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Distinct Roles of L-Selectin and Integrins α4β7 and LFA-1 in Lymphocyte Homing to Peyer's Patch-HEV In Situ: The Multistep Model Confirmed and Refined

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Summary

Circulating lymphocytes home to the mucosal lymphoid organs, Peyer's patches (PP), through high endothelial venules (HEV). In situ analyses revealed that transfused lymph node cells (LNCs) interact with PP-HEV in a series of overlapping adhesion events: L-selectin (CD62L) >α4β7 initiates interaction, L-selectin and α4β7 both participate in rolling, and Gαi-linked activation triggers arrest that requires both α4β7 and LFA-1. α4β7 dramatically reduces rolling velocity, and appears to be required for engagement of LFA-1. In contrast with resting LNC, preactivated LNC or α4β7^{hi} lymphoma cells require only α4β7 for arrest in PP-HEV. The predominant PP-HEV ligand for α4β7 but also apparently for L-selectin is the mucosal addressin MAdCAM-1. These results validate the concept of multimolecular adhesion/decision cascades in physiologic lymphocyte-endothelial recognition, define a novel role for α4 integrins as a "bridge" between selectin and β2 integrin-dependent events, and reemphasize the potential for direct adhesion through preactivated α4 integrins alone.

Introduction

The recruitment of lymphocytes from the blood is an essential response to chronic tissue inflammation, and is key to the regulation of physiologic lymphocyte trafficking and recirculation (Picker and Butcher, 1992). Lymphocyte extravasation is directed in vivo by mechanisms of selective lymphocyte–endothelial cell (EC) recognition, mechanisms that facilitate immune surveillance by directing recirculation pathways of naive and memory lymphocytes. This process supports regional immune responses by targeting lymphocyte effector cells to particular organs and tissues in the body as a function of the local microenvironment and inflammatory state (Picker and Butcher, 1992; Mackay, 1993). In an effort to explain the extraordinary specificity of lymphocyte (and other leukocyte) trafficking in vivo, we and others have proposed a general model of leukocyte–EC interaction as a multistep process, involving the following: primary adhesion, in some instances separable into molecularly distinct initial contact formation (sometimes referred to as tethering) and reversible rolling along the vessel wall; lymphocyte activation through G protein–linked receptors; subsequent activation-dependent sticking and arrest; followed by diapedesis in response to chemoattractant or haptotactic signals (Butcher, 1991; Shimizu et al., 1992; Springer, 1994). This hypothesis, in which lymphocyte recruitment results from an active decision process or algorithm requiring a yes/no decision at each step, has profound implications, providing a combinatorial mechanism for generating diversity and determining specificity in lymphocyte–EC interactions (Butcher, 1991). The multistep paradigm is well established for neutrophil interactions with inflamed venules in vivo (von Andrian and Arfors, 1993), and has also received substantial support from in vitro modeling of leukocyte adhesive interactions (Springer, 1994; Jones et al., 1994; Lawrence et al., 1995). However, it remains to be verified as a general model in vivo and, in particular, in the context of lymphocyte homing.

Previously, we used in situ videomicroscopy of exteriorized mouse Peyer's patches (PP) to demonstrate involvement of a rapid pertussis toxin (PTX)-sensitive signaling/activation event in lymphocyte interactions with high endothelial venules (HEV) (Bargatze and Butcher, 1993). Lymph node cells (LNCs) treated with PTX, which inhibits Gαi protein signaling, displayed efficient attachment and transient rolling in PP–HEV but were unable to make the transition to sticking and stable arrest. This study demonstrated the involvement of sequential rolling, activation, and sticking steps in lymphocyte recognition of HEV in this site.

Independent investigations involving short-term in vivo homing assays have implicated several defined adhesion and homing receptors in lymphocyte trafficking to PP. Of particular importance is the lymphocyte–PP homing receptor, the integrin α4β7 (Holzmann et al., 1989; Hu et al., 1992; Hamann et al., 1994). α4β7 plays a critical role in the targeting of lymphocytes to mucosal PP and lamina propria through interaction with the mucosal vascular addressin MAdCAM-1 (Berlin et al., 1993; Strauch et al., 1994), an immunoglobulin and mucin family member that is selectively expressed by venules in mucosa-associated lymphoid tissues and sites of inflammation (Briskin et al., 1993; Streeter et al., 1988). Antibodies to the lymphocyte L-selectin and to the β2 (CD18) integrin LFA-1 also reduce the localization of injected LNC to PP in vivo (Hamann et al., 1994). However, the respective functions of L-selectin, α4β7, and LFA-1 and their vascular ligands in lymphocyte homing to PP has not been reported.

Here, we have used in situ videomicroscopy, in conjunction with inhibitory antibodies to adhesion receptors, to dissect the molecular mechanisms of lymphocyte contact, rolling, and activation-dependent arrest in PP–HEV. We

demonstrate sequential and overlapping involvement of L-selectin, α4β7, and LFA-1 in recognition of PP–HEV by resting lymph node lymphocytes, but show that α4β7 is both necessary and sufficient for attachment and arrest of lymphocytes expressing preactivated or elevated α4β7 levels. We also present evidence implying a critical role for the mucosal addressin MAdCAM-1, not only in α4β7-mediated interactions but also in L-selectin-mediated attachment and rolling in this site. Our findings constitute direct demonstration of a multimolecular adhesion cascade leading to lymphocyte–EC recognition and arrest in a physiologic setting, and provide novel insights into the specialization and versatility of the adhesion receptors involved.

Results

Interactions of Injected LNCs with PP–HEV: Sequential L-Selectin-, α4β7-, and LFA-1-Mediated Steps

LNCs from young BALB/c mice were labeled with fluorescent dyes, injected intravenously, and their behavior during passage through PP–HEV was observed and recorded by video epifluorescence microscopy. As reported previously (Bargatze and Butcher, 1993) and reproduced here (Figure 1A), the majority of normal LNCs interacting with HEV displayed variable rolling and sticking behaviors (Figure 1A). To define the role(s) of individual adhesion receptors in the process, the effects of blocking anti-L-selectin, α4β7, or LFA-1 monoclonal antibodies (MAbs) on the frequency and characteristics of interactions were explored.

