rolling parameters in cremaster muscle venules</th>
<th>Pre</th>
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<th>Pre</th>
<th>Post</th>
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<th>Post</th>
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<td>39 ± 8</td>
<td>32 ± 8</td>
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<td>Vnull (μm/s)</td>
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<td>1453 ± 663</td>
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<td>1371 ± 824</td>
<td>1418 ± 367</td>
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<td>325 ± 184</td>
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<td>25 ± 7</td>
<td>25 ± 11**</td>
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* Baseline conditions (Pre) were assessed for 20–30 min, after which either a control mAb (clone 2-4A1) or the respective blocking mAb (50 μg) were injected i.v. Effects were determined 15 min later (Post). For chemokine experiments, the cremaster muscle was superfused for ≥45 min with prewarmed buffer containing CCL21 (100 nM, Post) after assessment of baseline conditions (Pre). For TNF-α experiments, measurements were taken 2-3 h after intrascrotal injection of 500 ng of TNF-α. The velocity of ≥20 noninteracting (Vnull) and ≥10 rolling (Vroll) cells was measured for each venule by frame-by-frame analysis of video tapes. Diameters, Vnull, rolling fractions, and WSR are shown as mean ± SD. Because Vnull was not normally distributed, median values are shown. Because rolling was nearly abolished after anti-L-selectin treatment, Vnull was not determined; RF, rolling fraction.

p < 0.001, **, p < 0.01; ns, not significant as compared to baseline (Pre) data.

FIGURE 3. CCL21 induces sticking of GFP+ T cells in cremaster muscle venules. Sticking (firm arrest for ≥30 s) of naive T cells in cremaster muscle venules of T-GFP mice was studied by epifluorescence intravital microscopy. A, The number of sticking GFP+ cells per millimeter-squared luminal surface area was assessed during a 30-min control period followed by superfusion with CCL21 ( ), CCL19 ( ), or buffer ( ). B, Pretreatment with TNF-α (500 ng, 2–3 h before the experiment) or prolonged superfusion with buffer or CXCL12 did not induce accumulation of GFP+ T cells, whereas superfusion of CCL21 or CCL19 induced significant T cell sticking (values for CCL19, CCL21, and CXCL12 superfusions represent ≥45 min time points). CCL21-mediated firm adhesion of GFP+ cells was abolished by prior injection of anti-LFA-1 mAb. Statistical analysis of the values obtained for CCL21 superfusion in comparison to the other treatment groups was performed using ANOVA. Bars represent mean ± SEM, n = at least six vessel trees/three animals per group; n.s., not significant.
reached a concentration in the venular lumen that was sufficient to induce arrest of more responsive leukocyte subsets (L. Scimone and U. H. von Andrian, unpublished observation). We next set out to determine which adhesion receptor mediated CCL21-induced firm adherence of GFP<sup>+</sup> cells. As the integrin LFA-1 (CD11a) is the major molecule mediating sticking of naive T cells in PLN HEV (28), we injected anti LFA-1 blocking mAb before cremaster muscle superfusion with CCL21. As shown in Fig. 3B, the number of firmly adherent GFP<sup>+</sup> cells observed under these conditions did not increase over baseline levels (p > 0.05 as compared with control), indicating that LFA-1 is the main adhesion molecule in CCL21-induced T cell sticking.

**CCL21 elicits accumulation of naive T cells in dorsal skin air pouches**

Although our intravitral microscopy analysis showed that the presence of CCL21 in a nonlymphoid tissue is sufficient to induce intravascular sticking of GFP<sup>+</sup> T cells, it remained to be determined whether this would lead to subsequent diapedesis and accumulation of naive T cell in the extravascular space. This question could not be answered by intravitral microscopy alone, because the maximal observation period during which animals can be kept in anesthesia was too short to assess the magnitude of T cell emigration, which is a relatively slow and inefficient process. Moreover, intravitral microscopy does not allow for phenotypic analysis of sticking and emigrated GFP<sup>+</sup> cells.

