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TRAFFIC SIGNALS ON ENDOTHELIUM FOR LYMPHOCYTE RECIRCULATION AND LEUKOCYTE EMIGRATION

Timothy A. Springer
The Center for Blood Research, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115

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INTRODUCTION

The circulatory and migratory properties of white blood cells have evolved to allow efficient surveillance of tissues for infectious pathogens and rapid accumulation at sites of injury and infection. Lymphocytes continually patrol the body for foreign antigen by recirculating from blood, through tissue, into lymph, and back to blood. Lymphocytes acquire a predilection, based on the environment in which they first encounter foreign antigen, to home to or recirculate through that same environment (39, 40). Granulocytes and monocytes cannot recirculate, but emigrate from the bloodstream in response to molecular changes on the surface of blood vessels that signal injury or infection. Lymphocytes can similarly accumulate in response to inflammatory stimuli. The nature of the inflammatory stimulus determines whether lymphocytes, monocytes, neutrophils, or eosinophils predominate, and thus exercises specificity in the molecular signals or "area codes" that are displayed on endothelium and control traffic of particular leukocyte classes.

Recent findings show that the "traffic signals" for lymphocyte recirculation and for neutrophil and monocyte localization in inflammation are strikingly similar at the molecular level. These traffic signal or area code molecules are

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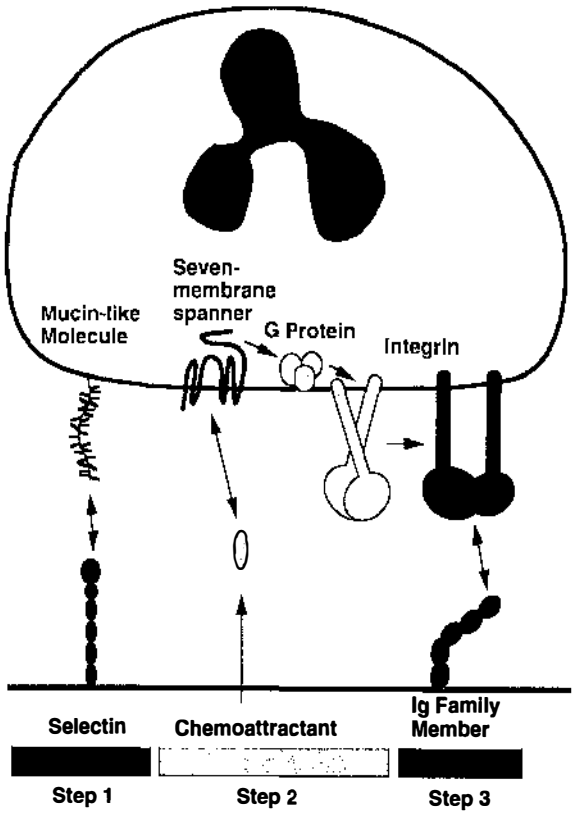


Figure 1 Three sequential steps provide the traffic signals that regulate leukocyte localization in the vasculature. Selectin molecules that bind carbohydrate ligands, often displayed on mucin-like molecules, are responsible for the initial tethering of a flowing leukocyte to the vessel wall and labile, rolling adhesions (*Step 1*). Tethering brings leukocytes into proximity with chemoattractants that are displayed on or released from the endothelial lining of the vessel wall. Chemoattractants bind to receptors that span the membrane seven times on the surface of leukocytes. These couple to G proteins, which transduce signals that activate integrin adhesiveness (*Step 2*). The integrins can then bind to immunoglobulin superfamily (IgSF) members on the endothelium, increasing adhesiveness and resulting in arrest of the rolling leukocyte (*Step 3*). Following directional cues from chemoattractants and using integrins for traction, leukocytes then cross the endothelial lining of the blood vessel and enter the tissue.

displayed together on endothelium but act on leukocytes in a sequence that was first defined for neutrophils and appears to hold true with slight modification for lymphocyte homing as well (Figure 1). The selectin (Step 1) allows cells to tether and roll, the chemoattractant (Step 2) tells cells to activate integrin adhesiveness and put on the brakes, and the Ig family member (Step 3) binds integrins and causes cells to come to a full stop. These three steps,

with multiple molecular choices at each step, provide great combinatorial diversity in signals. Accordingly, the selective responses of different leukocyte classes to inflammatory agents, as well as the preferential recirculation patterns of distinct lymphocyte subpopulations, can be explained by their distinct receptivity to combinations of molecular signals. Following an overview of leukocytes and endothelium, and of the molecules important in their interactions, I review the traffic signals that enable selective emigratory behavior of monocytes and neutrophils, and then elaborate how a paradigm of three or four sequential signals can be extended to lymphocyte recirculation. This review updates and extends about twofold a previous one (240). For recent reviews see 12, 25, 26, 36, 42, 73, 90, 97, 143, 159, 160, 167, 172, 196, 206, 223, 280.

THE FUNCTION OF LEUKOCYTE CLASSES CORRELATES WITH CIRCULATORY BEHAVIOR

Neutrophilic granulocytes are among the most abundant leukocytes in the bloodstream and the first to appear at sites of bacterial infection or injury. Neutrophils are produced at the prodigious rate of 10^9 cells/kg body wt/day in the bone marrow and have a half-life in the circulation of 7 h. Their lifespan after extravasation is hours or less (54). Their primary function is to phagocytose and eliminate foreign microorganisms and damaged tissue.

Monocytes are far less numerous in the blood than neutrophils, where their half-life is about 24 h (113). Like neutrophils, they are phagocytic and accumulate in response to traumatic injury or bacterial infection. However, monocytes differ from neutrophils, in that they accumulate at sites where T lymphocytes have recognized antigen, as in delayed type hypersensitivity reactions and graft rejection. Monocytes are important effector cells in antigen-specific T cell immunity, are activated by T cell products such as γ-interferon, and can organize around parasites into protective structures called granulomas. After extravasation, monocytes may also differentiate into longer-lived tissue macrophages or mononuclear phagocytes such as the Kupffer cells of the liver, which have a half-life of weeks to months.

In contrast to the neutrophil and monocyte, a lymphocyte may emigrate and recirculate many thousands of times during its life history. Recirculation of lymphocytes correlates with their role as antigen receptor–bearing surveillance cells. Lymphocytes function as the reservoir of immunological memory, and recirculate through tissues to provide systemic memory. Few of the body's lymphocytes are present at any one time in the bloodstream, where their half-life is 2 h. Distinct subsets of lymphocytes extravasate through the microvasculature in tissues such as skin and gut, and through specialized high endothelial venules (HEV) in lymphoid organs (39, 159, 196). After migrating

through tissue, lymphocytes find their way into the lymphatics. They percolate through draining lymph nodes in the lymphatic system and finally enter the thoracic duct, through which they return to the bloodstream. This journey is completed roughly every 1 to 2 days.

ENDOTHELIUM

By displaying specific signals, the endothelium is the most active player in controlling leukocyte traffic. Vascular endothelium is diversified at a number of levels. Large vessels differ from small vessels and capillaries, venular endothelium differs from arterial endothelium, and endothelial phenotype varies between tissues. The preferential migration of leukocytes from postcapillary venules may be related to factors such as shear stress, which is lower there and hence more favorable for leukocyte attachment than in capillaries or arterioles, or to events that occur when leukocytes pass through capillaries. However, when flow is controlled so that shear stress is equivalent in arterioles and venules (152), or when the direction of blood flow is reversed (182), attachment and emigration is far greater from venules, suggesting molecular differences in their endothelial surfaces. In agreement with this, P-selectin is much more abundant on postcapillary venules than on large vessels, arterioles, or capillaries (168), and induction of E-selectin and vascular cell adhesion molecule-1 (VCAM-1) expression in inflammation is most prominent on postcapillary venules (25). The mucin-like cluster of differentiation 34 (CD34) molecule is well expressed on capillaries and is absent from most large vessels (82), and CD36 is expressed on microvascular but not large vessel endothelium (251). The extracellular matrix may exert an influence on endothelial differentiation, as exemplified by modulation of adhesiveness (279). The high endothelium in lymphoid tissue, which expresses addressins for lymphocyte recirculation, is one of the most dramatic examples of endothelial specialization (196).

Inflammatory cytokines dramatically and selectively modulate the transcription and expression of adhesion molecules and chemoattractants in endothelial cells (203). Tumor necrosis factor (TNF) and interleukin-1 (IL-1) increase adhesiveness of endothelium for neutrophils and lymphocytes and induce intercellular adhesion molecule-1 (ICAM-1), E-selectin, and VCAM-1. IL-4, synergistically with other cytokines, increases adhesion of lymphocytes and induces VCAM-1 (164, 259). It is likely that the precise mixture of chemoattractants and cytokines produced at inflammatory sites in vivo determines which types of leukocytes emigrate. Thus injection into skin of IL-1α induces emigration of neutrophils and monocytes; as do lipopolysaccharide (LPS) and TNF-α, but with more prolonged emigration of the monocytes. IFN-γ induces emigration of monocytes but not neutrophils (113). IFN-γ and TNF-α, but not

IL-1α or LPS, recruit lymphocytes, and IL-4 is ineffective by itself but synergizes with TNF (33, 60, 118).

Acting more quickly than cytokines, vasoactive substances such as histamine and thrombin modulate endothelial function in seconds or minutes. They induce secretion of the storage granules of endothelial cells and platelets. Furthermore, they dilate arterioles and increase plasma leakage, which raises the hematocrit within microvessels, and thereby alters the rheology of blood so as to increase the collision of leukocytes with the vessel wall (218). Furthermore, arteriolar dilation and the ensuing increased blood flow in inflammatory sites are responsible for two of the cardinal signs of inflammation, rubor (redness) and calor (heat), and greatly enhance the discharge and, thus, accelerate the accumulation of leukocytes.

Area Code Molecules

SELECTINS Multiple protein families, each with a distinct function, provide the traffic signals for leukocytes. The selectin family of adhesion molecules (Figure 2) has an N-terminal domain homologous to Ca^{2+}-dependent lectins (25, 144, 167, 206, 238). The name selectin capitalizes on the derivation of lectin and select from the same Latin root, meaning to separate by picking out. Selectins are limited in expression to cells of the vasculature (Figure 2). L-selectin is expressed on all circulating leukocytes, except for a subpopulation of lymphocytes (85, 126, 151). P-selectin is stored preformed in the Weibel-Palade bodies of endothelial cells and the α granules of platelets. In response to mediators of acute inflammation such as thrombin or histamine, P-selectin is rapidly mobilized to the plasma membrane to bind neutrophils and monocytes (86, 140, 168). E-selectin is induced on vascular endothelial cells by cytokines such as IL-1, LPS, or TNF and requires de novo mRNA and protein synthesis (27).