L-Selectin >α4β7 Mediates Initial Lymphocyte Contact

Anti-L-selectin antibody significantly decreased the frequency of detectable interaction of injected LNC (Figure 1B): Only ~30% of anti-L-selectin-treated cells interacted at all, versus 70% of control cells. Those remaining cells that were capable of interacting in the presence of anti-L-selectin only infrequently displayed residual rolling, instead arresting rapidly: these may represent preactivated cells among the lymph node lymphocytes (whose interaction may be initiated and consummated by α4β7 without L-selectin contribution; see below). Thus, L-selectin appears to play a prominent role in initiating attachment of resting lymphocytes in PP–HEV. However, anti-L-selectin in combination with anti-α4 MAb or Fab fragments (Figure 1E) is even more effective at abrogating interaction, suggesting that α4β7 can also initiate lymphocyte contact, albeit at a lower frequency. These findings are consistent with in vitro studies that have demonstrated efficient L-selectin-mediated lymphocyte attachment and rolling, but also α4β7-mediated attachment of lymphocytes to MAdCAM-1 under flow, as well (Berg et al., 1993; Berlin et al., 1995). The present results suggest that for the major population of naive resting LNCs, which express high levels of L-selectin and low/intermediate levels of α4β7, L-selectin plays the predominant role in initiating physiologic interactions with PP–HEV.

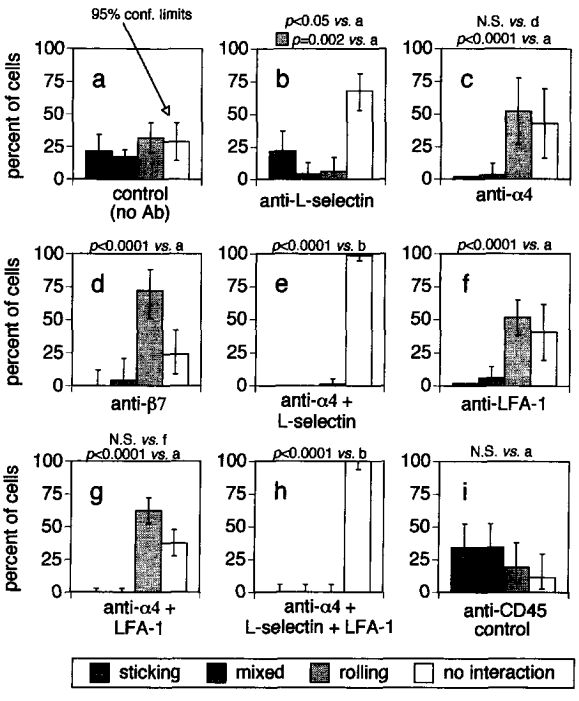

Figure 1. Effects of Anti-L-Selectin, α4, β7, and LFA-1 MAbs on Interactions of Normal LNC with PP–HEV In Situ

Fluorescent LNCs were injected intravenously, and their behavior during passage through HEV was recorded videomicroscopically for analysis. The mean behavior of each cell was scored as predominant sticking, intermediate, rolling, or noninteracting, and the population behavior is summarized in the histograms presented. Pretreatment or injection of MAbs was carried out as described in Experimental Procedures, and in Results. Histograms represent pooled analyses of 2–8 independent experiments, except for (D) and (I), which were from individual animals. Error bars in this and subsequent histograms represent 95% confidence intervals based on student's t (for $n > 2$) or binomial (when $n = 1$ or 2) distributions. N. S., not significant. χ square contingency tables were used to test the significance of differences in behavior between treatments or experiments. Comparisons focusing on one particular behavior, indicated next to the given p value, employed 2 × 2 tables (e.g., assessing the significance of differences in the frequency of rolling versus not-rolling behaviors in [b] versus control). Because of the variability in the behavior of lymphocytes in control experiments, comparisons to control were carried out by comparing pooled sample data with each of the five individual control experiments pooled in (a): experimental results were considered significantly different from control only if they were different at $p < 0.05$ from each of the control experiments. The most conservative p value resulting is presented in each case.

Both α4β7 and LFA-1 Are Required for Lymphocyte Arrest

As shown in Figure 1C, anti-α4 MAb treatment had no significant effect on the frequency of interaction with HEV, but converted behavior to rolling with almost total abrogation of lymphocyte arrest. Similar results were obtained with anti-β7 integrin MAb (Figure 1D). Thus, inhibition of α4β7 prevents engagement of activation-dependent adhesion mechanisms.

Surprisingly, anti-LFA-1 MAb also inhibited lymphocyte arrest almost completely, resulting in a predominant rolling phenotype as well (Figure 1F). Combined treatment

with anti-α4 and anti-LFA-1 also yielded predominant rolling behavior (Figure 1G). As expected, the combination of all three antibodies completely abrogated detectable interaction (Figure 1H). Control anti-CD45 MAb had no significant effect on cell behavior (Figure 1I). These results suggest that both α4β7 and LFA-1 are required for adhesion strengthening, leading to sticking and stable arrest of lymphocytes in PP–HEV.

α4β7 Participates in Lymphocyte Rolling

The velocity of lymphocyte rolling is a function of molecular interactions and densities, and of local hemodynamics, which vary significantly from vessel to vessel in vivo. To examine this parameter, therefore, we compared the speed of rolling lymphocytes in the same HEV during sequential treatments. Representative experimental results are illustrated in Figure 2. Anti-LFA-1-treated cells displayed slow rolling, which increased substantially in velocity following subsequent injection of anti-α4 (Figure 2A). Thus, α4β7 participates with L-selectin in lymphocyte rolling in PP–HEV, resulting in an increase in the apparent avidity of interaction. To illustrate this visually, video images of rolling lymphocytes pretreated with anti-LFA-1 alone (Figure 2B), or following subsequent injection of anti-α4 (Figure 2C) are presented. Injection of anti-L-selectin MAb into the same animal resulted in abrogation of all visible interactions (data not shown). For comparison with the rolling velocities observed in Figure 2, the velocity of noninteracting cells averaged from 880–8230 μ/s in different HEV (mean = 3812 ± 2783 SD; n = 7. HEV from 4 mice, 7–25 noninteracting cells analyzed per HEV).

The participation of α4β7 in lymphocyte rolling is independent of in situ PTX-sensitive lymphocyte activation, as PTX-pretreated lymphocytes display slow rolling that is dramatically increased in velocity upon injection of anti-α4 (Figure 2D). In contrast, anti-LFA-1 had no detectable effect on the rolling velocity of PTX-treated cells (Figure 2E), suggesting that in this setting participation of LFA-1 may be activation dependent and largely limited to adhesion strengthening. Together, these findings demonstrate that α4β7 plays an important role in increasing the avidity and reducing the velocity of rolling lymphocytes in PP–HEV. This may dramatically increase the opportunity for lymphocyte activation at the EC surface, and may also facilitate subsequent engagement of LFA-1.

