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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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Abbreviations used in this paper: PP, Peyer’s patch; 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; DlG, 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 addressins. Consistent with this finding, we found that CCL21 is expressed on PNA⁺ and PNA⁻ blood vessels in human diseases accompanied by lymphoid neogenesis. Importantly, the presence of naive T cells in perevitalis 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 intrinsic 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 2-A4 (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 mAb MECA-79 (rat IgM), and anti-LFA-1 mAb Tib 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 (HI100; BD Biosciences, Mountain View, CA); anti-human CD3, anti-human von Willebrand factor (vWF), and anti-human CD6 (DR25; DAKO, Carpinteria, CA); anti-human CD4 (MAT-4; Medscand Diagnostics, Malmö, Sweden); anti-human CCL21 (R&D Systems, Minneapolis, MN); and anti-human mAb PAL-E (Research Diagnostics, Flanders, NJ). Mouse mAb RIV9 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 (EB11 ligand chemokine/macroage-inflamatory 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-buffreed 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 ìg of mAb (9A9, 5H1, Mel-14, Tib-213, or 2-A4) 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 intracranially 2–3 h before the preparation.

The rolling fraction for each individual venule was determined as the percentage of leukocytes that transmigrated from the bloodstream to the vessel 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 (V) of individual rolling (Vrolling, at least 10 consecutive cells per venule) and noninteracting (Vnonr; 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 a mouse as described (19). After 6 days, PBS alone or PBS containing TNF-α (500 ng), CXCL12, CCL19 (1 or 2 ìg), or CCL21 (2 ìg) 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 ìm) 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 Committee 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 second- 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 vWF⁺ (or PAL-E⁺) blood vessels (×400 magnification). This was performed in agreement with the Helsinki Declaration and was approved by the Regional Committee 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 second- and third-step reagents was performed as described previously (20).
magnification, area = 5 × 10^4 μm²; median number of counted CD3⁺ cells/section: 288, range 200–488). Areas with densely packed lymphocytes within lymphoid aggregates were excluded from this evaluation, because unequivocal differentiation of CD45RA⁺ cells was not possible due to overlap of the fluorescent signal emitted by adjacent cells. First, CD3⁺ cells (green channel) and then CD45RA⁻/CD3⁺ cells (green and red channels) were counted on digitized pictures and expressed as the percentage of CD45RA⁻/CD3⁺ cells among total CD3⁺ 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 were compared using ANOVA. The effect of TNF-α on rolling velocities was analyzed using the Mann-Whitney U test. Accumulation of naive and central memory T cells in air pouches in response to chemokines and TNF-α was analyzed using ANOVA. Significance was assumed at p < 0.05.

Results

Phenotype of circulating T cells in 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 intravitreal 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 ≥5 days) induces complete loss of GFP in >90% of CD8⁺ and ~50% of CD4⁺ effector cells (14, 15). However, we also found that in vitro-generated CD8⁺ T cells with phenotypic and functional properties of central memory cells (24) maintain GFP expression, even after adoptive transfer in vivo (15, 25). Because it is not possible during intravitreal microscopy experiments to determine whether a GFP⁺ cell is naive or Ag-experienced, it was important to fully characterize GFP⁺ cells in the peripheral blood of T-GFP mice.

