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Sulfation of L-Selectin Ligands by an HEV-Restricted Sulfotransferase Regulates Lymphocyte Homing to Lymph Nodes

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Summary

Lymphocytes home to lymph nodes, using L-selectin to bind specific ligands on high endothelial venules (HEV). In vitro studies implicate GlcNAc-6-sulfate as an essential posttranslational modification for ligand activity. Here, we show that genetic deletion of HEC-GlcNAc6ST, a sulfotransferase that is highly restricted to HEV, results in the loss of the binding of recombinant L-selectin to the luminal aspect of HEV, elimination of lymphocyte binding in vitro, and markedly reduced in vivo homing. Reactivity with MECA 79, an adhesion-blocking mAb that stains HEV in lymph nodes and vessels in chronic inflammatory sites, is also lost from the luminal aspects of HEV. These results establish a critical role for HEC-GlcNAc6ST in lymphocyte trafficking and suggest it as an important therapeutic target.

Introduction

L-selectin on lymphocytes participates in their recruitment from the blood into lymph nodes (reviewed by Girard and Springer, 1995; Butcher and Picker, 1996). This receptor mediates the tethering and rolling of lymphocytes along high endothelial venules (HEV) within lymph nodes. This is the first event in a cascade of steps culminating in lymphocyte entry into the node. The adhesive interactions mediated by L-selectin occur through recognition of a discrete set of glycoproteins (collectively referred to as peripheral lymph node addressin [PNAd]) that are expressed on the luminal aspect of HEV (reviewed by Girard and Springer, 1995; Rosen, 1999). Several HEV-expressed, L-selectin reactive ligands (e.g., GlyCAM-1, CD34, podocalyxin, Sgp200, and MAdCAM-1) have been implicated as potential physiological ligands for L-selectin during lymphocyte homing. Consistent with the presence of a C-type lectin domain at the amino terminus of L-selectin, the HEV-expressed ligands of L-selectin require carbohydrate-based posttranslational modifications for recognition (reviewed by Kansas, 1996). A large body of evidence indicates that optimal binding by L-selectin requires sialylation, fucosylation, and carbohydrate sulfation of these ligands. The initial evidence implicating sulfation came from studies with chlorate, a metabolic inhibitor of this modification (Imai et al., 1993). Structural analysis of GlyCAM-1 (Hemmerich et al., 1994a, 1995; Hemmerich and Rosen, 1994) and histochemical studies with carbohydrate-specific antibodies (Paavonen and Renkonen, 1992; Mitsuoka et al., 1998; Clark et al., 1998) suggest that the posttranslational requirements for the HEV ligands may be met by sLe^x structures (i.e., Siaα2,3Galβ1,4-[Fucα1,3]GlcNAc) containing sulfate esters on the C-6 positions of GlcNAc and/or Gal. Each of these sulfation modifications is capable of enhancing the interaction of sLe^x with L-selectin (Scudder et al., 1994; Koenig et al., 1997; Bruehl et al., 2000). To date, most emphasis has been given to the GlcNAc-6-sulfate modification, because it is required for the binding of two classes of HEV-staining mAbs (i.e., MECA 79 and G72/G152) to L-selectin ligands (Streeter et al., 1988; Hemmerich et al., 1994b; Kimura et al., 1999; Mitsuoka et al., 1998). Notably, these antibodies inhibit L-selectin-dependent binding of lymphocytes to HEV.

The elucidation of the essential posttranslational modifications on L-selectin ligands has stimulated great interest in the enzymes that elaborate these modifications within high endothelial cells (HEC). Considerable evidence supports the involvement of the α(1,3)fucosyltransferase, Fuc-TVII, in the biosynthesis of the ligands (reviewed by Kansas, 1996). Most significantly, Fuc-TVII null mice exhibit highly diminished lymphocyte homing to lymph nodes, in correspondence with a dramatic reduction in ligand activity within HEV (Maly et al., 1996). Recent interest has focused on the carbohydrate sulfotransferases responsible for the Gal-6-O- and GlcNAc-6-O-sulfate modifications. Thus far, a single Gal-6-O-sulfotransferase (KSGal6ST) and two GlcNAc-6-O-sulfotransferases (designated GlcNAc6ST [Uchimura et al., 1998] and HEC-GlcNAc6ST [Bistrup et al., 1999]) have been implicated in ligand biosynthesis. The latter enzyme is also known as LSST (Hiraoka et al., 1999). These enzymes are expressed at the mRNA level in HEC (Uchimura et al., 1998; Bistrup et al., 1999; Hiraoka et al., 1999), are capable of effecting the appropriate sulfation modifications on CD34 and/or GlyCAM-1

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(Bistrup et al., 1999; Hiraoka et al., 1999), and can augment the L-selectin ligand activity of these glycoproteins under both equilibrium binding conditions and physiological flow conditions in a parallel plate chamber (Kimura et al., 1999; Bistrup et al., 1999; Hiraoka et al., 1999; Tangemann et al., 1999). HEC-GlcNAc6ST/LSST has stimulated the greatest interest, since its expression is highly restricted to HEC, whereas the other two enzymes are broadly expressed (Bistrup et al., 1999; Fukuta et al., 1997; Hiraoka et al., 1999; Uchimura et al., 1998). To determine whether HEC-GlcNAc6ST is indeed essential to lymphocyte homing, we have generated mice deficient in this enzyme. We demonstrate here that HEC-GlcNAc6ST deficiency dramatically impairs L-selectin ligand activity in HEV of lymph nodes and greatly reduces lymphocyte homing. Our results thus establish a critical role for this endothelial sulfotransferase in normal lymphocyte trafficking.

Results

Gene Targeting of HEC-GlcNAc6ST

The gene encoding HEC-GlcNAc6ST was incorporated into a targeting vector mutated by deletion of a 98 bp region and insertion of an IRES-lacZ reporter/neomycin resistance cassette within the coding region (nt 136–233) (Figure 1A). The deleted region is predicted to encode the PAPS 5′-phosphate binding site of the sulfotransferase (Hemmerich and Rosen, 2000). An appropriately targeted ES clone was injected into C57Bl/6 blastocysts to produce chimeric mice. Germline transmission was confirmed in F1 crosses of chimeric mice. Southern analysis (Figure 1B) and RT-PCR performed on the F2 progeny allowed identification of HEC-GlcNAc6ST wild-type (+/+), heterozygous (+/−), and gene-deleted (−/−) mice. The intercrosses between heterozygotes resulted in normal sized litters. Of 80 mice born, 28% were HEC-GlcNAc6ST −/− (9 females and 13 males), indicating that the absence of this enzyme did not cause embryonic lethality. The −/− mice were healthy and vigorous. RT-PCR analysis (Figure 1C) performed with RNA isolated from lymph nodes established that HEC-GlcNAc6ST mRNA was undetectable in −/− mice, whereas the mRNA levels for a broadly expressed carbohydrate sulfotransferase (i.e., GlcNAc6ST) were approximately equivalent between −/− and +/+ mice.

