

Topographic Distribution of Homing Receptors on B and T Cells in Human Gut-Associated Lymphoid Tissue

Relation of L-Selectin and Integrin $\alpha 4 \beta 7$ to Naive and Memory Phenotypes

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In mice, integrin $\alpha 4 \beta 7$ is the main receptor used by lymphocytes that home to the Peyer's patches, although L-selectin contributes to the initial interaction with high endothelial venules. Less is known about the expression and function of these adhesion molecules in humans. The distribution of L-selectin and $\alpha 4 \beta 7$ on various B- and T-cell subsets was examined in human Peyer's patches ($n = 8$) and appendix ($n = 4$), collectively called gut-associated lymphoid tissue. Multicolor immunophenotyping was performed on cryosections, and dispersed cells were examined by flow cytometry. In cryosections, $CD45RA^+$ T cells around and within interfollicular high endothelial venules, as well as surface (s)IgD⁺ B lymphocytes in the follicle mantles, often expressed abundant L-selectin but only intermediate levels of $\alpha 4 \beta 7$. $CD45R0^+$ T cells and sIgD⁻ B cells expressed higher levels of $\alpha 4 \beta 7$ and were often located near putative efferent lymphatics; only a small fraction (<20%) of such memory cells expressed L-selectin. By flow cytometry, considerably more T than B lymphocytes co-expressed L-selectin and $\alpha 4 \beta 7$ (40% versus 25% and 67% versus 39%, respectively). In samples with many L-selectin⁺ cells (>30%), more of these lymphocytes co-expressed $\alpha 4 \beta 7$ than in samples with few L-selectin⁺ cells. Because L-selectin and

$\alpha 4 \beta 7$ were co-expressed on lymphocytes located near high endothelial venules, and because such co-expression was relatively common when many L-selectin⁺ cells were present, both of these molecules might participate in homing to human gut-associated lymphoid tissue. Such homing is probably most pronounced for T lymphocytes that were found to express L-selectin and $\alpha 4 \beta 7$ more often than B lymphocytes. The selective and relatively high expression of $\alpha 4 \beta 7$ on memory cells located near efferent lymphatics indicated a different migratory capacity; after exit from gut-associated lymphoid tissue, such stimulated cells might home mainly to mucosal effector sites. (Am J Pathol 1997, 150:187-199)

Gut-associated lymphoid tissue (GALT) comprises Peyer's patches (PPs), appendix, and solitary lymphoid follicles scattered throughout the intestinal mucosa. B and T lymphocytes are primed against intestinal antigens in GALT and are subsequently seeded as memory or effector cells to distant mucosal effector sites, especially the gut lamina propria. Such homing is well established for IgA precursor B cells¹ and to some extent also for T cells.^{2,3} Cytokines or other factors in GALT and mesenteric lymph nodes modulate the expression of surface molecules so that the memory lymphocytes and blasts adhere to endothelial cells of venules in mucosal effector tissues and emigrate there.⁴ Distinct pairs of such receptors and endothelial ligands (addressins) co-

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operate to regulate tissue-specific lymphocyte extravasation.⁵⁻⁷ Hence, these molecules contribute significantly to immunological homeostasis by directing naive, activated, or memory cells to secondary lymphoid organs, inflamed tissues, or effector organs such as the intestinal lamina propria.⁷

Integrin $\alpha 4\beta 1$ was initially identified as a mouse PP homing receptor.⁸ It was shown to interact with vascular cell adhesion molecule (VCAM)-1,⁹ which, however, is absent from high endothelial venules (HEVs) in human PPs.^{10,11} Later, $\alpha 4\beta 7$ was found to be the main mouse PP homing receptor,¹² mediating lymphocyte interaction with the mucosal addressin cell adhesion molecule (MAdCAM)-1 present on HEVs in GALT.¹³ MAdCAM-1 is also expressed, albeit to a lesser extent, by HEVs in mesenteric lymph nodes and by ordinary venules in the gut lamina propria and lactating mammary gland.¹⁴ A third integrin, LFA-1 ($\alpha L\beta 2$), is constitutively expressed by all circulating leukocytes in normal humans; it is probably, without tissue specificity, involved in the final step (firm endothelial adherence and migration) of leukocyte extravasation.^{6,15} Human MAdCAM-1 has recently been cloned and its mRNA was detected in intestinal mucosa, GALT, and spleen.¹⁶ Its corresponding receptor, human $\alpha 4\beta 7$ (monoclonal antibody Act-1), is widely expressed by leukocyte subsets in peripheral blood, organized lymphoid tissue, intestinal lamina propria, and lung parenchyma.¹⁷⁻²² This integrin mediates *in vitro* adherence of human gut-derived blasts to HEVs in human and mouse GALT²³ as well as adhesion of human lymphoma cells to murine and human MAdCAM-1.^{20,16}

L-selectin (human Leu-8 or LECAM-1; mouse MEL-14) was early defined as the major homing receptor for peripheral lymph nodes²⁴; it also seems to participate in lymphocyte homing to PPs, as suggested by experiments both *in vivo*^{15,25,26} and *in vitro*.²⁷ L-selectin interacts with several different ligands, all of which may be expressed by HEVs in PPs: the mucin-like glycoproteins (Sgp50) designated GlyCAM-1²⁸; Sgp90, which is identical to the protein core of CD34²⁹; and the mucin-like part of murine MAdCAM-1.²⁷ L-selectin is mainly expressed by circulating naive (CD45RA⁺) T lymphocytes, except for a subset of putative skin-homing memory (CD45RO⁺) T lymphocytes.³⁰ Also, most circulating B lymphocytes are L-selectin⁺ (~90%)³¹ and have a naive phenotype because they express surface (s)IgD.^{32,33}

According to the current gut homing model for lymphocytes in mice, L-selectin and integrin $\alpha 4\beta 7$ operate in concert to recruit primarily naive cells to GALT,^{4,7,26} whereas only the latter contributes sig-

nificantly to extravasation in the lamina propria.^{15,26,27} It is unknown whether the same mechanisms regulate the migration of lymphocytes in the human gut. Therefore, the *in situ* expression of L-selectin and $\alpha 4\beta 7$ on naive and memory B and T cells, as well as the distribution of these molecules in relation to HEVs and putative efferent lymphatics, were examined in human GALT. The respective lymphocyte markers were quantitatively evaluated by flow cytometry on dispersed human GALT cells to complement the data obtained by multi-color immunohistochemical staining.

Materials and Methods

Tissue Specimens

Specimens of PPs ($n = 8$) were obtained during ileo-colonoscopy with a large biopsy forceps (FB-13Q; Olympus CF 1T 100I, Tokyo, Japan). The patients were 8 to 73 years old and had gastrointestinal complaints or suspected malignancy but a macroscopically normal bowel. Uninflamed appendix specimens ($n = 4$) were obtained from organ donors, 42 to 48 years old. The PP material constituted parallel samples for immunohistochemistry and flow cytometry from two patients, only samples for flow cytometry from one, and only for immunohistochemistry from five. The immunohistochemistry samples were within 30 minutes oriented on a thin slice of carrot, embedded in OCT compound (Tissue-Tek, Miles Laboratories, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at -70°C . Thin slices (0.5 cm) were excised from the proximal end of the appendix specimens and prepared for immunohistochemistry; the remainder was used for flow cytometry (see below). Cryosections were cut at $4\ \mu\text{m}$, dried overnight, acetone fixed (10 minutes), wrapped in aluminum foil, and stored at -20°C until use.