In agreement with previous results (14), all GFP⁺ cells were CD3⁺, and naive T cells (CD3⁺CD44low/⁻) were uniformly GFPbright, 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 forward/side light scatter (left plot). Nearly all CD44low/⁻ T cells were analyzed by three-color flow cytometry (right plot) and were identified by expression of CD3 after gating on live lymphocytes by forward/side light scatter (left plot).](image-url)
(88 ± 3%), a ligand for CCR7 (15). CD44<sup>high</sup> effector/memory cells fell in three separate categories: GFP<sup>-</sup>, GFP<sup>low</sup>, and GFP<sup>high</sup> (Fig. 1A). Because GFP<sup>-</sup> cells escape detection by intravital microscopy, we focused on the two GFP<sup>+</sup> subsets. Most GFP<sup>high</sup>CD44<sup>high</sup> 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<sup>low</sup>CD44<sup>high</sup> cells (R1 in Fig. 1B) was L-selectin<sup>-</sup> (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<sup>+</sup>CD44<sup>high</sup> subset, which we have shown previously to be of effector memory phenotype (25). CD44<sup>high</sup>GFP<sup>low</sup> cells constituted 1.0 ± 0.1% and 4.0 ± 0.6%, and CD44<sup>high</sup>GFP<sup>high</sup> 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<sup>+</sup> cells were CD44<sup>high</sup> memory cells.

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

Having established that the vast majority (~95%) of detectable (i.e., GFP<sup>high</sup>) 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<sup>+</sup> cells below the velocity of noninteracting GFP<sup>+</sup> cells in the same vessel. The baseline rolling fraction of GFP<sup>+</sup> cells in cremaster muscle venules (n = 46

![FIGURE 2.](http://www.jimmunol.org/) **GFP<sup>+</sup> T cells roll in cremaster muscle venules via L- and P-selectin.** Rolling of GFP<sup>+</sup> cells in the cremaster muscle of T-GFP mice was studied by epifluorescence intravital microscopy. A. The rolling fraction (the percentage of rolling cells in the total flux) is shown in venules of untreated cremaster muscles as a function of vessel diameter. B. Effect of anti-selectin mAbs (50 μg/mouse) on naive T cell rolling. The rolling index (rolling fraction after mAb injection/rolling fraction before mAb injection) was significantly reduced by anti-P- and L-selectin, but not anti-E-selectin (Wilcoxon signed rank test). C. Pretreatment (2–3 h) of cremaster muscle preparations with TNF-α (500 ng intrascrotally) had no effect on the frequency of GFP<sup>+</sup> T cell rolling, but (D) reduced the median rolling velocity (<i>V</i><sub>roll</sub>) in cumulative velocity curves. <i>V</i><sub>roll</sub> was measured for at least 10 consecutive leukocytes per venule; n = total number of cells/venules/mice. Values were compared using the Mann-Whitney U test and Kolmogorov Smirnov test. Bars in B and C represent mean ± SEM.
venules/12 animals) was 34 ± 2% and did not correlate with the venular diameter (Fig. 2A). To determine which adhesion molecules mediated naive T cell rolling in this setting, we compared the frequency of rolling GFP+ cells before and after i.v. injection of neutralizing mAbs (Fig. 2B). 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-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 microvascularity

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+ cells (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 adherence 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 adherence of these cells, suggesting that the chemokine...
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>+</sup>CD44<sup>+</sup>GFP<sup>+</sup> naive T cells (R3 in Fig. 4A) or CD44<sup>+</sup>CD44<sup>+</sup>GFP<sup>+</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 ~5-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>+</sup>CD44<sup>+</sup>GFP<sup>+</sup> naive T cells recovered from air pouches after the second injection, counted, and analyzed by flow cytometry. Representative FACS profiles (A) of migrated cells reveal the distinct ability of CCL21, but not TNF-α, to recruit GFP<sup>+</sup> T cells. Lymphocyte gates and regions (R1–3) were set based on subpopulation profiles described in Fig. 1 and used for enumerating migrated T cell subsets. B. Although TNF-α treatment enhanced the total number of leukocytes in air pouches, nearly all of the responsive cells were GFP<sup>+</sup>. C. Neither TNF-α nor CCL21 significantly increased the number of CD44<sup>+</sup>CD44<sup>+</sup>GFP<sup>+</sup> effector memory (R1, R3) cells. In contrast, CCL21 increased the absolute number (D) of CD44<sup>+</sup>CD44<sup>+</sup>GFP<sup>+</sup> naive (R3) and CD44<sup>+</sup>CD44<sup>+</sup>GFP<sup>+</sup> central memory (R2) cells in air pouches. E. In contrast to CCL21, neither CCL19 nor CXCL12 induced significant accumulation of CD44<sup>+</sup>CD44<sup>+</sup>GFP<sup>+</sup> (naive, R3) cells in air pouches. Symbols represent the number of naive T cells recovered from the air pouch of each individual mouse. *, p < 0.05, n.s., not significant.