Interactions of TK1 Lymphoma Cells and Preactivated Lymphocytes with PP–HEV: α4β7 Is Necessary and Sufficient for Binding

Resting LNCs express only modest levels of α4β7, and display their integrins in a relatively inactive state. To model interactions of preactivated or $\alpha4\beta7^{hi}$ cells, we initially assessed the in situ behavior of TK1 lymphoma cells. TK1 is a $CD8^+$, L-$selectin^{lo}$ to L-$selectin^{neg}$, $\alpha4\beta7^{hi}$, $LFA\text{-}1^+$ lymphoma that binds avidly to PP–HEV, and to isolated MAdCAM-1 both in static assays and under low physiologic shear in vitro (Berlin et al., 1993, 1995). As shown

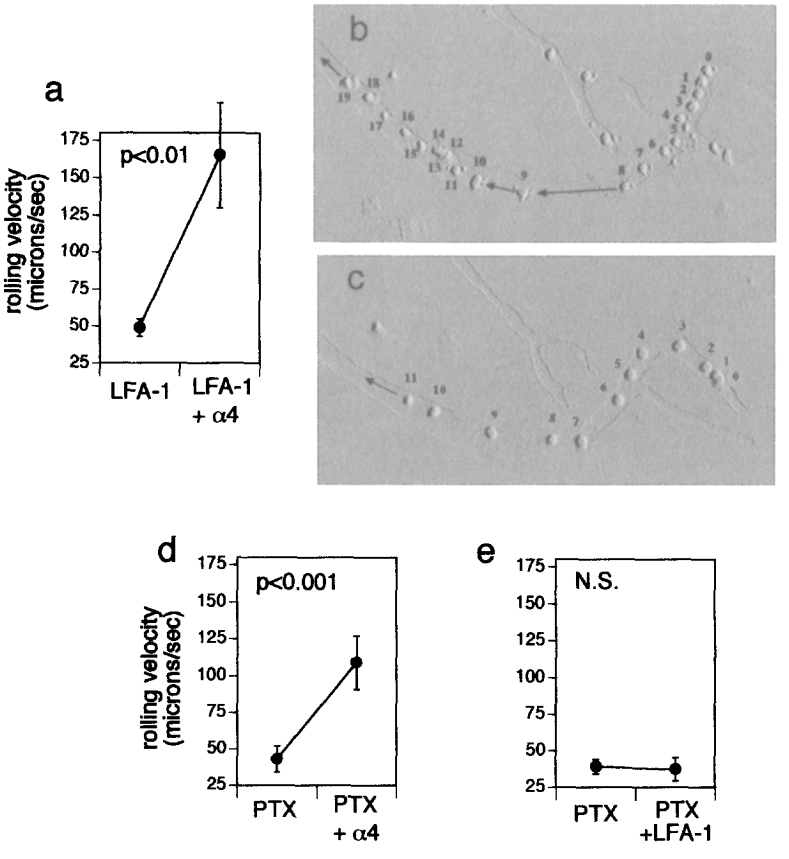

Figure 2. Effects of Anti-α4 and Anti-LFA-1 MAbs on the Velocity of LNC Rolling in HEV

Each graph presents paired data from a single HEV or set of HEVs in a given experiment.

(a) Results of a representative experiment in which the behavior of LNC was observed during sequential treatment with anti-LFA-1 (incubated with cells prior to injection, and coinjected in excess) and anti-α4 (subsequently injected intravenously).

(b and c) Composite photographs illustrating superimposed images of the position of individual cells selected from the experiment in (a), with images captured at 1 s intervals: the distance between images reflects the velocity of the cell. Rolling of an anti-LFA-1-treated cell is depicted in (b). The cell skipped over the sections indicated by the arrows. More rapid rolling of a representative lymphocyte following anti-α4 is shown in (c). For comparison with (a)–(c), the mean velocity of noninteracting cells in this HEV was 1,680 ± 437 μ/s (SD).

(d) The slow rolling of PTX-pretreated cells is converted to rapid rolling following anti-α4 injection.

(e) Anti-LFA-1 MAb has no effect on the rolling velocity of PTX-treated lymphocytes. The mean and standard error of rolling velocities determined from 10–20 individual cells per condition are presented. p values for comparisons of paired data within each experiment are given.

in Figure 3A, when injected intravenously TK1 cells stuck almost immediately to PP–HEV in vivo, often with little apparent rolling. Anti-α4 completely abrogated TK1 cell interactions (Figure 3B), whereas anti-L-selectin and anti-LFA-1 had no significant effect (Figures 3C, 3D). Figure 3E illustrates the inability of sequential anti-L-selectin and anti-LFA-1 to block TK1 cell adhesion and arrest, whereas subsequent injection of anti-α4 effectively inhibited interactions in the same HEV.

We also assessed the effects of experimental integrin activation on interactions of LNC (Figure 4). Lymphocytes were activated either with phorbol myristate acetate (PMA) (100 ng/ml, for 5 min at 37°C), which triggers integrins while leaving variable levels of L-selectin on the cell surface; or by replacement of Mg^{2+} with Mn^{2+} in the medium, which activates integrins and leaves L-selectin expression intact. As reported previously (Bargatze and Butcher, 1993), preactivated lymphocytes, like TK1 cells, tend to arrest rapidly in HEV with only very brief (<1 s) or no initial rolling (Figure 4A). Intermediate/mixed and rolling behaviors are usually diminished, but in some experiments a significant if variable fraction of rolling cells was also observed: when present, these cells often rolled loosely and rarely arrested, suggesting that they may lack or express only low levels of α4β7, rendering them incapable of arrest in PP. Cells that display a noninteracting phenotype may additionally lack L-selectin, reducing their ability to attach and establish rolling.