Antibodies

The primary antibodies for *in situ* and flow-cytometric immunostaining are listed in Table 1. The secondary immunohistochemical reagents were biotin-, indocarbocyanin (Cy3)-, or fluorescein isothiocyanate (FITC)-conjugated subclass-specific goat anti-mouse IgG or IgM (Southern Biotechnology, Birmingham, AL), aminocoumarin (AMCA)-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), and Texas Red (British Research Laboratories, Gaithersburg, MD)- or AMCA-conjugated streptavidin (Vector). All reagents were applied at 10 to 50 $\mu\text{g}/\text{ml}$, except for the Cy3-conjugated antibody

Table 1. Antibodies Used as Primary Reagents

Designation/ clone	Specificity	Isotype/label	Working concentration ($\mu\text{g/ml}$ or dilution)	Source
Leu-4	CD3	IgG1/PerCP	Purified Ig: 2.5,* 5.0	Becton Dickinson
BMA030	CD3	IgG2a	Purified Ig: 2.5*	Behringwerke, Marburg, Germany
RIV9	CD3	IgG3	Purified Ig: 1/10	Sanbio, Am Uden, The Netherlands
A0452	CD3	Rabbit Ig	Affinity isolated Ig: 1/20	DAKO, Glostrup, Denmark
Leu-3a and -3b	CD4	IgG1-FITC	Purified Ig: 2.5*	Becton Dickinson
Leu-2a	CD8	IgG1-PE	Purified Ig: 2.5*	Becton Dickinson
HD37	CD19	IgG1	Supernatant: 1/20,* 1/10	DAKO
HIB19	CD19	IgG1-Cy-Chrome	Purified Ig: 5.0*	PharMingen, San Diego, CA
L26	CD20	IgG2a	Supernatant: 1/40	DAKO
4B4	CD29(β 1)	IgG1	Purified Ig: 0.5,* 1.0	Coulter Co., Hialeah, F
Leu-18	CD45RA	IgG1/FITC	Purified Ig: 2.5,* 5.0	Becton Dickinson
Leu-CD45R0	CD45R0	IgG1/PE	Purified Ig: 2.5,* 5.0	Becton Dickinson
UCHL-1	CD45R0	IgG2a	Supernatant: 1/50* 1/10	P. C. L. Beverly, Oxford, UK
L25.3	CD49d(α 4)	IgG2b	Purified Ig: 2.5*	Becton Dickinson
B-5G10	CD49d(α 4)	IgG1	Ascites: 1/5000,* 1/1000	M. E. Hemler, Dana-Farber Institute, Boston, MA
Act-1	α 4 β 7	IgG1	Purified Ig: 3.75,* 1.0	A. I. Lazarovits, London, Canada
Leu-8	L-selectin	IgG2a/PE/FITC	Purified Ig: 2.5,* 5.0	Becton Dickinson
4G8	L-selectin	IgG1	Purified Ig: 1.0	R&D Systems, Abingdon, UK
Anti-IgD	δ -chain	IgG-FITC	Purified Ig: 40.0	Sigma
IgD26	δ -chain	IgG1	Supernatant: 1/100,* 1/10	D. Y. Mason, Oxford, UK
A0082	vWf	Rabbit Ig	Ig fraction: 1/1600	DAKO
Leu-7	CD57	IgM	Purified Ig: 2.5*	Becton Dickinson
R-505	Cytokeratin	Rabbit Ig	Antiserum: 1/100	Authors' laboratory
X39	KLH	IgG1/PerCP/PE/FITC	Purified Ig: 2.5,* 5.0	Becton Dickinson
X40	KLH	IgG2a/PE/FITC	Purified Ig: 2.5,* 5.0	Becton Dickinson
X75.3	KLH	IgG2b	Purified Ig: 2.5,* 5.0	Becton Dickinson
MG106	None	IgG1-Tri-Color	Purified Ig: 5.0*	Caltag Laboratories, San Francisco, CA
Goat serum	None	IgG-FITC	Purified Ig: 10.5	Sigma

PerCP, peridinin chlorophyll; PE, phycoerythrin; KLH, keyhole limpet hemocyanin.

*Concentration used in flow cytometry.

(1 $\mu\text{g/ml}$) and the AMCA-conjugated antiserum (75 $\mu\text{g/ml}$). For flow cytometry, phycoerythrin- or FITC-conjugated goat anti-mouse IgG1 and IgG2a were used at 3 to 10 $\mu\text{g/ml}$.

Immunohistochemistry

In serial cryosections, every 10th section was fixed in 10% formalin and stained by hematoxylin and eosin (H&E) to ensure normal histology and to locate HEVs and putative lymphatics. Adjacent sections (cut immediately before and after the H&E-stained ones) were subjected to three-color immunostaining as previously detailed.³⁴ This was achieved by combining two murine monoclonal antibodies of different subclasses with one of the primary rabbit antisera. The former were directed against lymphoid markers whereas the latter, except for anti-CD3 and anti-IgD, identified tissue elements such as epithelium (cytokeratin) or endothelium (von Willebrand factor, vWf). The sections were incubated with murine antibodies of different isotypes for 1 hour, the corresponding secondaries were next mixed with the primary rabbit antiserum and applied for 1.5 hours, and then the

final incubations (30 minutes) were performed with streptavidin-Texas Red and AMCA-conjugated anti-rabbit IgG (or only with streptavidin-AMCA when FITC- and Cy3-conjugated reagents were employed in the second step). Control sections were incubated with isotype- and concentration-matched primary murine antibodies of irrelevant specificities followed by relevant secondary reagents.

Microscopy

By light microscopy, the PP specimens were shown to contain two to seven lymphoid follicles with intervening interfollicular zones and relatively poorly defined marginal zones (Figure 1a). The HEVs were easily distinguished from other vessels in the interfollicular zones by their thick, cobblestone-like endothelium. Moreover, thin-walled vessels with flat endothelium and hardly discernible smooth muscle walls were seen around several follicles (Figure 1a); these corresponded to efferent lymphatics described in the rat.³⁵ The structure of the appendix specimens was fairly similar, except for the mucosal lamina