CARBOHYDRATES AND MUCIN-LIKE MOLECULES All selectins appear to recognize a sialylated carbohydrate determinant on their counter-receptors (26, 143, 206). E-selectin and P-selectin recognize carbohydrate structures that are distinct, but are both closely related to the tetrasaccharide sialyl Lewis x and its isomer sialyl Lewis a (Figure 2). The actual ligand structures for E- and P-selectin are more complex than sialyl Lewis a or x, as shown by display of the ligand for E-selectin, but not P-selectin, on fucosyl transferase-transfected cells that express sialyl Lewis x (141). The affinity of E-selectin for soluble sialyl Lewis x or a is quite low, with K_d = 0.2–0.8 mM (183), which suggests that a higher affinity ligand may yet be identified. P-selectin is specific for carbohydrate displayed on the P-selectin glycoprotein ligand (PSGL-1), suggesting either that PSGL-1 expresses a specific carbohydrate structure or that

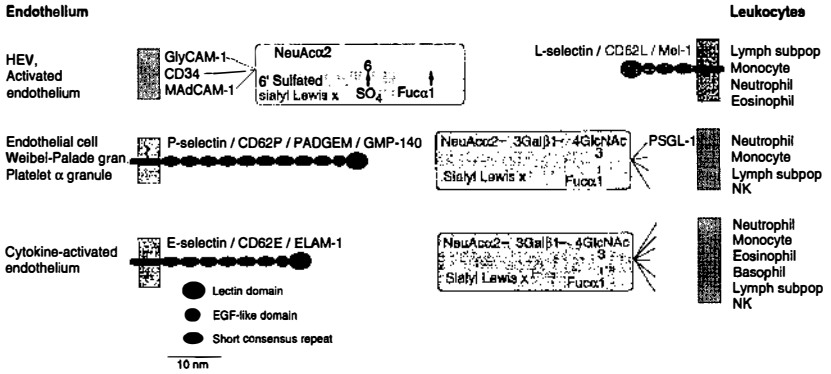


Figure 2 Selectins and their ligands. The selectins are shown to scale, based on electron micrographs of P-selectin (260), the X-ray structure of E-selectin lectin and EGF domains (91), and estimates of the sizes of the short consensus repeats (SCR) (238). P-selectin is shown palmitylated on a transmembrane cysteine (84). The carbohydrates are not to scale. Sialyl Lewis a and x contain Galβ1–3(Fucα1–4)GlcNAc and Galβ1–4(Fucα1–3)GlcNAc linkages, respectively.

PSGL-1 protein forms part of the ligand binding site (173). The affinity of P-selectin for PSGL-1 is high with a K_d = 70 nM(260). Structure-function studies suggest that the Ca^{2+}-binding site and a cluster of basic residues on E-selectin coordinate with the fucosyl and sialic acid carboxylate moieties, respectively, of sialyl Lewis x (78, 91).

The carbohydrate ligands for L- and P-selectin are O-linked to specific mucin-like molecules. Mucins are serine- and threonine-rich proteins that are heavily O-glycosylated and have an extended structure. L-selectin recognizes at least two mucins in HEV (Figure 3): glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), which is secreted (144), and CD34, which is on the cell surface (17). The carbohydrate ligand for L-selectin is related to sialyl Lewis a and x (19, 83), contains sialic acid and sulfate, and is O-linked to mucin-like structures of HEV (206). Structural studies on the carbohydrates of GlyCAM-I show that 6′ sulfated sialyl Lewis x (Figure 2) is a major oligosaccharide capping group and is a candidate for the ligand structure (101).

The mucin-like P-selectin glycoprotein ligand (PSGL-1) is a disulfide-linked dimer of 120-kDa subunits (173) that is sensitive to O-glycoprotease, which selectively cleaves mucin-like domains (186, 245). PSGL-1 (Figure 3) was isolated by screening for cDNA that expressed ligand activity (212). COS cells must be transfected both with the PSGL-1 cDNA and α-3/4 fucosyl transferase cDNA to express P-selectin ligand activity. By contrast, COS cells cotransfected with cDNA for α-3/4 fucosyl transferase and another mucin-like molecule that is expressed by neutrophils, CD43, lack P-selectin ligand activity.

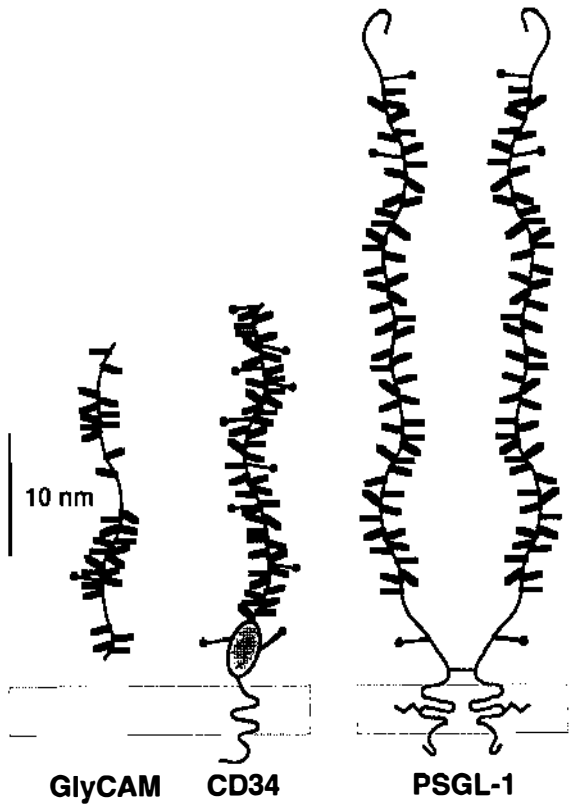


Figure 3 Mucin-like carriers of selectin ligands. The GlyCAM (144) and CD34 (17, 227) molecules synthesized by peripheral lymph node HEV and MAdCAM-1 molecule synthesized by mucosal HEV (see Figure 5) bear O-linked carbohydrates that bind to L-selectin. CD34 has a globular domain that may be Ig-like (13) and is resistant to O-glycoprotease (250). The PSGL-1 molecule on neutrophils bears O-linked carbohydrates that bind to P-selectin (186, 212). A cysteine in the transmembrane region is predicted to be palmitylated. O-linked sites and N-linked sites are shown as bars and lollipops, respectively. The length of the mucin-like domains, and the percent of serines and threonines that are O-glycosylated, are proportioned to measurements for CD43 (45 nm per 224 amino acids and 75–90% of O-glycosylation) (65).

FUNCTION OF SELECTINS AND THEIR LIGANDS Selectins mediate functions unique to the vasculature, the tethering of flowing leukocytes to the vessel wall, and the formation of labile adhesions with the wall that permit leukocytes subsequently to roll in the direction of flow. One study demonstrated this with purified P-selectin incorporated into supported planar lipid bilayers on one wall of a flow chamber (147). At wall shear stresses within the range of those found in postcapillary venules, neutrophils formed labile attachments to the

P-selectin in the bilayer and rolled in response to fluid drag forces. In other studies, intravascular infusion of a soluble L-selectin/IgG chimera inhibited neutrophil rolling attachments in vivo (153), as did infusion of anti-L-selectin monoclonal antibodies (266). More recent studies have shown that neutrophils roll on E-selectin in purified form (148) or on the endothelial cell surface both in vitro (1) and in vivo (188), that monoclonal antibody (mAb) to P-selectin decreases neutrophil rolling in vivo (28), and that neutrophil rolling in the microvasculature of mice genetically deficient in P-selectin is almost completely absent (165).

P- and L-selectin may cooperate with one another because inhibition of either almost completely inhibits neutrophil rolling in vivo (153, 165, 266, 267). E- and L-selectin also appear to cooperate (135, 148, 200, 265). A class of ligand that is closely associated with L-selectin on the neutrophil surface is required for the initial tethering during flow to E-selectin bilayers, after which another class of ligands that mediates rolling takes over (145).

Selectins can mediate tethering of a flowing cell in the span of a millisecond. Other adhesion receptors require minutes to develop similar adhesive strength and do not mediate rolling (51, 147). It has been hypothesized that selectins differ from other adhesion molecules not in affinity (K_{eq}), but in having much more rapid association (K_{on}) and dissociation (K_{off}) rate constants (147), as has recently been confirmed (Table 1). Rolling is intermittent and appears mediated by random association and dissociation of selectin-ligand bonds, a small number of which tether a leukocyte to the vessel wall at any one time. A rapid association rate facilitates the initial tethering in flow. A rapid dissociation rate ensures that even with multiple selectin-ligand bonds, it will not take long before the bond that is most upstream randomly dissociates, allowing the cell

Table 1 Fast on and off rates of a selectin and affinity modulation of an integrin

	K_{on} ($M^{-1}s^{-1}$)	K_{off} (s^{-1})	K_d (μM)
P-selectin	1.4×10^{7}[a]	1[b]	0.07[c]
LFA-1 low affinity[d]	3×10^{2}	0.03	100
LFA-1 high affinity[e]	ND[f]	ND	0.6

[a] Calculated from $K_{on} = K_{off}/K_d$

[b] At very low P-selectin densities in lipid bilayers, neutrophils, attach transiently, i.e. they subsequently detach rather than roll. Measurements of the cellular dissociation rate suggest that the $t_{1/2}$ for dissociation of a single selectin-ligand bond is about 0.7 s (R Alon & T Springer, unpublished data).

[c] For binding of monomeric, truncated P-selectin to neutrophils (260).

[d] k_{on}, k_{off}, and K_d were measured by competitive inhibition by monomeric, truncated ICAM-1 of binding of Fab to LFA-1 on resting lymphocytes (157).

[e] Same as d but for phorbol ester-stimulated lymphocytes. Approximately 20% of the cell surface LFA-1 was in the high affinity state (157).

[f] ND = not determined.

to roll forward a small distance until it is held by the next most upstream bond (96, 147).

The elongated molecular structure of selectins and mucins and their segmental flexibility (65, 260) are predicted to enhance their accessibility for binding to counter-structures on closely opposed cells (147). P-selectin and PSGL-1 are currently the most elongated adhesion molecules known (Figure 3 and 4) and can bridge together two cells with plasma membranes about 0.1 μm apart. Expression on cytoplasmic protrusions further enhances accessibility. L-selectin is clustered on microvilli of neutrophils (79, 200), which project about 0.3 μm above the surface of a cell with a diameter of 7 μm, and contain 90% of the L-selectin (D Bainton et al, unpublished data). In keeping with this topographic distribution, rolling in vivo requires the integrity of the L-selectin cytoplasmic domain and is inhibited by cytochalasin B (125). Lymphocytes bind through microvilli to HEV (7, 261). Conversely, the mucin-like CD34 molecule (227) is concentrated on filopodia of nonspecialized endothelial cells found in the microvasculature of most tissues (82). These filopodia are concentrated near junctions between endothelial cells, and electron micrographs of granulocytes binding to the microvasculature in inflammatory sites suggest that the earliest binding event is to these filopodia (62).