To determine whether and to what extent naive T cells can leave the circulation in nonlymphoid tissues upon encounter of CCL21, we generated air pouches in the dorsal skin of T-GFP mice. Six days later, groups of six to eight animals were injected twice with either PBS alone or with PBS containing TNF-α (500 ng), or chemokines (2 µg for CCL21, 1 or 2 µg for CCL19 and CXCL12) into air pouches. Eight hours later, air pouches were thoroughly lavaged, leukocytes in the lavage fluid were enumerated and their phenotype was analyzed by flow cytometry as described in Fig. 1 (Fig. 4).

Virtually no CD44<sup>high</sup>GFP<sup>low</sup> naive T cells (R3 in Fig. 4A) or CD44<sup>high</sup>GFP<sup>high</sup> central memory cells (R2 in Fig. 4A) were found when PBS was injected into air pouches alone or with TNF-α (Fig. 4, A–D), even though TNF-α caused a massive inflammatory response as evidenced by a ~3-fold increase in the total leukocyte number recovered (p < 0.05 vs PBS; Fig. 4B). In contrast, injection of CCL21 did not change the overall number of recovered cells (which were mostly myeloid cells). However, CCL21 provoked the appearance of two distinct populations of naive and central memory T cells, which represented 4.8 ± 2.0% and 1.4 ± 0.6% of gated lymphocytes, respectively. On average, 751 ± 182 naive (R3) and 394 ± 114 central memory (R2) T cells migrated to an air pouch after injection of CCL21 (Fig. 4D). These numbers were significantly higher than those after injection of PBS alone or PBS plus TNF-α (naive T cells: 28 ± 14 and 11 ± 11, respectively; central memory T cells: 53 ± 26 and 58 ± 46, respectively; p < 0.01; Fig. 4D). There was no significant difference in the frequency or total number of CD44<sup>high</sup>GFP<sup>low</sup> (R1 in Fig. 4A) cells after PBS, TNF-α, or CCL21 injection (Fig. 4C).

We also investigated the accumulation of GFP<sup>+</sup>CD44<sup>low</sup> cells after the administration of CXCL12 and CCL19 into s.c. air pouches (n = 7; Fig. 4E). To adjust for differences in the molecular mass of these chemokines (CCL21: 16–17 kDa; CCL19: 9.4 kDa; CXCL12: 8 kDa, according to the manufacturer’s specifications), in five of the seven animals in each group, 1 µg of CCL19 or CXCL12 was injected, whereas recipients of CCL21 always received 2 µg. Two additional mice received 2 µg of CCL19 or CXCL12 twice. Because there was no difference in the response to these different doses of CCL19 and CXCL12, results are pooled in Fig. 4E. Somewhat surprisingly, neither CCL19 nor CXCL12 induced a statistically significant increase in the number of GFP<sup>+</sup>CD44<sup>low</sup> cells in the air pouches (Fig. 4E; CXCL12: 40 ± 32; CCL19: 118 ± 51, p > 0.05 vs PBS). Taken together, the local generation of an appropriate chemokine, i.e., CCL21, appears to be required for naive T cell entry into tissues.

**CCL21 is expressed in blood vessel endothelial cells in RA synovial tissue (RAST) and UC**

Having determined that the administration of exogenous CCL21 induces naive T cell recruitment into normal mouse tissues, we asked whether the ectopic production of this chemokine might play a role in human disease. Tissue sections derived from RAST, UC, psoriasis and, as a control, normal skin and colon were immunostained for CCL21. Sections were also stained for vWF, which is highly expressed by endothelial cells (EC) in blood vessels, but
CCL21 protein and mRNA are expressed by vWF\(^+\) blood vessels in chronic inflammatory diseases in humans. Frozen sections of (A and F) normal colon, (B) tonsils, (C and E) ulcerative colitis, and (D and G–I) RAST were subjected to immunostaining (A–E and I) and/or in situ hybridization (F–H). A. Immunostaining of normal colon for vWF (green) and CCL21 (red); B–D, staining for CD3 (green), CCL21 (red), and vWF (blue). Arrows in A–D depict vWF\(^+\) blood vessels. vWF\(^+\) lymphatic vessels in all tissues showed strong staining with anti-CCL21 (A–C, arrowheads). Staining of a serial section to D revealed that the CCL21\(^+\) blood vessel (arrow) did not express PNAd (not shown). E. Staining for PNAd (green), CCL21 (red), CD3 (blue). The arrow depicts a PNAd-positive EC. Note that whereas not all of the ECs are PNAd\(^+\), all express CCL21. F. CCL21 mRNA in normal colon was detected using a DIG-labeled CCL21 antisense probe and mRNA was visualized with Fast Red (red). Sections were subsequently stained with anti-vWF (green), vWF\(^+\) lymphatic vessels showed strong CCL21 mRNA expression (arrowhead), whereas vWF\(^+\) blood vessels were negative for CCL21 (arrow). G and H, Serial sections of RAST were used for in situ hybridization with (G) antisense red or (H) a sense control probe for CCL21. Immunostaining of an adjacent section (I) with anti-vWF reveals strong vWF expression in the CCL21\(^+\) vessel (arrow). Original magnification: \(\times 400\) (A–D and F–I), \(\times 600\) (E).