Leukocyte Distribution and Lymphoid Organ Histology in HEC-GlcNAc6ST Null Mice

Leukocyte levels in the blood (total leukocytes, lymphocytes, neutrophils, and monocytes) were unaltered between HEC-GlcNAc6ST −/− and +/+ mice (Figure 2A), and the levels of L-selectin expression on all leukocytes were comparable (data not shown). In the case of peripheral lymph nodes (PN), there was a 65% reduction in lymphocyte cell number in −/− mice (Figure 2B). The differences in cell numbers did not appear to be due to selective depletion of L-selectin^{hi} cells (data not shown). Lymphocyte numbers in mesenteric lymph nodes (MN), Peyer's patches (PP), spleen, and thymus were not significantly different in the −/− and +/+ mice (Figures 2B and 2C). The diminished lymphocyte count in −/− PN was reflected in a corresponding reduction in their size and weight, whereas those of the spleens were equivalent (data not shown). Histologically, PN from −/− mice showed an architecture that was similar to those from +/+ mice, including the presence of HEV in paracortical regions (Figure 2D and see below). Primary follicles were evident in the −/− mice, although they were generally smaller. Immunohistochemical analysis confirmed that B and T cells were concentrated in follicles and the paracortex, respectively, as they are in lymph nodes of +/+ mice (data not shown). Furthermore, staining for SLC, an HEV-associated chemokine (Gunn et al., 1998), was unaltered in the −/− mice (data not shown). There were no obvious histological abnormalities in the other lymphoid organs (spleen, MN, PP, and thymus).

Deficient Expression of L-Selectin Ligands and MECA 79 Epitopes in HEV of HEC-GlcNAc6ST Null Mice

HEV with distinctive high-walled endothelial cells were evident in lymph nodes and Peyer's patches of HEC-GlcNAc6ST −/− mice (Figures 3–5). Staining with soluble L-selectin chimera molecules has been widely used as a direct measurement of L-selectin ligand activity within HEV (Watson et al., 1990; Maly et al., 1996). We employed an IgM chimera of L-selectin as our histochemical probe (Maly et al., 1996; Bistrup et al., 1999). Strikingly, lymph nodes from HEC-GlcNAc6ST −/− mice exhibited no detectable staining on the luminal and lateral aspects of the HEC (Figures 3 and 4). However, a distinct abluminal layer of staining was present. In contrast, lymph nodes from +/+ mice exhibited staining on all aspects of the HEC. To verify that the abluminal staining represented bona fide ligand activity, we preincubated the chimera with EDTA, which inactivates the C-type lectin domain of L-selectin. EDTA completely eliminated the HEV staining in +/+ mice as well as the residual abluminal staining in the HEV of −/− mice (Figure 3). Digestion with sialidase, a treatment that inactivates L-selectin ligands on HEV (Rosen et al., 1985), also eliminated the abluminal ligands associated with the HEV of −/− mice as well as the staining of HEC in +/+ mice (data not shown).

To further characterize the HEV in HEC-GlcNAc6ST null mice, we stained lymph node sections with an E-selectin/IgM chimera (Figure 4). Previous work indicates that this selectin is capable of blocking the function of L-selectin ligands on HEV (Mebius and Watson, 1993) but does not require sulfation for this interaction (Tangemann et al., 1999). When the E-selectin chimera was applied to sections from wild-type mice, the HEV were stained in a pattern identical to that observed with the L-selectin/IgM chimera. In the HEC-GlcNAc6ST −/− mice, the pericellular pattern of E-selectin/IgM staining was retained, in contrast to the abluminal staining with the L-selectin/IgM chimera.

The MECA 79 antibody has been widely used as a probe for L-selectin ligands on HEV in secondary lymphoid organs (reviewed by Rosen, 1999). Experiments with chlorate originally established that this antibody recognizes a sulfate-dependent epitope (Hemmerich et al., 1994b), and recent studies have shown that the pertinent modification is GlcNAc-6-sulfate (Kimura et al., 1999; Bruehl et al., 2000). Unlike L-selectin, MECA 79 does not require sialylation or fucosylation for its

