Rapid Acquisition of Tissue-specific Homing Phenotypes by CD4+ T Cells Activated in Cutaneous or Mucosal Lymphoid Tissues

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Abstract

Effector and memory T cells can be subdivided based on their ability to traffic through peripheral tissues such as inflamed skin and intestinal lamina propria, a property controlled by expression of 'tissue-specific' adhesion and chemoattractant receptors. However, little is known about the development of these selectively homing T cell subsets, and it is unclear whether activation in cutaneous versus intestinal lymphoid organs directly results in effector/memory T cells that differentially express adhesion and chemoattracant receptors targeting them to the corresponding nonlymphoid site. We define two murine CD4+ effector/memory T cell subsets that preferentially localize in cutaneous or intestinal lymphoid organs by their reciprocal expression of the adhesion molecules P-selectin ligand (P-lig) and α4β7, respectively. We show that within 2 d of systemic immunization CD4+ T cells activated in cutaneous lymph nodes upregulate P-lig, and downregulate α4β7, while those responding to antigen in intestinal lymph nodes selectively express high levels of α4β7 and acquire responsiveness to the intestinal chemokine thymus-expressed chemokine (TECK). Thus, during an immune response, local microenvironments within cutaneous and intestinal secondary lymphoid organs differentially direct T cell expression of these adhesion and chemoattractant receptors, targeting the resulting effector T cells to the inflamed skin or intestinal lamina propria.

Key words: T cell • homing • chemokine • adhesion • lymph node

Introduction

To maximize the chances of antigen encounter, naive lymphocytes recirculate through specialized secondary lymphoid organs such as the spleen, lymph nodes, and Peyer's patches (PP), which efficiently trap, concentrate, and present antigens. However, effector lymphocytes often function in nonlymphoid sites of inflammation and infection. Moreover, effector and memory lymphocytes generally display selective tropism for specific peripheral tissues such as the skin or intestinal lamina propria (ILP; references 1 and 2).

Lymphocyte homing from blood into tissues is mediated by a series of sequential interactions between the lymphocyte and the vascular endothelium in specialized postcapillary venules (1). Tissue specificity is imparted by the use of different combinations of adhesion and chemoattractant receptors at distinct anatomical sites. For example, high level expression of α4β7 integrin, whose ligand mucosal addressin cell adhesion molecule (MAdCAM)-1 is expressed on postcapillary venules in the ILP, targets one population of memory T cells to this site (3, 4). In addition, a subset of these intestinal α4β7hi cells also expresses CCR9 (5, 6), the specific receptor for the chemokine thymus-expressed chemokine (TECK), which is expressed by small intestinal epithelium (6). In contrast, memory and effector cell recruitment to inflamed skin requires expression of lymphocyte surface ligands for vascular selectins, either P- or E-selectin in the mouse (7).

Little is known about the development of these tissue-specific lymphocyte subsets. Expression of specific homing phenotypes may be directed by unique microenvironments within cutaneous versus intestinal secondary lymphoid organs during initial T cell activation (8). Alternatively, homing and chemoattractant receptors may be upregulated stochastically in secondary lymphoid organs and subject to selection during effector and memory cell recirculation by survival of cells trafficking through antigen-rich tissues (1,

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9). Indeed, even the kinetics with which lymphocytes acquire tissue-specific tropism during the primary immune response remains unexplored.

We have examined the distribution of selectin binding CD4$^+$ T cells in vivo and found that expression of P-selectin ligand (P-lig) and $\alpha 4\beta 7$ integrin define largely nonoverlapping subsets of CD4$^+$ memory T cells which differentially localize in cutaneous and intestinal lymphoid tissues, respectively. Using an adoptive transfer system, we've shown that within 2 d of immunization, cells responding in intestinal mesenteric lymph nodes (MLNs) begin to express high levels of the integrin $\alpha 4\beta 7$ and gain TECK responsiveness, while those activated in subcutaneous peripheral lymph nodes (PLNs) upregulate surface ligands for P-selectin. Importantly, these phenotypic changes occur when antigen–specific cells remain sequestered within secondary lymphoid organs, before their reentry into the recirculating lymphocyte pool. Thus, we conclude that responding T cells are rapidly programmed within secondary lymphoid organs to express tissue-specific homing receptors expected to target them to distinct nonlymphoid tissues.