Only weakly expressed or absent in lymphatic vessels (29). Normal colon (Fig. 5A) and skin (not shown) contained abundant vWF\(^+\) blood vessels, but no staining for CCL21 was seen in these vessels. In contrast, vWF\(^+\) lymphatic vessels showed a prominent cytoplasmic staining pattern for CCL21 consistent with previous reports that lymphatic EC in normal tissues express this chemokine (30). As a positive control for CCL21 expression in blood vessels we used human tonsils, in which CCL21 staining was consistently found on vWF\(^+\) vessels (Fig. 5B). These vessels were also PNAd\(^+\), and therefore represented HEV (not shown). Importantly, numerous PNAd\(^-\) vWF\(^+\) vessels in both UC (Fig. 5, C and E) and RAST (Fig. 5D) expressed CCL21. No staining was seen with an isotype-matched control mAb (not shown). Some sections were also double-stained using anti-CCL21 Ab as well as mAb PAL-E, which specifically visualizes blood vessel, but not lymphatic, endothelium (31). Identical results were obtained as with anti-vWF Ab (data not shown).

Next, we analyzed the cellular source of CCL21 mRNA, because CCL21 protein can be produced by extravascular cells and can be transported to and across HEV in PLN (21, 32). In situ hybridization of normal colon mirrored our findings with Ab stainings inasmuch as the CCL21 mRNA signal was only observed in vWF\(^+\) vessels (Fig. 5F). In contrast, in situ hybridization of RAST samples revealed that vWF\(^+\) EC expressed CCL21 mRNA (Fig. 5, G–I). Thus, the microvessels in chronically inflamed synovium acquired the ability to synthesize CCL21, a property that is normally restricted to HEV in PLN and PP. Indeed, CCL21\(^+\) vWF\(^+\) blood vessels were found in areas where the formation of lymphoid follicles was prominent, and some of these vessels were also PNAd\(^+\) (not shown). In addition, CCL21\(^+\) blood vessels were also common outside organized lymphoid structures, where they constituted 85 ± 10% (RAST) and 66 ± 6% (UC) of vWF\(^+\) vessels. In contrast, CCL21\(^+\) vessels made up only 16.6 ± 0.5% of vWF\(^+\) vessels in psoriasis (\(p < 0.05\) compared with RAST and UC, one-way ANOVA), an inflammatory skin disease, in which lymphoid neogenesis does not commonly occur (33).

In addition to endothelial cells, a strong positive signal for CCL21 mRNA and protein was also detected in many stromal cells in UC and, especially, RAST (Fig. 5). These cells were HLA-DR\(^+\), but most did not express the dendritic cell (DC) marker CD11c (not shown).

Naive T cells are present in RAST and UC

We next examined the relationship between CCL21 expression and the presence of naive T cells in these diseases. Anti-CD45RA in combination with anti-CD3 was used to detect naive T cells by immunostaining of frozen sections. Within ectopic lymphoid tissue in RAST and UC, densely packed CD3\(^+\) T cells were observed (data not shown). Strikingly, CD45RA\(^+\) CD3\(^+\) cells were abundant in these tissues, even outside organized lymphoid follicles where they were frequently located in small clusters around a central blood vessel (Figs. 5, C–E, and 6, A and B). At these sites, CD45RA\(^+\) cells constituted 14.8 ± 6.1% and 6.0 ± 1.7% of the total CD3\(^+\) T cell population in RAST and UC, respectively. In contrast, naive T cells were rarely found in psoriasis (Figs. 6C and 7), where 0.5 ± 0.5% of CD3\(^+\) T cells expressed CD45RA (\(p < 0.05\) vs RAST and UC). CCL21 expression in blood vessels correlated positively with the influx of naive T cells (Fig. 7C).