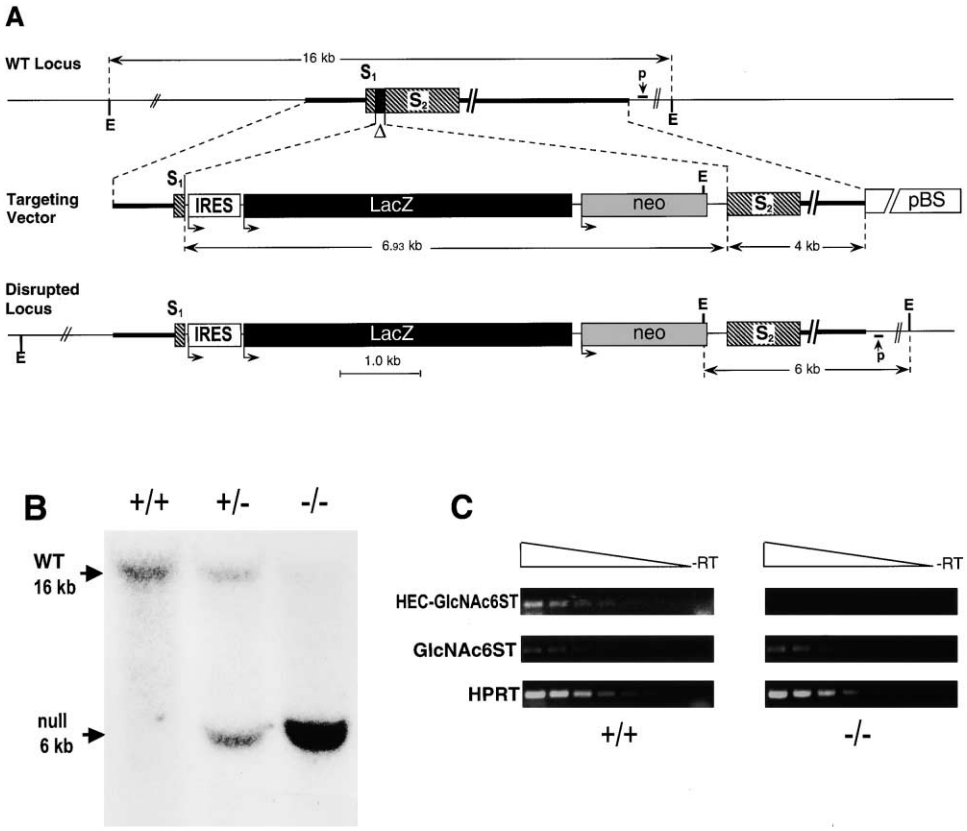

Figure 1. Inactivation of the HEC-GlcNAc6ST Locus

(A) Structure of wild-type and mutant HEC-GlcNAc6ST loci. Thicker solid lines denote genomic sequence contained within the targeting construct. A 98 bp region (Δ) of the HEC-GlcNAc6ST coding sequence corresponding to the putative PAPS 5′-phosphate binding site of the enzyme was replaced by a 6.93 kb IRES-lacZ reporter and neomycin resistance cassette (IRES-lacZ-*neo*). S_1 denotes HEC-GlcNAc6ST coding sequence upstream of the deleted segment (Δ), and S_2 denotes HEC-GlcNAc6ST coding sequence downstream of Δ. E indicates restriction sites for EcoRV. The blots were hybridized with a mixture of two overlapping probes, indicated by p. pBS denotes Bluescript vector sequence.

(B) Southern blot analysis of wild-type and mutant HEC-GlcNAc6ST loci. Southern blots were prepared using EcoRV-digested genomic DNA from F2 progeny of a chimeric male derived from an ES clone. Arrows indicate fragments corresponding to the wild-type (WT) and null alleles with the sizes indicated.

(C) Expression of HEC-GlcNAc6ST transcripts in gene-targeted mice. Total RNA was prepared from lymph nodes of wild-type (+/+) and HEC-GlcNAc6ST null (−/−) mice and was used to synthesize ds cDNA. Fragments corresponding to HEC-GlcNAc6ST, GlcNAc6ST, and HPRT were amplified by PCR from 2-fold serial dilutions of the cDNA preparations. The reaction products (746, 339, and 300 bp, respectively) were analyzed by agarose gel electrophoresis and ethidium bromide staining. Specific products were not seen when reverse transcriptase was omitted from the preparation of cDNA.

binding to HEV-ligands, suggesting that this antibody recognizes a subsite of the relevant oligosaccharide structures (Hemmerich et al., 1994b; Maly et al., 1996). The HEC-GlcNAc6ST −/− mice allowed us to determine whether this particular GlcNAc-6-O-sulfotransferase is responsible for the in situ expression of the MECA 79 epitope. As shown in Figures 5A and 5B, MECA 79 staining was dramatically reduced in PN HEV of −/− mice. However, when we allowed increased time for chromogen development and omitted counterstaining with hematoxylin, we noted abluminal staining of HEV in both MN (Figure 5D) and PN (data not shown) of −/− mice. In contrast, MECA 79 stained all aspects of the HEC in the +/+ mice (Figures 5A and 5C). The original characterization of the MECA 79 mAB by Streeter et al. (1988) reported abluminal staining in Peyer's patch HEV, a pattern that we confirmed in the +/+ mice (Figure 5E). This abluminal staining was unaltered in the HEC-GlcNAc6ST −/− mice (Figure 5F). To verify that the expression of other adhesion molecules was not perturbed in the lymph nodes of HEC-GlcNAc6ST −/− mice, we stained for MAdCAM-1 (Figures 5G and 5H) and CD31 (data not shown) and found identical patterns of HEV staining in +/+ and −/− mice.

Impaired Lymphocyte Adhesion to HEV from HEC-GlcNAc6ST Null Mice

Exogenous lymphocytes can bind to HEV in cryostat-cut lymph node sections under shear conditions (Stamper and Woodruff, 1976). The binding interaction is completely dependent on the presence of both L-selectin and its ligands on HEV (Gallatin et al., 1983; Arbonés et al., 1994; Rosen et al., 1985; Maly et al., 1996). We wanted to determine whether the deficiency in L-selectin ligands demonstrated in the HEC-GlcNAc6ST −/− mice with soluble probes was apparent in a functional cell binding assay. Indeed, lymphocyte adherence to PN HEV of HEC-GlcNAc6ST −/− mice was almost

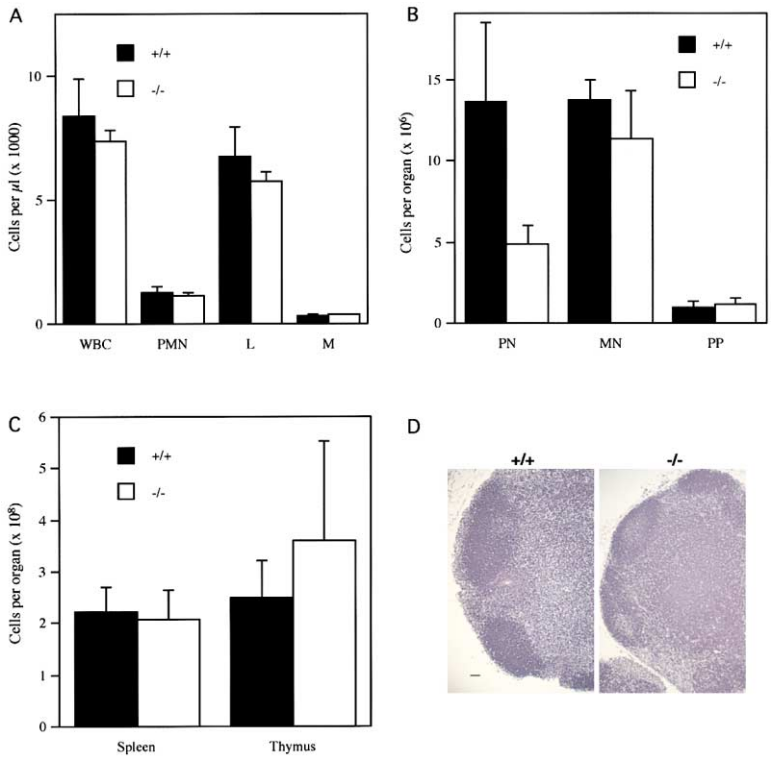

Figure 2. Leukocyte Distribution in Blood and Lymphoid Organs of HEC-GlcNAc6ST Null Mice

(A) Leukocytes were counted in the blood of wild-type (+/+) and HEC-GlcNAc6ST null (−/−) mice. WBC, PMN, L, and M indicate total white blood cell, neutrophil, lymphocyte, and monocyte counts, respectively. Eight +/+ and eight −/− mice were compared.

