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Intrinsic Differences in L-Selectin Expression Levels Affect T and B Lymphocyte Subset-Specific Recirculation Pathways[1]

Mimi L. K. Tang,[2] Douglas A. Steeber,[2] Xiu-Qin Zhang, and Thomas F. Tedder[3]

Lymphocyte migration into lymphoid organs is regulated by tissue-specific adhesion molecules such as L-selectin and the $\alpha_4\beta_7$ integrin. Whether L-selectin also regulates lymphocyte subset-specific migration into specific lymphoid tissues was examined in this study by comparing the migration of CD4$^+$ T cells, CD8$^+$ T cells, and B cells from L-selectin-deficient and wild-type mice. T cells were the predominant lymphocyte subset entering PLN, MLN, Peyer's patches, and spleen during short term (1-h) migration assays. However, both B cell and CD4$^+$ and CD8$^+$ T cell entries into PLN, MLN, and Peyer's patches were dramatically impaired (73–98%) by loss of L-selectin. Lymphocyte expression of $\alpha_4\beta_7$ integrin did not compensate for the loss of L-selectin, since both B and T cells predominantly migrated into the spleen in the absence of L-selectin. The more efficient migration of T cells into peripheral lymphoid tissues relative to that of B cells was partly explained by the finding that T cells expressed L-selectin at 50 to 100% higher levels than B cells. In addition, a 50% reduction in L-selectin expression by lymphocytes from hemizygous L-selectin$^{+/-}$ mice resulted in a 50 to 70% decrease in short term lymphocyte migration into peripheral lymphoid tissues relative to that of wild-type lymphocytes. Thus, the differential migration of T and B lymphocyte subsets to lymphoid tissues is regulated in part by subset-specific differences in L-selectin expression levels. *The Journal of Immunology,* **1998, 160: 5113–5121.**

Lymphocyte migration to peripheral lymph nodes (PLN),[4] mesenteric lymph nodes (MLN), and Peyer's patches is mediated by lymphocyte L-selectin interacting with specific ligands on high endothelial venules (HEV) in these tissues (1–4). The $\alpha_4\beta_7$ integrin also expressed by lymphocytes interacts with mucosal HEV determinants to mediate lymphocyte migration into Peyer's patches and MLN (5–7). Migration of lymphocytes into PLN, MLN, and Peyer's patches is reduced markedly in L-selectin-deficient mice (4, 8–10), while lymphocyte migration into MLN and Peyer's patches is diminished markedly in β_7 integrin-deficient mice (11). These tissue-specific lymphocyte recirculation pathways establish a framework for understanding lymphocyte migration into individual lymphoid tissues, since most blood-derived T and B cells express both L-selectin and $\alpha_4\beta_7$ (12). However, the preferential migration of lymphocyte subsets into specific peripheral lymphoid tissues suggests that subset-specific mechanisms regulate the extraction of lymphocyte subsets into given tissues rather than the mere presence or absence of tissue-specific lymphocyte-endothelial adhesion receptors (12, 13).

Studies of lymphocyte migration in sheep indicate that different lymphocyte subsets have selective affinities for individual lymphoid tissue vascular beds. Specifically, CD4$^+$ cells appear to enter PLN from the blood via HEV more efficiently than other lymphocyte subsets (13–16). Similarly, CD4$^+$ cells reportedly recirculate much more efficiently than CD8$^+$ cells under physiologic conditions in vivo (13, 16–18). Based on the different migration properties of CD4$^+$ cells, CD8$^+$ cells, and B cells, the existence of subset-specific lymphocyte-endothelial cell recognition systems has been postulated (13–17, 19–21). Studies in humans and rats have also indicated subset-specific differences in lymphocyte migration (22–26).

Studies in mice further support the existence of lymphocyte subset-specific adhesion mechanisms that regulate the differential entry of lymphocyte subsets into lymphoid tissues. In mice, T cells distribute preferentially to PLN, whereas B cells are reported to distribute preferentially to Peyer's patches and spleen (27–29). Similar proportions of CD4$^+$ and CD8$^+$ cells migrate into PLN, although CD4$^+$ T cells migrate into Peyer's patches more efficiently than CD8$^+$ T cells (29). The different migration characteristics of T and B cells are evident regardless of whether the cells are from spleen, PLN, MLN, or Peyer's patches (27). Although previous studies of L-selectin-deficient mice have documented L-selectin's role in tissue-specific lymphocyte migration into secondary lymphoid tissues (4, 8–10), such studies have not evaluated whether the absence of L-selectin reduces the migration of all lymphocyte populations or only the migration of a specific lymphocyte subset(s). The finding that the tissue distribution of CD4$^+$ cells and memory T cells is affected more severely than that of other lymphocyte subsets in L-selectin-deficient mice (8) suggests that L-selectin could be involved in lymphocyte subset-specific migration. Therefore, the role of L-selectin in the regulation of lymphocyte subset migration was examined in the current study. Our findings demonstrate that CD4$^+$ and CD8$^+$ T cells have the highest propensity to migrate into PLN, MLN, Peyer's patch, and spleen and that L-selectin regulates this migration. Furthermore, intrinsic differences in expression levels of cell surface L-selectin

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[4] Abbreviations used in this paper: PLN, peripheral lymph node; MLN, mesenteric lymph node; HEV, high endothelial venule; L-selectin$^{-/-}$, L-selectin deficient; PE, phycoerythrin; R_i, the ratio of calcein-labeled test cells to PKH26-labeled internal control cells injected into mice for migration assays; R_o, the ratio of calcein-labeled test cells to PKH26-labeled internal control cells within each tissue after migration assays.

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were found to regulate stoichiometrically the differential migration of T and B cell subsets into lymphoid tissues.

Materials and Methods

Animals

L-selectin$^{-/-}$ mice were generated as previously described (4) and backcrossed onto the C57BL/6 background for seven to nine generations. Lack of cell surface L-selectin expression was verified by immunofluorescence staining of blood leukocytes with a FITC-conjugated anti-mouse L-selectin mAb (30). All mice used were 2 mo of age and were housed in a specific pathogen-free barrier facility. Control mice were age-matched wild-type mice generated from heterozygous breedings of L-selectin$^{+/-}$ mice or were C57BL/6 mice purchased from The Jackson Laboratory (Bar Harbor, ME). Equivalent results were obtained for both groups of control mice, and both groups are referred to subsequently as wild-type mice. All studies and procedures were approved by the animal care and use committee of Duke University Medical Center (Durham, NC).

Lymphocyte isolation, mAbs, and flow cytometry

Blood was aspirated from the retro-orbital venous plexus of anesthetized mice. Single-cell suspensions from spleen, PLN (bilateral axillary, inguinal, and brachial nodes were pooled), MLN (superior mesenteric cords were pooled), and Peyer's patches were prepared as previously described (8) and filtered through nylon gauze to remove debris before washing twice in PBS. Erythrocytes in splenocyte suspensions and peripheral blood were lysed with 0.83% ammonium chloride solution or 2% acetic acid, respectively. Total cell numbers were enumerated using a hemocytometer.