CHEMOATTRACTANTS Chemoattractants are important in activation of integrin adhesiveness and in directing the migration of leukocytes. In chemotaxis, cells move in the direction of increasing concentration of a chemoattractant, which typically is a soluble molecule that can diffuse away from the site of its production, where its concentration is highest (70, 274). Leukocytes, which can sense a concentration difference of 1% across their diameter, move steadily in the direction of the chemoattractant. There is much interplay between adhesion molecules and chemoattractants because adhesion to a surface is required to provide the traction necessary for migration directed by chemoattractants, and chemoattractants can activate adhesiveness.

The alternative mechanism to chemotaxis is haptotaxis. In haptotaxis, cells migrate to the region of highest adhesiveness (45). Thus on a gradient of an adhesive ligand affixed to the surface of other cells or to the extracellular matrix, and in the absence of a chemotactic gradient, motile cells will tend to accumulate in the region of highest ligand density. Both chemotaxis and haptotaxis can contribute to cell localization, but haptotaxis has yet to be demonstrated in vivo.

Classical leukocyte chemoattractants act broadly on neutrophils, eosinophils, basophils, and monocytes (Table 2). A recently described family of chemoattractive cytokines, termed chemokines, are 70–80 residue polypeptides and have specificity for leukocyte subsets (12, 172). Two subfamilies of chemokines have been defined by sequence homology and by the sequence

Table 2 Leukocyte chemoattractants

Chemoattractant	Origin	Responding cells
Classical chemoattractants[a]		
N-formyl peptides	Bacterial protein processing	Monocyte, neutrophil, eosinophil, basophil
C5a	Complement activation	Monocyte, neutrophil, eosinophil, basophil
Leukotriene B4	Arachidonate metabolism	Monocyte, neutrophil
Platelet-activating factor (PAF)	Phosphatidylcholine metabolism	Monocyte, neutrophil, eosinophil
CXC chemokines[b]		
IL-8/NAP-1	T lymphocyte, monocyte, endothelial cell, fibroblast, keratinocyte, chondrocyte, mesothelial cell	Neutrophil, basophil
CTAP-III/β-thromboglobulin/ NAP-2	Successive N-terminal cleavage of platelet basic protein released from α-granules	Neutrophil, basophil, fibroblast
gro/MGSA	Fibroblast, melanoma, endothelial cell, monocyte	Neutrophil, melanomas, fibroblast
ENA-78	Epithelium	Neutrophil
CC chemokines[c]		
MCP-1	T lymphocyte, monocyte, fibroblast, endothelial cell, smooth muscle	Monocyte, T lymphocyte subpopulation, basophil
MIP-1α	Monocyte, B and T lymphocyte	Monocyte, T lymphocyte subpopulation, basophil, eosinophil
RANTES	T lymphocyte, platelet	Monocyte, T lymphocyte subpopulation, eosinophil
I-309	T lymphocyte, mast cell	Monocyte

References:
[a] (70, 232), [b] (12, 29, 43, 137, 172), [c] (2, 12, 43, 124, 172, 208, 214–216, 253, 255).

around two cysteine residues (Table 2). The CXC or α chemokines tend to act on neutrophils and nonhematopoietic cells involved in wound healing, whereas the CC or β chemokines tend to act on monocytes and in some cases on eosinophils and lymphocyte subpopulations.

It has long been debated whether chemoattractants can act in the circulation, where they would be rapidly diluted and swept downstream by blood flow. Tethering and rolling of leukocytes through selectins would enhance exposure to chemoattractants by prolonging contact with the vessel wall. However, retention of chemoattractants at their site of production by noncovalent interactions with molecules on the vessel wall and within the inflammatory site may also be important. Heparin-binding sites on chemokines provide a mech-

anism for retention in the extracellular matrix (109) to enhance concentration gradients and perhaps to present chemokines on the endothelium to circulating leukocytes (207, 253).

CHEMOATTRACTANT RECEPTORS Leukocyte chemoattractant receptors have multiple functions. They not only direct migration, but also activate integrin adhesiveness, and stimulate degranulation, shape change, actin polymerization, and the respiratory burst (232). Chemoattractant receptors are G protein-coupled receptors that span the membrane seven times. Ligand binding to the seven membrane-spanner is coupled to exchange of GTP for GDP bound to the associated G protein heterotrimer and results in activation by the G protein α and $\beta\gamma$ subunits of signaling effectors such as phospholipase C-β_2 (277). This results in release of diacylglycerol and inositol phosphates, and mobilization of Ca^{2+}. Neutrophils and lymphocytes express $G\alpha_{i2}$ and $G\alpha_{i3}$ subunits (18, 232). The Gα subunits of the α_i class are ADP-ribosylated and irreversibly inactivated by pertussis toxin. All the biological effects of leukocyte chemoattractants are inhibited by pertussis toxin. Coupling through $G\alpha_i$ subunits has been confirmed by reconstitution in transfected cells (277). The lipid mediators LTB4 and PAF are as active as formylated bacterial peptides C5a and IL-8 in stimulating chemotaxis, but less active in stimulating the respiratory burst and other functions of neutrophils (232); this correlates with their ability to couple to distinct Gα subunits in transfected cells (6).

Cloning of the receptors for formylated bacterial peptides C5a and PAF has shown that they are expressed on both neutrophils and monocytes, whereas the receptor for IL-8 is expressed only on neutrophils (181). The receptor for MCP-1 is expressed on monocytic cells but not on neutrophils (52). Thus the specificity of chemoattractants is regulated by the cellular distribution of their receptors.

INTEGRINS Integrins are perhaps the most versatile of the adhesion molecules. Integrin adhesiveness can be rapidly regulated by the cells on which they are expressed. Each integrin contains a noncovalently associated α and β subunit, with characteristic structural motifs (Figure 4). Five integrins are important in the interaction of leukocytes with endothelial cells. Their cellular distribution, ligand specificity, and structure are summarized in Table 3 and Figure 4.

ACTIVATION OF INTEGRINS The adhesiveness of LFA-1 and VLA-4 on T lymphocytes is activated by cross-linking of the antigen receptor and other surface molecules (73, 223, 238). Increased adhesiveness occurs within a few minutes, is not accompanied by any change in quantity of surface expression, and appears to result from both conformational changes that increase affinity for ligand, and altered interaction with the cytoskeleton (73, 81, 88). However,

Table 3 Integrins in leukocyte-endothelial interactions

Subunits	Names	Distribution	Ligands
Leukocyte integrins[a]			
$\alpha^L\beta_2$	LFA-1, CD11a/CD18	B and T lymphocyte, monocyte, neutrophil	ICAM-1, ICAM-2, ICAM-3
$\alpha^M\beta_2$	Mac-1, CR3, CD11b/CD18	Monocyte, neutrophil	ICAM-1, iC3b, fibrinogen, factor X
$\alpha^x\beta_2$	p150,95, CR4, CD11c/CD18	Monocyte, neutrophil, eosinophil	iC3b, fibrinogen
α^4 Integrins[b]			
$\alpha^4\beta_1$	VLA-4, CD49d/CD29	B and T lymphocyte, monocyte, neural crest-derived cells, fibroblast, muscle	VCAM-1, fibronectin
$\alpha^4\beta_7$	LPAM-1, CD49d/CD-	B and T lymphocyte subpopulations	MAdCAM-1, VCAM-1, fibronectin

References
[a] (133, 238) [b] (24, 31, 50, 100, 105, 107, 108, 110, 211, 238).

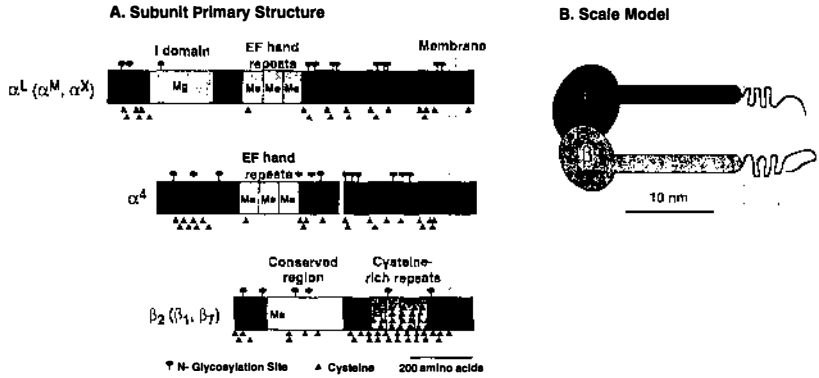


Figure 4 Integrins that bind endothelial ligands. (*A*) Schematics of representative integrin α and β subunits. The structures of α^L (142) and β_2 (134) integrin subunits are shown as representative of α^M and α^X or β_1 and β_7, respectively; cysteines are identical, and glycosylation sites vary but are sparse in the I domain and EF hand repeats. The EF hand repeats are divalent metal-binding motifs that may bind Ca^{2+} or Mg^{2+} (labeled Me). A binding site for Mg^{2+} and Mn^2 but not Ca^{2+} has been identified in the I domain (171). The α^4 integrin subunit has a posttranslational proteolytic cleavage site (252). A putative divalent cation binding site has been defined in the conserved domain of the integrin β_3 subunit and is shown for β_2 (156). (*B*) Scale model of an integrin based on electron micrographs of the integrins gpIIbIIIa (44) and VLA-5 ($\alpha^5\beta_1$) (184).

it is unlikely that recognition by T cell receptors of antigen on endothelial cells (204) is a step in lymphocyte trafficking because traffic of lymphocytes that can and cannot recognize specific antigen is increased in antigen-induced inflammation. Although evidence has been presented that binding of neutrophils to selectins can activate adhesiveness of integrins (155), other evidence has failed to confirm this (148, 158; T Diacovo & T Springer, unpublished data).

Thus far the best candidates for activation of integrin adhesiveness within the vasculature are chemoattractants. Adhesiveness of Mac-1 and LFA-1 on neutrophils and monocytes is activated by N-formylated peptide and IL-8 (38, 74, 154, 154, 229, 276). In contrast to LFA-1 on lymphocytes and neutrophils, Mac-1 on neutrophils is increased about tenfold on the surface by chemoattractant-stimulated fusion of secretory granules with the plasma membrane (221); however, this is neither sufficient nor necessary for increased adhesiveness (195, 262). The transient nature of the activation of integrin adhesiveness (76, 154) provides a mechanism for de-adhesion and, perhaps, for retraction of the trailing edge of a leukocyte from the substrate during cell migration.

Conformational changes in LFA-1 and Mac-1 that are associated with increased adhesiveness are suggested by the reaction of mAb and antigen-binding fragments (Fab), i.e. they react only with these molecules after cellular

activation (72, 127, 138, 201). After chemoattractant activation of neutrophils, saturation binding shows that 10% of the surface Mac-1 molecules express an activation epitope, yet mAb to this epitope completely blocks binding to ligands such as ICAM-1 and fibrinogen, which suggests that ligand binding is mediated by a subpopulation of activated Mac-1 molecules (72). The I domain of leukocyte integrins is important in ligand binding (71, 171) and expresses activation epitopes (72, 138). Recent measurements of the affinity of cell surface LFA-1 for soluble, monomeric ICAM-1 (Table 1) have directly demonstrated that cellular activation increases the affinity of a subpopulation of LFA-1 molecules by approximately 200-fold (157).