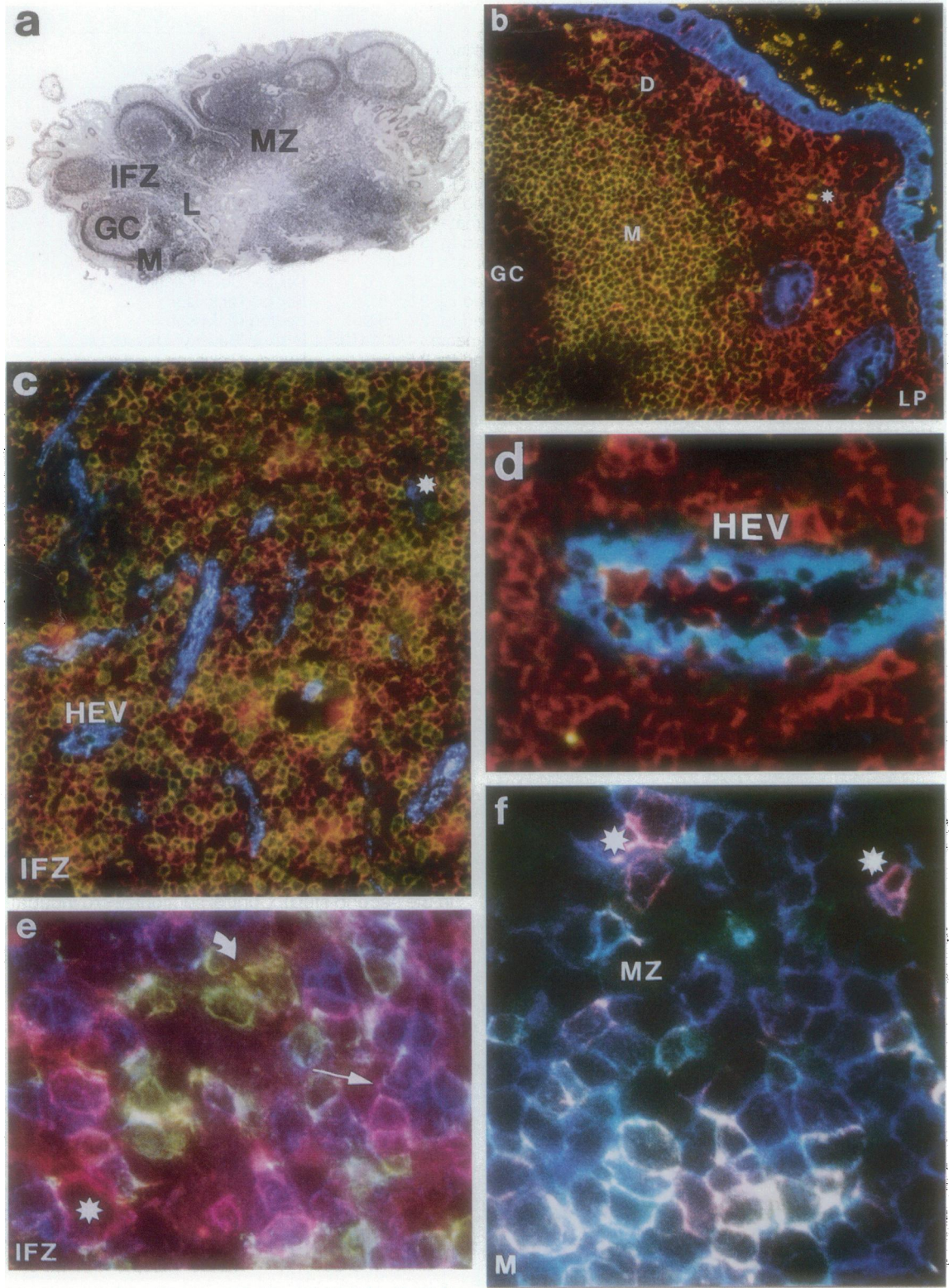


Table 2. *Topographic Distribution of Various Lymphoid Cell Surface Markers and Adhesion Molecules in Human GALT*

Compartment	CD3	CD19/20	CD45RA	CD45R0	slgD	L-selectin	$\alpha 4\beta 7$	$\alpha 4$	$\beta 1$
Dome	+	+*	+	+	+	(-)	++	++	(+)
Germinal center	+	+++	++	+	(-)	(-)	(-)	(-)	(+)
Mantle zone	+	+++	+++	(-)	+++	+ / +++	+	++	(+)
Marginal zone	(+)	++†	+	(+)	(-)	(+)	+	+	(+)
Interfollicular zone	+++	+†	++	+	+	++	+ / +++	++	(+) / +

Arbitrary grading of frequency was as follows: (-), <1%; (+), <10%; +, 10 to 50%; ++, 51 to 90%; +++, >90%. Estimates were obtained by semiquantitative evaluation of immunostained cryosections.

*Many cells positive for CD19 but negative for CD20.

† Occasional cells positive for CD19 but negative for CD20.

propria that intervened between the follicles and their surrounding interfollicular zones.

Immunofluorescence microscopy was performed at $\times 125$, $\times 312.5$, or $\times 1000$ with a Leitz DMRXE microscope-camera system (Leica, Wetzlar, Germany) equipped with a Ploem-type vertical illuminator for selective observation of green, red, or blue and for dual red/green observation. The distribution of lymphoid cell markers was evaluated by two- or three-color staining for different combinations of CD3, CD19, CD20, CD45RA, CD45R0, IgD, L-selectin, $\alpha 4\beta 7$, and the $\alpha 4$ and $\beta 1$ integrin subunits. Co-staining was in addition performed for cytokeratin and vWf. The lymphocyte marker distribution was recorded in relation to epithelium and vessels. Because cells in organized lymphoid tissue are closely packed, enumeration of phenotypes based on surface membrane staining is difficult. Therefore, the phenotypic proportions and distribution of positive cells were recorded on a semiquantitative basis at $\times 125$ or $\times 312.5$ magnification (Table 2). B or T lymphocytes expressing integrins, L-selectin, IgD, or different CD45 isoforms were evaluated by switching between the red and green filters, the dual red/green filter, and the selective blue filter. Thus, red, green, or yellow (red plus green) cells were examined for co-expression of blue marker at $\times 1000$ magnification.

Double- and triple-fluorescence exposures were obtained on Ektachrome 800/1600 daylight film,

whereas routinely stained sections were recorded on 64 Tungsten daylight film. In the triple exposures, a purple cell profile represented mixed red (Texas Red or Cy3) and blue (AMCA) fluorescence, yellow represented mixed red and green (FITC), light blue (turquoise) represented mixed green and blue, and white represented all three colors superimposed.