Abs used in these studies included unconjugated and FITC-, phycoerythrin (PE)-, or biotin-conjugated mAbs reactive with L-selectin (LAM 1-116 mouse mAb) (30), $\alpha_4\beta_7$ (DATK-32, American Type Culture Collection, Rockville, MD) (31), Thy1.2 (5a-8; Caltag, San Francisco, CA), B220 (RA3-6B2; Caltag), CD4 (L3T4; PharMingen, San Diego, CA), and CD8 (53-6.72; PharMingen). Secondary Abs used were FITC-conjugated goat anti-rat IgG (Southern Biotechnology Associates, Birmingham, AL) and streptavidin PerCP (Becton Dickinson, San Jose, CA). Isotype-matched rat IgG Abs (PharMingen) were used as controls.

Expression of L-selectin or $\alpha_4\beta_7$ by lymphocyte subsets was assessed by two-color fluorescence cytometry. Single cell suspensions of lymphocytes were incubated either with unconjugated DATK-32 mAb followed by FITC-conjugated goat anti-rat IgG secondary Ab or with FITC-conjugated LAM1–116 mAb. Cells labeled with DATK-32 mAb and goat anti-rat IgG secondary Ab were incubated with PBS containing 5% normal rat serum to block secondary Ab binding sites. Subsequently, all cell preparations were stained with PE-conjugated Abs against Thy1.2, CD4, CD8, or B220. Mean log fluorescence intensities of L-selectin and $\alpha_4\beta_7$ expressed by gated T cells and B cells were then determined by flow cytometry.

Concurrent expression of $\alpha_4\beta_7$ and L-selectin on T and B cell subsets was examined by three-color fluorescence cytometry. Single cell suspensions were incubated sequentially with DATK-32 mAb, PE-conjugated goat anti-rat IgG Abs, 5% normal rat serum as a blocking agent, FITC-conjugated LAM1-116 mAb, and biotinylated mAbs against CD4, CD8, Thy1.2, or B220, followed by streptavidin PerCP. Concurrent L-selectin and $\alpha_4\beta_7$ expression was determined for gated subsets of PerCP-labeled lymphocytes by flow cytometric analysis.

In all cases, after blood leukocytes were labeled, the erythrocytes were lysed with a Coulter wholeblood Immuno-Lyse kit according to the manufacturer's instructions (Coulter, Miami, FL). For phenotype experiments, 10,000 cells with the forward and side light scatter properties of mononuclear cells were analyzed on a FACScan flow cytometer (Becton Dickinson) with fluorescence intensity shown on either a 3-decade (Figs. 3 and 4) or a 4-decade (Fig. 2) log scale. Fluorescence contours are shown as 50% log density plots.

In vivo migration assays

For single-color migration assays, single-cell suspensions of splenocytes from wild-type or L-selectin$^{-/-}$ mice were labeled with calcein-AM (Molecular Probes, Eugene, OR) as previously described (32). Briefly, cells ($5–10 \times 10^7$) were incubated in 2 ml of RPMI 1640 medium (Life Technology, Gaithersburg, MD) containing 1 μM calcein-AM on ice for 30 min, with gentle mixing every 5 min. Cells were then washed twice in PBS, counted, and resuspended at 1×10^8 cells/ml in PBS. In most experiments, calcein-labeled splenocytes ($1–4 \times 10^7$ cells in 250–400 μl) were injected into the lateral tail vein of individual wild-type mice. At the appropriate times, single-cell suspensions of tissues were prepared, and aliquots of cells were labeled with PE-conjugated Abs against Thy1.2, B220, CD4, or CD8. Five thousand calcein-labeled cells with the forward and side light scatter properties of mononuclear cells were analyzed by flow cytometry, although for some tissues smaller numbers of L-selectin-deficient cells were collected for analysis because of decreased numbers of migrating cells within these tissues. The total number of calcein-labeled cells recovered from individual lymphoid tissues was determined by multiplying the total cell counts for individual tissues by the frequency of labeled cells. The percentage of injected calcein-labeled cells that migrated to individual tissues was then determined.

Two-color migration experiments were performed as previously described (4, 8). Test cells (either L-selectin$^{+/-}$ or wild-type splenocytes) were labeled with 1 μM calcein-AM as described above. Internal control wild-type splenocytes were labeled with PKH26 (Sigma, St. Louis, MO). Briefly, splenocytes (5×10^7) were resuspended in 1 ml of PKH26 diluent, immediately added to an equal volume of a 3-μM PKH26 dye solution, and allowed to incubate at room temperature for 2 min. Labeling was stopped by the addition of 2 ml of FCS. Cell suspensions were washed twice with PBS, counted, and then stored on ice. PKH26-labeled cells (2×10^7) and calcein-labeled cells (2×10^7) were mixed in a total volume of 400 μl for injection into individual wild-type mice. An aliquot of the injected cell mixture was also analyzed by flow cytometry to calculate the injected ratio of calcein- to PKH26-labeled cells (R_i). After 1 h, single-cell suspensions of tissues were prepared, and the percentages of calcein- and PKH26-labeled cells were determined by flow cytometric analysis. A minimum of 5000 PKH26-labeled cells were collected for each sample. The ratio of calcein-/PKH26-labeled cells within tissues or blood (R_o) was calculated, and results were expressed as the ratio of R_o/R_i in each tissue, as previously described (4, 8).

Statistical analysis

All data are shown as the mean ± SEM, unless otherwise indicated. Paired comparisons between groups were conducted using Student's *t* test.

Results

Short term migration of T and B cells from L-selectin$^{-/-}$ mice

The role of L-selectin in the migration of T and B cells from the circulation into lymphoid tissues was examined in short term (1-h) in vivo migration assays. Calcein-labeled splenocytes from L-selectin$^{-/-}$ or wild-type mice were injected into the tail veins of individual wild-type mice. Although the spleens of L-selectin-deficient mice are larger than the spleens of wild-type mice, the distribution of lymphocyte subsets remains relatively unchanged in spleens of 2-mo-old L-selectin-deficient mice (4, 8, 33). Since the relative number of T and B cells varied in each donor mouse, the effect of injecting varying numbers of splenocytes ($1–4 \times 10^7$) on T and B cell migration was assessed. Increasing the number of injected splenocytes beyond $\geq 2 \times 10^7$ did not have a significant effect on the number or the relative frequency of T and B cells migrating into tissues in 1-h migration assays (data not shown). Therefore, the number of splenocytes injected into mice was adjusted whenever possible to provide similar frequencies of T and B cells within samples of cells that were being compared directly.