Materials and Methods

Mice and Adoptive Transfers.   BALB/c and DO11.10xRAG2$^{-/-}$ mice were bred and housed at the Veterans Administration Hospital in Palo Alto, CA. Mice used in experiments shown in Figs. 1 and 2 were >1 y old. For all other experiments, mice were 1–3 mo old. BALB/c mice were given between 5–10 × 10^6 erythrocyte-depleted splenocytes from sex-matched DO11.10xRAG-2$^{-/-}$ animals by retroorbital injection 24 h before immunization. For 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) labeling, cells were incubated for 10 min at 37°C in HBSS (5 × 10^6 cells per milliliter) containing 500 nM CFSE (Molecular Probes).

Immunizations.   Mice were immunized by intraperitoneally injection of 100 ug LPS (*Escherichia coli* serotype 055:B5; Sigma-Aldrich) alone or with 500 ug OVA (Sigma-Aldrich) in 200 ul PBS. Peripheral blood was collected at the indicated times after immunization by retroorbital bleeding. MLNs and PLNs (pooled inguinal and brachial) were harvested 2 d after immunization and disaggregated between frosted slides.

Flow Cytometry.   The following directly conjugated mAbs were used: FITC-anti-CD45RB (clone 16A; BD PharMingen); APC-anti-CD4 (clone RM4–5; BD PharMingen); PE-anti-$\alpha 4\beta 7$ (clone DATK32; BD PharMingen); PE-anti-IFN-γ (clone XMG1.2; BD PharMingen); FITC-anti–IL-4 (clone BVD6–24G2; Caltag); and a P-selectin-IgM fusion protein was used to stain for P-lig (10). Second and third step reagents used were biotin-goat anti–human IgM (Caltag) and CyChrome-streptavidin (BD PharMingen).

Intracellular Cytokine Analysis.   Lymphocytes were stained for expression of P-lig and CD4 and stimulated for 4 h with PMA (50 ng/ml; Sigma-Aldrich) plus ionomycin (1 ug/ml; Sigma-Aldrich) in DMEM containing 10% bovine calf serum and monensen (10 ug/ml; Sigma-Aldrich). Stimulated cells were fixed and permeabilized with the cytofix-cytoperm kit (BD PharMingen) according to the manufacturer's instructions and stained with anticytokine antibodies.

*Chemotaxis.*   Transwell chemotaxis assays, and calculation of percent migration for flow cytometry defined populations based on their frequency in migrated and input cells was performed essentially as described previously (11). Four replicate wells were used per chemokine in each experiment. Chemokines used were as follows: 100 nM mouse IFN-inducible protein (IP)-10 (Peprotech EC); and 250 nM mouse TECK (R&D Systems).

Results

$\alpha 4\beta 7$ and P-lig Define Memory CD4$^+$ T Cell Subsets. To determine if intestinal and cutaneous lymph nodes were enriched in memory T cells displaying homing receptors expected to target them to the corresponding peripheral tissue, we examined $\alpha 4\beta 7$ and functional selectin ligand expression on naive and memory CD4$^+$ T cells isolated from murine spleen, PLNs, MLNs, and PP. We used >1-y-old mice for this analysis due to their increased frequency of memory T cells (similar results were obtained in young mice; data not shown), and focused on functional P–lig expression because E-selectin binding T cells were much less frequent than P-selectin binding memory cells in mouse lymphoid tissues and are reported to be a subset of P-selectin binding cells in the mouse (7). As expected, naive CD4$^+$CD45RB^{hi} cells from all tissues examined were uniformly $\alpha 4\beta 7^{intermediate}$ and P–lig$^-$. By contrast, $\alpha 4\beta 7$ and P-lig expression were heterogeneous on memory cells, and were more or less mutually exclusive, clearly defining three memory T cell subsets (Fig. 1). Moreover, these subsets were present in different proportions in the various lymphoid organs examined. While both $\alpha 4\beta 7^{hi}$ and P-lig$^+$ populations were present in the spleen and PLNs, few P-lig$^+$ cells were located in the PP and MLNs, and the $\alpha 4\beta 7$hi population was dramatically enriched in these tissues (Fig. 1 and Table I). Thus, P-lig and $\alpha 4\beta 7$ define nonoverlapping subsets of CD4$^+$ memory T cells that preferentially localize in cutaneous and intestinal lymphoid tissues, respectively.