(B and C) Lymphocyte counts were determined in mechanically dissociated lymphoid organs from six +/+ and six −/− mice. In (A)–(C), error bars indicate SEM values. In (A), none of differences between the +/+ versus −/− mice were statistically significant ($p > 0.05$). In (B) and (C), the difference in the number of lymphocytes in pooled PN between +/+ and −/− mice was significant ($p < 0.02$).

(D) Paraffin sections were prepared from peripheral lymph nodes of +/+ and −/− mice and stained with hematoxylin and eosin. Bar represents 100 μM.

completely absent compared to the substantial binding to PN HEV of +/+ mice (Figure 6A). This latter interaction was dependent on L-selectin, since it was completely inhibited by a function-blocking antibody (i.e., MEL-14) or by inclusion of EDTA (Figure 6A).

Since the interaction between L-selectin and its HEV ligands is essential for lymphocyte homing to lymph nodes (Gallatin et al., 1983; Arbonés et al., 1994; Maly et al., 1996), we next asked whether migration to lymph nodes in vivo would be reduced in the HEC-GlcNAc6ST −/− mice. We labeled wild-type lymphocytes with an intravital fluorescent dye and injected them into the tail veins of either wild-type (+/+) or HEC-GlcNAc6ST −/− mice. After 1 hr, the number of labeled cells that had accumulated in various lymphoid organs was determined by flow cytometry as the ratio of fluorescent lymphocytes to total number of resident lymphocytes (Maly et al., 1996). As shown in Figure 6B, lymphocyte homing

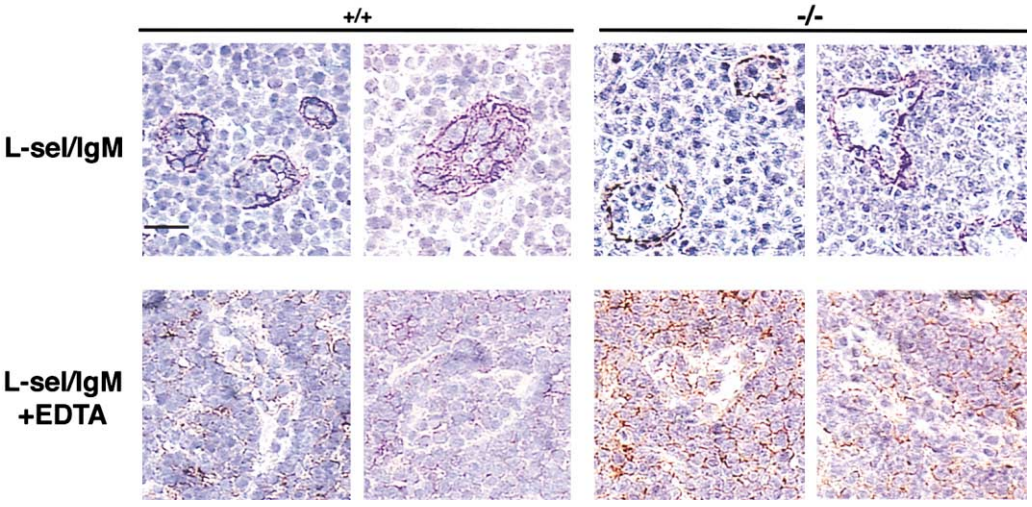

Figure 3. Expression of L-selectin Ligands on Peripheral Lymph Node HEV

Sections of peripheral lymph nodes from +/+ and HEC-GlcNAc6ST null (−/−) mice were stained with the L-selectin/IgM chimera in the presence or absence of EDTA (10 mM). The reaction product is reddish brown. Counterstaining was with hematoxylin. The HEV in −/− mice showed abluminal staining. The reticular staining seen in the presence of EDTA condition was not seen without EDTA and was judged to be nonspecific. Bar represents 20 μm.

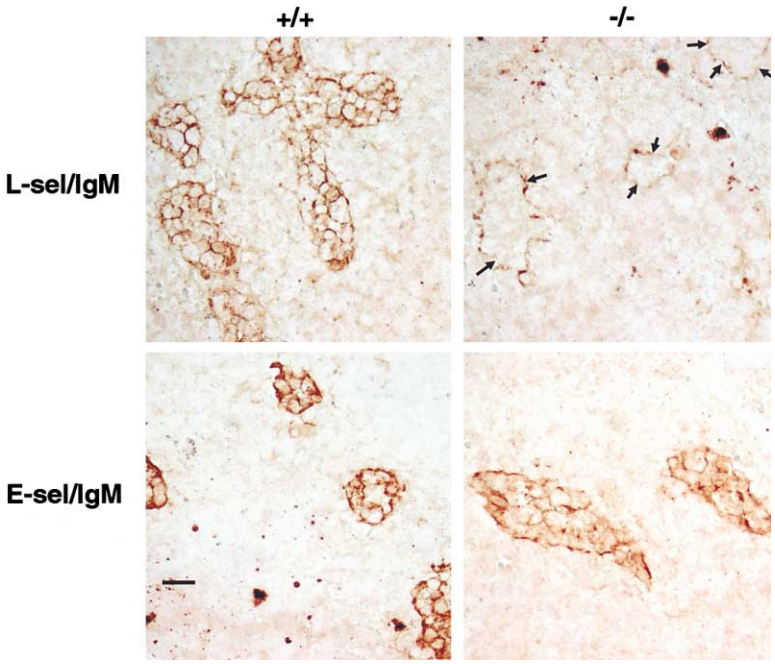

Figure 4. Expression of Ligands for L- and E-selectin on Peripheral Lymph Node HEV

Sections of peripheral lymph nodes from +/+ and HEC-GlcNAc6ST −/− mice were stained with the L-selectin/IgM chimera or E-selectin/IgM chimera. Arrows indicate abluminal staining of HEV with the L-selectin/IgM chimera in the −/− mice. The sections were not counterstained. Bar represents 20 μm.

in the −/− mice was significantly reduced to both peripheral lymph nodes and mesenteric lymph nodes, to 50% and 69% of +/+ levels, respectively. Normal levels of homing to Peyer's patches and spleen were observed in the −/− mice.