It should be cautioned that CD45RA is also expressed on recently activated CD8\(^+\) cytotoxic effector cells in humans (24, 34). Thus, it is possible that some of the CD45RA\(^+\) CD3\(^+\) cells in human tissue samples were not naive. To address this question, we also examined CD45RA expression on CD4\(^+\) cells, because Ag-experienced CD4\(^+\) T cells are thought to be more uniformly...
CD45RA− (24). These studies revealed that 46 ± 7% and 27 ± 5% of the CD45RA+CD3+ cells were also CD4+ in RAST and UC, respectively, confirming the correlation between CCL21 expression and the recruitment of naive T cells, at least for the CD4+ subset (data not shown).

Discussion

In the present report, we show that the chemokines CCL21, CCL19, and CXCL12 have striking differences in their potency to recruit naive T cells when exogenously applied to nonlymphoid tissues. CCL21, but not CXCL12, was capable of attracting GFP+CD44low cells in cremaster muscle venules and in s.c. air pouches. CCL19 had similar effects as CCL21 in the cremaster muscle model, but did not lead to significant accumulation of naive T cells in air pouches. Concordantly, we demonstrate that CCL21 protein and mRNA are expressed in PNAdvascular endothelial cells in RA and UC, two diseases in humans with which lymphoid neogenesis is associated. CCL21 expression on blood vessels correlated with the presence of naive T cells in the inflammatory infiltrate. In contrast, blood vessels in psoriasis, in which lymphoid neogenesis is not usually observed, were largely CCL21−, and naive T cells were sparse. These findings indicate that blood vessel-expressed CCL21 may participate in the formation of ectopic lymphoid tissue by attracting T lymphocytes.

Some common autoimmune diseases, including RA, multiple sclerosis, Hashimoto’s thyroiditis, diabetes mellitus, and chronic inflammatory bowel diseases, as well as some infectious diseases are associated with the accumulation of lymphocytes and DCs in target organs, resulting in the de novo formation of organized lymphoid tissue (reviewed in Ref. 35). Although it has been suggested that these LN-like structures causally contribute to the perpetuation of autoimmune diseases, relatively little is known about the molecular mechanisms that lead to their formation. In particular, homing pathways of lymphocytes into these tissues have yet to be characterized.

Naive T cells draining from chronically inflamed skin have been observed in sheep, and PNAd+ venules have been demonstrated at the site of inflammation (36). Moreover, expression of PNAd and/or MAdCAM-1 on HEV-like vessels is commonly found in lymphoid neogenesis (reviewed in Ref. 4). These vessels represent a likely port of entry for naive T cells in chronically inflamed tissues. However, naive T cell traffic via this route could only occur at relatively late stages, i.e., after HEV have formed. An alternative scenario would be that, at least in the early course of chronic inflammation, naive T cells gain access to the periphery via regular blood vessels that do not (yet) express HEV-specific vascular addressins. This hypothesis is supported by the fact that inflamed venules in many vascular beds up-regulate L-selectin ligands, which are distinct from PNAd and MAdCAM (5, 6). Moreover, transgenic approaches have shown that the continuous presence of CCL21 during embryonal development, and postnatally, leads to the recruitment of naive T cells, which are indispensable for ectopic lymphoid tissue formation (10, 11, 12), indicating that their recruitment may precede, and, in fact, induce HEV formation. Thus, although HEV are likely to facilitate lymphocyte influx, their presence may be a consequence of, rather than a prerequisite for, lymphoid neogenesis.

Although the observations discussed above highlight the role of T lymphocytes, including naive T cells, in chronic inflammatory diseases, the recruitment mechanisms that act on different T cell subsets under pathologic conditions are incompletely understood. Similarly, although it is widely accepted that naive T cells are excluded

![Figure 6](image1.png)

**FIGURE 6.** Naive T cells infiltrate in RAST and UC, but not psoriasis. Frozen sections of RAST (A), UC (B), and psoriasis (C) were analyzed for the presence of naive T cells in the inflammatory infiltrate. Naive T cells were identified by simultaneous staining for CD3 (green) and CD45RA (red) and appear yellow in merged images. Blood vessels were identified by staining for vWF (blue). Original magnification: ×400.