Anti-α4 or β7 MAb blocked activated lymphocyte sticking and arrest, and dramatically if variably reduced the frequency of interaction (Figures 4B, 4C; Figure 4D, middle). Residual interacting cells displayed rolling consistent with retention of cell surface L-selectin, and were more apparent with Mn^{2+} (Figure 4D, middle) than with PMA-activated cells (presumably reflecting significant shedding of L-selectin following PMA activation). In contrast, anti-LFA-1 MAb alone (Figure 4D, left) or in combination with

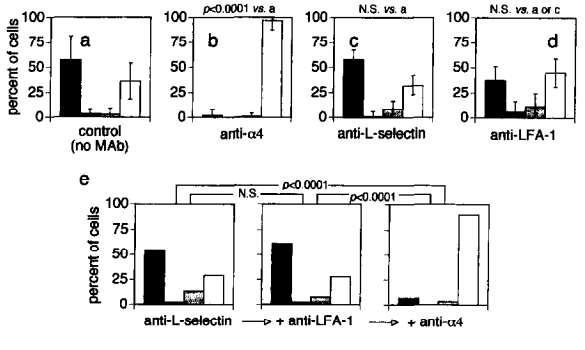

Figure 3. Anti-α4 but Not Anti-L-Selectin or Anti-LFA-1 Inhibits TK1 Cell Interactions with Peyer's Patch HEV

TK1 cells were labeled, injected intravenously, and visualized in PP–HEV in the presence of the indicated MAbs. Data analyses and presentation in (a)–(d) are as for Figure 1. In (e), the behavior of TK1 cells was analyzed in a single HEV during sequential treatment with anti-L-selectin, anti-LFA-1, and finally anti-α4. In this and subsequent figures in which sequential behaviors were analyzed in individual HEV, error bars are omitted.

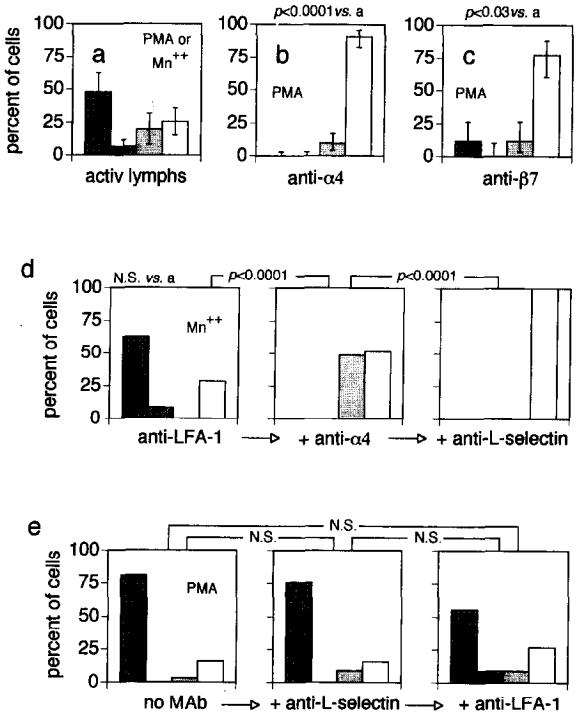

Figure 4. Effects of Anti-L-Selectin, α4, β7, and LFA-1 MAbs on Activated LNC Interactions with PP–HEV

Lymphocytes were labeled and activated by brief pretreatment with PMA, or by replacement of Mg^{2+} with Mn^{2+} in the medium, and were then injected intravenously for analyses of in situ behavior as in Figures 1 and 3.

(a) Pooled data from three experiments with PMA-activated LNC, and two with Mn^{2+}-activated cells.

(b and c) PMA-activated cells.

(d) Mn^{2+}-activated LNC were preincubated and injected with anti-LFA-1 MAb, and analyzed in the same vessels following subsequent injection of anti-α4 and then anti-L-selectin MAbs.

(e) Mn^{2+}-activated LNC were injected and analyzed following subsequent injection of anti-L-selectin and then anti-LFA-1 MAbs.

anti-L-selectin (see Figure 4E, middle) had no substantial effect on the characteristic sticking behavior of interacting activated cells. The predominant role of α4β7 is illustrated in Figure 4D, which presents the results of sequential antibody treatments on the behavior of Mn^{2+}-activated lymphocytes in the same HEV. Lymphocytes pretreated and injected with anti-LFA-1 MAb retained the ability to arrest; subsequent injection of anti-α4 blocked sticking but left residual rolling that was in turn abrogated by anti-L-selectin. The minimal effect of sequential anti-L-selectin and anti-LFA-1 treatments is illustrated in Figure 4E.

We conclude that when expressed at high functional levels (which may require some degree of pretriggering of integrins, but would also be influenced by receptor density), α4β7 can mediate lymphocyte recognition of PP–HEV with no requirement for L-selectin or LFA-1 participation, or probably for additional local activating signals (although under physiologic circumstances such signals may be required for maintenance of adhesion, or for diapedesis, or both).

MAdCAM-1 Is Involved in Both α4β7 and L-Selectin-Dependent Interactions

In vitro studies have implicated MAdCAM-1 as a vascular receptor for α4β7, and also (under certain circumstances) for lymphocyte L-selectin. When expressed by HEV, MAdCAM-1 can be modified by peripheral node addressin (PNAd)-associated L-selectin-binding carbohydrate epitopes, which allows it to support L-selectin-mediated attachment and rolling under physiologic shear in vitro (Berg et al., 1993). Although PNAd-associated glycotopes can modify multiple mucin-like (and possibly other) HEV glycoproteins (Berg et al., 1991a), the abundance of MAdCAM-1 on PP–HEV raised the possibility that MAdCAM-1 might play a prominent role as an L-selectin ligand in this site.

To address this question, we initially assessed the effect of anti-MAdCAM-1 MAb MECA-367 on attachment, rolling, and arrest of lymphocytes in PP–HEV. MECA-367 recognizes the N-terminal immunoglobulin domain of MAdCAM-1 (M. J. Briskin et al., unpublished data) and inhibits α4β7 binding (Berlin et al., 1993). As shown in Figure 5A, intravenous injection of MECA-367 inhibited both rolling and sticking of subsequently injected lymphocytes, in a manner most comparable with anti-L-selectin or anti-L-selectin plus anti-α4 treatments (compare with Figures 1B, 1E), suggesting inhibition of L-selectin-mediated attachment and rolling. Control class-matched MAb MECA-32 against a widespread endothelial cell surface antigen also expressed by HEV had no effect on in situ interactions (Figure 5B). Importantly, monovalent Fab fragments of MECA-367 prevented lymphocyte sticking and arrest much like anti-α4β7 or anti-β7 treatment, but had little or no effect on the frequency of rolling (Figure 5C; 5E, middle). The ability of whole but not monovalent Fab fragments of MECA-367 MAb to inhibit lymphocyte rolling suggested that the effect might require cross-linking of MAdCAM-1. Consistent with this, injection of whole MAb MECA-89 against an independent epitope on MAdCAM-1 (MECA-89 recognizes the second immunglobulin domain (M. J. Briskin et al., unpublished data], and has no effect on α4β7 binding to MAdCAM-1 in vitro [Nakache et al., 1988]) did finally inhibit rolling in the animal initially treated with MECA-367 Fab fragments (Figure 5C).