To investigate the basis of the residual homing seen in the HEC-GlcNAc6ST −/− mice, we pretreated lymphocytes with MEL-14 mAb prior to injection into animals. As shown in Table 1, MEL-14 strongly inhibited the accumulation of labeled lymphocytes into PN and MN of null mice to 3% and 9% of the control values, respectively. Comparable effects of MEL-14 were observed in wild-type mice (Table 1). To confirm that the residual lymph node homing in the HEC-GlcNAc6ST −/− mice was L-selectin dependent, we tested the ability of lymphocytes from L-selectin null mice to home to the lymphoid organs of HEC-GlcNAc6ST −/− mice. Consistent with the MEL-14 experiments, L-selectin null lymphocytes showed negligible homing to PN of HEC-GlcNAc6ST −/− mice. These data clearly establish the importance of the sulfotransferase in the homing to LN but suggest the existence of additional ligands that are formed independently of this enzyme (see below).

Discussion

With the realization that L-selectin is a lectin-like receptor, there has been considerable interest in defining the key posttranslational modifications of its HEV-expressed ligands. An extensive collection of in vitro experiments from many laboratories has implicated sialylation, fucosylation, and sulfation for optimal receptor/ligand interactions. In vivo support for the importance of sialylation has come from experiments in which intravenous administration of sialidase suppressed lymphocyte homing to lymph nodes in mice (Rosen et al., 1989). As reviewed in the Introduction, the critical contribution of α(1,3) fucosylation to ligand function has been established by the genetic disruption of the Fuc-TVII locus in mice. The purpose of the present study was to test the importance of GlcNAc-6-sulfate modifications on HEV-ligands. Heretofore, the strongest evidence for the in vivo significance of this sulfation modification has come from studies with the MECA 79 mAb. This antibody recognizes GlcNAc-6-O-sulfate-containing structures within L-selectin ligands (see above). When injected intravenously into mice, MECA 79 substantially inhibits homing to lymph nodes (Streeter et al., 1988) by blocking tethering and rolling of lymphocytes along the HEV (von Andrian, 1996). With the molecular identification of HEC-GlcNAc6ST/LSST (Bistrup et al., 1999; Hiraoka et al., 1999), gene targeting became a feasible approach to evaluate the true relevance of the GlcNAc-6-sulfate modification. Even though there are several related enzymes that can synthesize the GlcNAc-6-sulfate structure (reviewed by Hemmerich and Rosen, 2000), HEC-GlcNAc6ST was the prime candidate for the critical sulfotransferase based on its preferential expression in HEC.

The HEC-GlcNAc6ST −/− mice showed an unambiguous physiologic phenotype in that homing to lymph nodes was markedly reduced, particularly in peripheral lymph nodes. Indeed, peripheral lymph nodes of −/− mice were significantly smaller and contained fewer lymphocytes than those of +/+ mice. That the homing defect was due to a deficiency in L-selectin ligands was supported by in vitro adherence assays and by staining experiments with an L-selectin/IgM chimera, in which luminally disposed ligands on HEV were undetectable.

Luminal staining of HEV with the MECA 79 mAb was also eliminated in the lymph nodes of HEC-GlcNAc6ST −/− mice, consistent with a reduction in L-selectin ligand activity at these sites. Since MECA 79 binding to L-selectin ligands does not depend on their fucosylation or sialylation (see above), the failure of this mAb to stain the luminal aspects of HEV in the null mice indicates

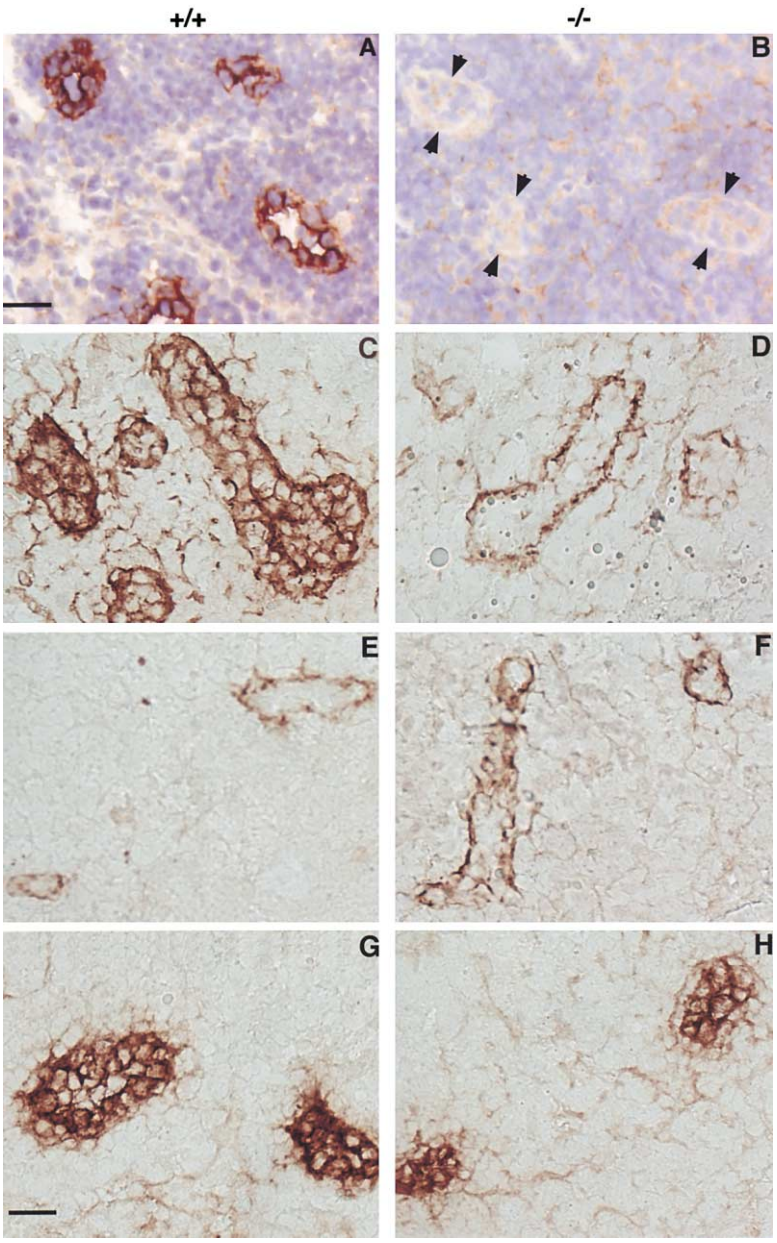

Figure 5. Staining for MECA 79 and MAdCAM-1 Epitopes on Lymphoid Organ Sections

Sections of PN (A and B), MN (C, D, G, and H), and PP (E and F) from +/+ and HEC-GlcNAc6ST −/− mice were stained with MECA 79 (A–F) or MECA 367 (MAdCAM-1, G and H). Hematoxylin counterstaining was used in (A) and (B). In (B), arrowheads denote the boundaries of three separate HEV. In (C)–(H), hematoxylin counterstaining was omitted to permit visualization of the abluminal staining pattern. No staining was observed with class-matched control immunoglobulins (data not shown). Bars represent 20 μm.