After in vitro activation and polarization, P-lig is expressed on Th1 but not Th2 CD4$^+$ T cells (12). Since intestinal lymphoid tissues have been reported to preferentially induce Th2 cell development (13), this provides a potential explanation for the lack of P–lig$^+$ cells in the MLNs and PP. To address this experimentally, we isolated cells from lymphoid tissues, and examined P-lig expression in conjunction with intracellular cytokine analysis. Surprisingly, Th1 (IFN-γ^+IL-4$^-$) and Th2 (IFN-γ-IL-4$^+$) cells were present in similar proportions in all these lymphoid tissues (Fig. 2 A). Additionally, P-lig was expressed by the majority of Th1, Th2, and Th0 (IFN-γ^+IL-4$^+$) cells in the PLNs, but was present on a much smaller fraction of these populations from the intestinal MLNs and PP (Fig. 2 B). Therefore, in contrast to models suggested by in vitro studies, expression of P-lig does not distinguish Th1 and Th2 cells in vivo, nor can selective Th1 expression account for the paucity of P-lig$^+$ T cells in the intestinal lymphoid tissues.

T Cells Rapidly Acquire Tissue-specific Homing Properties after Activation in Intestinal versus Cutaneous Lymphoid Organs.   Specialized microenvironments within intestinal and cutaneous secondary lymphoid organs may induce or select for patterns of adhesion and chemoattractant receptor
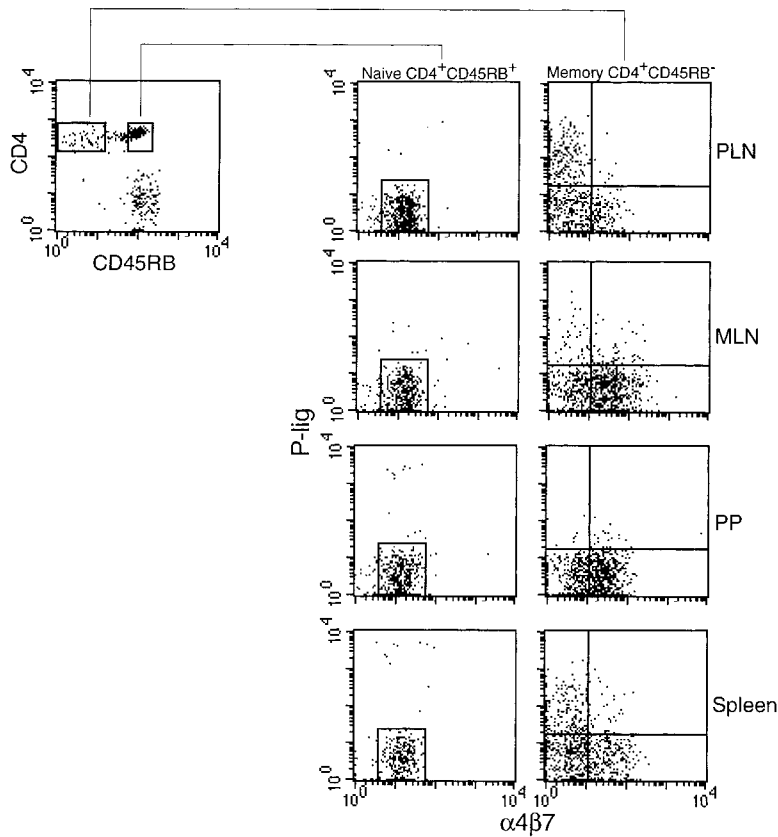
Figure 1. α4β7 and P-lig define three CD4+ memory T cell populations that differentially localize in intestinal and cutaneous lymphoid organs. (Left) CD4 and CD45RB expression by lymphocytes isolated from the cutaneous PLNs of a >1-y-old BALB/c mouse. The gates used to define 'naive' (CD4+CD45RB+) and 'memory' (CD4+CD45RB−) T cells are shown. (Right) α4β7 and P-lig expression by gated naive and memory CD4+ T cells isolated from the indicated lymphoid tissues of a >1-y-old BALB/c mouse.