Isolation of Mononuclear GALT Cells

Mononuclear cells from three PP and four appendix samples were prepared by enzymatic tissue digestion as previously described.³⁶ In brief, tissue fragments ($\sim 2 \times 2$ mm) were incubated at least eight times in dispase (1.5 mg/ml; Boehringer Mannheim, Mannheim, Germany) and thereafter passed sequentially through 200- and 45- μ m sterile nylon filters for single-cell dispersion. From each sample, 5×10^6 to 20×10^6 cells were isolated, lymphocytes constituting 50 to 80%. Viability was 95 to 98% as evaluated with exclusion of bright yellow cells in a mixture of ethidium bromide (12 μ g/ml; Sigma Chemical Co., St. Louis, MO) and acridin orange (3.6 μ g/ml; Sigma) dissolved in phosphate-buffered (pH 7.5) isotonic saline (PBS). Preliminary experiments had shown that 1 hour of treatment of peripheral blood lymphocytes with dispase (37°C) resulted in complete loss of L-selectin from the cell surface while the other markers remained intact. However,

Figure 1. In situ immunofluorescence staining for L-selectin and $\alpha 4\beta 7$ in cryosections from human PPs. **a:** Morphology of PP with seven follicles visualized by H&E staining. GC, germinal centers; M, mantle zones; MZ, marginal zones; IFZ, interfollicular (T-cell) zone; L, perfollicular putative lymphatic vessel. Original magnification, $\times 25$. **b:** Three-color staining decorating cytokeratin-positive epithelium (blue), $\alpha 4\beta 7$ (red), and L-selectin (green) in PP field including part of lymphoid follicle with subepithelial dome (D) and adjacent lamina propria (LP). The mantle zone (M) contains many L-selectin⁺ cells that usually also express some $\alpha 4\beta 7$ (yellow profiles), but many cells are $\alpha 4\beta 7$ ⁻ (purely green). The L-selectin⁻ cells (purely bright red) in the dome and lamina propria represent mostly B-cell blasts and plasma cells. Eosinophils show yellow nonspecific staining (*). Original magnification, $\times 250$. **c:** Three-color staining for vWf (blue), CD45RA (red), and L-selectin (green) in interfollicular zone. Several HEVs are decorated blue. Most surrounding lymphocytes are CD45RA⁺/L-selectin⁺ (yellow profiles), but some CD45RA⁺/L-selectin⁻ are also present (purely red). Relatively few CD45RA⁺/L-selectin⁺ putative memory cells can be seen (purely green, exemplified by asterisk). Original magnification, $\times 250$. **d:** Two-color staining for von Willebrand factor (blue) and L-selectin (red) in PP field containing a HEV that expresses von Willebrand factor (blue). Some L-selectin⁺ cells adhere to the endothelium. Original magnification, $\times 400$. **e:** Three-color staining for CD3 (blue), CD20 (green), and $\alpha 4\beta 7$ (red) in interfollicular zone. Purple cells are CD3⁺/ $\alpha 4\beta 7$ ⁺, and yellow cells are CD20⁺/ $\alpha 4\beta 7$ ⁺; where cells are juxtaposed, blending of all three colors produces white contact zones. Variable expression of $\alpha 4\beta 7$ by CD3⁺ cells appears in the range from bright purple to blue (thin arrow). Note two CD20⁺ cells (thick arrow), one $\alpha 4\beta 7$ ⁻ cell (purely green), and one $\alpha 4\beta 7$ ⁺ cell (yellow). An $\alpha 4\beta 7$ ^{hi}CD20⁻CD3⁻ B-cell blast is purely red (asterisk). Original magnification, $\times 1000$. **f:** Three-color staining for CD20 (blue), IgD (green), and L-selectin (red) in mantle zone (M) and marginal zone (MZ) of lymphoid follicle. Co-expression of all three markers indicates a naive B-cell phenotype (white), whereas the CD20⁺/slgD⁻/L-selectin⁻ phenotype (blue) represents memory B cells located in the marginal zone (upper half). Some CD20⁻ cells in the mantle zone (lower half) with a naive phenotype (slgD⁺) lack L-selectin (turquoise) and a few CD20⁺/slgD⁻ memory B cells (*) are positive for L-selectin. Original magnification, $\times 1000$.

L-selectin was regained after reconstitution for 10 hours in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% v/v fetal calf serum (Gibco); such incubation for up to 24 hours did not significantly affect surface expression of other molecules (CD45, integrins, or B- and T-cell markers) on isolated peripheral blood lymphocytes. Therefore, cells dispersed from GALT were reconstituted for 10 to 15 hours before further treatment.

Flow Cytometry

The antibody reagents were diluted to appropriate working concentrations (Table 1) in PBS containing 1.25% w/v bovine serum albumin. Immunostaining was performed in V-shaped 96-well plates (Costar, Cambridge, MA) as follows: the cell suspension (1×10^5 cells per well in 50 μ l RPMI with 2% v/v fetal calf serum and 0.1% w/v NaN_3) was incubated in the dark (20 minutes at 4°C with gentle stirring) either with a mixture (50 μ l) of three fluorochrome-conjugated primary antibody reagents or with two unconjugated primaries. The cells were washed twice in medium, and those incubated with fluorochrome conjugates were resuspended in 400 μ l of medium and kept on ice (for 1 to 2 hours) in the dark until analysis. With unlabeled primaries, phycoerythrin- or FITC-conjugated secondary antibodies were added and incubated as described and then washed twice. In wells to be additionally stained with Cy-Chrome-conjugated antibody to CD19 or with peridinin-chlorophyll-conjugated antibody to CD3 (Table 1), a blocking step with heat-inactivated mouse serum (10% in PBS) was performed before this fourth incubation. After two final washes, the cells were resuspended in 400 μ l of medium and stored at 4°C until use (within 1 hour).

The suspensions were analyzed in a FACScan flow cytometer (Becton-Dickinson) equipped with the LYSYS II software programme. For two-color analyses, the instrument was set up with CaliBRITE beads (Becton Dickinson, Mountain View, CA) and compensation was adjusted visually. For three-color analyses, the set-up was performed with anti-CD3 (peridinin chlorophyll) or anti-CD19 (Cy-Chrome) combined with anti-CD4 (FITC) and anti-CD8 (phycoerythrin) relative to the respective negative controls (Table 1). Compensation was adjusted first between the FL1 and FL2 channels and then between the FL2 and FL3 channels (peridinin chlorophyll as well as Cy-Chrome were detected in the FL3 channel). A wide lymphocyte gate was set in the forward/side scatter dot plot, and 10,000 cells were acquired in list mode for each analysis. Two-color analysis

was performed within this lymphocyte gate, whereas gates set in the forward scatter/FL3 dot plot were used to define T- and B-lymphocyte populations analyzed for the expression of two additional markers.

Results

Immunohistochemical Observations

Topographic Distribution of Lymphocyte Phenotypes and Homing Receptors

The *in situ* mapping of lymphocyte markers is summarized in Table 2. Some of these data have been published previously.^{11,22} B lymphocytes (CD19^+ or CD20^+) were localized in the germinal centers, mantle zones, marginal zones, domes, and M-cell pockets (marginal zones are defined as collections of $\text{slgD}^- \text{CD20}^+$ memory cells around the slgD^+ follicle mantles).³⁷ Cells positive for CD19 but negative for CD20 were relatively numerous in the domes, and some were also seen in the marginal and interfollicular zones. Moreover, occasional B cells in the interfollicular zones expressed IgD. T lymphocytes (CD3^+) were mainly localized in the latter zones, domes, follicle-associated epithelium, and M-cell pockets and were only few and scattered in the germinal centers.