that the GlcNAc-6-sulfate modification was not present at this site. Although the most obvious explanation for the reduction in L-selectin ligand activity is the absence of the GlcNAc-6-sulfate modification, the possibility of indirect effects must be considered. For example, diminished carbohydrate sulfation resulting from the deletion of HEC-GlcNAc6ST could induce changes in sialylation and fucosylation of HEV glycoproteins. Our staining results with the E-selectin/IgM chimera argue against this possibility. This probe stained lymph node HEV of +/+ mice in a pattern indistinguishable from that seen with the L-selectin chimera, consistent with previous observations (Mebius and Watson, 1993). Our analysis showed that while the L-selectin staining pattern was markedly altered in the −/− mice, the E-selectin staining pattern was essentially retained. E-selectin binding to its physiologic ligands (e.g., PSGL-1) on leukocytes requires sialylation and fucosylation (reviewed by Kansas, 1996), and we infer that similar requirements exist for its staining of HEV. Indeed, we demonstrated that sialidase treatment of sections eliminated E-selectin staining of HEV. With respect to sulfation, the GlcNAc-6-sulfate modification imparted to recombinant GlyCAM-1 by HEC-GlcNAc6ST does not affect its binding to E-selectin (Tangemann et al., 1999). Thus, it is highly likely that E-selectin binds to the sLe^x-like structures within L-selectin ligands independently of their sulfation status, as do certain sLe^x-specific mAbs (Mitsuoka et al., 1998). We therefore conclude that the sialylation and fucosylation of the relevant glycoproteins were not aberrant in HEC-GlcNAc6ST –/– mice.

A number of interesting comparisons can be made between the present results and those obtained with other gene-targeted mice. The HEC-GlcNAc6ST −/− mice showed no changes in leukocyte levels in the blood, as was also observed in L-selectin −/− mice

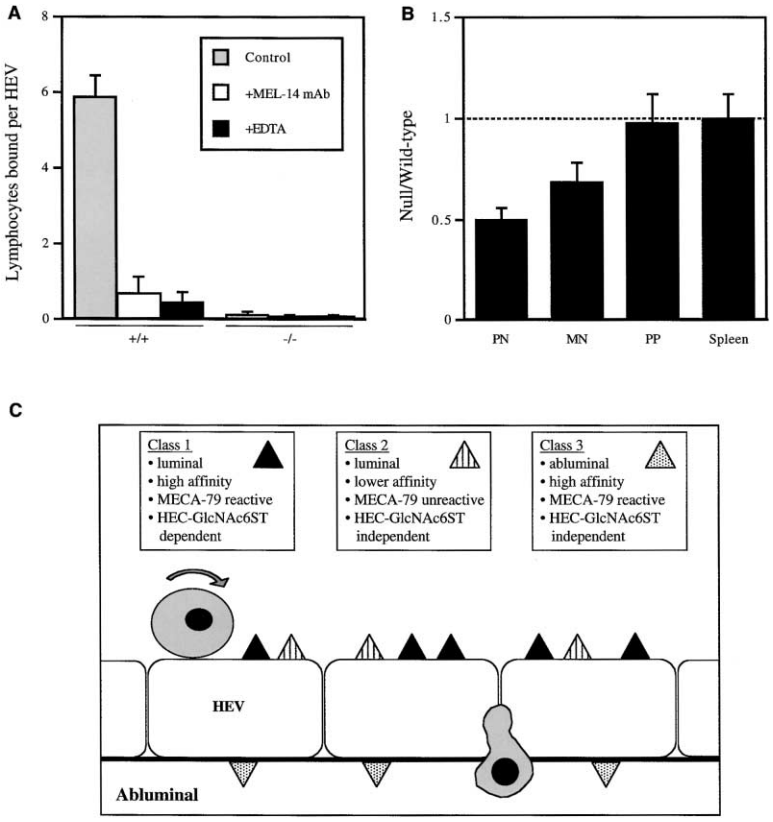

Figure 6. In Vitro Adhesion and In Vivo Homing of Lymphocytes

(A) Sections of peripheral lymph nodes from +/+ and HEC-GlcNAc6ST −/− mice were used in the Stamper-Woodruff in vitro adherence assay. EDTA (10 mM) or anti-L-selectin mAb (MEL-14) were included to determine the contribution of L-selectin to adhesion. Exogenous lymphocytes that had attached to HEV in the sections were enumerated, and the mean number of bound lymphocytes per HEV was determined. Error bars denote SEMs derived from the analysis of four independent sections per treatment. The difference in the number of lymphocytes that attached to +/+ versus −/− sections was significant ($p < 0.029$).

(B) Fluorescently labeled lymphocytes were injected intravenously into +/+ and HEC-GlcNAc6ST −/− mice. After 1 hr, peripheral lymph nodes (PN), mesenteric lymph nodes (MN), Peyer's patches (PP), and spleen were isolated and dissociated into single-cell suspensions. The number of fluorescent cells was determined by flow cytometry as a percentage of total lymphocytes within each organ. Homing in the null animals (−/−) is shown as a fraction of that observed in the wild-type animals, which is set to 1 (see Experimental Procedures). Data were pooled from 4 separate experiments in which a total of 13 +/+ and 15 −/− animals were used. Error bars denote SEMs. The −/− mice showed significantly reduced homing to both PN and MN relative to that in the +/+ mice ($p < 0.0006$ and 0.017, respectively). Homing to PP and spleen was not significantly different between −/− and +/+ mice.

(C) Model of three classes of HEV-associated ligands for L-selectin. Class 1 and 3 ligands can be directly visualized by staining with MECA 79 and L-selectin/IgM. By contrast, class 2 ligands are hypothetical and remain to be directly shown (see Discussion).