Surprisingly, the integrin VLA-4, by contrast to LFA-1 and Mac-1, appears to be capable of supporting rolling. Lymphocytes can tether in flow and subsequently roll on VCAM-1. If activated while rolling by phorbol ester or TS2/16 mAb to the β_1 subunit, the lymphocytes arrest and develop firm adhesion. Activated lymphocytes tether as efficiently as resting lymphocytes but do not roll. Fibronectin can support development of firm adhesion in static conditions but not in tethering or rolling in flow. VCAM-1 is less efficient than selectins in mediating tethering and rolling (R Alon et al, submitted).

IgSF MEMBERS ON ENDOTHELIUM AS INTEGRIN LIGANDS In a paradigm first established with ICAM-1 binding to LFA-1, several immunoglobulin superfamily (IgSF) members, expressed on endothelium, bind to integrins expressed on leukocytes (Figure 5). ICAM-1, ICAM-2, and ICAM-3 are products of distinct and homologous genes and were all initially identified by their ability to interact with LFA-1 (68, 210, 243). ICAM-1 has also been found to bind to Mac-1 through a distinct site in its third Ig domain (74, 75, 230) (Figure 5). Induction of ICAM-1 on endothelium and other cells by inflammatory cytokines may increase cell-cell interactions and leukocyte extravasation at inflammatory sites, whereas constitutive expression of ICAM-2 may be important for leukocyte trafficking in uninflamed tissues, as in lymphocyte recirculation. ICAM-3 is restricted to leukocytes. All three ICAMs contribute to antigen-specific interactions, thus inhibition with mAb to all three is required to completely block LFA-1-dependent antigen-specific T cell responses (67).

VCAM-1 is inducible by cytokines on endothelial cells, and on a more restricted subset of nonvascular cells than ICAM-1 (25). A single VCAM-1 gene gives rise through alternative splicing to a seven domain isoform and to a second isoform that contains either six domains or three domains and a glycosyl phosphatidylinositol membrane anchor (130, 176, 259) (Figure 5). VCAM-1 is a ligand for the integrin $\alpha^4\beta_1$ (VLA-4) and binds weakly to $\alpha^4\beta_7$ (50, 77, 211). In contrast to the shorter isoforms, the seven domain isoform of VCAM-I has two binding sites for VLA-4 in highly homologous domains 1 and 4 (191, 192, 268, 269).

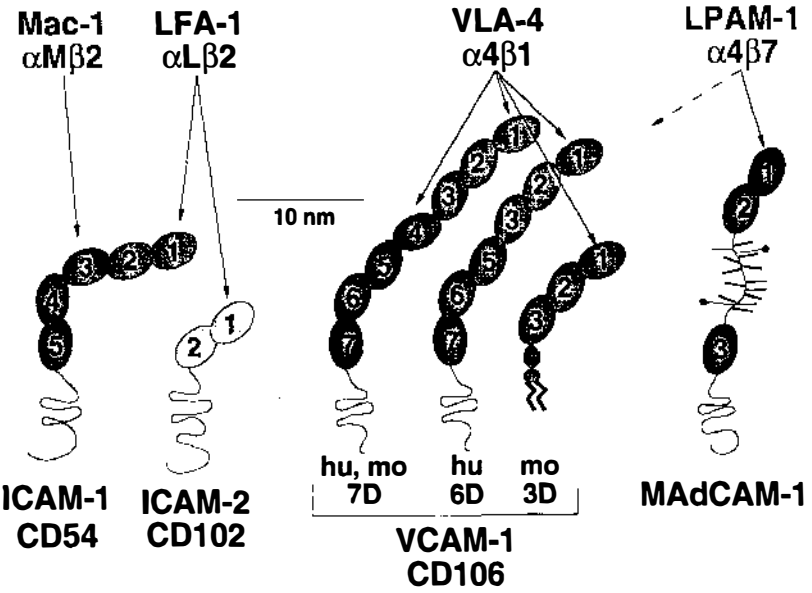


Figure 5 Ig superfamily adhesion receptors on endothelium, and their integrin-binding sites. Members of the Ig superfamily share the immunoglobulin domain, composed of 90 to 100 amino acids arranged in a sandwich of two sheets of anti-parallel β-strands, which is stabilized by one or (in the N-terminal domain of the molecules shown) two disulfide bonds. The immunoglobulins and T cell receptors are the only known members of this family that undergo somatic diversification. The function of the IgSF in adhesion evolutionarily predates specialization for antigen recognition. The shape and size of the ICAM-1 molecule, with its unpaired Ig domains and bend, was determined by electron microscopy (131, 242), as was that of VCAM-1 (192). Immunoglobulin domains are ellipsoids with a length of 4 nm parallel to the β-strands and 2.5 nm in the other dimensions. The mucin-like region of MAdCAM-1 is modeled as described in the legend to Figure 3. N-linked glycosylation sites in the Ig domains of this and the other molecules are not shown. References for structures (in parentheses) and for localization of the domains to which integrins bind (in brackets) follow: ICAM-1 (226, 244) [75, 242]; ICAM-2 (243); VCAM-1 (176, 190, 205) [191, 192, 268, 269]; MAdCAM-1 (34).

An addressin for lymphocyte recirculation to mucosa is expressed on Peyer's patch HEV and on other venules (248). Now termed mucosal addressin cell adhesion molecule (MAdCAM-1), it contains three Ig-like domains and a mucin-like region interposed between domains 2 and 3 (34) (Figure 5). MAdCAM-1 binds the integrin $\alpha^4\beta_7$ but not $\alpha^4\beta_1$ (24, 108). Furthermore, carbohydrates attached to the mucin-like domain of MAdCAM-1 bind L-selectin and mediate lymphocyte rolling (20). Thus MAdCAM-1 has a dual function as an integrin and selectin ligand.

OTHER MOLECULES CD31 is an IgSF member expressed on leukocytes, platelets, and at cell-cell junctions on endothelium (3, 4, 177, 185, 228, 247, 254).

CD31 can bind homophilically to itself and also heterophilically to an uncharacterized counter-receptor. mAb cross-linking of CD31, similarily to many but not all other lymphocyte surface molecules, can trigger integrin adhesiveness (254). Interaction between CD31 on endothelial junctions and CD31 on leukocytes appears to be required for transmigration but not for integrin-mediated binding of leukocytes to endothelium (178). CD31-CD31 interaction may represent a fourth step in transendothelial migration that overlaps the integrin-mediated step and may contribute to the maintenance of the permeability barrier function of endothelia during transmigration.

CD44 is a widely distributed molecule in the body that is homologous with cartilage-link protein, is extensively alternatively spliced, and can bear heparin sulfate or chondroitin sulfate side chains (98). The best understood function of CD44 is as a major surface receptor for hyaluronate (11, 64). Alternatively spliced forms of CD44 are important in tumor metastasis (92) and in localization of antibody-secreting cells (9). CD44 (H-CAM, Hermes) was at one time mistakenly thought to be the human equivalent of murine mel-14 (L-selectin). It participates in vitro in lymphocyte interaction with HEV and activated endothelium (119, 189). However, lack of cell surface CD44 has no effect on lymphocyte recirculation in vivo (41).

Toward a Multi-Step Model of Neutrophil Emigration in Inflammation

INTEGRINS AND SELECTINS Patients who are genetically deficient in the leukocyte integrins, owing to mutations in the common β_2 integrin CD18 subunit, provided early evidence that adhesion molecules are required for leukocyte extravasation in vivo (8, 133). Leukocyte adhesion deficiency-I (LAD-I) patients have life-threatening bacterial infections, and neutrophils in these patients fail to cross the endothelium and accumulate at inflammatory sites, despite higher than normal levels of neutrophils in the circulation. In vitro, LAD-I neutrophils or normal neutrophils treated with mAb to the leukocyte integrins are deficient in binding to and migrating across resting or activated endothelial monolayers (35, 231). Even though capable of binding to activated endothelium through selectins, LAD-I neutrophils fail to transmigrate (231). mAb to the leukocyte integrin β_2 subunit, and in some cases the integrin α^M subunit, have been found to have profound effects in vivo (97). These mAb prevent the neutrophil-mediated injury that occurs when ischemic tissue is reperfused, and thus can prevent death from shock after blood loss, limb necrosis after frostbite or after amputation and replantation, and tissue necrosis from myocardial ischemia and reperfusion. mAb to leukocyte integrins and to ICAM-1 can also inhibit lymphocyte and monocyte-mediated antigen-specific responses in vivo, including delayed-type hypersensitivity, granuloma formation, and allograft rejection (97).

Whereas mAb to the leukocyte integrin β_2 subunit blocked accumulation of leukocytes in tissue in response to chemoattractants, and prevented stable adhesion of leukocytes in the local vasculature, it had no effect on the number of rolling leukocytes on the vessel wall (10). Furthermore, leukocyte integrins were found to mediate binding of neutrophils to endothelial monolayers in a parallel wall flow chamber at subphysiologic, but not at physiologic, shear stresses found in postcapillary venules (146, 231).

Parallel studies showed that selectins were required for leukocyte accumulation in vivo and acted at an early step. Antagonists of L-selectin and E-selectin inhibit neutrophil and monocyte influx into skin, peritoneal cavity, and lung in response to inflammatory agents (122, 123, 151, 180, 272). mAb to L-selectin was shown to inhibit neutrophil accumulation on cytokine-stimulated endothelium at physiologic shear stress (229). Stimulation of neutrophils with chemoattractants results in shedding into the medium within minutes of L-selectin, with kinetics similar to upregulation of surface expression of the integrin Mac-1. Based on this, and the evidence reviewed above, it was hypothesized that selectins might act at a step prior to integrins (132).