The germinal centers were negative for $\alpha 4\beta 7$, the mantle zones negative or weakly positive, and the domes as well as the marginal and interfollicular zones contained moderately to strongly positive cells. This integrin expression pattern was largely followed by the $\alpha 4$ subunit, whereas staining for the $\beta 1$ subunit was dull on lymphoid cells but bright on large nonlymphoid cells and fiber-like structures in all compartments of GALT as well as in epithelial cells outside putative M cells. L-selectin was mainly expressed by cells in the follicle mantles (Figure 1b) and in interfollicular zones (Figure 1c) but rarely at other sites. The distribution of CD45RA principally followed that of L-selectin, but the former phenotype dominated over the latter (Figure 1c). CD45RO was most prominent on lymphocytes within the follicle-associated epithelium and on cells in the domes and interfollicular zones.

Homing Receptors Expressed by B-Cell Subsets

Mantle zone cells consisted of $\text{slgD}^+ \text{CD19}^+ \text{CD20}^- \text{CD45RA}^+$ B lymphocytes that were either positive or negative for L-selectin; the number of L-selectin⁺ cells varied considerably among the specimens. Mantle zone cells were either weakly positive or negative for

$\alpha 4\beta 7$ as well as for the integrin $\alpha 4$ subunit but were usually negative for the $\beta 1$ subunit. B cells (usually of CD19⁺CD20⁺slgD⁻ memory phenotype) in the marginal and interfollicular zones expressed $\alpha 4\beta 7$ with variable intensity (Figure 1e), whereas L-selectin was present on only rare slgD⁻ B lymphocytes in these areas (Figure 1f). In addition, occasional cells strongly positive for $\alpha 4\beta 7$ and CD19 but negative for CD3, CD20, and L-selectin were seen at these sites; they were deemed to represent B-cell blasts²² (Figure 1e).

Homing Receptors Expressed by T-Cell Subsets

Most L-selectin⁺ cells were seen in the interfollicular zones, and they were mainly CD3⁺CD45RA⁺ (naive) T lymphocytes. This subset expressed $\alpha 4\beta 7$ at intermediate levels although more strongly than the mantle B lymphocytes. The number of L-selectin⁺ naive T lymphocytes varied less than that of naive B lymphocytes. CD3⁺CD45R0⁺ (memory) T lymphocytes were, with few exceptions, negative for L-selectin but more positive for $\alpha 4\beta 7$ and the integrin $\alpha 4$ subunit than naive cells. In the domes and germinal centers, T lymphocytes were CD45R0⁺, expressed $\alpha 4\beta 7$ relatively strongly, and usually lacked L-selectin. Most T lymphocytes, regardless of memory or naive phenotype, were virtually negative for the integrin $\beta 1$ subunit, although a few were distinctly positive.

Homing Receptors in Relation to HEVs and Efferent Lymphatics

In the interfollicular zones, HEVs were positive for vWf. L-selectin⁺ cells were seen around such vessels, within their lumina, and between the endothelial cells (Figure 1d). Most of these L-selectin⁺ lymphocytes were of the naive (CD45RA⁺) T-cell phenotype (Figure 2c), but some were B lymphocytes (usually slgD⁺). Such L-selectin⁺ cells were not seen in the vessels of adjacent lamina propria. The L-selectin⁺ cells were usually weakly positive or negative for $\alpha 4\beta 7$. However, when the L-selectin⁺ cells were numerous, they more often showed distinct co-expression of this integrin. Few CD45R0⁺ T lymphocytes were present in the same areas (Figure 2b). Therefore, cells in and around HEVs were predominantly naive, L-selectin⁺ T lymphocytes with low or moderate levels of $\alpha 4\beta 7$. Conversely, this marker was more strongly expressed by lymphocytes at the periphery of the follicles near putative lymphatics with thin walls and flat endothelium that was negative for vWf (Figure 2d). These lymphocytes were usually memory T (CD45R0⁺) or B (slgD⁻) lymphocytes.

Flow Cytometric Analyses

In samples examined both by immunohistochemistry and by flow cytometry, the estimated phenotype proportions were generally in good agreement except for B cells that were better represented by the former than by the latter method. The samples contained virtually identical proportions of B (CD19⁺) and T (CD3⁺) lymphocytes with a median 47% of each subset (Figure 3 and Table 3). Almost 50% of the CD19⁺ lymphocytes were slgD⁺ (naive) and 93% were CD45RA⁺, whereas less than 2% expressed CD45R0 (Table 3); the latter marker is reported to be temporarily expressed by B cells undergoing terminal differentiation.³⁸ Few B lymphocytes (25%) (Figure 4, Table 4) expressed L-selectin compared with the large fraction in peripheral blood (90%).³¹ The proportions of naive (CD45RA⁺) and memory (CD45R0⁺) T lymphocytes varied considerably although their medians were quite similar (49 and 51%, respectively; Table 3). The total L-selectin⁺ cell fraction was also highly variable (Tables 3 and 4), but it was relatively large in samples with many CD45RA⁺ T cells. L-selectin was more often expressed by T lymphocytes (40%) than by B lymphocytes (25%), although the latter were particularly heterogeneous with regard to this marker (Table 4).

Integrin $\alpha 4\beta 7$ was less often expressed by B than by T lymphocytes (39% versus 63%; Figure 5 and Table 4), and the same was true for the $\alpha 4$ subunit (62% versus 88%) and the $\beta 1$ subunit (50% versus 86%) (Figures 4 and 5 and Table 4). The $\beta 1$ expression levels were equal to those of naive $\beta 1^{\text{low}}$ peripheral blood lymphocytes.¹¹ However, a small $\beta 1^{\text{high}}$ fraction was seen among memory T lymphocytes (CD45R0⁺) as well as among the L-selectin⁻ B lymphocytes (Figure 5). CD45R0⁺ lymphocytes (mainly memory T cells) were 65% $\alpha 4\beta 7^+$, 90% $\alpha 4^+$, and 88% $\beta 1^+$, whereas the CD45RA⁺ lymphocytes (B cells and naive T cells) were 48% $\alpha 4\beta 7^+$, 50% $\alpha 4^+$, and 45% $\beta 1^+$ (Figure 5 and Table 4). Moreover, the mean $\alpha 4\beta 7$ fluorescence intensity for the CD45R0⁺ fraction was twice that for the CD45R0⁻ fraction (Figure 5).

Most L-selectin⁺ lymphocytes were naive, as they rarely were found in the CD45R0⁺ (9%; Table 4) or CD19⁺slgD⁻ fractions (<20%; one experiment performed). The L-selectin⁺ lymphocytes were a median 50% $\alpha 4\beta 7^+$ as shown for B cells in Figure 4, in contrast to the CD45R0⁺ T-cell subset (65%) (Figure 5 and Table 4). However, in two samples with many L-selectin⁺ cells (>30%), a higher proportion co-expressed $\alpha 4\beta 7$ or the $\alpha 4$ subunit (>60%).