![Figure 7](image2.png)

**FIGURE 7.** The expression of CCL21 on blood vessels correlates with the influx of naive T cells in chronic inflammatory diseases. Serial sections of tissue samples from psoriasis, RA, and UC were immunostained as described in Figs. 5 and 6, and analyzed for the percentage of CCL21+ vessels among total vWF-high blood vessels (A), and the percentage of CD45RA+ cells in the total CD3+ T cell population (B). *p < 0.05, one-way ANOVA. C, The number of CCL21+ blood vessels was plotted against CD45RA+ T cells. Symbols represent results from individual samples of RA (○), UC (□), and psoriasis (■).
from nonlymphoid tissues under normal and acute inflammatory conditions, the molecular mechanisms behind this exclusion have yet to be examined. To set a baseline for the adhesion behavior of naive T cells in nonlymphoid organs, we used our well-established T-GFP model (14). T-GFP mice are especially suited to address this question, because >95% of GFP<sup>high</sup> cells in the peripheral blood of young animals are of a naive phenotype, i.e., they are CD44<sup>low</sup>, express high levels of CCR7 (Fig. 1), and are readily detectable by intravital microscopy. We used the cremaster muscle as a model because the endothelial traffic molecules involved in leukocyte accumulation in that tissue have been defined in detail by intravital microscopy (26, 27). Thus, the unavoidable surgical trauma associated with tissue preparation induces sequential expression of endothelial adhesion molecules. Initially, P-selectin is up-regulated, which mediates leukocyte rolling during the first hour. Thereafter, endothelial L-selectin ligands also contribute to rolling (26). We found that ~35% of GFP<sup>high</sup> cells interact with venules in the cremaster muscle in a largely L-selectin-dependent manner. The two major known endothelial L-selectin ligands, PNAd and MadCAM-1 (3, 28), are not expressed in acutely inflamed cremaster muscle venules, and the molecular nature of L-selectin ligands in the periphery remains to be identified.

T cell-endothelial cell interactions in nonlymphoid organs in vivo were studied previously by intravital microscopy using CD2-EGFP transgenic mice, in which enhanced GFP (EGFP) was expressed under the control of the CD2 promoter (37). In these mice both naive and effector/memory T cells express EGFP. A small subset of CD8<sup>+</sup> T cells (~1% of circulating leukocytes) expressed high levels of EGFP, permitting in vivo detection. These cells rolled in an α<sub>i</sub>-integrin-dependent manner in TNF-α and IFN-γ-treated cremaster muscle venules. Because all naive T cells and central memory cells in T-GFP mice are GFP<sup>+</sup>, whereas CD8<sup>+</sup> EGFP<sup>high</sup> cells in CD2-EGFP mice were probably composed of Ag-experienced effector/memory cells which might have downregulated L-selectin expression, the present findings and the observations reported in CD2-EGFP mice do not contradict each other.

Interestingly, we found a partial reduction in the rolling fraction of GFP<sup>+</sup> cells after injection of blocking Abs against P-selectin. The protein backbone of the principal P-selectin counterreceptor, P-selectin glycoprotein ligand (PSGL)-1, is expressed by all leukocytes, but requires extensive posttranslational glycosylation and sulfation to be functional (38). Highly functional (i.e., soluble P-selectin binding) lymphocyte-expressed PSGL-1 is primarily observed on effector Th1 cells and CTL, but is normally absent on naive T cells in vitro (15, 39). However, the present in vivo findings are consistent with earlier observations that activated platelets bind up to 50% of human and murine peripheral blood T cells in a P-selectin-dependent manner (40), indicating that functional, but presumably low affinity and/or density, P-selectin ligands are expressed on many naive T cells. Alternatively, the modest effect of anti-P-selectin could have been indirect, e.g., by blocking the adhesion of GFP<sup>−</sup> leukocytes, such as neutrophils, which express functional L-selectin ligands on their surface. Thus, anti-P-selectin and anti-L-selectin might have additionally interfered with so-called secondary tethering, where L-selectin on naive T cells interacts with PSGL-1 on leukocytes that are already bound to the endothelium in a P-selectin-dependent fashion (41, 42).