Further studies showed that selectins mediate rolling, and function prior to development of firm adhesion through integrins. At sites of inflammation, leukocytes first attach to the vessel wall in a rolling interaction, then become arrested or firmly adherent at a single location on the vessel wall before diapedesis (56). This process was fully reconstituted with purified components of the endothelial surface (147). At physiologic shear stresses, neutrophils attach to and form labile rolling adhesions on phospholipid bilayers containing purified P-selectin, but not on bilayers containing ICAM-1. Chemoattractants stimulate strong, integrin-mediated adhesion to bilayers containing ICAM-1 under static conditions but not in shear flow. At physiologic shear stresses, if both P-selectin and ICAM-1 are present in the phospholipid bilayer, resting neutrophils attach and roll identically as on bilayers containing P-selectin alone. However, when chemoattractant is added to the buffer flowing through the chamber, the rolling neutrophils arrest, spread, and firmly adhere through the integrin-ICAM-1 interaction. Chemoattractant does not enhance interaction of neutrophils with bilayers containing P-selectin alone, but rather inhibits it. These findings show that purified adhesion molecules and chemoattractants representing the endothelial signals can reproduce the key events in leukocyte localization in vivo, and prove that the selectin-mediated step is a prerequisite for the chemoattractant and integrin-mediated steps (147). Complementary in vivo studies showed that mAb to L-selectin, or L-selectin/IgG chimeras, decreased the number of rolling leukocytes (153, 266) and the number of leukocytes that subsequently became firmly adherent, whereas mAb to the β_2 integrin subunit only decreased firm adherence of leukocytes. This suggests that L-selectin acts at a step prior to leukocyte integrins (266). In static assays,

a factor derived from cytokine-stimulated endothelium induced shedding of L-selectin, and if transmigration was blocked with CD18 mAb, induced release of neutrophils from inverted endothelial monolayers, also suggesting that L-selectin acted prior to leukocyte integrin-mediated emigration (229). In elegant confirmation of a three-step model in a static assay of neutrophil adhesion to histamine-stimulated endothelium, juxtacrine cooperation between P-selectin and platelet-activating factor (PAF) was found (158). P-selectin tethered neutrophils to endothelium and thereby augmented stimulation by PAF of CD18-dependent neutrophil adhesion. Stimulation of adhesiveness was by PAF and not by P-selectin, as shown with PAF receptor antagonists.

The requirement for the carbohydrate ligands of selectins for leukocyte emigration in vivo has received strong support from studies of two patients with a genetic defect in biosynthesis of fucose and who therefore lack the ligands for E-selectin and P-selectin (80, 264). The defect, designated LAD-II, has many clinical similarities to LAD-I including strikingly depressed neutrophil emigration into inflammatory sites.

CHEMOATTRACTANTS Chemoattractants are required for transendothelial migration in vitro and in vivo and can induce all steps required for transmigration in vivo. Injection of chemoattractants into skin or muscle leads to robust emigration of neutrophils from the vasculature and accumulation at the injection site (58). Injection of lipopolysaccharide or cytokines that induce IL-8 synthesis also elicits neutrophil emigration. Moreover, mAb to IL-8 markedly inhibits neutrophil emigration into lung and skin in several models of inflammation (179, 220).

The effects of pertussis toxin provide further evidence for the importance of $G\alpha_i$ protein-coupled receptors in leukocyte emigration in vivo. Pretreatment of neutrophils with pertussis toxin inhibits emigration into inflammatory sites (187, 234).

Chemoattractants impart directionality to leukocyte migration. By contrast to intradermal injection, intravascular injection of IL-8 does not lead to emigration (99). Cytokine-stimulated endothelial monolayers grown on filters secrete IL-8 into the underlying collagen layer. Neutrophils added to the apical compartment emigrate into the basilar compartment, but not when the IL-8 gradient is disrupted by addition of IL-8 to the apical compartment (109). Although IL-8 acts as an adhesion inhibitor in some assays (87), the results are partially attributable to disruption of a gradient of IL-8 on activated endothelial monolayers when exogenous IL-8 is added on the same side as the neutrophils.

Chemoattractants act on the local tissue, as well as on leukocytes. Neutrophil chemoattractants injected into the same skin site hours apart will stimulate neutrophil accumulation the first but not the second time, whereas a second

injection into a distant site will stimulate accumulation. Desensitization occurs for homologous chemoattractants only (57, 59). Thus chemoattractants must act on and homologously desensitize a cell type that is localized in tissue. In some cases this localized cell may be the mast cell. Some chemoattractants stimulate the mast cell (which localizes in tissue adjacent to the vasculature), or its better studied relative the basophil, to release histamine (29, 137) and TNF (270). Histamine induces P-selectin and TNF induces E-selectin on endothelium. Thus chemoattractants may indirectly increase selectin expression on endothelium, as well as directly activate integrin adhesiveness on leukocytes.

A Three-Step Area Code for Signaling Neutrophil and Monocyte Traffic

The above evidence indicates that emigration from the vasculature of neutrophils and monocytes is regulated by at least three distinct molecular signals (Figure 1, Figure 6A). A key feature is that selectin-carbohydrate, chemoattractant-receptor, and integrin-Ig family interactions act in sequence, not in parallel. This concept has been confirmed by the observation that inhibition of any one of these steps gives essentially complete, rather than partial, inhibition of neutrophil and monocyte emigration. An important consequence of a sequence of steps, at any one of which there are choices of multiple receptors or ligands that have distinct distributions on leukocyte subpopulations or endothelium, is that it provides great combinatorial diversity for regulating the selectivity of leukocyte localization in vivo, as has been emphasized in several reviews (36, 143, 223, 240, 280).

The term area code for models for cell localization in the body (106, 239) is particularly apt because it is now known that at least three sequential steps are involved. The concepts of area codes and traffic signals can be combined by thinking of how telephone traffic is routed by digital signals. Each type of leukocyte responds to a particular set of area code signals. Inflammation alters the expression and location of the signals on vascular endothelium. It is as if leukocytes carry "cellular phones." An example of how this model works is shown for the two cell types for which the signals are best understood, neutrophils and monocytes (Figure 6B). Chemoattractants provide the greatest number of molecular choices (or "digits") and the greatest cellular specificity.

Refinements to the three-step model are in order. First, selectins actually mediate two steps, initial tethering to the vessel wall and rolling (Figure 6A), which can be distinguished for E-selectin by dependence on different classes of neutrophil ligands (145). Thus selectins can cooperate, and some selectin-ligand combinations may be more important in tethering and others in rolling. Second, the steps are overlapping, rather than strictly sequential (Figure 6A). Although L-selectin is shed from neutrophils soon after activation (132), the

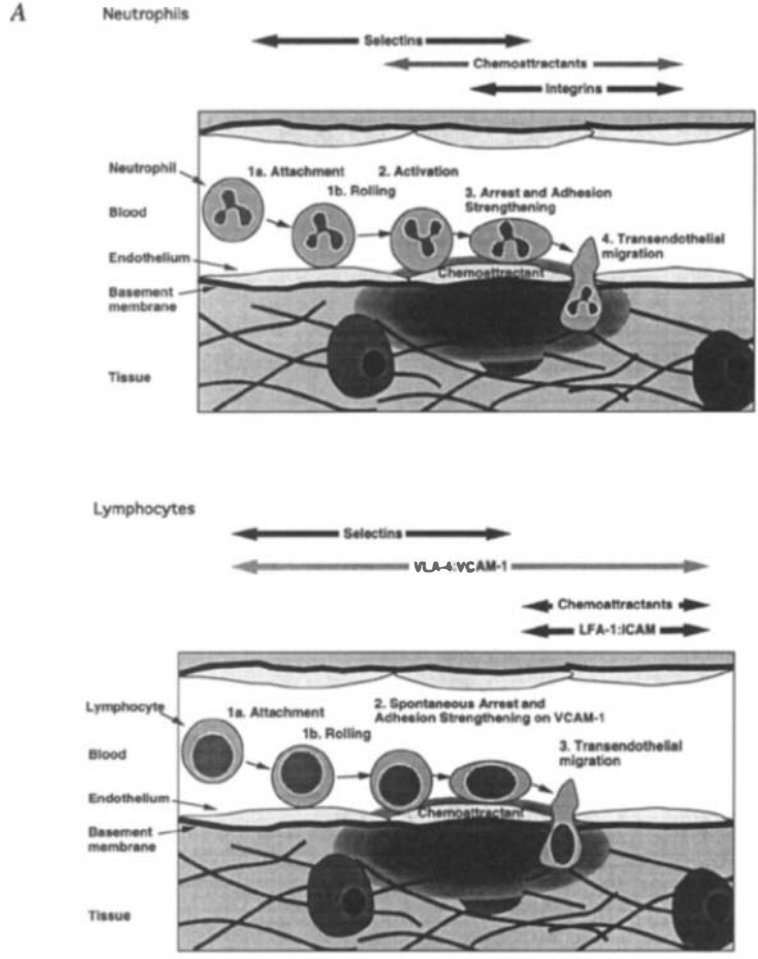
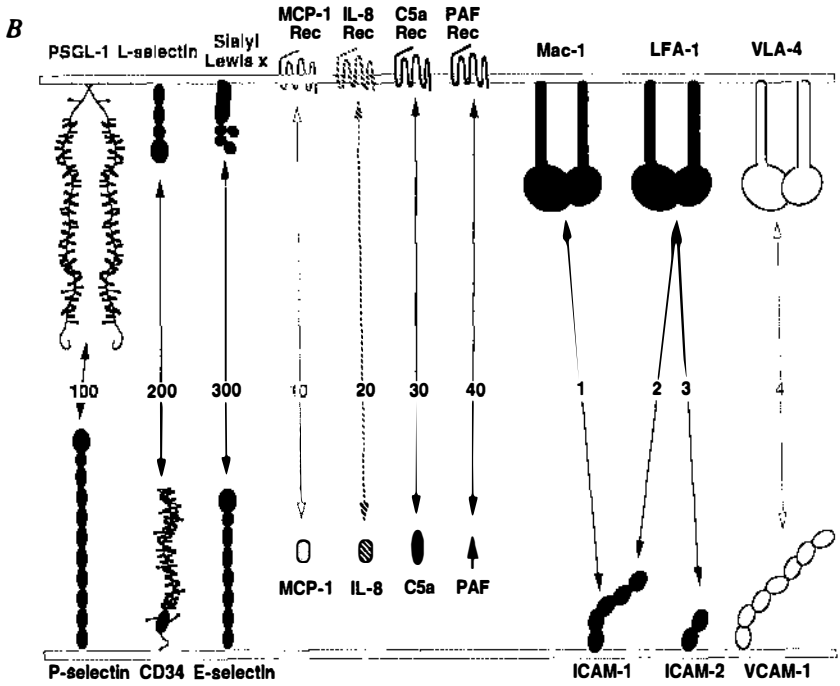


Figure 6 The three-step area code model. (*A*) Selectins, chemoattractants, and integrins act in sequence with some overlap. The sequence in which these signals act on neutrophils and lymphocytes may differ. (*B*) Combinatorial use of different molecules at each step can generate a large number of different area codes, and specificity for distinct leukocyte subpopulations. All of the known selectin and integrin interactions are shown in the hundreds and ones place, respectively; however, only a subset of the chemoattractants is shown in the tens place (see Table 1) due to space limitations. The area codes symbolize how specificity for monocytes, neutrophils, or both can be generated at inflammatory sites.

kinetics of shedding by neutrophils in whole blood (min) are much slower than the transition from rolling to integrin-mediated attachment (ms-s) (266). L-selectin is shed more slowly from lymphocytes than from neutrophils (121, 235). Furthermore, ligands for P-selectin (173) and E-selectin (145) remain on



Monocyte Area Codes	111, 211, 311, 112, 212, 312, 113, 213, 313, 114, 214, 314, 134, 234, 334, 144, 244, 344
Neutrophil Area Codes	121, 221, 321, 122, 222, 322, 123, 223, 323
Monocyte and Neutrophil Area Codes	131, 231, 331, 132, 232, 332, 133, 233, 333, 141, 241, 341, 142, 242, 342, 143, 243, 343
Null Area Codes	124, 224, 324

the neutrophil surface after activation. Thus interactions with selectins will continue after activation of integrins, probably persisting until transendothelial migration is completed. Chemoattractants are required not only for activation of integrin adhesiveness, but also for directional cues during the subsequent step of transendothelial migration. Finally, β_1 integrins that bind to extracellular matrix components are undoubtedly required during migration through the subendothelial basement membrane.