**FIGURE 4.** Accumulation of naive T cells in air pouches in response to CCL21. A. s.c. air pouch was induced in the back of T-GFP mice. Six days later, PBS, TNF-α (100 ng in PBS), CCL21 (2 μg in PBS), CCL19, or CXCL12 (1 or 2 μg) was injected twice (at 0 and 4 h) in each of six to eight mice/group. Cells that had migrated into the air pouch were collected 4 h after the second injection, counted, and analyzed by flow cytometry. Representative FACS profiles (A) of migrated cells reveal the distinct ability of CCL21, but not TNF-α, to recruit GFP<sup>+</sup> T cells. Lymphocyte gates and regions (R1–3) were set based on subpopulation profiles described in Fig. 1 and used for enumerating migrated T cell subsets. B. Although TNF-α treatment enhanced the total number of leukocytes in air pouches, nearly all of the responsive cells were GFP<sup>+</sup>. C. Neither TNF-α nor CCL21 significantly increased the number of CD44<sup>+</sup>CD44<sup>+</sup>GFP<sup>+</sup> effector memory (R1, R3) cells. In contrast, CCL21 increased the absolute number (D) of CD44<sup>+</sup>CD44<sup>+</sup>GFP<sup>+</sup> naive (R3) and CD44<sup>+</sup>CD44<sup>+</sup>GFP<sup>+</sup> central memory (R2) cells in air pouches. E. In contrast to CCL21, neither CCL19 nor CXCL12 induced significant accumulation of CD44<sup>+</sup>CD44<sup>+</sup>GFP<sup>+</sup> (naive, R3) cells in air pouches. Symbols represent the number of naive T cells recovered from the air pouch of each individual mouse. *, p < 0.05, n.s., not significant.
FIGURE 5. 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 C3D (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), and C3D (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: ×400 (A–D and F–I), ×600 (E).

only weakly expressed or absent in lymphatic vessels (29). Normal col on (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

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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 PNAd vascular 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](http://www.jimmunol.org/) 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](http://www.jimmunol.org/) 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+ 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 naïve 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 naïve 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, PNA<sub>d</sub> 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 naïve 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>4</sub> integrin-dependent manner in TNF-α and IFN-γ-treated cremaster muscle venules. Because all naïve T cells and central memory T 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 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 CTLs, but is normally absent on naïve 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 naïve 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 naïve 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 naïve T cells, only arrest when integrins are functionally activated by a chemoattractant (reviewed in Ref. 2). Although naïve T cells express α<sub>4</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 naïve 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). They further suggest that the lack of the appropriate chemokine in the periphery is a rate-limiting factor for naïve T cell arrest, and that the failure to adhere can be overcome by the application or induction of endothelial presentation of CCL21.

The influx of naïve 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 naïve T cells. Such a phenomenon has been observed in mice with transgenic expression of CCL2 (monocyte chemoattractant protein-1/α) under the control of the mouse mammary tumor long-terminal 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 naïve T cells to air pouches remains to be determined.

The differential effects of CCL21 and CCL19 on naïve 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 naïve 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 naïve 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 adherence 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 naïve 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-like 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^+ /PAL-E^+ blood vessels with flat 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 transcytosed 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 prerequisite for T cell activation (44, 52). Consequently, CCR7 and its ligands are key elements in the multistep model of leukocyte-endothelial cell interactions in inflammation: distinct roles for L-selectin and integrins αβ2 and LFA-1 in lymphocyte homing to Peyer’s patch HEV in situ: the multistep model confirmed and refined. Immunity 3:99.