The greatest numbers of injected wild-type splenocytes were recovered from the spleen (8.8 ± 1.1% of injected cells) after 1 h, with lower numbers in PLN (0.38 ± 0.08%), MLN (0.41 ± 0.07%), and Peyer's patches (0.03 ± 0.01%; Table I and Fig. 1*A*). T cells were the predominant lymphocyte population migrating into PLN, MLN, Peyer's patches, and spleen (Fig. 1, *B* and *C*). Similar relative percentages of injected T and B cells were found in the blood. The relative differences in numbers of T and B cells migrating into tissues compared with the numbers of T and B cells injected indicated that T cells were 17 ± 2, 9 ± 2, and 4 ± 1 times more likely than B cells to migrate into PLN, MLN, and Peyer's patches, respectively.

Compared with that of splenocytes from wild-type mice, the migration of L-selectin$^{-/-}$ cells into PLN, MLN, and Peyer's patches was significantly reduced ($p < 0.05$; Fig. 1*A* and Table I). The percentages of injected L-selectin$^{-/-}$ T cells that migrated into PLN, MLN, and Peyer's patches were reduced by 98, 97, and 77%, respectively, compared with those of wild-type cells (Fig.

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Table I. *Number and frequency of calcein-labeled lymphocytes migrating into secondary lymphoid tissues*[a]

	Total Cells ($\times 10^{-3}$)		T Cells ($\times 10^{-3}$)		B Cells ($\times 10^{-3}$)	
	Wild type	L-selectin$^{-/-}$	Wild type	L-selectin$^{-/-}$	Wild type	L-selectin$^{-/-}$
Short term (1 h)						
PLN	130 ± 25	3.2 ± 0.8†	120 ± 23	2.7 ± 0.7†	6.6 ± 2.2	0.2 ± 0.1*
	(2.00 ± 0.38)[c]	(0.04 ± 0.01)†	(1.80 ± 0.35)	(0.03 ± 0.01)†	(0.08 ± 0.02)	(0.002 ± 0.001)†
MLN	140 ± 24	6.3 ± 1.4†	110 ± 23	4.4 ± 1.1†	14 ± 5	1.2 ± 0.3*
	(1.82 ± 0.32)	(0.08 ± 0.01)†	(1.59 ± 0.32)	(0.06 ± 0.01)†	(0.16 ± 0.04)	(0.016 ± 0.003)†
Peyer's patches	11 ± 2	3.4 ± 0.8*	7.6 ± 1.5	2.2 ± 0.6†	2.2 ± 0.7	0.3 ± 0.1*
	(1.11 ± 0.25)	(0.27 ± 0.05)*	(0.71 ± 0.19)	(0.19 ± 0.04)*	(0.18 ± 0.05)	(0.035 ± 0.01)*
Blood[b]	300 ± 44	220 ± 46	120 ± 22	120 ± 24	130 ± 29	73 ± 24
	(6.18 ± 0.91)	(4.74 ± 0.84)	(2.71 ± 0.46)	(2.75 ± 0.49)	(2.76 ± 0.58)	(1.49 ± 0.44)
Spleen	3,100 ± 410	4,100 ± 900	1,800 ± 210	2,900 ± 610*	950 ± 200	790 ± 250
	(5.50 ± 0.66)	(5.04 ± 1.02)	(3.39 ± 0.47)	(3.76 ± 0.02)	(1.66 ± 0.37)	(0.49 ± 0.22)
Injected	34,000 ± 2,500	31,000 ± 2,400	18,000 ± 1,400	19,000 ± 1,000	13,000 ± 1,400	8,900 ± 1,300
Long term (48 h)						
PLN	430 ± 63	5.4 ± 0.7†	370 ± 52	4.9 ± 0.6†	46 ± 14	0.3 ± 0.1†
MLN	610 ± 140	60 ± 14†	480 ± 115†	42 ± 10†	75 ± 16	12 ± 4†
Peyer's patches	39 ± 10	29 ± 6	13 ± 4	8.9 ± 3.1	21 ± 4	17 ± 3
Blood	220 ± 32	280 ± 48	110 ± 11	160 ± 23	110 ± 20	110 ± 24
Spleen	2,500 ± 270	5,400 ± 840†	1,500 ± 200	4,000 ± 750†	800 ± 93	1,100 ± 220
Injected	35,000 ± 2,200	35,000 ± 2,200	18,000 ± 1,900	19,100 ± 760	14,000 ± 1,300	12,000 ± 2,000

[a] Calcein-labeled splenocytes from L-selectin$^{-/-}$ or wild-type mice ($n \geq 6$ each) were injected into individual recipient mice. After 1 or 48 h, single-cell suspensions of tissues from recipient mice were labeled with PE-conjugated mAbs against Thy1.2 or B220, and the percentage of calcein-labeled lymphocytes that were Thy1.2$^+$ T cells or B220$^+$ B cells was determined by two-color fluorescence cytometry. Absolute numbers of cells were then calculated from individual tissue cell counts and are expressed as means ± SEM.

[b] Mean values for blood are given as cells/ml.

[c] Mean frequency of calcein-labeled lymphocytes within each tissue.

* Differences between L-selectin$^{-/-}$ and wild-type lymphocytes were significant, $p<0.05$; †$p<0.01$.

1*B*). L-selectin$^{-/-}$ B cell migrations into PLN, MLN, and Peyer's patches were reduced by 95, 86, and 80%, respectively, compared with those of wild-type B cells (Fig. 1*C* and Table I). Thus, the migrations of both T and B cells into PLN, MLN, and Peyer's patches at 1 h are predominantly dependent on L-selectin expression.

The rate of lymphocyte entry into tissues was also assessed by determining the proportion of calcein-labeled cells in each tissue after 1-h migration assays. These determinations also compensated for any size differences between tissues, since they are a function of the number of cells recovered from each tissue sample. T cells migrated into PLN, MLN, Peyer's patches, and spleen at 22-, 10-, 4-, and 2-fold higher frequencies than B cells, respectively (Table I). The rate of T lymphocyte migration into PLN and MLN was almost double the rate of entry into Peyer's patches. By contrast, the rate of B lymphocyte migration into PLN was almost half the rate of entry into MLN and Peyer's patches. Nonetheless, the loss of L-selectin expression reduced the rates of T cell entry into PLN, MLN, and Peyer's patches by 98, 96, and 73%, respectively. Similarly, the loss of L-selectin expression reduced the rates of B cell entry into PLN, MLN, and Peyer's patches by 98, 90, and 81%, respectively. Therefore, migration of both lymphocyte subsets was dependent on L-selectin expression.