expression that target effector T cells to the appropriate nonlymphoid tissues. However, the low frequency of antigen-specific T cells during the early stages of the immune response has precluded any direct examination of this process in vivo. To boost the frequency of antigen-specific T cells to detectable levels, we employed an adoptive transfer system in which naive OVA-specific TCR transgenic T cells are injected into nontransgenic hosts and tracked with the clonotype-specific mAb KJ1–26 (14).

Adoptive transfer recipients were immunized with a single intraperitoneal injection of OVA+LPS. LPS was chosen as an adjuvant for its known proinflammatory effects and its ability to potentiate immune responses against soluble proteins. Consistent with the 'antigen-trapping' of T cells in secondary lymphoid tissues (15), the antigen-specific KJ1–

26+ T cells disappeared from the recirculating pool of T cells in the blood within 1 d of immunization, where they were not detected again in significant numbers until day 3 (Fig. 3 A). Cell division analysis using CFSE-labeled transgenic cells reveals that during this period of sequestration from the blood, antigen-specific T cells were activated and proliferating in both subcutaneous PLNs and MLNs, where by day 2 after immunization most cells in both tissues had divided ∼2–4 times (Fig. 3 B). Thus, this adoptive transfer and immunization protocol provides a unique opportunity to compare the surface phenotype of antigen-specific CD4+ T cells activated in the PLNs or MLNs, before these populations have intermingled through effector cell recirculation. Accordingly, we analyzed expression of P-lig and α4β7 on antigen-specific T cells 2 d after immunization

Table I. *The Distribution of P-lig and α4β7 Among Gated Memory T Cells in Secondary Lymphoid Tissues*

Tissue	Percentage of P-lig+α4β7−	Percentage of P-lig−α4β7+	Percentage of P-lig−α4β7−	Percentage of P-lig+α4β7+
PLNs	37.1 ± 3.5	18.9 ± 1.6	42.7 ± 3.0	1.3 ± 0.7
MLNs	4.3 ± 0.7	62.3 ± 4.4	29.0 ± 5.8	4.5 ± 1.7
PP	1.2 ± 0.2	63.5 ± 5.5	32.6 ± 5.9	2.7 ± 0.5
Spleen	17.4 ± 2.0	29.2 ± 4.8	50.3 ± 5.5	3.1 ± 1.3