In additional experiments designed to isolate the contribution of L-selectin, LNCs were injected with an excess of anti-α4 MAb. As shown above, under these conditions, lymphocytes display L-selectin-dependent rolling in PP–HEV. Anti-MAdCAM-1 MAb MECA-367 was injected and, after several minutes, interactions with HEV were largely abrogated, confirming the inhibition of L-selectin interaction (Figure 5D). In a second experiment, Fab fragments of anti-α4 MAb were used to block α4 participation. As shown in Figure 5E, injection of MECA-367 Fab fragments had no additional effect on the residual L-selectin-dependent rolling, but whole MECA-89 again inhibited remaining interactions.

Thus, intact but not monovalent anti-MAdCAM-1 MAbs against two distinct epitopes inhibit L-selectin-dependent lymphocyte rolling in PP–HEV. One possibility was that cross-linking resulted in removal of MAdCAM-1 from the HEV surface. To address this, we assessed the effects of anti-MAdCAM-1 MAbs on binding of Mn^{2+}-activated lymphocytes, whose interactions are mediated through α4β7 without requirement for L-selectin (as demonstrated above).

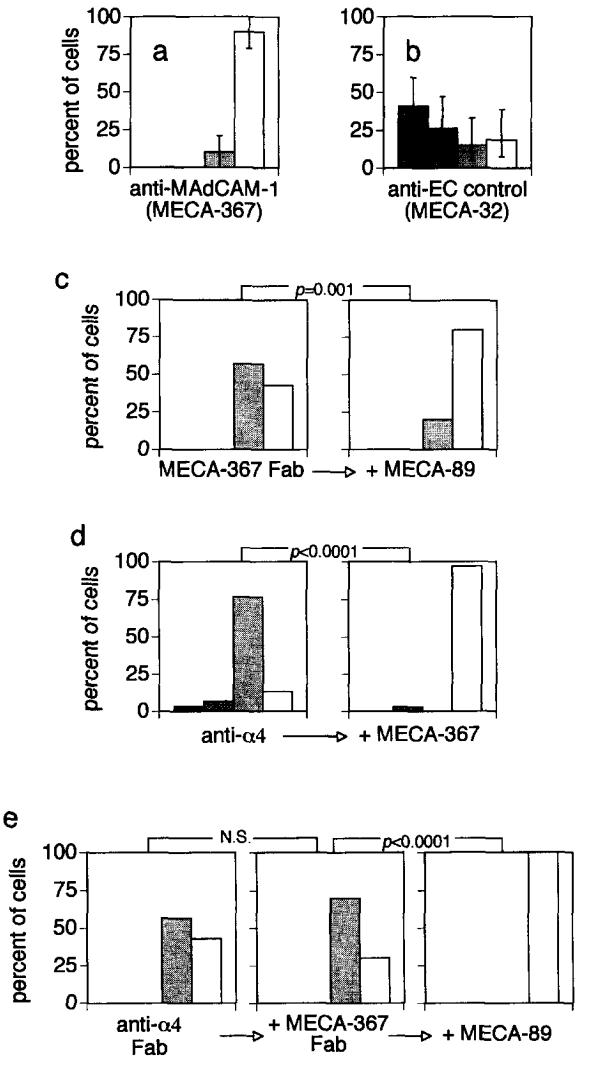

Figure 5. Effects of Anti-MAdCAM-1 MAbs on Interactions of LNC in PP–HEV

Analyses and data presentation as in Figures 1 and 3.

(a) Anti-MAdCAM-1 MAb MECA-367 prevents sticking and inhibits rolling of LNC.

(b) Control class-matched antibody MECA-32 against a pan-endothelial cell antigen has no significant effect on behavior.

(c) Monovalent Fab fragments of MECA-367 inhibited sticking but left residual rolling that was inhibited by subsequent injection of whole anti-MAdCAM-1 MAb MECA-89 (against a second epitope).

(d) Lymphocytes pretreated with anti-α4 display rolling that is inhibited by subsequent injection of whole anti-MAdCAM-1 MAb MECA-367.

(e) Rolling of lymphocytes pretreated with anti-α4 Fab fragments was not blocked by monovalent Fab fragments of MECA-367, but was inhibited by subsequent injection of anti-MAdCAM-1 MAb MECA-89. Antibodies MECA-367 or MECA-89 (or Fabs) to MAdCAM-1, or control class-matched antibody MECA-32 against a pan-endothelial cell antigen, were injected intravenously and allowed to saturate vessels for ~4–7 min prior to injection of labeled LNC (in [b] and [c]), or prior to analysis of previously injected lymphocyte behavior (in [d] and [e]).

As illustrated in Figure 6A, MECA-367 almost completely abrogated interactions of Mn^{2+}-preactivated LNC, again consistent with the inhibition of L-selectin- as well as α4β7-dependent events. In contrast with MECA-367, MECA-89 had no significant inhibitory effect on activated LNC interactions (Figure 6B), but subsequent injection of anti-α4 Fab completely abrogated binding in the presence of MECA-89. Thus, pretreatment with MECA-89 still permits α4β7-dependent adhesion, demonstrating that MAdCAM-1 must still be present. The absence of detectable interaction in the presence of MECA-89 and anti-α4 MAbs also confirms further that, as above, L-selectin-dependent adhesion is lost in the presence of MECA-89.