Lymphocyte Recirculation: Distinct Traffic Patterns for Naive and Memory Lymphocytes

Patrolling the body in search of foreign antigen, lymphocytes follow circuits through nonlymphoid and lymphoid tisues (Figure 7). The peripheral lymph nodes draining skin and muscle, and the gut-associated lymphoid tissues such

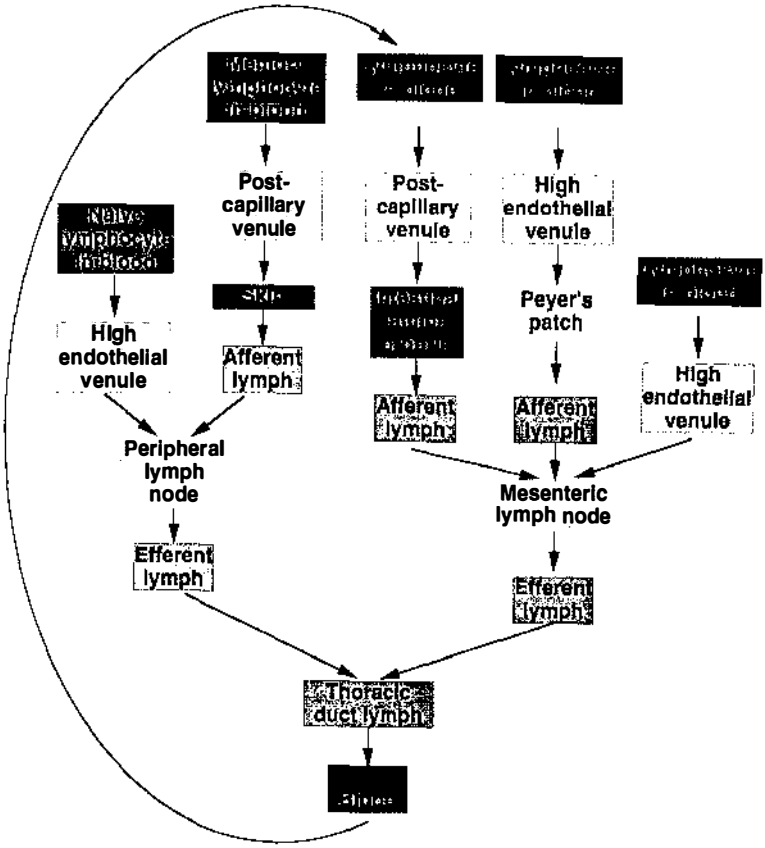


Figure 7 Lymphocyte recirculation routes.

as Peyer's patch, differ in the types of antigens to which lymphocytes are exposed. When collected from lymph draining gut or skin, lymphocytes from adult animals, but not newborns, show a twofold or higher preference to recirculate to the type of organ from which they came and to reappear in the draining lymph (39, 40, 117, 159). This suggests that priming by specific antigen in a particular environment may induce expression of surface receptors that enable preferential recirculation to the type of secondary organ where specific antigen was first encountered. Evidence exists for separate streams of lymphocytes that recirculate through the skin, gut, and lung and that drain into their associated lymphoid tissues (159, 196).

Our understanding of the mechanisms of this selectivity has been advanced by the discovery that "naive" and "memory" lymphocytes prefer different

recirculation pathways (162). When naive lymphocytes encounter antigen, those lymphocytes with receptors specific for the antigen are stimulated to clonally expand and are converted to memory lymphocytes that have altered expression of adhesion receptors and circulatory patterns. Lymphocytes that emigrate in the hind leg of a sheep through flat endothelium in the skin and drain through the afferent lymphatics to the popliteal lymph node are all of the memory phenotype. By contrast, lymphocytes in the efferent lymph from the popliteal lymph node, derived mostly from traffic through HEV, are predominantly of the naive phenotype. Thus, at least for peripheral tissues and lymph nodes, memory lymphocytes emigrate preferentially through tissue endothelium, whereas naive lymphocytes enter the lymph node through HEV (Figure 7). Memory lymphocytes are more sensitive to specific antigen than naive lymphocytes and thus better able to respond to antigen in peripheral tissues, which have fewer antigen-presenting cells than do lymph nodes (160).

Traffic through HEV

The high or cuboidal-shaped endothelial cells found in HEV are specialized for emigration of lymphocytes into peripheral lymph nodes that drain skin and the lymphoid tissues of the mucosa: Peyer's patches, tonsils, and appendix. Emigration into the spleen, by contrast, involves sinusoidal endothelia and molecular mechanisms that are distinct and not yet characterized. About 25% of lymphocytes that circulate through an HEV will bind and emigrate, a much higher percent than through nonspecialized flat venules (30, 275). HEV phenotype is developmentally regulated. The carbohydrate ligands for L-selectin are absent from peripheral lymph node HEV at birth but are displayed at adult levels by 6 weeks (196). If peripheral lymph nodes are deprived of afferent lymph, the HEV convert from a high to a flat-walled endothelial morphology, lose expression of L-selectin ligands, and lose the ability to support lymphocyte traffic (169, 170). Introduction of antigen into the node leads to a full restoration of HEV phenotype and function. Furthermore, intense antigenic stimulation can induce formation of HEV in diverse non-lymphoid tissues (161, 196).

Molecular Mechanisms Defined by the HEV-Binding Assay

When lymphocyte suspensions are overlayed on thin sections cut from frozen lymph nodes, the lymphocytes specifically bind to the morphologically distinct HEV (241). Specific differences have been demonstrated between binding to peripheral lymph node and Peyer's patch HEV (37, 275). T lymphocytes bind one and one-half-fold better than B lymphocytes to peripheral lymph node HEV in vitro, and show a similar preference to recirculate to this site in vivo. B lymphocytes bind two- to threefold better to Peyer's patch than to peripheral lymph node HEV and show similar preference in recirculation in vivo. These

preferences are reflected in the preponderance of T cells in peripheral lymph nodes, and the preponderance of B lymphocytes in Peyer's patch, where they are important in secretion of IgA and IgM into the mucosa (246). Certain lymphoma cells possess marked preference for binding to Peyer's patch or peripheral lymph node HEV in vitro (37) and for metastasis in vivo to mucosal or peripheral lymphoid tissue, respectively (16). Assay of lymphoma cell binding to HEV in the Stamper-Woodruff assay has led to the identification of two important adhesion pathways.

MOLECULES INVOLVED IN BINDING TO PERIPHERAL NODE HEV The L-selectin molecule was initially defined in the mouse with the Mel-14 mAb as a molecule on lymphocytes required for binding to peripheral lymph node, but not Peyer's patch, HEV (85). Conversely, the MECA-79 carbohydrate antigen was defined with mAb that bound specifically to peripheral lymph node HEV and blocked lymphocyte binding. The isolated MECA-79 antigen, termed the peripheral node addressin (249), binds to L-selectin on lymphocytes (22). An L-selectin/IgG chimera was also found to specifically bind to HEV in peripheral lymph node and to block lymphocyte binding (273). The L-selectin/IgG chimera was used to isolate two distinct mucin-like ligands, GlyCAM-1, which is secreted by HEV (144), and CD34, a surface molecule on HEV (17). MECA-79 mAb recognizes a carbohydrate determinant that is expressed on multiple protein species in HEV, including GlyCAM-I and CD34 and, compared to L-selectin, recognizes an overlapping but distinct set of glycoproteins (22, 144). Sialylation and sulfation of the O-linked side chains of the GlyCAM-1 and CD34 molecules are required for activity in binding to L-selectin (22, 111, 206). HEV differ from other tissues in carbohydrate processing; GlyCAM-1 and CD34 expressed in transfectants, and CD34 expressed in other vascular endothelia, do not bind L-selectin chimera under conditions in which binding to HEV is detectable (144). However, an L-selectin ligand with a presumably lower affinity is certainly present on most endothelia, as shown by L-selectin-dependent rolling in vivo and binding in vitro (125, 153, 229, 236, 237, 265, 266).

MOLECULES INVOLVED IN BINDING TO PEYER'S PATCH HEV Elegant screens for mAb with specificity for Peyer's patch HEV, and ability to block lymphocyte binding to HEV, yielded mAb MECA-367 to the mucosal addressin now termed MAdCAM-1 (248). MAdCAM-1 is expressed on endothelia in mucosal tissues, not only on HEV in Peyer's patch, but also on venules in intestinal lamina propria and in the lactating mammary gland (213, 248). MAdCAM-1 has both IgSF domains and a mucin-like domain (34) (Figure 5).

Similar elegant screens for mAbs with specificity for lymphoma cells that bound to Peyer's patch HEV and with the ability to block binding to HEV in the Stamper-Woodruff assay yielded mAbs to the α^4 subunit of the Peyer's

patch homing receptor (104). The α^4 subunit was found to be associated with a novel β subunit, β_p (105), which is identical to β_7 (108). The integrin $\alpha^4\beta_7$, but not $\alpha^4\beta_1$ binds to Peyer's patch HEV (108), and $\alpha^4\beta_7$ binds directly to MAdCAM-1 (24).

An Area Code Model for Lymphocyte Migration Through HEV

PERIPHERAL LYMPH NODE HEV Although the L-selectin:mucin and $\alpha^4\beta_7$: MAdCAM-1 interactions were identified in parallel assays, recent studies suggest that multiple steps are involved in lymphocyte interaction with HEV and raise the possibility that these interactions function in distinct, rather than parallel, steps in this process. Soon after its discovery as a peripheral lymph node homing receptor, L-selectin also was found to be present on neutrophils and eosinophils and to be important in emigration of, at least, neutrophils (151). As expected from their strong expression of L-selectin, neutrophils and other leukocytes can bind avidly to HEV in the Stamper-Woodruff assay, yet do not normally home to peripheral lymph nodes in vivo. Injection of *Escherichia coli* supernatant induces acute emigration of neutrophils through HEV of the draining lymph node. Thus signals other than those mediated by L-selectin can regulate the class of leukocyte that home into a lymph node (151). Although peripheral node HEV is far richer than any other site in the body in expression of the carbohydrate receptor for L-selectin (112), this is insufficient to explain the specificity of lymphocyte homing to this organ. The findings suggest that L-selectin is required for lymphocyte emigration through peripheral lymph node HEV and may help regulate recirculation of the L-selectin-positive subset of lymphocytes; however, L-selectin is insufficient to determine the specificity of the cell types that emigrate, and other currently undefined molecules are required to achieve specificity.