Percentage of CD4+CD45RB− cells isolated from the PLNs, MLNs, PP or spleen with the indicated surface phenotype (defined by quadrant gate shown in Fig. 1). Data are mean and SD of values obtained from three age-matched (>1-y-old) BALB/c mice.
MLN, mesenteric LN; PLN, peripheral LN; PP, Peyer's patches.
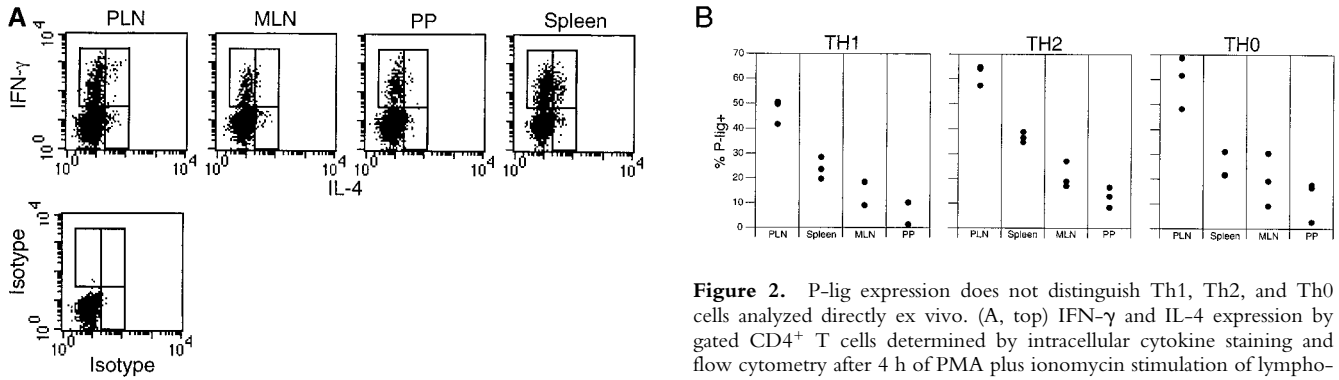
Figure 2. P-lig expression does not distinguish Th1, Th2, and Th0 cells analyzed directly ex vivo. (A, top) IFN-γ and IL-4 expression by gated CD4^{+} T cells determined by intracellular cytokine staining and flow cytometry after 4 h of PMA plus ionomycin stimulation of lymphocytes isolated from the indicated organs. (Bottom) Staining of stimulated lymphocytes from PLNs with isotype control antibodies. (B) Percentage of P-lig^{+} cells among gated Th1, Th2, and Th0 CD4^{+} T cells isolated from the indicated organs. Each data point represents a measurement taken from an individual animal. A total of three >1-y-old BALB/c mice were analyzed.
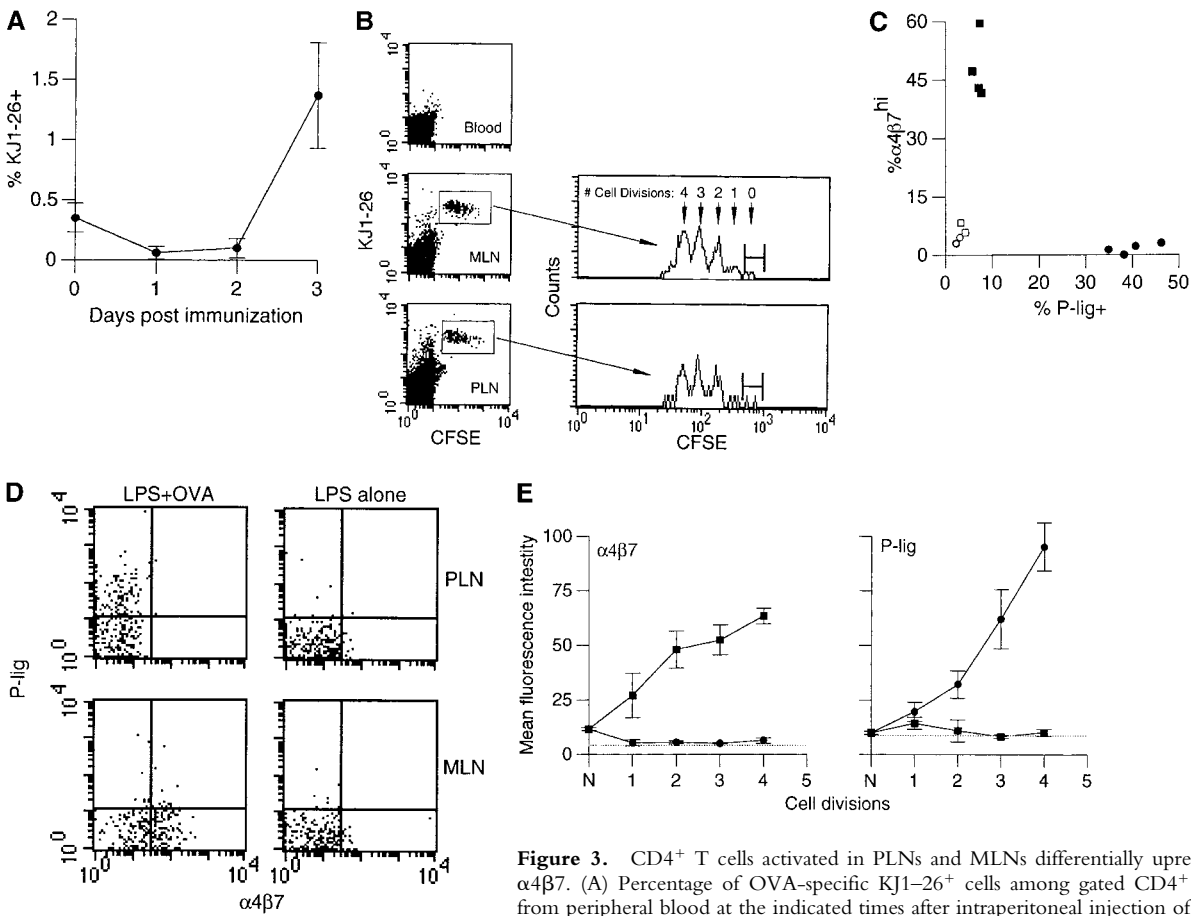
Figure 3. CD4^{+} T cells activated in PLNs and MLNs differentially upregulate P-lig and α4β7. (A) Percentage of OVA-specific KJ1–26^{+} cells among gated CD4^{+} T cells isolated from peripheral blood at the indicated times after intraperitoneal injection of OVA plus LPS. Data are mean and SD of values obtained from five mice at each time point. (B) Representative flow cytometry data of cellular CFSE content and KJ1–26 staining on gated CD4^{+} T cells isolated from the indicated tissues 2 d after intraperitoneal