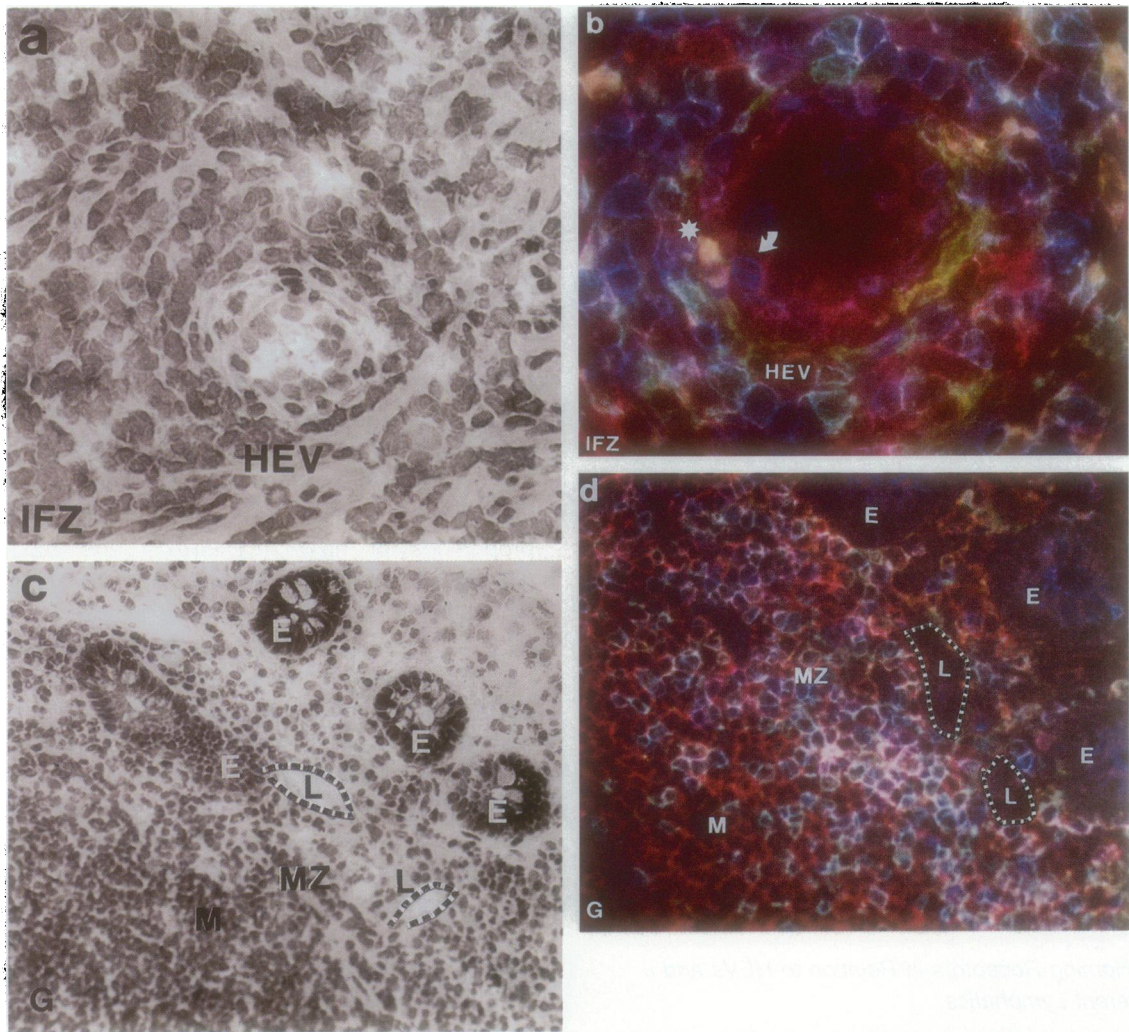


Figure 2. In situ immunofluorescence staining for $\alpha 4\beta 7$ and CD45RO in cryosections from human PPs. **a:** Morphology of HEV with characteristic endothelium in interfollicular zone (IFZ) as visualized by H&E staining. Original magnification, $\times 400$. **b:** Three-color staining for CD3 (blue), CD45RO (green), and $\alpha 4\beta 7$ (red) in comparable field as **a**. Naive lymphocytes ($CD3^+/CD45RO^-$) with low levels of $\alpha 4\beta 7$ (faint purple) dominate around HEV, one crossing the vessel wall (arrow). Occasional memory cells ($CD3^+/CD45RO^+$) are virtually negative for $\alpha 4\beta 7$ (turquoise), and a few $\alpha 4\beta 7^+$ B cells ($CD3^-/CD45RO^-$) can also be seen (purely red). Eosinophils show yellow nonspecific staining (*). Original magnification, $\times 1000$. **c:** Morphology of periphery of lymphoid follicle and adjacent lamina propria. G, germinal center; M, mantle zone; MZ, marginal zone; L, putative lymphatic vessels; E, crypt epithelium in adjacent lamina propria. Original magnification, $\times 250$. **d:** Three-color staining for CD3 (blue), CD45RO (green), and $\alpha 4\beta 7$ (red) in comparable field as **b**. Several memory T cells ($CD3^+/CD45RO^+$) with strong co-expression of $\alpha 4\beta 7$ (white) are seen in the marginal zone near lymphatics. Most other T cells show a naive phenotype ($CD3^+/CD45RO^-$) with low levels of $\alpha 4\beta 7$ (faint purple). Germinal center is negative for all markers, whereas mantle zone cells are negative or weakly positive for $\alpha 4\beta 7$ (purely red); occasional cells with strong expression represent B-cell blasts. Original magnification, $\times 400$.

Discussion

Extensive studies on the roles of L-selectin and $\alpha 4\beta 7$ in gut homing have been performed in mice, with particular emphasis on mechanisms involved in the extravasation of lymphocytes in PPs.^{8,12,13,15,25-27} Several reports exist on the expression and function of $\alpha 4\beta 7$ also in humans,¹⁶⁻²² but the simultaneous expression of L-selectin and $\alpha 4\beta 7$ has been studied only on peripheral blood lymphocytes.³⁹ According to current theories of lymphocyte recirculation, PPs and appendix are assumed to contain both recently

arrived naive lymphocytes and memory cells (or blasts) destined to leave GALT for subsequent lodging in mucosal effector organs. The present work provides information on co-expression of L-selectin and $\alpha 4\beta 7$ on B- and T-cell subsets and describes their location in relation to sites of entrance or exit in human GALT. Our data can be interpreted in view of the functional information available for mice. The results showed 1) L-selectin⁺ lymphocytes were primarily found around, within, or traversing HEVs in the interfollicular zones as well as in follicular mantles, 2)