Long term migration of T and B cells from L-selectin$^{-/-}$ mice

The role of L-selectin in the accumulation of calcein-labeled splenocytes in lymphoid tissues was examined in long term (48 h) in vivo migration assays. The greatest numbers of injected wild-type lymphocytes were recovered from the spleen (7.2 ± 1.0%), with smaller numbers from PLN (1.2 ± 0.2%), MLN (1.7 ± 0.4%), and Peyer's patches (0.12 ± 0.04%; Fig. 1*D* and Table I). The relative differences in numbers of T and B cells migrating into tissues compared with the numbers of T and B cells injected indicated that T cells localized 9 ± 1 and 6 ± 1 times more efficiently than B cells in PLN and MLN, respectively (Table I). By contrast, more B cells accumulated in Peyer's patches than T cells (T/B ratio = 0.5 ± 0.1; Table I). Similar frequencies of injected T and B cells were found in the blood, but more T cells than B cells accumulated in the spleen (Fig. 1, *E* and *F*). Therefore, T cells localized predominantly in PLN, MLN, and spleen, while B cells were preferentially retained in Peyer's patches relative to T cells.

The loss of L-selectin dramatically impaired both T and B cell (99% decrease) localization in PLN at 48 h compared with that of wild-type splenocytes (Fig. 1, *E* and *F*, and Table I). The percentage of injected L-selectin$^{-/-}$ lymphocytes that localized in MLN was reduced by approximately 93% ($p < 0.01$) for T cells and by approximately 77% ($p < 0.01$) for B cells relative to that of their wild-type counterparts (Fig. 1, *E* and *F*). There was only a 38% reduction in the percentage of injected L-selectin$^{-/-}$ T cells recovered from Peyer's patches, while localization of L-selectin$^{-/-}$ and that of wild-type B cells were equivalent (Fig. 1, *E* and *F*). Localization of both T cells and B cells in the spleen was increased by loss of L-selectin, although significantly ($p < 0.01$) more L-selectin$^{-/-}$ T cells migrated to the spleen than B cells. Therefore, loss of L-selectin expression severely altered both T cell and B cell localization, with T cells being affected to a greater extent than B cells.

Migration of CD4$^+$ and CD8$^+$ T cells into secondary lymphoid tissues

The role of L-selectin in CD4$^+$ and CD8$^+$ T cell migration to lymphoid tissues was examined 1 and 48 h after injection of L-selectin$^{-/-}$ or wild-type splenocytes. There was no difference in the migration of wild-type CD4$^+$ and CD8$^+$ cells at either time point, except CD4$^+$ cells migrated approximately twofold faster than CD8$^+$ cells to Peyer's patches in 1-h migration assays ($p < 0.05$; Table II). PLN and MLN migrations were markedly reduced (>96%) for both CD4$^+$ and CD8$^+$ T cells by the loss of L-selectin in 1-h migration assays (Table II). Peyer's patch migration of CD4$^+$ and CD8$^+$ T cells was also significantly reduced (76–81%) by L-selectin loss. After 48 h, the lack of L-selectin expression reduced both CD4$^+$ and CD8$^+$ cell localization in PLN and MLN

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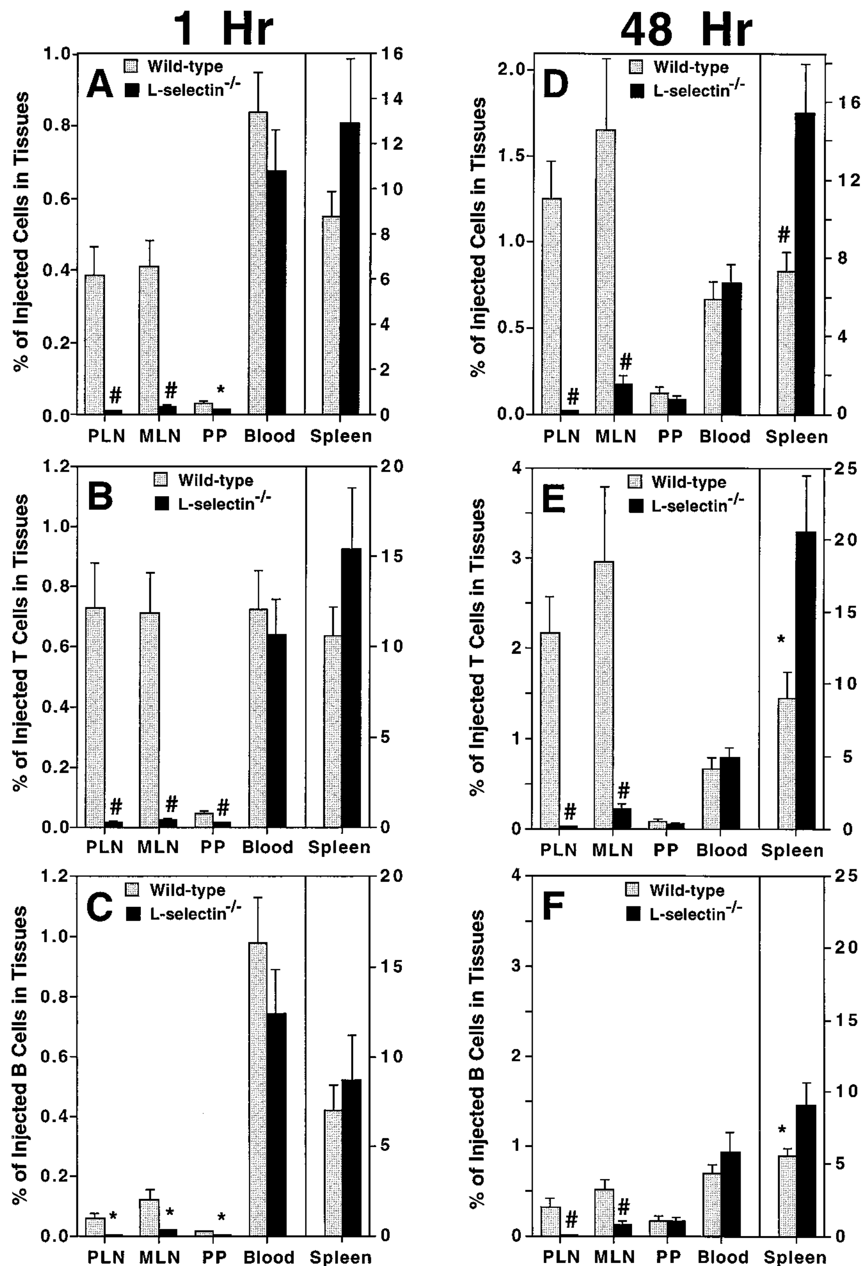

FIGURE 1. Short term (*A–C*) and long term (*D–F*) in vivo migration of L-selectin$^{-/-}$ and wild-type splenocytes. One hour or 48 h after calcein-labeled splenocytes were injected into individual wild-type mice, single-cell suspensions were prepared from the indicated tissues. Cells were labeled for Thy1.2 or B220 expression and examined by flow cytometry. *A* and *D*, The percentage of injected calcein-labeled splenocytes that migrated into individual tissues. *B* and *E*, The percentage of injected T cells that entered each tissue; *C* and *F*, the percentage of injected B cells that entered each tissue. Values for the short term migration represent the mean (±SEM) results obtained in seven or more independent experiments, and those for the long term migration represent results from six or more independent experiments. The mean numbers of migrating cells are shown in Table I. PLN, bilateral axillary, inguinal, and brachial nodes were pooled; MLN, superior mesenteric cords were pooled. PP, Peyer's patches. Differences between splenocytes from L-selectin$^{-/-}$ and wild-type mice were significant: * indicates $p < 0.05$; # indicates $p < 0.01$.