Numerous in vivo and in vitro studies have shown that rolling leukocytes, including naive T cells, only arrest when integrins are functionally activated by a chemoattractant (reviewed in Ref. 2). Although naive T cells express α<sub>i</sub>-integrins and LFA-1 which mediate effective sticking in HEV (3, 28), they failed to arrest in the cremaster muscle and to migrate into s.c. air pouches, even under severe, TNF-α-induced inflammatory conditions. However, accumulation of GFP<sup>+</sup> cells could be provoked by exogenous application of CCL21 in both the cremaster muscle and air pouch models. It should be cautioned that ~5% of GFP<sup>high</sup> cells in peripheral blood of young mice display a memory cell phenotype (CD44<sup>high</sup>). These cells also express CCR7 (Fig. 1) and, hence, resemble central memory T cells (24). Thus, we cannot formally exclude that a disproportionally high fraction of the GFP<sup>+</sup> cells that arrested in response to CCR7 ligands in cremaster muscle venules were Ag-experienced. However, phenotypic analysis of cells isolated from air pouches in response to CCL21 revealed the presence of GFP<sup>high</sup>CD44<sup>low</sup> cells. This finding argues strongly that the mere presence of CCL21 is indeed capable of attracting naive T cells to the periphery. These results are also consistent with our previous demonstration that the intracutaneous injection of CCL21 or CCL19 can transiently restore the defect in T cell homing in plt/plt mice (21, 32).

The influx of naive T cells into s.c. air pouches in response to CCL21 is in apparent contrast to a recent report showing absence of lymphoid tissue formation in KCCL21 mice with transgenic expression of CCL21 in the epidermis under the control of the keratin-14 promoter (11). A possible explanation for this phenomenon could be that transgenic expression of CCL21 in KCCL21 mice by a large number of keratinocytes may result in high systemic levels of CCL21, which might have desensitized circulating naive T cells. Such a phenomenon has been observed in mice with transgenic expression of CCL2 (monocyte chemoattractant protein-1/03E) under the control of the mouse mammary tumor long-term repeat (43). Given these considerations, the phenotype of KCCL21 mice is not necessarily in conflict with our current observations. Whether the same adhesion mechanisms as observed in cremaster muscle venules apply for homing of naive T cells to air pouches remains to be determined.

The differential effects of CCL21 and CCL19 on naive T cell accumulation in the air pouch model were surprising, given that both chemokines induced firm adhesion of GFP<sup>+</sup> cells in cremaster muscle venules. Moreover, we have previously shown that s.c. injected CCL19 and CCL21 are similarly transported to and across LN HEV, where both are capable of reconstituting naive T cell recruitment in plt/plt mice (21, 32). One has to consider that the LN represents a highly specialized microenvironment, where lymph-borne chemokines reach HEV via the fibroblastic reticular cell conduit (44). Such a mechanism may not exist for skin microcirculation, which could conceivably result in differential transport of the two chemokines to the vascular lumen. Alternatively, it is also possible that both CCL19 and CCL21 triggered integrin activation, but only CCL21 may be able to provide additional signals necessary for the subsequent transmigration of naive T cells into the pouch cavity. The molecular mechanisms behind this striking difference remain to be identified.

In contrast to CCL21, CXCL12 was incapable of inducing firm adhesion and accumulation of GFP<sup>+</sup> cells in the cremaster muscle and the air pouch model, respectively. This failure of CXCL12 to induce recruitment of naive T cells was unexpected, because immobilized CXCL12 is as efficient at inducing integrin activation on rolling T cells in vitro as CCL21 (45). Moreover, addition of CXCL12 to the luminal surface of endothelial monolayers in flow chamber experiments resulted in retention of this chemokine on the endothelial surface and efficient T cell recruitment (46). However, our data are consistent with the recent observation that transgenic expression of CXCL12 in pancreatic islets, in contrast to
CCL21 and CCL19, results in little accumulation of T cells (12). Thus, CXCL12 apparently has different effects on naive T cell recruitment in vitro and in vivo. However, we cannot exclude that the concentration of CXCL12 on the luminal surface of the endothelium under the conditions used were insufficient to mediate the arrest of fast rolling naive T cells (despite the fact that they induced sticking of Ag-experienced T cell populations; L. Scimone and U. H. von Andrian, unpublished observation).