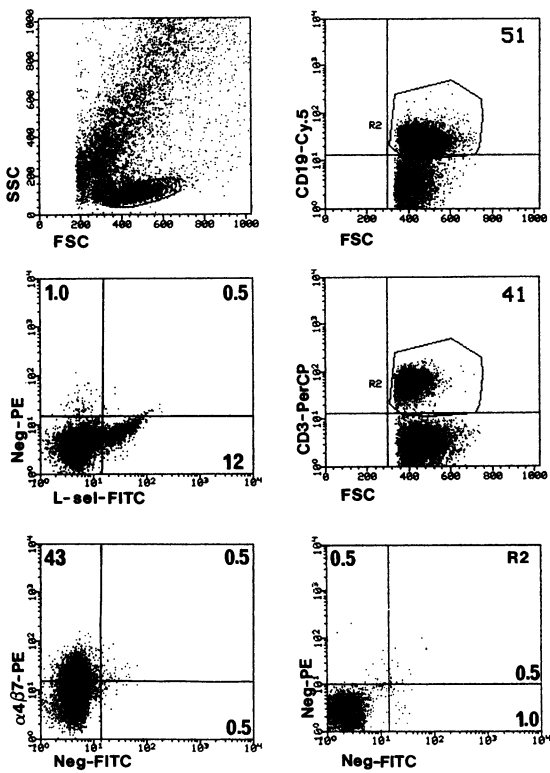


Figure 3. Flow-cytometric analysis of dispersed cells from PP to demonstrate gating principles and controls. Upper left panel: Forward/side scatter dot plot of ungated cells. The gate set for analysis of lymphoid cells is indicated. Middle and lower left panels demonstrate cursors set to separate positively stained cells from those incubated with irrelevant primary control antibodies in two-step staining procedures. Upper and middle right panels illustrate gates set for analysis of CD19⁺ and CD3⁺ cells, respectively. Lower right panel illustrates cursors set for CD19⁺ or CD3⁺ cells (R2) incubated with irrelevant primary control antibodies and corresponding secondary phycoerythrin- and FITC-conjugated antibodies in a four-step staining procedure. Numbers in quadrant corners indicate percentage of positive cells.

naive T lymphocytes (CD45RA⁺) predominated in the L-selectin⁺ population, but naive B lymphocytes (sIgD⁺) were also well represented, and 3) integrin

Table 3. Lymphocyte Phenotype Proportions (Percent) Obtained by Flow Cytometry of Dispersed Human GALT Cells

Phenotype	GALT
CD19	47 (40–60)
IgD	20 (12–27)
CD3	47 (40–60)
CD45RA	69 (43–77)
CD45R0	24 (13–49)
L-selectin	30 (5–72)
IgD/CD19**	49 (25–55)
CD45RA/CD19	93 (92–96)
CD45R0/CD19	1.5 (1–4)
CD45RA/CD3	49 (19–60)
CD45R0/CD3	51 (29–81)

Data are expressed as median and range for all experiments (PP and appendix). The last five lines represent the ratios of the former to the latter marker.

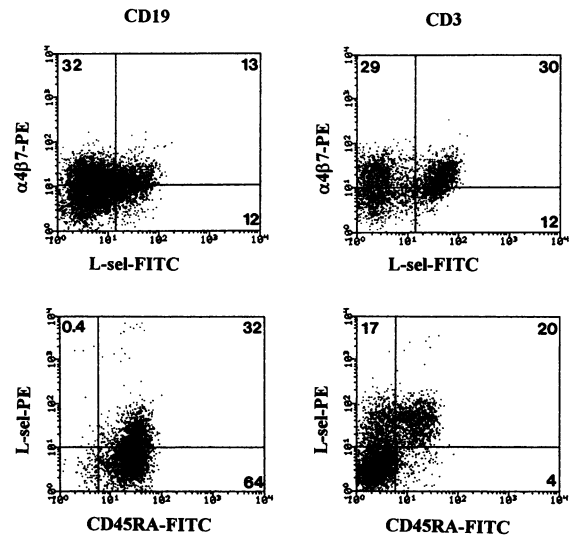


Figure 4. Flow-cytometric analysis of dispersed cells from PP (upper panels) and appendix (lower panels). Examination of CD19⁺ cells (left panels) and CD3⁺ cells (right panels) for expression of $\alpha 4\beta 7$ and CD45RA in relation to L-selectin in samples with many (>30%) L-selectin⁺ cells. Upper panels: CD19⁺ cells are less positive for $\alpha 4\beta 7$ and L-selectin than CD3⁺ cells, and L-selectin⁺ cells express intermediate levels of $\alpha 4\beta 7$ compared with L-selectin⁻ cells in these samples. Lower left panel: Approximately one-third of CD19⁺ cells express L-selectin whereas virtually all are positive for CD45RA. Lower right panel: Of the CD3⁺ cells expressing CD45RA, most are also positive for L-selectin. In this particular sample, a relatively large fraction of T cells are positive for L-selectin but negative for CD45RA, thus representing a memory population. Numbers in quadrant corners indicate percentage of positive cells.

$\alpha 4\beta 7$ was expressed at low or intermediate levels by naive GALT lymphocytes (often L-selectin⁺) near HEVs but more strongly on memory cells (usually L-selectin⁻) at the periphery of the follicles near putative efferent lymphatics.

In samples prepared for both immunohistochemistry and flow cytometry, the overall subset proportions determined by these two methods were in good agreement except that the dispersed mononuclear cells often contained relatively fewer B cells. Germinal center cells cultured at 37°C are known to undergo apoptosis within 16 hours.⁴⁰ Because such culturing was necessary to reconstitute surface L-selectin, apoptosis might have caused some selective depletion of B cells. Moreover, loss of activated cells (eg, germinal center B cells) has been observed when lymphoid tissue is dispersed to prepare suspensions.⁴¹ Nevertheless, the presence of 1 to 3% CD57⁺ cells in our suspensions (data not shown) supported germinal center representation because this phenotype is present only in that compartment of secondary lymphoid tissue.⁴² We confirmed this by immunohistochemistry in GALT (unpublished observation). Therefore, samples containing at least 40%

Table 4. Proportions (Percent) of Naive and Memory B (CD19⁺) and T (CD3⁺) Cells Expressing L-Selectin and $\alpha 4$ Integrins Obtained by Flow Cytometry of Dispersed Human GALT Cells

Phenotype	L-selectin	$\alpha 4\beta 7$	$\alpha 4$	$\beta 1$
CD19	25 (3–84)	39 (30–47)	62 (35–98)	50 (38–95)
CD3	40 (30–66)	63 (60–67)	88 (30–98)	86 (51–95)
IgD	30 (16–93)	ND	ND	ND
CD45RA	30 (15–87)	48 (45–50)	50 (44–94)	45 (40–50)
CD45R0	9 (2–50)	65 (50–80)	90 (60–93)	88 (85–90)
CD45RA ⁺ CD3 ⁺	(80–90)*	ND	ND	ND

Data are expressed as median and range for all experiments (PP and appendix). ND, not determined. CD45RA⁺ subset represents naive T cells as well as most B cells.

*Only two experiments performed.

B lymphocytes as well as a small fraction of CD57⁺ cells were deemed acceptable for flow-cytometric analysis. The high fraction of L-selectin⁺ cells found in two samples further supported this conclusion because such cells are rarely found in the gut lamina propria.^{43,44} The sum of B- and T-cell proportions obtained by FACScan analysis rarely amounted to 100%, probably because some dendritic cells and macrophages were present in the suspensions. However, the sum was never below 90%, suggesting little contribution from such unwanted cell types.