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by about 98 and 92%, respectively (Table II). L-selectin loss only reduced CD4$^+$ and CD8$^+$ cell migration into Peyer's patches by about 39%. The loss of L-selectin expression resulted in increased localization of both CD4$^+$ and CD8$^+$ cells in the spleen (Table II). Therefore, CD4$^+$ and CD8$^+$ T cells were similarly dependent on L-selectin expression for migration into lymphoid tissues.

T and B cells express different densities of L-selectin

Since the preferential migration of T cells to lymphoid tissues relative to B cells could result from differences in adhesion molecule expression between these lymphocyte subsets, L-selectin and $\alpha_4\beta_7$ expression were assessed by two-color immunofluorescence flow cytometry. Almost all the T and B cells from blood expressed L-selectin and $\alpha_4\beta_7$ (Table III). Most of the T and B cells from PLN, MLN, Peyer's patches, and spleen also expressed L-selectin and $\alpha_4\beta_7$ (Table III). However, fewer CD4$^+$ cells expressed L-selectin than CD8$^+$ cells (Table III). CD4$^+$ cells from Peyer's patches expressed L-selectin at particularly low frequencies. Similarly, fewer CD4$^+$ cells expressed $\alpha_4\beta_7$ than B cells or CD8$^+$ cells.

Concurrent expression of L-selectin and $\alpha_4\beta_7$ on individual lymphocytes in secondary lymphoid tissues was examined using three-color fluorescence cytometric analysis. The majority of T and B cells (80–94%) in blood, PLN, and MLN were L-selectin/$\alpha_4\beta_7$ double positive (Fig. 2). Although most T and B cells in Peyer's patches were L-selectin/$\alpha_4\beta_7$ double positive, the single positive T and B cells were generally $\alpha_4\beta_7{}^+$. In the spleen, similar proportions (~55–60%) of T and B cells were L-selectin/$\alpha_4\beta_7$ double positive, although there was a slightly higher frequency of L-selectin$^+$/$\alpha_4\beta_7{}^-$ T cells than B cells (15 vs 7%). Therefore, the majority of circulating T and B cells express both L-selectin and $\alpha_4\beta_7$, although variability in expression occurs for lymphocytes already in lymphoid tissues.

T cells always expressed L-selectin at significantly higher cell surface densities than B cells (Fig. 3, *A* and *C*, and Table III).

Table II. *$CD4^+$ and $CD8^+$ T cell migration into lymphoid tissues*[a]

	$CD4^+$ Cells ($\times 10^{-3}$)		$CD8^+$ Cells ($\times 10^{-3}$)	
	Wild type	L-selectin$^{-/-}$	Wild type	L-selectin$^{-/-}$
Short term (1 h)				
PLN	71 ± 14	2.0 ± 0.5[†]	44 ± 8	0.9 ± 0.2[†]
MLN	74 ± 15	3.1 ± 0.7[†]	41 ± 8	1.2 ± 0.3[†]
Peyer's patches	5.7 ± 1.3	1.7 ± 0.4*	2.1 ± 0.5	0.6 ± 0.1*
Blood[b]	73 ± 14	85 ± 17	40 ± 8	29 ± 6
Spleen	1,100 ± 180	1,800 ± 410	630 ± 68	970 ± 230
Injected	9,900 ± 940	11,000 ± 690	6,900 ± 550	7,900 ± 220
Long term (48 h)				
PLN	180 ± 25	3.0 ± 0.4[†]	170 ± 26	1.8 ± 0.3[†]
MLN	240 ± 57	25 ± 6[†]	220 ± 58	18 ± 4[†]
Peyer's patches	8.8 ± 2.8	5.6 ± 1.8	5.1 ± 1.6	3.4 ± 1.2
Blood	64 ± 7	96 ± 14	40 ± 5	54 ± 9
Spleen	940 ± 150	2,500 ± 400[†]	560 ± 100	1,400 ± 270*
Injected	9,600 ± 970	11,000 ± 480	7,300 ± 780	8,000 ± 270

[a] Calcein-labeled splenocytes from L-selectin$^{-/-}$ or wild-type mice ($n \geq 6$ each) were injected into the tail veins of individual recipient mice as in Table I. After 1 or 48 h, single-cell suspensions of tissues from recipient mice were labeled with PE-conjugated mAbs, and the percentage of $CD4^+$ or $CD8^+$ calcein-labeled lymphocytes was determined by two-color fluorescence cytometry. Absolute numbers of cells were calculated from individual tissue cell counts. Values represent the mean ± SEM number of cells.

[b] Values for blood are given as cells/ml.

* Differences between L-selectin$^{-/-}$ and wild-type lymphocytes were significant, $p<0.05$; [†] $p<0.01$.

Relative mean fluorescence staining levels for L-selectin expression by T cells were consistently 1.5 to 2.0 times those of B cells, regardless of the tissue of origin ($p < 0.001$; Fig. 3*C*). By contrast, mean $\alpha_4\beta_7$ expression levels on T cells were 50 to 71% those of B cells in all tissues ($p < 0.001$; Fig. 3, *B* and *C*, and Table III). This difference in $\alpha_4\beta_7$ expression levels was also found for T and B lymphocytes from L-selectin$^{-/-}$ mice (data not shown). Regardless of the tissue source, the mean linear fluorescence staining intensity of $CD8^+$ cells for L-selectin was 29 to 40% greater than that for $CD4^+$ cells (Table III). However, $\alpha_4\beta_7$ expression levels were equivalent for $CD4^+$ and $CD8^+$ cells. These findings demonstrate intrinsic subset-specific differences between T and B cell regulation of L-selectin and $\alpha_4\beta_7$ cell surface expression that are independent of the secondary lymphoid tissue of origin.