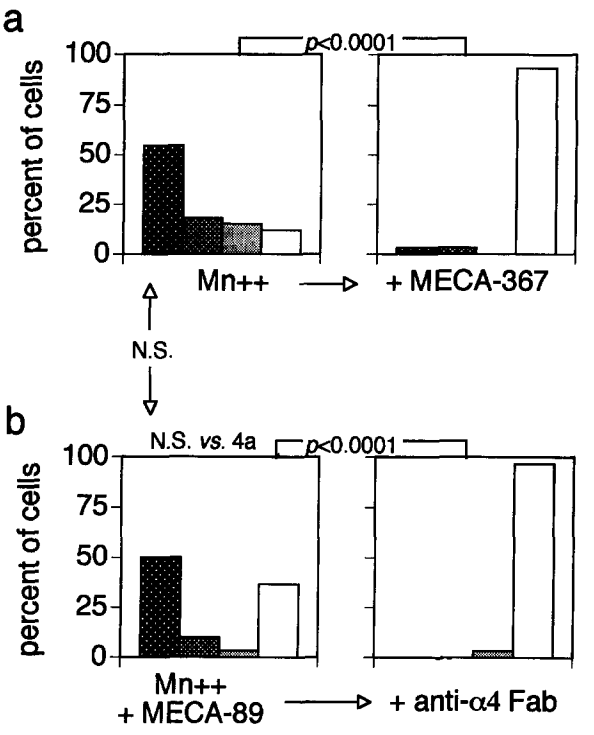

Figure 6. Effects of Anti-MAdCAM-1 MAbs on Interactions of Mn^{2+}-Activated LNC in PP-HEV

Analyses and data presentation as in Figures 4 and 5.

(a) Anti-MAdCAM-1 MAb MECA-367 blocks both sticking and rolling of preactivated LNC.

(b) Anti-MAdCAM-1 MAb MECA-89 had no significant effect on the behavior of activated cells (N. S. versus Figure 4A), but all interactions were inhibited following subsequent injection of anti-α4 Fab fragments.

Discussion

To examine the relevance of the multistep model of leukocyte–EC recognition to lymphocyte trafficking, we have studied the interactions of lymphocytes with PP–HEV in situ, focusing on the roles of adhesion receptors in the events leading to stable arrest. Our findings reveal striking differences in the mechanisms controlling adhesion of resting lymph node lymphocytes versus preactivated or $\alpha4\beta7^{hi}$ lymphoid cells, and we will discuss these situations separately.

LNCs from young mice are comprised largely of naive T and B cells, which, like their human counterparts, express L-selectin and moderate levels of α4β7 and LFA-1 (Picker and Butcher, 1992; Kilshaw and Murant, 1991; Berlin et al., 1993; Erle et al., 1994; Schweighoffer et al., 1993; D. Andrew, L. Rott, and E. C. B., unpublished data). The majority of injected LNCs, presumably representing primarily such naive lymphocytes, interacts with PP–HEV through a complex series of events mediated by sequential but overlapping engagement of L-selectin, α4β7, and LFA-1. L-selectin dominates in the initiation of primary contact. α4β7 can also initiate adhesion at lower efficiency. Both L-selectin and α4β7 participate in activation-independent rolling, with α4β7 playing an apparently essential role in increasing the avidity and reducing the velocity of rolling cells. Engagement of α4β7 is required for activation-dependent adhesion strengthening and arrest, which also requires participation of the β2 integrin LFA-1. These results represent a demonstration of a multistep adhesion cascade in physiologic lymphocyte–EC interactions.

One of the striking features of this adhesion cascade is the overlap in functions of the receptors involved. Although each component is required for efficient arrest, overlapping roles appear to permit some residual interaction when any one of the components is inoperative. This overlap between sequential functions may explain in part the less than complete inhibition of LNC homing to PP by antibodies to any one of the receptors involved (Hamann et al., 1994), although this may also reflect in part heterogeneity in lymphocyte subsets, as well. The ability of α4β7 to initiate interactions of resting lymphocytes (albeit inefficiently compared with L-selectin) may also help explain the population of PP by lymphocytes in L-selectin-deficient mice (Arbonés et al., 1994). Overlapping roles may be important evolutionarily, as well, permitting significant trafficking even in the context of altered or deficient activity of individual components of the adhesion cascade.

α4β7 appears to occupy a unique bridging position in the process of LNC homing: α4β7 is apparently required for efficient conversion of L-selectin-mediated primary interaction to assisted arrest, thus ensuring its participation in the "decision process" leading to lymphocyte recruitment into PP. This is in contrast with observations of neutrophils, which lack α4 integrins and, when rolling through selectins, can directly engage β2 integrins in response to in situ activation (von Andrian et al., 1992). The ability of α4β7 to participate in activation-independent rolling may relate to the expression of this integrin in a constitutively functional state: although α4β7 binding to MAdCAM-1 can be dramatically enhanced by lymphocyte activation, even resting lymphocytes bind reasonably well in vitro (Berlin et al., 1993). Relatively low avidity and rapid on–off rates of resting α4β7 may also be required to permit rolling (versus sticking).

The apparent requirement for LFA-1 during sticking of most LNC is perhaps surprising, especially in light of the ability of experimentally activated α4β7 to mediate lymphocyte arrest in the absence of LFA-1 participation (discussed below). It is possible that on naive lymphocytes,

which express relatively low levels of α4β7, physiologic levels of α4β7 triggering are insufficient to mediate arrest under high shear. Kinetic aspects of integrin activation might also be important; i.e., LFA-1 may be more rapidly triggered in response to local activating signals than α4β7. Alternatively, engagement of LFA-1 might be required for efficient triggering of α4β7 to its activated state in this setting.

The present studies confirm the inability of lymphocyte LFA-1 to initiate functional contact with endothelium in a physiologic in vivo setting: anti-α4β7 treatment completely prevents engagement of lymphocyte LFA-1 in PP–HEV. Particularly surprising is the inability of LFA-1 to participate even when lymphocyte attachment and rolling is initiated through L-selectin. In this setting, the relatively high velocity of rolling via L-selectin alone (i.e., when α4β7 is blocked) may present reduced opportunities for in situ triggering of secondary adhesion mechanisms; it may simply be too rapid to permit engagement of LFA-1 because of a slower on rate for LFA-1 than for α4 integrins; or too loose to enforce contact of the planar cell body bearing LFA-1 with the endothelial surface under the high shear characteristic of HEV. The differences in requirements for LFA-1 versus α4 integrin involvement in lymphocyte–EC interactions are striking, and may reflect in part the specialized concentration of α4β7 integrins and the relative exclusion of β2 integrins from microvillous sites of initial cell contact under flow (Berlin et al., 1995; Erlandsen et al., 1993).