The interfollicular zones with their HEVs are the sites of entrance for both B and T lympho-

cytes in GALT.⁴⁵ These zones contained mainly L-selectin⁺CD45RA⁺ naive T lymphocytes and a small fraction of sIgD⁺ naive B lymphocytes that usually also expressed L-selectin. Lymphocytes positive for this marker were often found between the endothelial cells of HEVs. Because L-selectin is found mainly on naive cells but becomes down-regulated (at least temporarily) after activation,⁴⁶ its presence along with CD45RA on a large fraction of T lymphocytes in GALT suggested that they had recently extravasated. The variable proportion of this phenotype among the specimens could reflect that the magnitude of lymphocyte recruitment to GALT is dynamic and depends on the level of L-selectin ligands on HEVs.^{27–29} We found that naive L-selectin⁺ cells expressed lower levels of $\alpha 4\beta 7$ than memory cells (usually L-selectin⁻), except in samples with many L-selectin⁺ cells. The low levels of $\alpha 4\beta 7$ and the lack of L-selectin on many naive mantle zone B lymphocytes are difficult to explain but might reflect down-regulation (at least of $\alpha 4\beta 7$) on unstimulated cells. Nevertheless, $\alpha 4\beta 7$ might be the most important $\alpha 4$ integrin mediating firm lymphocyte adherence in GALT.¹⁵ This possibility was supported by the virtual absence of VCAM-1 (the endothelial ligand for $\alpha 4\beta 1$) on GALT HEVs^{10,11} and by the detection of MAcCAM-1 mRNA in human GALT.¹⁶ Our data supported the notion that both L-selectin and $\alpha 4\beta 7$ contribute to extravasation in GALT. However, because more T than B lymphocytes expressed these markers, continuous recruitment to GALT appears to be dominated by T lymphocytes.

We have previously shown that $\alpha 4\beta 7$ is expressed relatively strongly on B lymphocytes and blasts with a memory phenotype, that is, putative activated¹⁷ cells.²² In the present study, this subset was generally shown to be negative for L-selectin, except for occasional CD20⁺ memory (sIgD⁻) B cells. Also, T cells showed variable expression of $\alpha 4\beta 7$; the memory subset (CD45R0⁺) had higher levels than the naive fraction (CD45R0⁻). The generally higher lev-

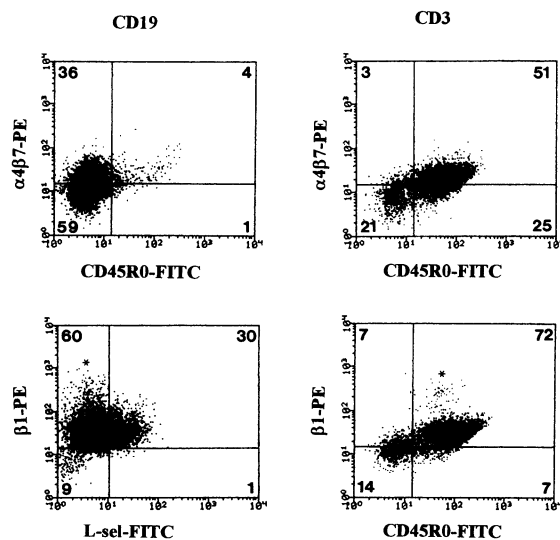


Figure 5. Flow-cytometric analysis of dispersed cells from PP (upper panels) and appendix (lower panels) and examination of CD19⁺ (left panels) and CD3⁺ cells (right panels) for expression of $\alpha 4\beta 7$ and integrin $\beta 1$ subunit in relation to CD45R0 and L-selectin. Left panels: Dot plots represent gated CD19⁺ cells. Upper left: CD19⁺ cells are more often negative than positive for $\alpha 4\beta 7$. A small fraction of cells express CD45R0. Lower left: A small fraction of putative memory B lymphocytes (L-selectin⁻) are strongly positive for $\beta 1$ (*), whereas most L-selectin⁺ B cells express low levels of $\beta 1$. Right panels: Dot plots represent gated CD3⁺ cells. Upper right: A much larger fraction of memory (CD45R0⁺) than naive (CD45R0⁻) lymphocytes express $\alpha 4\beta 7$. Lower right: Memory cells also express more of the integrin $\beta 1$ subunit than the naive subset. A small fraction of memory cells are $\beta 1^{\text{high}}$ (*). Numbers in quadrant corners indicate percentage of positive cells.

els of this integrin on memory than on naive lymphocytes suggested that the former subset may adhere more readily to putative endothelial ligands such as MAdCAM-1.¹⁵ $\alpha 4\beta 7^{\text{high}}$ memory B and T cells could be on their way to exit from GALT because they were mainly located near putative efferent lymphatics. Such vessels eventually drain into peripheral blood via mesenteric and thoracic duct lymph.⁴⁷ When memory $\alpha 4\beta 7^{\text{high}}$ cells reach the blood circulation, they likely extravasate preferentially at mucosal effector sites expressing MAdCAM-1,¹⁶ although some might re-enter GALT because integrin $\alpha 4\beta 7$ promotes adherence to HEVs in the absence of L-selectin.¹⁵

The $\alpha 4$ subunit was present on more cells than $\alpha 4\beta 7$, probably reflecting that $\alpha 4$ also associates with $\beta 1$; most circulating lymphocytes are in fact known to express both $\alpha 4\beta 1$ and $\alpha 4\beta 7$.²⁰ By flow cytometry, 80% of dispersed GALT lymphocytes expressed $\beta 1$ but only at a level comparable to the $\beta 1^{\text{low}}$ fraction of circulating lymphocytes¹¹; $\beta 1$ was expressed at very low levels *in situ* as well. The small fraction of $\beta 1^{\text{high}}$ memory T and B lymphocytes detected by flow cytometry might nevertheless represent a subset that up-regulates $\beta 1$ instead of $\beta 7$ in GALT, but the biological significance of this is unclear.

Some memory T and B lymphocytes in GALT (<20%) expressed L-selectin, although most (including putative B-cell blasts) had high levels of $\alpha 4\beta 7$ but no L-selectin. This was in agreement with previous findings showing that CD45R0⁺ T-cell blasts generated in human appendix, as well as human antibody-forming cells elicited by oral immunization, were mainly negative for L-selectin.^{48,49} If the relatively few L-selectin⁺ $\alpha 4\beta 7^{\text{high}}$ memory cells observed in GALT ever emigrate, which is likely because such cells occur in peripheral blood,³⁹ they might preferentially return to this site; the MAdCAM-1 variety present on the endothelium of lamina propria venules is unable to interact with L-selectin, at least in mice, thereby contrasting that expressed by HEVs in GALT.²⁷ At present, no study has reported interactions of human MAdCAM-1 with L-selectin, but the virtual absence of L-selectin⁺ cells in intestinal lamina propria^{43,44} suggests that MAdCAM-1 expressed there does not bind L-selectin.

In conclusion, the data reported in our study are in good agreement with previously published observations in mice and humans and support the theory that both L-selectin and $\alpha 4\beta 7$ contribute to lymphocyte extravasation in GALT, whereas only the latter molecule contributes substantially to the homing of memory cells from GALT to mucosal effector sites.

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