L-selectin expression levels regulate lymphocyte migration

L-selectin expression levels on leukocytes from hemizygous L-selectin$^{+/-}$ mice are approximately 50% of wild-type levels (4). This difference is consistent for $CD4^+$ and $CD8^+$ T cell and $B220^+$ B cell populations (Fig. 4*A*). Therefore, migration of splenocytes from L-selectin$^{+/-}$ mice was examined in short term in vivo migration assays to determine whether levels of L-selectin expression significantly affect lymphocyte migration. Calcein-labeled L-selectin$^{+/-}$ or wild-type splenocytes were mixed with equal numbers of internal control PKH26-labeled wild-type splenocytes and injected into the tail veins of recipient wild-type mice. After 1 h, single-cell suspensions of tissues from recipient mice were prepared. The ratio of calcein-labeled test cells (L-selectin$^{+/-}$ or wild-type) to PKH26-labeled control cells was determined for each tissue (R_o) and normalized by dividing by the injected ratio of calcein-labeled test cells to PKH26-labeled control cells (R_i). Migrations of L-selectin$^{+/-}$ splenocytes to PLN, MLN, and Peyer's patches at 1 h were reduced by 69, 68, and 47%, respectively, compared with those of wild-type splenocytes (Fig. 4*B*). Therefore, migrations of lymphocytes to PLN, MLN, and Peyer's patches correlated directly with expression levels of L-selectin.

The cellularity of lymphoid tissues in hemizygous L-selectin$^{+/-}$ mice was assessed to determine whether reduced L-selectin expression results in long term migration defects. The numbers of

Table III. *Lymphocyte expression of L-selectin or $\alpha_4\beta_7$ integrin*[a]

	% of Cells That Are L-selectin$^+$ (MFI of expression)[b]				% of Cells That Are $\alpha_4\beta_7^+$ (MFI of expression)			
Tissue:	B cells	T cells	$CD4^+$ cells	$CD8^+$ cells	B cells	T cells	$CD4^+$cells	$CD8^+$cells
Blood	96 ± 1	96 ± 1	96 ± 1	99 ± 1[†]	96 ± 1	78 ± 4*	90 ± 2	91 ± 1
	(102 ± 8)	(163 ± 15)[†]	(138 ± 13)	(178 ± 19)[†]	(42 ± 3)	(30 ± 2)[†]	(29 ± 5)	(33 ± 3)
PLN	88 ± 6	94 ± 3	88 ± 4	96 ± 3*	97 ± 1	76 ± 2[†]	73 ± 1	79 ± 1[†]
	(102 ± 14)	(146 ± 15)[†]	(113 ± 9)	(151 ± 16)[†]	(34 ± 1)	(17 ± 1)[†]	(16 ± 1)	(18 ± 2)
MLN	83 ± 6	89 ± 2	82 ± 3	97 ± 1[†]	97 ± 1	76 ± 4[†]	75 ± 3	81 ± 2
	(87 ± 15)	(146 ± 12)[†]	(117 ± 10)	(161 ± 17)[†]	(34 ± 1)	(19 ± 1)[†]	(19 ± 1)	(19 ± 1)
Peyer's patch	82 ± 3	46 ± 3[†]	36 ± 2	71 ± 4[†]	98 ± 2	96 ± 4	72 ± 4	85 ± 2[†]
	(76 ± 10)	(125 ± 11)[†]	(105 ± 11)	(141 ± 15)*	(32 ± 1)	(21 ± 1)[†]	(21 ± 1)	(21 ± 1)
Spleen	78 ± 3	78 ± 4	72 ± 2	94 ± 2[†]	85 ± 2	73 ± 2[†]	72 ± 2	82 ± 3*
	(88 ± 9)	(164 ± 13)[†]	(129 ± 9)	(180 ± 9)[†]	(28 ± 1)	(20 ± 1)[†]	(20 ± 1)	(20 ± 2)

[a] Adhesion molecule expression by lymphocyte subsets of wild-type mice was assessed by two-color immunofluorescence cytometry as in Figure 3. All values represent the means ± SEM obtained for lymphocytes from three or more mice.

[b] MFI, mean fluorescence intensity. Values were obtained by determining the mean linear fluorescence channel numbers for cells expressing each adhesion molecule.

* Differences between B and T cells or $CD4^+$ and $CD8^+$ T cells were significant, $p<0.05$; [†] $p<0.01$.

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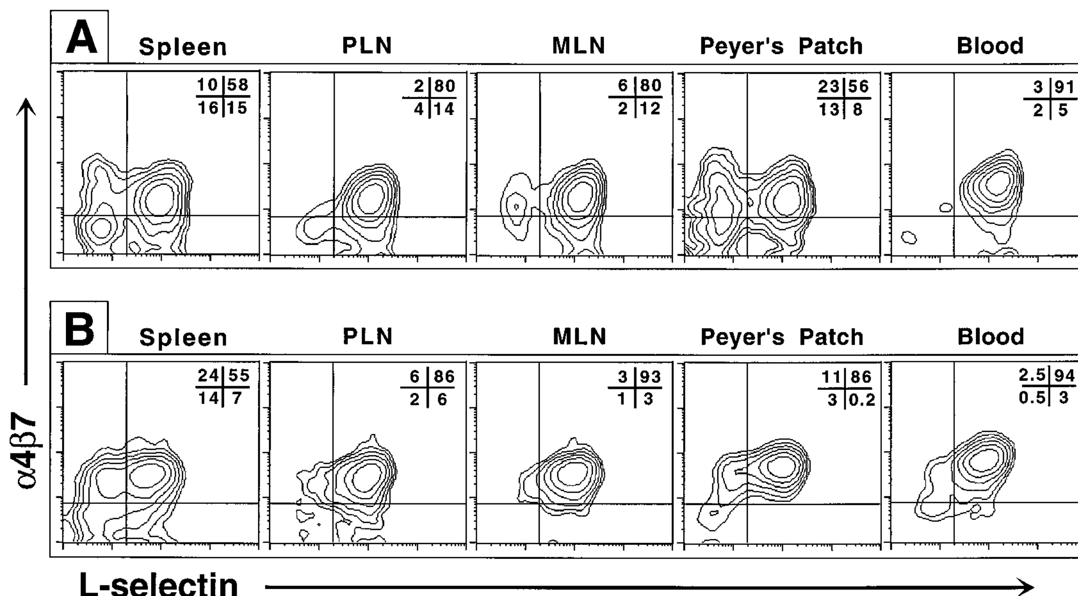

FIGURE 2. Expression of L-selectin and $\alpha_4\beta_7$ integrin by T cells (*A*) and B cells (*B*) from wild-type mice. Single-cell suspensions from tissues were stained for L-selectin (FITC-conjugated LAM1-116 mAb) and $\alpha_4\beta_7$ (DATK-32 and PE-conjugated goat anti-rat IgG) expression. T (Thy1.2$^+$) and B (B220$^+$) cells were identified by staining with biotinylated mAbs and avidin-PerCP, and the gated population of cells was then examined for the expression of L-selectin and $\alpha_4\beta_7$ by flow cytometry. Values represent the percentage of gated cells located within the four delimited quadrants. These results are representative of those obtained in two independent experiments.

lymphocytes in the spleens (86 ± 6 × 10^6), PLN (4.9 ± 0.8 × 10^6), MLN (6.8 ± 1.2 × 10^6), Peyer's patches (1.0 ± 0.2 × 10^6), and blood (4.4 ± 1.1 × 10^6/ml) of four L-selectin$^{+/-}$ mice were similar to those of their wild-type littermates. The frequencies of CD4$^+$ (29 ± 4%), CD8$^+$ (15 ± 1%), and B220$^+$ (47 ± 4%) cells in blood were also similar to those of wild-type littermates. Therefore, although reduced L-selectin levels delayed significantly the kinetics of lymphocyte entry into lymphoid tissues, this did not cause an obvious redistribution of lymphocyte subsets.