In vivo studies strongly suggest that lymphocyte emigration through HEV is a multi-step process that utilizes area code models similar to those of other leukocytes. mAb to L-selectin almost completely blocks emigration of lymphocytes from blood into peripheral lymph nodes (85, 94). However, mAb to the integrin LFA-1 also markedly reduces or almost completely abolishes lymphocyte migration into peripheral lymph nodes (41, 95). Thus molecules of step 1 and 3 (see Figure 1) are required for homing to peripheral lymph nodes in vivo. LFA-1 on blood lymphocytes requires activation for binding to its counter-structures ICAM-1 and ICAM-2 (238), which are expressed on HEV (69, 76). Binding of L-selectin does not trigger activation of LFA-1 because lymphocytes attach and roll in flow on purified peripheral node addressin identically whether or not purified ICAM-1 is present on the substrate; an additional stimulus is required before lymphocytes will arrest and strengthen adhesion through LFA-1 (M Lawrence et al, in preparation).

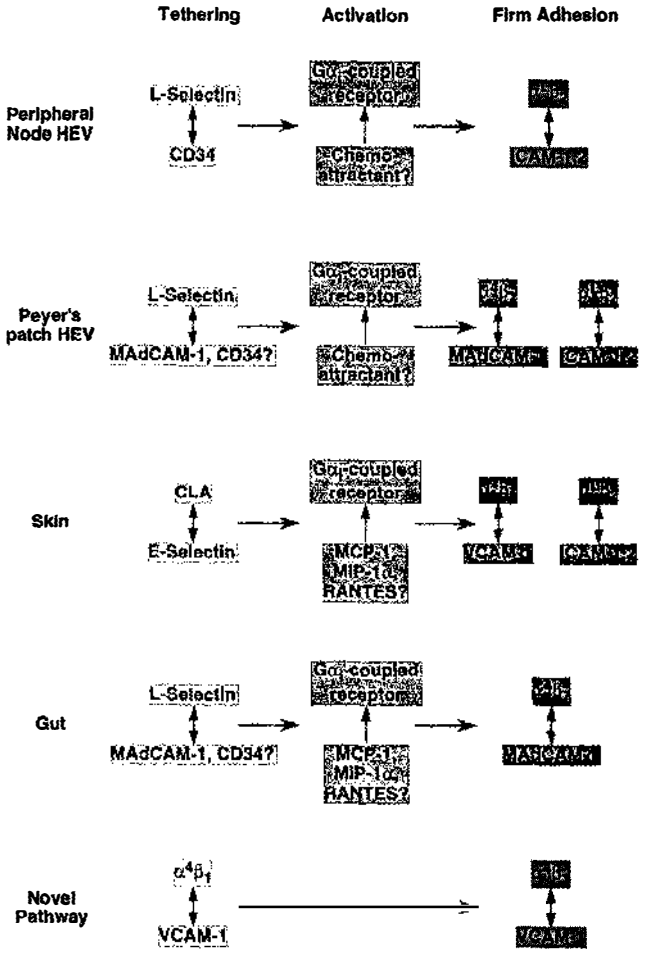


Figure 8 The three- or four-step area code paradigm for lymphocytes. For skin and gut, the pathways shown may mediate recirculation and increased accumulation in inflammation. The novel pathway shown at the bottom may be important when VCAM-1 expression on endothelium is induced by cytokines and may cooperate with the other illustrated pathways. For each organ, the interacting molecules are shown on the top for lymphocytes and on the bottom for endothelia. See text for support for the molecular assignments at each step, based primarily on in vivo data.

G protein–coupled receptors are required for lymphocyte recirculation and likely provide the signals required to activate the adhesiveness of LFA-1. Pertussis toxin causes lymphocytosis and profoundly depresses lymphocyte recirculation (271). Murine lymphocytes treated with pertussis toxin in vitro and reinfused fail to emigrate into either peripheral lymph nodes or Peyer's patches (175). This suggests that G protein–coupled receptors of the α_i class are required for lymphocyte emigration through HEV. Results with mice with a transgene for the ADP-ribosylating subunit of pertussis toxin selectively expressed in the T lineage suggest that $G\alpha_i$ proteins are not only required for emigration from the bloodstream, but also for emigration from the thymus (48, 49). Despite lack of emigration, pertussis toxin–treated lymphocytes bind normally to lymph node HEV in vitro. These findings provided the basis for an early proposal for a two-step model, in which G protein–coupled receptors function subsequent to binding of lymphocytes to HEV (233).

Thus emigration of lymphocytes through peripheral node HEV requires three sequential area code signals that are analogous to those involved in neutrophil emigration from the bloodstream (Figure 8). Identification of a putative lymphocyte chemoattractant secreted by peripheral lymph node HEV, and a chemoattractant receptor that is predicted to be selectively expressed on the naive subset of lymphocytes that recirculate through peripheral node HEV, will be a subject of intense research interest in coming years.

PEYER'S PATCH mAb to L-selectin block 50% of lymphocyte emigration from blood to Peyer's patch and to the remainder of the intestine (93, 94). This is consistent with the lower level of L-selectin ligand in Peyer's patch HEV than in peripheral lymph node HEV (15, 163, 273). mAb to certain epitopes on the integrin α^4 and β_7 subunits inhibit recirculation by approximately 50% of lymphocytes to Peyer's patch and intestine, but have no effect on recirculation to peripheral lymph node; furthermore, mAb specific for the $\alpha^4\beta_7$ complex are equally as effective as mAb to α^4 (93). Moreover, recirculation is inhibited by mAb to MAdCAM-1 (248), implicating $\alpha^4\beta_7$ binding to MAdCAM-1 in recirculation to mucosal tissue. mAb to LFA-1 block recirculation to Peyer's patch by 50 to 80%, but have no effect on recirculation to the remainder of the intestine (41, 95). Thus both LFA-1 and $\alpha^4\beta_7$ contribute to emigration into mucosal lymphoid tissue.

G protein–coupled receptors act subsequent to a rolling interaction in Peyer's patch HEV. In contrast with peripheral lymph nodes, Peyer's patches may be visualized by intravital microscopy (30). Normally, lymphocytes roll along Peyer's patch HEV for only a few seconds, then arrest and emigrate. However, prior treatment of lymphocytes with pertussis toxin completely blocks arrest and emigration, and prolongs the rolling indefinitely, so that the lymphocytes pass

out of the Peyer's patch rather than emigrate (14). It remains to be established, but seems likely, that a chemoattractant presented or secreted by Peyer's patch binds to a $G\alpha_i$-coupled receptor on lymphocytes and activates LFA-1 and $\alpha^4\beta_7$ to mediate arrest and emigration (Figure 8). Lymphoma cells or lymph node lymphocytes can bind to Peyer's patch HEV or purified MAdCAM-1 without any apparent need for activation; however, activation increases the strength of binding to MAdCAM-1 (24, 108). The pertussis toxin studies suggest that activation of blood lymphocytes is required for the last step of arrest and emigration (14, 233). Truncation of the cytoplasmic domain of β_7 greatly decreases binding to HEV. Thus interactions with the cytoplasmic domain can regulate the avidity of $\alpha^4\beta_7$ for MAdCAM-1 (63), similar to regulation of the avidity of LFA-1 for ICAM-1 (102, 103) by the β_2 integrin subunit cytoplasmic domain.

Recirculation of Memory Lymphocytes

DISTINCT PATHWAYS THROUGH SKIN AND GUT Memory lymphocytes are imprinted so that they are more likely to return to the type of tissue, such as skin or mucosa, where they first encountered antigen (39, 40, 159). The surface phenotypes of gut and skin-homing memory cells are distinct (163). Furthermore, staining of lymphocytes in sections of skin and gut with mAb shows distinctive expression of adhesion molecules that may contribute to selective extravasation in these tissues, or to subsequent localization within these tissues in specific anatomic compartments (Table 4).

Table 4 Naive and memory lymphocyte subsets[a]

Molecule	Naive lymphocytes	Memory lymphocytes
CD45RO	Negative	Positive
CD45RA	High	Low
CD2	Low	High
LFA-3	Negative	Positive
L-selectin	Positive	Positive and negative subsets
α^4	Low	High
	Memory lymphocytes subsets	
	Gut associated	Skin associated
CLA	Negative	Positive
$\alpha^E\beta 7$ (HML-1)	Positive	Negative
$\alpha^4\beta_7$[b]	High	Low
$\alpha^4\beta_1$	Low	High
α^6	Low	High

[a] References: (107, 163, 219), [b] but see (129).

SKIN HOMING LYMPHOCYTES Lymphocytes that extravasate in the skin and appear in afferent lymph have a distinct pattern of expression of adhesion molecules (163) (Table 4). Furthermore, as shown by staining of tissue sections, T lymphocytes localized in the skin, but not in the gut, express a carbohydrate termed cutaneous lymphocyte–associated antigen (CLA) (198). CLA is closely related to sialyl Lewis a and x (21) and is a ligand for E-selectin (23). Binding of a subpopulation of memory lymphocytes that bears CLA to E-selectin may contribute to the tropism of this subset to the skin (89, 197, 224). E-selectin is induced on dermal endothelial cells in delayed type hypersensitivity (61) and in chronically inflamed skin (197). Cloned T cells derived from challenged skin express high levels of CLA and bind to E-selectin, whereas T cell clones derived from blood lymphocytes do not (5). Both types of clones bind to P-selectin.

GUT HOMING LYMPHOCYTES The most organized lymphoid structures in the wall of the gut are the Peyer's patches. They underlie follicle-associated epithelia that contain M cells, which are specialized for uptake of antigen from the gut lumen. Other lymphocytes localize more diffusely in the lamina propria underlying the digestive epithelium and in the epithelial layer. Studies on gut afferent lymph reveal the presence of memory and naive lymphocytes (163); whether there is differential migration of naive and memory lymphocytes through Peyer's patch HEV and lamina propria postcapillary venules, both of which contribute to gut afferent lymph (Figure 7), remains unclear. Gut-homing memory lymphocytes display a surface phenotype distinct from skin-homing lymphocytes (Table 4). When injected into the bloodstream, memory lymphocytes from gut afferent lymph display a strong preference to return to gut afferent lymph, whereas naive lymphocytes redistribute randomly (163). Gut afferent memory lymphocytes display an α^4 high, β_1 integrin low phenotype, suggesting they are $\alpha^4\beta_7^+$ (163) in common with a subpopulation of memory lymphocytes in blood (219). Expression of MAdCAM-1 on Peyer's patch HEV and postcapillary venules in lamina propria (248), and 50% inhibition by mAb to α^4 and β_7 of migration into Peyer's patches and intestine (93), suggest a role for $\alpha^4\beta_7$ interaction with MAdCAM-1 in both sites.