As mentioned above, the behavior and mechanisms of adhesion of preactivated LNC or α4β7^{hi} TK1 cells are quite distinct from those of the majority of resting LNC. In previous studies (Berlin et al., 1995), we found that α4β7 can mediate direct L-selectin-independent interactions of TK1 cells and of activated lymph node lymphocytes with MAdCAM-1 in vitro, and with venules in the small intestinal lamina propria in situ. In contrast, resting LNC failed to interact detectably with lamina propria venules, which are MAdCAM-1^{+} but lack detectable L-selectin-binding peripheral node addressin-associated glycotopes (Berlin et al., 1995). The present studies reveal that, in PP–HEV as well, α4β7 was both necessary and sufficient for binding of TK1 cells and of PMA or Mn^{2+}-activated LNC. The dramatic effect of integrin triggering on the role of α4β7, with loss of requirement for L-selectin, underscores the potential of lymphocyte activation to regulate both the mechanisms and sites of lymphocyte homing. The ability of α4β7 expressed at high functional levels to initiate and consummate adhesion may help explain the distinctive gut-homing properties of mucosal immunoblasts, which traffic extremely efficiently to the intestinal lamina propria (Picker and Butcher, 1992). It may also underlie the selective trafficking of gut–recirculating memory T cells (Mackay, 1993), many of which are α4β7^{hi} but lack L-selectin (C. Mackay and E. C. B., unpublished data). On the other hand, the inefficiency of contact initiation by α4 integrin on most resting lymphocytes may help ensure that naive cells traffic preferentially through L-selectin-binding lymphoid organ HEV.

The N-terminal immunoglobulin domains of MAdCAM-1 are required for α4β7 binding (M. J. Briskin et al., unpublished data), but MAdCAM-1 also contains a mucin-like domain that can be modified by lymph node or PP–HEV with the anti-PNAd MAb MECA-79 epitope, associated with HEV ligands for the L-selectin. We have shown previously that purified HEV MAdCAM-1 supports L-selectin-mediated lymphocyte contact and rolling under shear in vitro (Berg et al., 1993). Many HEV glycoproteins can be so modified, however (Berg et al., 1991a; Tamatani et al., 1993; Hemmerich et al., 1994), and we have emphasized that only in situ analyses can reveal which of these is physiologically relevant in L-selectin-mediated attachment and rolling (Berg et al., 1991a). Furthermore, the importance of particular ligands is likely to vary depending upon the organ or microenvironment involved.

Our present results, however, suggest that MAdCAM-1 dominates L-selectin as well as α4β7 recognition of PP–HEV in situ. Whole anti-MAdCAM-1 MAbs dramatically inhibit L-selectin-dependent rolling on PP–HEV. This effect may require cross-linking, leading to local redistribution or conformational changes in MAdCAM-1 (with altered carbohydrate presentation), as Fab fragments of MAb MECA-367, which inhibit α4β7-mediated interactions, had no effect on L-selectin-dependent rolling. Moreover, inhibition was conferred by MAbs to two distinct epitopes, MECA-367 and 89, which recognize the first and second immunoglobulin domains in MAdCAM-1, respectively. Neither of these MAbs significantly inhibit L-selectin-mediated rolling on purified MAdCAM-1 in vitro (E. L. Berg, C. Berlin, and E. C. B., unpublished data), thus arguing against direct antibody blockade of the carbohydrate-presenting mucin domain. Finally, MAdCAM-1 is neither internalized nor cleaved from the HEV surface, as antibody MECA-89 against the second immunoglobulin domain inhibits L-selectin-dependent rolling but not the direct α4β7-depen-

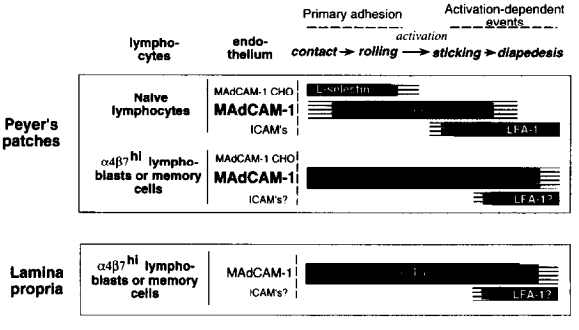

Figure 7. Schematic Summary of the Proposed Sequential but Overlapping Roles of L-Selectin, α4β7, and LFA-1 in LNC (Primarily Naive) Lymphocyte Interactions with PP–HEV; and of the Dominant Involvement of α4β7 in Interactions of Activated α4β7^{hi} Cells (Gut-Homing Immunoblasts?) in PP–HEV and in Venules of the Small Intestinal Lamina Propria

As discussed in the text, α4β7^{hi} memory lymphocytes may also use α4β7 to initiate contact in MAdCAM-1^{+} venules, and may or may not require local activation for subsequent arrest. We consider the model presented to be the simplest that fits the MAb inhibition studies presented here; however, more complicated models, some of which are discussed briefly in the text, cannot be formally excluded.

dent attachment of Mn^{2+}-activated lymphocytes in vivo. These findings provide strong evidence that MAdCAM-1 plays a prominent role in presentation of L-selectin-binding glycotopes in PP–HEV, either directly or through close association with other L-selectin ligands.

Although our current findings are consistent with previous in vitro and short-term in vivo homing studies implicating L-selectin, α4β7, and LFA-1 in lymphocyte trafficking to PP (Bargatze et al., 1990; Hamann et al., 1994), it must be considered that the observed behavior of lymphocytes may be influenced not only by the normal programming of PP–HEV, but also potentially by inflammatory signals associated with manipulation and exteriorization of the intestines in the current model.

Figure 7 presents a schematic summary of the adhesion/activation cascades implicated here in lymphocyte homing to PP, and previously in trafficking to lamina propria (Berlin et al., 1995). The schematic emphasizes the sequential but overlapping roles of L-selectin, α4β7, and LFA-1 in interactions of predominantly naive LNC; and the self-sufficient involvement of α4β7 in preactivated $\alpha4\beta7^{hi}$ lymphoblast homing to PP–HEV and to lamina propria venules suggested by our findings.