Discussion

In this study, the role of L-selectin in the regulation of T and B lymphocyte subset migration into secondary lymphoid tissues was examined. L-selectin-dependent lymphocyte-HEV interactions were evaluated directly by comparing the migration of L-selectin$^{-/-}$ and wild-type splenocytes in short term in vivo assays. Wild-type T cells migrated more efficiently than B cells into PLN,

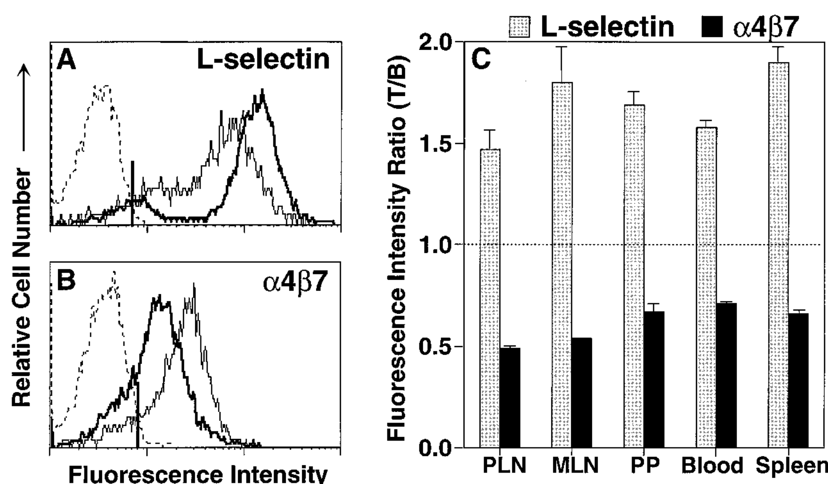

FIGURE 3. Levels of L-selectin and $\alpha_4\beta_7$ expression by T and B cells from wild-type mice. Splenocytes were stained with mAbs directed against either L-selectin (*A*; FITC-conjugated LAM1-116 mAb) or $\alpha_4\beta_7$ (*B*; DATK-32 and FITC-conjugated goat anti-rat IgG Abs). T cells (heavy lines) and B cells (thin lines) were detected with PE-conjugated mAbs reactive with Thy1.2 or B220, respectively. Negative control staining (dashed lines) was obtained using directly conjugated isotype-matched control Abs (*A*) or rat serum and FITC-conjugated goat anti-rat IgG Abs (*B*). The vertical bars indicate the level of staining at which cells were considered to be positive for the indicated Ag. *C*, Ratios of mean linear fluorescence intensity of adhesion molecule expression by Thy1.2$^+$ T cells relative to that by B220$^+$ B cells. The ratio of relative L-selectin or $\alpha_4\beta_7$ expression on T cells compared with that on B cells was obtained by dividing the mean fluorescence intensity for L-selectin or $\alpha_4\beta_7$ staining on Ag-positive T cells by the mean fluorescence intensity on Ag-positive B cells. Values represent the mean ratios (±SEM) obtained from tissues of three or more mice. The dotted line represents a ratio of 1.0, which would indicate equivalent fluorescence intensities for T and B cells.

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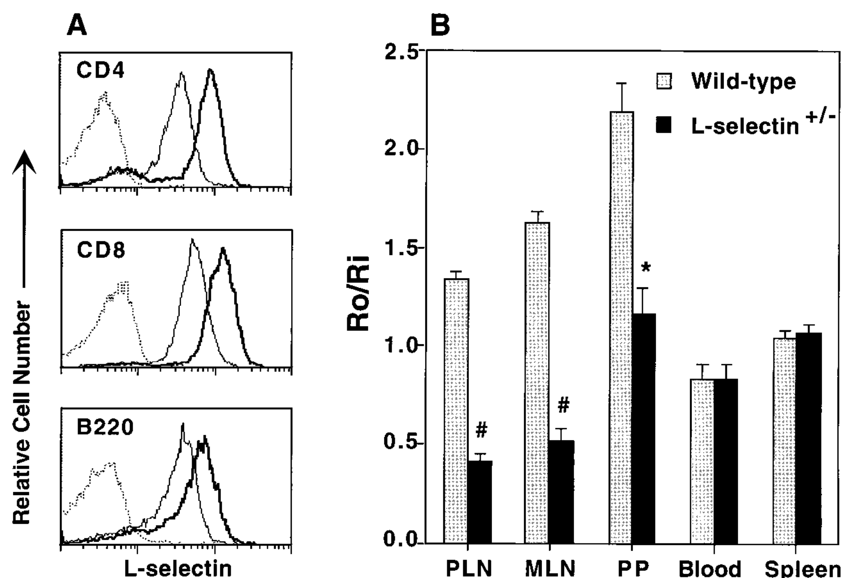

FIGURE 4. L-selectin expression levels affect lymphocyte migration. *A*, Cell surface expression of L-selectin on $CD4^+$, $CD8^+$, or $B220^+$ subsets of splenocytes from wild-type (heavy lines) and hemizygous L-selectin$^{+/-}$ (thin lines) mice. Splenocytes were stained simultaneously with PE-conjugated mAbs reactive with CD4, CD8, or B220 and FITC-conjugated LAM1-116 mAb. Negative control staining (dashed lines) for each subset of cells was obtained using a FITC-conjugated isotype-matched control mAb. Histograms represent the expression level of L-selectin on each gated cell population. *B*, Short term in vivo migration of splenocytes from L-selectin$^{+/-}$ mice. Calcein-labeled splenocytes from L-selectin$^{+/-}$ or wild-type littermates were mixed with an equal number of internal control PKH26-labeled wild-type splenocytes and injected into the tail veins of wild-type mice. After 1 h, lymphocytes were isolated from the indicated tissues and analyzed by fluorescence cytometry. The ratio of calcein-labeled test cells to PKH26-labeled internal control cells within each tissue (R_o) was normalized by dividing by the injected ratio of calcein-labeled test cells to PKH26-labeled control cells (R_i) to generate R_o/R_i ratios as described in *Materials and Methods*. Values represent the mean (±SEM) R_o/R_i ratios obtained in four or more independent experiments. The mean linear fluorescence intensity of L-selectin staining on L-selectin$^{+/-}$ splenocytes used in each experiment was 48 ± 3.6% of the wild-type level. Differences between L-selectin$^{+/-}$ and wild-type splenocytes were significant: * indicates $p < 0.05$; # indicates $p < 0.01$.