(Arbonés et al., 1994). In contrast, Fuc-TVII deficient mice exhibit a very pronounced leukocytosis (Maly et al., 1996), very likely because this enzyme makes an essential contribution to the physiological ligands for all three selectins (Maly et al., 1996). There are notable differences between the HEC-GlcNAc6ST −/− mice and L-selectin −/− mice. The reduction in lymphocyte number in PN of the HEC-GlcNAc6ST −/− mice (65%) was less than that observed in the L-selectin-deficient mice (70%–90%) (Arbonés et al., 1994; Catalina et al. 1996). Consistent with these results, the deficiency in homing to lymph nodes in the HEC-GlcNAc6ST −/− mice was not complete, whereas homing of L-selectin −/− lymphocytes to both PN and MN has been shown to be strongly diminished (Wagner et al., 1998). Importantly, the residual homing to the lymph nodes of the HEC-GlcNAc6ST −/− mice was still based on L-selectin, as revealed by two lines of evidence. First, MEL-14 inhibited this homing almost completely, and second, L-selectin null lymphocytes were almost completely unable to home to the lymph nodes of HEC-GlcNAc6ST −/− mice.

One interpretation of our results is that there are two classes of luminal ligands for L-selectin on lymph node HEV, designated as class 1 and class 2 in Figure 6C.

Table 1. Involvement of L-Selectin in Lymphocyte Homing to Lymphoid Organs of HEC-GlcNAc6ST Null and Wild-Type Mice

Organ	Effect of MEL-14 mAb on Homing (Fraction of Untreated Control)		Relative Homing of L-Sel −/− versus L-Sel +/+ Lymphocytes in HEC-GlcNAc6ST −/− Mice
	+/+ mice	−/− mice	
PN	0.05 ± 0.04	0.03 ± 0.01	0.03 ± 0.002
MN	0.15 ± 0.01	0.09 ± 0.005	0.13 ± 0.014
PP	0.45 ± 0.08	0.33 ± 0.01	0.50 ± 0.039

In vivo homing was performed as described in the Experimental Procedures. For the Mel-14 experiements, 10 +/+ and 10 HEC-GlcNAc6ST −/− mice were split into two groups of 5 each. In one group the donor (wt) lymphocytes were pretreated with MEL-14 mAb prior to injection; in the other group there was no antibody treatment. The fractional content of fluorescent cells was determined for each lymphoid organ. The effect of the MEL-14 mAb was determined by comparing the ratio of the fraction with mAb to the mean of the fraction in the control mice. Mean ratios ± standard errors are shown. The data obtained from the control mice are incorporated into Figure 6B. To determine the relative homing of L-sel −/− versus L-sel +/+ lymphocytes, 5 HEC-GlcNAc6ST −/− mice were injected with a mixture of fluorescently labeled wt (CMTMR dye) and L-selectin −/− (CMFDA dye) splenocytes. Fractional content of fluorescent cells for each source population was determined for each organ. The ratio of the two fractions yielded the relative homing. Means of the ratios ± standard errors are shown.

The first class of ligands, which can be directly demonstrated by staining with MECA 79 and L-selectin/IgM, requires the activity of HEC-GlcNAc6ST. In contrast, class 2 ligands are not detectable by either MECA 79 or the L-selectin chimera. Yet, these ligands may be reasonably inferred in order to explain the L-selectin-dependent homing to lymph nodes in the absence of HEC-GlcNAc6ST. The subset of MECA 79 independent ligands that support lymphocyte rolling on tonsillar HEV (Clark et al., 1998) may fall into this class. Class 2 ligands may be structurally related to the L-selectin ligands on Peyer's patch HEV that underlie the tethering and rolling of leukocytes in these vessels (Bargatze et al., 1995; Kunkel et al., 1998) and are essential for lymphocyte homing to this lymphoid organ (Hamann et al., 1991; also see Table 1). These luminal ligands on PP HEV, like the *postulated* class 2 ligands on PN HEV, are MECA 79 unreactive (Streeter et al., 1988) and do not require HEC-GlcNAc6ST for activity, as deduced from the normal homing of lymphocytes to PP in HEC-GlcNAc6ST null mice (Figure 6B). Furthermore, these PP ligands also appear to be of relatively low affinity in that they are not stained by an L-selectin chimera (Watson et al., 1990) and do not support in vitro adherence of lymphocytes (Gallatin et al., 1983). Confirmation of the class 2 ligands will require the direct visualization of L-selectin-dependent rolling of lymphocytes on the luminal aspects of HEV within lymph nodes of HEC-GlcNAc6ST null mice. Should the existence of class 2 ligands be established, it will remain to be determined whether they (and their PP counterparts) rely on other sulfation modifications or function independently of sulfation.

Another class of ligands designated class 3 (Figure 6C) was directly revealed by the present analysis. These ligands, detected by staining with both the L-selectin/IgM chimera and MECA 79, were present on the abluminal aspect of lymph node HEV. These ligands are present in the HEV of wild-type mice but were previously unappreciated because of the very prominent pericellular staining by these reagents. With the loss of apical and lateral staining in lymph nodes of HEC-GlcNAc6ST –/– mice, the abluminal signal was apparent. Interestingly, Streeter et al. (1988) reported that MECA 79 staining of Peyer's patch HEV is restricted to an abluminal location. We confirmed this abluminal pattern in the wild-type mice and further showed that this staining was retained in the HEC-GlcNAc6ST –/– mice. Thus, MECA 79 epitopes, presumably due to the action of a GlcNAc-6-O-sulfotransferase other than HEC-GlcNAc6ST, are associated with the abluminal aspect of HEV in lymph nodes and Peyer's patches. GlcNAc6ST (Uchimura et al., 1998; Kimura et al., 1999) is a candidate for this activity.

Although L-selectin has traditionally been thought of in terms of supporting lymphocyte tethering and rolling on the apical aspects of endothelium, recent studies by Hickey et al. (2000) have provided evidence that L-selectin may function during later stages of the recruitment cascade. The abluminal ligands revealed by the present study raise the possibility that L-selectin may be involved in regulating the migration of extravasated lymphocytes within the lymph node parenchyma. The residual L-selectin dependent homing to lymph nodes in the HEC-GlcNAc6ST null mice may (at least in part) reflect this novel function for L-selectin.