Previous studies in mouse models have demonstrated that lymphoid neogenesis is associated with CCL21 expression on HEV-likes vessels (13, 47). In addition, the expression of CCL21 protein on endothelial cells in RAST, chronic inflammatory liver disease, and certain autoimmune skin diseases has been reported recently (48–50). However, in these studies the exact nature of endothelial cells, i.e., whether they represented HEV, was not determined. The present work extends these findings by showing expression of CCL21 protein not only in PNA+ HEV-like vessels, but also in PNA– vWF+/PALS– lymph vessel endothelium, in two autoimmune diseases, RA and UC. In addition, we demonstrate by in situ hybridization that blood vessel endothelium expresses CCL21 mRNA. This finding is of importance, as endothelial cells can pick up chemokines from surrounding tissues, which can be further transcytokted to the luminal surface (21, 32). We also show that CCL21 expression on blood vessels positively correlated with the presence of CD45RA+ T cells in the inflammatory infiltrate.

What might be the pathophysiologic consequences of ectopic CCL21 expression in the course of autoimmune diseases? Is the acquisition of CCL21 production by endothelial cells a mere epiphenomenon of the inflammatory process, or does it represent a critical step in the process of lymphoid neogenesis? The guidance of T cells and APC to T cell areas in secondary lymphoid organs is tightly controlled by constitutively expressed chemokines (2, 51). In particular, the entry of naive T cells from the bloodstream into PLN is mediated by HEV-displayed CCL21 (and probably CCL19). Stromal cells express high amounts of CCL21 in the T cell zone in murine PLN (52). From this it has been suggested that these stromal cells may establish migrational “corridors” where both CCR7+ naive T cells and CCR7+ DC are brought together by the high local concentration of CCL21, and so can establish physical interactions which are requisite for T cell activation (44, 52). Consequently, CCR7 and its ligands are key elements in the spatial and temporal regulation of naive T cell priming. Hence, CCL21 expression by blood vessels might indeed attract CCR7+ naive and central memory T cells to goal organs in certain autoimmune diseases. Our demonstration of CCL21 mRNA and protein expression by stromal cells in RAST and UC suggests that in analogy to secondary lymphoid organs, T cells and APC might come into physical contact and, consequently, autoactive naive T cells may be primed directly at the site of inflammation. Interestingly, activation of naive CD4+ T cells induces LTAββββ expression on their surface (12, 53, 54). LTAββββ is a major factor in the formation of organized lymphoid tissues (55, 56). Thus, a plausible scenario for the development of lymphoid neogenesis could be that it is initiated by de novo presentation of CCL21 on endothelial cells, which, as we show in this study, triggers the influx of naive T cells. When naive T cells become stimulated, they provide a rich source of LTAββββ, which would further enhance the expression of CCL21 and other lymphocyte trafficking molecules, thus establishing a positive feedback loop.

Taken together, our data provide a number of strong arguments for the role of endothelium-expressed CCL21 in lymphoid neogenesis: 1) naive T cells can roll via L- and P-selectin, but are incapable of sticking on acutely inflamed endothelium in cremaster muscle; 2) the presence of CCL21 or CCL19, but not CXCL12, in cremaster muscle is sufficient to trigger integrin activation and sticking of naive T cells in this nonlymphoid site; 3) only the presence of CCL21, but not CCL19 or CXCL12, in air pouches leads to extravasation of naive T cells within 8 h, a period that is much shorter than the >6 days needed for the development of HEV in the pouch wall induced by chronic inflammatory stimuli (57); 4) lymphoid neogenesis in transgenic mice is inducible by chronic overexpression of CCL21 in the absence of other inflammatory stimuli, but is dependent upon (presumably CCL21-mediated) T cell recruitment (10); 5) in human RA, UC, and psoriasis, naive T cell accumulation correlates both quantitatively and spatially with the frequency and localization of CCL21+ microvessels; and 6) the presence of CD8+ T cells in rheumatoid synovitis in a human synovium-SCID chimera model was necessary for germinal center formation and maintenance (58). These findings suggest that CCL21 expression by blood vessels is an important early event in the formation of lymphoid tissue in tertiary organs, leading to the influx of naive T cells and possibly other CCR7+ lymphocytes.

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