MLN, Peyer's patches, and spleen at 1 h (Fig. 1 and Table I). Nonetheless, both T and B cell migrations into PLN and MLN were reduced severely in the absence of L-selectin. T and B cell migration into Peyer's patches was also predominantly L-selectin dependent, although approximately 30% of L-selectin$^{-/-}$ splenocytes still entered this tissue (Table I). By contrast, the loss of L-selectin resulted in significantly increased T and B cell migration into the spleen. Migration of $CD4^+$ and $CD8^+$ cells was similar, and both subsets required L-selectin expression for migration (Table II). Therefore, although T cells migrate into all lymphoid tissues at a faster tempo than B cells, both cell populations are predominantly dependent on L-selectin expression for entry into these tissues.

The more efficient migration of T cells into peripheral lymphoid tissues relative to that of B cells is partly explained by the finding that T cells consistently expressed L-selectin at 50 to 100% higher levels than B cells regardless of their tissue source (Fig. 3 and Table III). Conversely, T cells expressed $\alpha_4\beta_7$ integrin at significantly lower levels than B cells regardless of their tissue source (Fig. 3 and Table III). These results contrast markedly with previous studies showing variability in L-selectin expression among lymphocyte subsets (34, 35). Nonetheless, intrinsic differences in L-selectin expression levels observed on T and B cells in the current studies correlated directly with lymphocyte migration across HEV. A 50% reduction in L-selectin expression by lymphocytes from hemizygous L-selectin$^{+/-}$ mice resulted in a 50 to 70% decrease in short term lymphocyte migration into peripheral lymphoid tissues relative to wild-type lymphocytes (Fig. 4). These results demonstrate that the preferential migration of T lymphocytes into secondary lymphoid tissues may be directly regulated by the higher levels of endogenous L-selectin expression. Factors in addition to L-selectin expression levels are also likely to influence subset-specific lymphocyte migration, since a twofold reduction in L-selectin on T cells from L-selectin$^{+/-}$ mice did not reduce the level of T cell migration to that of normal B lymphocytes.

Most circulating lymphocytes simultaneously expressed both L-selectin and $\alpha_4\beta_7$ integrin (Fig. 2). However, lymphocyte utilization of $\alpha_4\beta_7$ for entry into lymphoid tissues is relatively inefficient (4, 8). In the absence of L-selectin expression, T and B cells migrated preferentially to the spleen rather than to MLN or Peyer's patches (Fig. 1). In addition, despite having lower levels of $\alpha_4\beta_7$ expression (Fig. 3 and Table III), T cells demonstrated a fivefold higher rate of migration into Peyer's patches than B cells in the absence of L-selectin expression ($p < 0.004$; Table I). However, higher levels of $\alpha_4\beta_7$ expression by B cells may facilitate B cell retention in Peyer's patches relative to that of T cells during 48-h migration assays (Fig. 1 and Table I). Since lymphocyte entry into Peyer's patches is entirely dependent upon lymphocyte-HEV interactions (36) and L-selectin loss significantly inhibits lymphocyte migration into Peyer's patches in short term assays (Fig. 1 and Table I), increased retention or utilization of L-selectin-independent adhesion pathways must account for the accumulation of Peyer's patch lymphocytes in long term assays (Fig. 1) and in L-selectin$^{-/-}$ mice (4, 8). Specific lymphocyte subsets do not appear to selectively enter Peyer's patches in the absence of L-selectin, since Peyer's patch lymphocytes from wild-type and L-selectin-deficient mice predominantly have the phenotypic characteristics of naive lymphocytes, and increased numbers of $\alpha_4\beta_7^{high}$, $CD44^{high}$, or $CD18^{high}$ memory cells are not observed (our unpublished observations) (8). Therefore, L-selectin expression is required for the efficient migration of T and B cells into peripheral lymphoid tissues, although other factors influence their long term localization in Peyer's patches.

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Our recent in vivo studies examining leukocyte rolling in Peyer's patches of L-selectin-deficient mice and β_7 integrin-deficient mice (37) support the conclusion that L-selectin is a dominant factor in this process. Although wild-type and β_7 integrin-deficient mice have similar frequencies of rolling leukocytes in Peyer's patches, rolling flux is reduced by about 80% in L-selectin-deficient mice. β_7 integrins presumably mediate the rolling of the remaining cells at characteristic slow velocities. Consistent with this, $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins can each mediate rolling and firm adhesion during in vitro assays (38–40). In situ studies have also identified distinct roles for L-selectin and the $\alpha_4\beta_7$ and LFA-1 integrins in lymphocyte binding to Peyer's patch HEV (41). Collectively, these studies indicate that although $\alpha_4\beta_7$ ligands are present on Peyer's patch HEV, these ligands are unable to support efficient attachment and firm adhesion of most T and B cells when they lack L-selectin. This inability probably reflects a requirement for the simultaneous engagement of multiple adhesion receptors to support efficient leukocyte rolling on vascular endothelium in lymphoid tissues, as occurs at sites of inflammation (42, 50). Whether the requirement for L-selectin expression in this process relates to its role as a signal transduction molecule remains to be determined experimentally (30, 43, 44).

Different patterns of B cell and $CD4^+$ and $CD8^+$ T cell migration have been reported for sheep, humans, rats, and mice. Many of these differences can be attributed to differences in experimental approach, differences in the assay systems used, or differences in the source of cells being assayed. However, the results of this study suggest that these variances may also relate to species-specific differences in L-selectin expression levels. There is remarkable heterogeneity in the levels and patterns of L-selectin expression by lymphocytes among different animal species (12, 30, 45, 46). Therefore, variability in published experimental results is expected given that small differences in L-selectin expression are likely to significantly affect lymphocyte migration patterns. Future studies of lymphocyte migration must take these considerations into account.

The current findings demonstrate that lymphocyte subset-specific migration into secondary lymphoid tissues is regulated in part by expression levels of L-selectin. Moreover, higher L-selectin expression by T cells is likely to be a major factor in their preferential migration to secondary lymphoid tissues relative to that of B cells. Indeed, the finding that L-selectin deficiency affects most significantly the migration of $CD4^+$ T cells and memory T cells (8) is consistent with the findings of the current study. Regulation of L-selectin expression is clearly a complex and dynamic process, as L-selectin expression levels change rapidly in response to numerous stimuli, including cellular activation (47, 48). In addition, naive lymphocytes predominantly express high levels of L-selectin, while memory cells are more heterogeneous in L-selectin expression (8, 49). The finding that a twofold difference in L-selectin expression can dramatically alter lymphocyte migration in vivo establishes an important role for adhesion receptor expression levels in the regulation of lymphocyte migration. Consequently, our current results suggest that even relatively small changes in L-selectin expression levels may have dramatic effects on the efficiency of adhesion molecule-ligand interactions and leukocyte entry into tissues. This paradigm is likely to apply to other adhesion molecules as well.

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