A subpopulation of gut lymphocytes distinct from those in lamina propria localize within the epithelium on the external surface of the basement membrane and express the human mucosal lymphocyte (HML-1) integrin $\alpha^E\beta_7$ (47, 128, 193). The α^E integrin subunit contains an I domain and a novel proteolytic cleavage site preceded by a stretch of acidic residues, just N-terminal to the I domain (222). Binding of intraepithelial lymphocytes (IEL) to epithelial cell monolayers in vitro is inhibited by mAb to α^E, which suggests that $\alpha^E\beta_7$ may help mediate localization of IEL in epithelia in vivo (46). Intraepithelial T

lymphocytes may undergo thymus-independent differentiation in situ, and their recirculation pattern is undefined. HML-1 is expressed on a subpopulation (2–6%) of blood T cells, which are in the memory subset and are CLA^- and L-$selectin^-$ (199). Transforming growth factor β (TGF-β) together with mitogen induces expression of HML-1 on peripheral T cells and increases expression on IEL (128, 193). TGF-β also induces switching of B lymphocytes to production of the IgA class of immunoglobulin (55), the predominant class secreted in the mucosa. These dual effects on differentiation of mucosa] lymphocytes suggest the possibility that TGF-β may be an environment-specific cytokine that imprints lymphocytes, when first exposed to antigen, to recirculate selectively to the gut.

Alteration of Lymphocyte Trafficking in Inflammation

Antigen injected into the tissue of sensitized individuals induces localized accumulation of lymphocytes. These lymphocytes, and those accumulating in tissues in autoimmune disease, are almost all memory cells (120, 202). The phenotype of these cells is quite similar to that of lymphocytes trafficking through these sites under basal conditions. This suggests that the signals for lymphocytes trafficking may be qualitatively the same in the basal and inflammatory states and that they are upregulated in inflammation. Accumulation of lymphocytes induced by specific antigen, or by injection of IFN-γ or TNF-α, is significantly inhibited by mAb to either the LFA-1α or the integrin α^4 subunit (53, 114, 115, 217, 278). A combination of mAb to LFA-1 and α^4 gives almost complete inhibition of lymphocyte emigration and the resulting induration and plasma leakage (116). mAb to E-selectin and VCAM-1 also inhibit lymphocyte accumulation in delayed type hypersensitivity in skin (225). Multiple signals are thus required for augmented trafficking of lymphocytes into skin in inflammation (Figure 8). Both antigen responsive and nonresponsive lymphocytes traffic into sites of antigenic stimulation (166). Antigen-specific lymphocytes may accumulate in the site because stimulation through their antigen receptors increases adhesiveness of integrins and causes them to be retained, whereas nonresponsive lymphocytes more rapidly enter the lymphatics and leave the site.

The interaction between VCAM-I and VLA-4 mediates rolling and firm adhesion (R Alon et al, *J. Cell. Biol.*, in press), thus it does not fit neatly into the three-step paradigm established for neutrophils. mAb to LFA-1 or VLA-4 alone do not completely inhibit lymphocyte accumulation in inflammation, and patients with LAD-I show delayed-type hypersensitivity reactions. This suggests that the functions of VLA-4 and LFA-1 are partially overlapping in the step of firm adhesion, but they may also act in series, as in VLA-4-mediated rolling followed by LFA-1-mediated firm adhesion. VLA-4 may act together

with selectins to augment T lymphocyte tethering and rolling in the vasculature. All or most memory T lymphocytes lack L-selectin (32, 126, 163, 257). The CLA^+ subset can bind E-selectin, and T lymphocytes can also bind P-selectin (66, 174). Peripheral blood T lymphocytes are substantially less efficient than neutrophils in tethering in hydrodynamic flow to E-selectin and P-selectin (T Diacovo et al, unpublished data); therefore, cooperation of VCAM-I with E-selectin or P-selectin, or among all three molecules, may be important in enhancing lymphocyte accumulation in inflammation.

Inflammation also affects traffic through HEV. Antigen injected into tissue drains to the regional lymph node and greatly increases blood flow to the node and traffic of naive lymphocytes through HEV (161). Furthermore, memory lymphocytes now appear to enter the node directly; this is associated with induction of VCAM-I on non-HEV vascular endothelia within the node (161). Entry is inhibited by mAb to α^4, which suggests a role for interaction of VCAM-I with $\alpha^4\beta_1$ (114, 161).

Lymphocyte chemoattractants are interesting candidates for the step 2 signal (see Figure 1) for lymphocyte accumulation at inflammatory sites. Pertussis toxin treatment inhibits lymphocyte emigration in response to antigen in delayed-type hypersensitivity (234). Identification of lymphocyte chemoattractants has been hampered by the low motility of lymphocytes compared to monocytes or neutrophils (194), and by the low signal-to-background ratio, typically less than 2, in most chemotaxis assays. Recent interest has focused on chemokines (Table 2). A number of chemokines, all of which were isolated based on chemoattractive activity for neutrophils or monocytes, or by cloning genes of unknown function, have subsequently been tested and found to be chemoattractive for lymphocyte subpopulations (12, 172). These include IL-8 (139) (but see 136, 150), RANTES (216), MIP-1β (253), MIP-1α and β (215, 255), and IP-10 (256). There are differences among reports in the subsets found to be chemoattracted, and some reports use lymphocytes preactivated by T cell receptor cross-linking, which may be relevant to migration within inflammatory sites, but not to emigration from blood. Of interest, MIP-1β can induce binding of the naive, $CD8^+$ subset to VCAM-1, either in solution or when immobilized on a substrate, mimicking presentation by an endothelial cell surface (253; S Shaw, personal communication); the specific effect is modest, equal to background binding. The RANTES cytokine, by contrast to MIP-1 β, selectively attracts the memory T lymphocyte subset (216).

Vascular endothelium may present chemoattractant to lymphocytes in a functionally relevant way, as well as provide a permeability barrier that stabilizes the chemoattractant gradient. A transendothelial chemotaxis assay more accurately simulates lymphocyte emigration from the bloodstream than filter chemotaxis assays and yields signals >10 times background (43). Since

lymphocytes, responding to specific antigen in tissue, signal emigration of further lymphocytes into the site, a chemoattractant was sought in material secreted by mitogen-stimulated mononuclear cells. Purification to homogeneity guided by the transendothelial lymphocyte chemotaxis assay revealed that MCP-1, previously thought to be solely a monocyte chemoattractant, is a major lymphocyte chemoattractant (43). Subsequent studies using the transendothelial chemotaxis assay have confirmed that lymphocytes respond to RANTES and MIP-1α (CC chemokines), but do not respond to IL-8 or IP-10 (CXC chemokines) (209). MCP-1, RANTES, and MIP-1α selectively attract the memory T lymphocyte subset and the CD4 and CD8 subsets. All also attract monocytes, but not neutrophils, with MCP-1 being more potent than RANTES or MIP-1α as a monocyte chemoattractant. The physiologically relevant transendothelial assay suggests that CC chemokines tend to attract both monocytes and lymphocytes, in agreement with the long-standing clinical observation that lymphocyte emigration into inflammatory sites is always accompanied by emigration of monocytes. The converse is not true. Monocytes sometimes emigrate in the absence of lymphocytes, correlating with activity of chemoattractants such as C5a and PAF on monocytes, but not on lymphocytes. Teleologically, it is important that monocytes accompany lymphocytes into inflammatory sites in order to present antigen and to carry out effector functions in which monocytes are activated by T lymphocytes. MCP-1 is abundantly expressed at sites of antigen challenge and autoimmune disease (149, 172, 263), and together with MIP-1 α and RANTES, is an excellent candidate to provide the step 2 signal required to activate integrin adhesiveness and emigration of both monocytes and lymphocytes in vivo (Figure 8).

The finding that resting T lymphocytes that tether and roll on VCAM-I can spontaneously arrest and develop firm adhesion on VCAM-1 (R Alon et al, *J. Cell Biol.*, in press) has provocative implications for the multi-step model. It suggests that the VLA-4:VCAM-I interaction not only can mediate the steps of rolling and firm adhesion, but may also short-circuit the step of stimulation by chemoattractants of firm adhesion through integrins. This is intriguing; although a twofold stimulation of adhesiveness of VLA-4 to VCAM-1 has been demonstrated by MIP-1β in one system (253), with the chemoattractant that is most effective in eliciting transendothelial chemotaxis of T lymphocytes, MCP-1, it is difficult to detect stimulation of integrin adhesiveness on lymphocytes (M Carr & T Springer, unpublished data). Therefore, an alternative pathway may exist in which VCAM-1 can mediate both tethering and arrest of lymphocytes, perhaps in cooperation with other endothelial molecules, prior to stimulation by chemoattractants. After arrest, chemoattractants would guide transendothelial migration, and perhaps stimulate further increases in the ad-

hesiveness of the integrins VLA-4 and LFA-1, which are important in migration across the endothelium and basement membrane.

CONCLUDING REMARKS

A three-step or area code model of leukocyte emigration from the bloodstream, established and validated in vitro and in vivo with neutrophils (Figure 1 and 6B), appears extendible with only slight modification to all subclasses of leukocytes including lymphocytes (Figure 8). Multiple adhesion and chemoattractant receptors are used combinatorially in a series of steps that enable leukocytes to progress from initial tethering in flow to firm adhesion and emigration. The distinct distribution of receptors on leukocyte subsets for signals that are displayed on endothelium regulates selection of the subclasses of leukocytes that emigrate at inflammatory sites and the distinctive recirculation behavior of lymphocyte subsets.

Many important developments await. Strong evidence suggests that G protein–coupled receptors are required for lymphocyte recirculation, but many of the putative lymphocyte chemoattractants specific to HEV, mucosa, and skin, and the receptors for these chemoattractants on lymphocytes, remain to be identified. Specific mucin-like molecules that present carbohydrate ligands to selectins have recently been identified. Are there similar mucin-like molecules on lymphocytes that present carbohydrates to P-selectin or E-selectin, and do these differ from the PSGL-1 molecule on neutrophils? It is likely that endothelial cells express molecules that retain chemoattractants on the luminal surface, preventing them from being washed away by blood flow, as already suggested for MIP-1β and IL-8. Are these molecules specifically regulated? The mucin-like ligands of selectins have many features such as extended structure, sulfation, and negative charge in common with proteoglycans, and thus might have a second function of binding chemokines through their heparin-binding sites and presenting them to leukocytes. Presenting molecules might be required not only to prevent chemoattractants from being washed away by blood flow, but also to generate maximal chemoattractant activity, analogous to proteoglycans that must bind fibroblast growth factor to enable signaling through a second receptor molecule. It will be interesting to determine whether chemoattractant receptors on leukocytes couple to distinct G proteins and signaling effectors, allowing for selectivity in which integrins are upregulated in avidity. For example, do chemoattractants differ in ability to upregulate adhesiveness of two integrins such as LFA-1 and VLA-4 expressed on the same cell? Finally, after the area code is dialed and cells emigrate across the endothelium, much remains to be learned about the "7 digit code" that

regulates leukocyte migration and localization within specific anatomic compartments.

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