immunization of DO11.10 adoptive transfer recipients with OVA plus LPS. The horizontal marker in the histograms represents the CFSE fluorescence intensity of naive CD4^{+}KJ1–26^{+} T cells isolated from animals immunized with LPS alone (data not shown). (C) Expression of α4β7 and P-lig by gated CD4^{+}KJ1–26^{+} cells isolated from PLNs (● and ○) and MLNs (■ and □) 2 d after intraperitoneal immunization of DO11.10 adoptive transfer recipients with OVA plus LPS (black symbols) or LPS alone (white symbols). Each data point represents a measurement from an individual animal. (D) Representative flow cytometry data of α4β7 and P-lig staining on gated CD4^{+}KJ1–26^{+} cells isolated from PLNs (top) and MLN (bottom) 2 d after immunization of DO11.10 adoptive transfer recipients with OVA plus LPS (left) or LPS alone (right). The quadrant gate used to define the P-lig^{+} and α4β7^{hi} populations in C is indicated. (E) Mean fluorescence intensity (MFI) of α4β7 (left) and P-lig (right) staining on gated CD4^{+}KJ1–26^{+} cells isolated from the MLNs (■) or PLNs (●) 2 d after intraperitoneal immunization of DO11.10 adoptive transfer recipients with OVA plus LPS as a function of cell division (as determined by CFSE content). Data are mean and SE of values obtained from four (α4β7) or five (P-lig) mice. N represents the MFI of α4β7 or P-lig staining on naive cells isolated from animals immunized with LPS alone. Dotted lines represent background MFI of cells stained with an isotype control (left) or unstained cells (right).