The insights from the present in situ study provide an important context for considering the potential roles of selectins, α4 integrins, and β2 integrins in the tissue- and inflammation-specific regulation of lymphocyte trafficking in general. We hypothesize, for example, that the integrin α4β1 (VLA-4) may be able to support roles analogous to those revealed for α4β7, but in the context of trafficking of other lymphocyte subsets in nonmucosal tissues in the body. Thus, as one example, α4β1–VCAM-1 interactions may be able to play a bridging role between cutaneous lymphocyte antigen–E-selectin-mediated primary attachment and LFA-1-assisted arrest in memory lymphocyte trafficking to inflamed skin (Berg et al., 1991b; Picker and Butcher, 1992), whereas preactivated $\alpha4\beta1^{hi}$ immunoblasts may be able to arrest at $VCAM\text{-}1^{+}$ venular sites of inflammation without selectin or β2 integrin involvement. α4 integrins are not required for lymphocyte entry into peripheral lymph nodes, however (Issekutz, 1991; Hamann et al., 1994); in this site, the extremely high expression of PNAd glycotopes on LN–HEV may allow L-selectin to support sufficiently avid rolling interactions so that a bridging contribution of α4 integrins is not needed for arrest. The lessons learned here may also prove applicable to the trafficking of eosinophils, mast cells, and monocytes, which, like lymphocytes, coordinately express selectin, α4 integrin, and β2 integrin adhesion receptors. Consistent with this hypothesis, recent studies have revealed that α4 integrins participate in eosinophil rolling in inflamed rabbit mesenteric venules (Sriramarao et al., 1994).

In conclusion, our findings confirm the relevance of a multistep model to the regulation of lymphocyte traffic, and extend our understanding of the model by dissecting two extreme variants of the process: PP homing of resting LNC, which involves sequential engagement of three adhesion receptors and at least one as-yet-unidentified activating signal; and the simpler interactions of preactivated $\alpha4\beta7^{hi}$ cells, which can be mediated by α4β7 alone.

Experimental Procedures

Antibodies

MAbs utilized were the following: anti-α4 PS/2, anti-LFA-1 α chain FD441.8, anti-L-selectin MEL-14, and anti-CD45 MAb 30G12 (American Type Culture Collection); anti-α4β7 heterodimer DATK32 and anti-β7 MAb FIB30 (Andrew et al., 1994); anti-mouse endothelial cell antigen MECA-32 (Hallmann et al., 1995); and anti-MAdCAM-1 MECA-367 and MECA-89 (Streeter et al., 1988).

LNCs and TK1 Lymphoma Cells

The AKR/Cum TK1 cell line has been described (Butcher et al., 1980; Hu et al., 1992; Berlin et al., 1993) and was cultured in RPMI 1640 with 5% iron-supplemented bovine calf serum (Hyclone Labs, Logan, Utah). TK1 variants exhibiting plastic adherence were excluded from these assays. Pooled mesenteric and peripheral LNCs were isolated from 6- to 8-week-old BALB/c mice; handling and fluorochrome labeling were as described (Bargatze and Butcher, 1993). Lymphocytes were used within 75 min of isolation.

Activation of Lymphocyte Integrins

For Mn^{2+} activation, fluorochrome-labeled LNCs were incubated at room temperature in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS) plus 10 mM HEPES (pH 7.0; HBSS/HEPES) containing 2 mM EDTA, and were then diluted 3-fold with Ca^{2+}/Mg^{2+}-free HBSS/HEPES, pelleted, and resuspended in HBSS/HEPES containing 2 mM $CaCl_2$ and 2 mM $MnCl_2$, followed immediately by injection. The effects of Mn^{2+} activation began to wear off within about 5 min in vivo, so that all data shown with Mn^{2+}-activated LNC was collected within this time frame. Previous in vitro studies have confirmed that this protocol triggers both α4β7 and LFA-1 activation on mouse LNC in our hands (Berlin et al., 1993; J. J. Campbell and E. C. B., unpublished data).

For activation with PMA (Sigma), PMA was added to a 100 ng/ml final concentration, and cells were incubated an additional 5 min at 37°C immediately before injection as described (Bargatze and Butcher, 1993).

In Situ Videomicroscopic Analyses of Lymphocyte Interactions with PP–HEV

In situ videomicroscopic analyses were carried out as described (Bargatze and Butcher, 1993). In brief, mice were anesthetized and an exteriorized bowel segment was positioned for epifluorescence microscopy and video recording of an individual PP on the small intestine. Fluorochrome-labeled cells (~2.5×10^7) (either TK1 or LNC) in 0.5 ml DMEM were injected intravenously into the tail vein. In some experiments, TK1 cells or lymphocytes were pretreated with anti-lymphocyte MAbs (100 μg/ml) at room temperature for 20 min (not washed) before injection with an excess of MAb (250–500 μg). In others, lymphocytes were injected and observed for 3–7 min, and then 250 or 500 μg of MAb was infused intravenously with or without additional cells, allowing visualization of the effect of MAb in the same vessels. Video recording was continuous; analyses of interactions were routinely initiated immediately after infusion of sample cells. When antibody was administered after initial cell infusion, the antibodies were allowed to circulate and achieve saturation for 2 min (for anti-lymphocyte antibodies) or ~5 min (for anti-MAdCAM-1 MAb) prior to further analysis of cell behavior. All fluorescent sample cells entering the HEV during the analysis period were analyzed, until at least 25 interacting cells were characterized (range 25–100 cells per experiment).

Individual cells were analyzed on a second-by-second basis for 10 s after initiation of HEV interaction or until the cell passed from the HEV. Interactions of ≥1 s were considered significant and were scored. Each 1 s period of interaction was assigned a value of 0 for sticking behavior, defined arbitrarily as static binding to the HEV wall during at least 2 consecutive seconds; or of 1, indicating predominant rolling interaction including stop-and-go behavior with static binding of <1 s. If during an interaction sequence a cell "skipped," releasing transiently from the vessel wall, only the time interval during which rolling or

sticking took place was scored. For each cell, the values assigned to each second of interaction were averaged, yielding an indicator of the mean behavior of the cells within the continuous range from 0 to 1. Inherent in this scoring procedure is a bias toward more interactive behaviors, so that very brief <1 s initial rolling displayed by some lymphocytes was not reflected in the mean score. Histograms are presented, displaying the frequency of cells with predominant sticking (0–0.2), intermediate or mixed (>0.2–0.8), predominant rolling (>0.8–1.0), or noninteractive behaviors; each category of behavior is assigned a consistently shaded bar in all histograms presented (see Figure 1 legend). Error bars in histograms represent 95% confidence intervals based on student's t (for $n > 2$) or (when $n = 1$ or 2) binomial distributions. Contingency tables were used to assess the significance of differences in population behaviors between conditions, based on the χ square distribution. (See also Figure 1 legend.)

For determination of noninteracting cell velocities, seven PP–HEV from four different animals were analyzed. In each venule, the velocity of 7–25 noninteracting injected lymphoid cells was assessed from the position of the cell in consecutive video frames (representing 1/30 s each). Velocities were highly variable with a given vessel, and from vessel to vessel.

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