during this period of sequestration in the lymph nodes. While cells isolated from animals immunized with LPS alone maintained their naive phenotype, we found that even at this very early time point a major fraction of the cells activated in the MLNs expressed high levels of $\alpha4\beta7$ (30–60%), whereas P-lig was preferentially expressed by a similar proportion of cells activated in subcutaneous PLNs (Fig. 3 C and D). CFSE analysis was also used to examine $\alpha4\beta7$ integrin and P-lig expression as a function of cell division (Fig. 3 E). Cells that had undergone the same number of divisions in the PLNs or MLNs still displayed differential expression of P-lig and $\alpha4\beta7$, and the distinct homing phenotypes of these cells cannot therefore be accounted for by subtle differences in the extent of T cell activation and proliferation in these tissues.

Chemokines are also thought to contribute to tissue-specific lymphocyte homing. Outside of the thymus, the chemokine TECK is expressed selectively by epithelial cells of the small intestine, where it is believed to participate in the homing or retention of lymphocytes in the ILP and intestinal epithelium (6). Accordingly, a subset of circulating $\alpha4\beta7^{hi}$ memory T cells and nearly all lymphocytes isolated from the ILP and intestinal epithelium of human jejunum express the TECK receptor CCR9 (5, 6). Therefore, we examined the TECK responsiveness of antigen-specific T cells activated in the MLNs or PLNs 2 d after systemic OVA immunization. In parallel with their specific expression of $\alpha4\beta7$, only the cells activated in the MLNs migrated to TECK in this assay, whereas cells activated in both locations responded to the chemokine IP-10, which is broadly expressed at many inflammatory sites (reference 16; Fig. 4). Thus, naive CD4$^+$ T cell activation in cutaneous or intestinal lymphoid tissues results in the rapid induction or selection of effector cells displaying patterns of adhesion and chemoattractant receptors expected to target them to inflamed skin or the ILP, respectively.

Discussion

Several experimental observations have suggested that the site of antigen presentation dictates the resulting homing phenotype of the effector and memory T cells generated.

First, lymphocyte blasts isolated from intestinal and cutaneous lymphoid organs or lymphatics preferentially home or recirculate through their tissue of origin in adoptive recipients (17, 18). Second, antigen-specific T cells isolated from human blood after oral or subcutaneous immunization differentially express the intestinal homing receptor $\alpha4\beta7$ (19). Third, CD45RA$^+$CD45RO$^+$ 'transitional' T cells isolated from human tonsils and appendix display differential homing receptor expression (8). Finally, experiments in mice and humans have shown that functional T cell memory for enteric or cutaneous antigens is contained in $\alpha4\beta7^{hi}$ and E-selectin ligand$^+$ populations, respectively (20, 21). However, none of these studies directly addressed the developmental and/or homeostatic mechanisms that give rise to tissue-specific effector and memory T cell subsets. In this study, we directly visualize CD4$^+$ T cells during the naive to effector transition, and find that the tissue-selective homing receptors $\alpha4\beta7$ and P-lig are selectively and rapidly upregulated on cells activated in intestinal and cutaneous lymphoid tissues, respectively. Further, we show that responsiveness to the intestinal chemokine TECK is upregulated by activated T cells exclusively in intestinal but not subcutaneous lymphoid organs. Most importantly, T cells acquire these homing phenotypes within 2 d of activation, before they leave their site of initial antigen encounter, ruling out the possibility that these phenotypes are randomly or stochastically induced and selected during successive rounds of recirculation in antigen-rich peripheral tissues.

During activation and differentiation in secondary lymphoid organs, CD4$^+$ T cells integrate TCR, costimulatory and cytokine signals to make fate decisions, ultimately resulting in the generation of effector and memory cells. One well-studied fate decision made by CD4$^+$ T cells is the Th1 versus Th2 decision, in which effector cells become either proinflammatory IFN-γ producing Th1 cells or antiparasitic IL-4 producing Th2 cells (22). Initially, several adhesion and chemokine receptors, including P-lig, were described as either Th1- or Th2-specific based on expression by cells polarized during in vitro activation (12). However, data presented in Fig. 2 and elsewhere (23, 24) have shown that many of these associations are not observed on Th1 and Th2 cells examined directly ex vivo, therefore it ap-
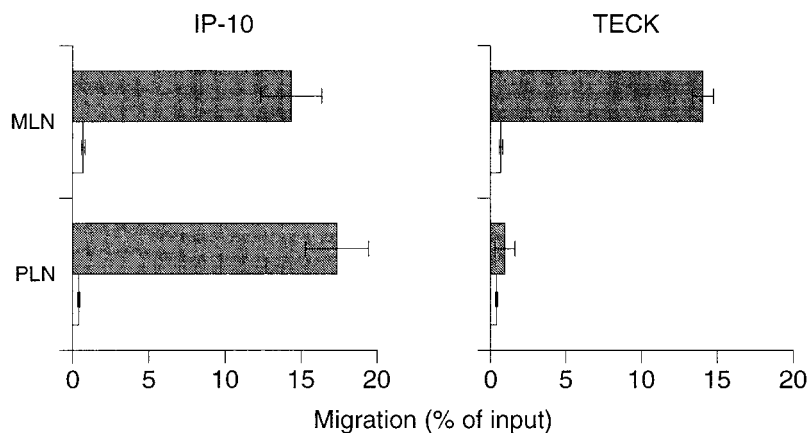
Figure 4. CD4$^+$ T cells activated in PLNs or MLNs differentially respond to the intestinal chemokine TECK. Migration of CD4$^+$KJ1–26$^+$ cells isolated from the MLNs or PLNs 2 d after intraperitoneal immunization of DO11.10 adoptive transfer recipients with OVA plus LPS to medium alone (white bars) or to the indicated chemokines (gray bars). Data are mean and SD of multiple measurements taken in one experiment, which is representative of 4.

pears that homing receptor expression can be regulated independently of the Th1 versus Th2 decision. Interestingly, another tissue-dependent lymphocyte fate decision is that made by antigen-specific B cells, which selectively undergo isotype switching to IgA in mucosal lymphoid tissues (25). Our results demonstrate that the local lymphoid microenvironment controls not only these elements of lymphocyte effector specialization but also imprint or select specific tissue homing properties.

The molecular nature of the signals that direct expression of tissue-specific homing receptors in vivo remain unknown. However, the rapid acquisition of tissue-specific homing properties in secondary lymphoid organs suggests that these phenotypes may be induced during the initial T cell interaction with tissue-derived APC. Accordingly, several phenotypic and functional differences between intestinal and cutaneous APC have been described previously (26). These different APC, by delivering unique sets of costimulatory and cytokine signals, may therefore help direct expression of the appropriate adhesion and chemoattractant molecules. Of particular interest is the histological observation that subsets of dendritic cells in intestinal PP selectively express the $\alpha4\beta7$ ligand MAdCAM-1 (27). It is therefore tempting to speculate that this MAdCAM-1 expression in intestinal lymphoid organs may contribute in some way to the selective upregulation of $\alpha4\beta7$ on T cells stimulated in these tissues.

The ability to target effector lymphocytes to specific tissues helps increase the efficiency of pathogen clearance and prevents pathologic inflammation at uninvolved sites. Additionally, since many pathogens display selective tissue tropism, tissue-specific recirculation of memory lymphocytes increases their likelihood of a second antigen encounter, ensuring a robust anamnestic response. In this paper, we have for the first time visualized the development of distinctive homing receptor–defined T cell subsets in vivo, demonstrating that this process occurs very quickly after antigen-recognition during primary responses within intestinal and skin-associated secondary lymphoid organs. As inflammatory and autoimmune diseases generally result from the infiltration of peripheral tissues by pathogenic lymphocytes, further discerning the mechanisms that target effector lymphocytes is critical for understanding the etiology of these diseases and is likely to yield novel therapeutic targets.

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