Taken together, the present results establish the importance of the GlcNAc-6-sulfate modification to the function of a major class of L-selectin ligands (class 1) within lymph node HEV and establish the role of HEC-GlcNAc6ST in the elaboration of these ligands. The contribution of HEC-GlcNAc6ST may extend to vascular beds other than lymph node HEV. Sulfate-dependent endothelial ligands for L-selectin have been demonstrated on activated endothelial cells in vitro (Zakrzewicz et al., 1997; Tu et al., 1999). The nature of the sulfation modification and the responsible sulfotransferases are not presently known. Extensive studies performed in human and various animal models have demonstrated the induction of MECA 79 staining vessels at many sites of chronic inflammation (Girard and Springer, 1995; Rosen, 1999), including human allografts undergoing rejection (Toppila et al., 1999, Kirveskari et al., 2000) and peribronchial tissue of human asthmatics (Toppila et al., 2000). An inflammatory model in AKR mice has been studied in the most detail (Michie et al., 1995). These mice exhibit a hyperplastic thymus, which is associated with the presence of MECA 79$^+$ HEV-like vessels in the medulla. The importance of L-selectin and its ligands in this model has been confirmed by showing that lymphocyte migration to the hyperplastic thymus is substantially inhibited by injection of an L-selectin mAb or MECA 79 (Michie et al., 1995). Recently, Hiraoka et al. (1999) reported that LSST (i.e., HEC-GlcNAc6ST) mRNA is expressed in these HEV-like vessels. An important question for future investigation is whether HEC-GlcNAc6ST is similarly induced within activated endothelia in inflammatory lesions in humans, especially in diseases where conspicuous MECA 79 staining is seen.

Experimental Procedures

Generation of HEC-GlcNAc6ST-Deficient Mice

An approximately 4.9 kb genomic fragment including the protein coding region of the HEC-GlcNAc6ST gene was isolated from a mouse genomic library and subcloned into the BamHI site of the pBluescript II SK(−) vector. A 98 bp fragment corresponding to a segment of the protein-coding region was replaced by an IRES-lacZ reporter and neomycin resistance cassette (IRES-lacZ-*neo*). This mutation was designed to produce a loss of function mutation by deletion of amino acids 46–78 as well as insertion of the 6.5 kb reporter/resistance cassette. The IRES-lacZ-*neo* cassette was flanked by 0.9 kb of mouse genomic DNA at its 5′ aspect and by 4.0 kb of mouse genomic DNA at its 3′ aspect. The targeting vector was linearized and electroporated into mouse embryonic (ES) stem cells. ES cells were selected for G418 resistance, and colonies carrying the homologously integrated IRES-lacZ-*neo* DNA were identified by PCR amplification with a *neo*-specific primer and a primer specific for a sequence outside of the targeting vector homology region. Colonies that gave rise to the correct size PCR products by agarose gel electrophoresis were confirmed by Southern blot analysis using genomic probes adjacent to the 5′ and 3′ regions of homology. The presence of a single IRES-lacZ-*neo* cassette was confirmed by Southern blot analysis using a neomycin gene fragment as a probe. Male chimeric mice were produced by injection of the targeted ES cells into C57BL/6 blastocysts. Chimeric mice were bred with C57BL/6 mice to produce F1 heterozygotes. Germline transmission was confirmed by PCR and Southern analysis. F1 heterozygous males and females were mated to produce F2 wild-type, heterozygous, and homozygous mutant animals. The animals were maintained in a barrier facility.

For the Southern analysis of the F2 mice, genomic DNA was purified from the liver of HEC-GlcNAc6ST +/+, +/−, and −/− mice. Thirty micrograms of DNA from each sample was digested with

EcoRV, transferred to a nylon membrane, and hybridized using end-labeled overlapping 3′ external genomic probes (5′-CAAGAGTGTT TTCTAAATACAGTATTGTAGAAAGTAATTGCCAATAGCATGAGTCT GGA-3′ and 5′-TAAACCTATGGAATGAATAAAGGCATGCTTGACA AAAAGTCATATCCAGACTCATGCTA-3′). For RT-PCR analysis of the F2 mice, total RNA was purified from peripheral lymph nodes and mesenteric lymph nodes of HEC-GlcNAc6ST +/+ and −/− mouse by lysis and extraction with RNAZol (Tel-Test Inc, Friendwood, TX). Double-stranded (ds) cDNA was synthesized from the total RNA using random hexamers for priming and AMV reverse transcriptase. PCR reactions were carried out with the cDNAs and the following primer pairs: HEC-GlcNAc6ST (5′ primer, 5′-CATGGG TCAGCATGCCTTCCATACTAACGCCA-3′; 3′ primer, 5′-CTCTGC TTGGGGCTGAGGAGAAAGGGGCACTC-3′), GlcNAc6ST (5′ primer, 5′-GCCTACCGCAAGGAGGTCGTCGGACTGGTGGAC-3′; 3′ primer, 5′-ACTGTTAACCCGCTCATAGCCCAGCACGGCCAT-3′), HPRT (5′ primer, 5′-CCTGCTGGATTACATCAAAGCACTG-3′; 3′ primer, 5′-TCC AACACTTCGTGGGGTCCT-3′).

The PCR products from 2-fold serial dilutions of cDNAs were analyzed by agarose gel electrophoresis.

Statistical Analyses

The statistical comparisons performed in Figures 2 and 6 were calculated with the two-tailed Mann-Whitney test. The choice of this nonparametric test, instead of the Student's t test, was dictated by the fact that the variances between comparison groups were not equivalent (Glantz, 1987).

Analysis of Blood and Lymphoid Organs

Blood was obtained from mice (5–10 weeks old) by cardiac puncture, placed in Microtainer tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ), and stored at 4°C until analysis. A complete hematological profile was obtained using a Hemavet 850 automated hematological analyzer (CDC Technologies, Oxford, CT). Freshly isolated lymphoid organs (5- to 7-week-old mice) were weighed directly. The peripheral lymph nodes consisted of pooled axillary, brachial, and inguinal nodes. For cell counts, the lymphoid organs were placed in cold PBS and teased with two 23G needles to create a uniform single-cell suspension. The resulting suspension was rinsed with PBS through a 100 μm nylon cell strainer (Falcon, Franklin Lakes, NJ). Viable (trypan blue negative) nucleated cells (the vast majority of which were lymphocytes) were counted by hemocytometer.

Histology Procedures

Conventional Histology

Ten-week-old mice were sacrificed, peripheral lymph nodes (axillary and brachial) were removed and fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.3) at room temperature (RT) for 5 hr and then overnight at 4°C. After alcohol dehydration, the tissue was embedded in paraffin and 2 μm sections were cut and stained with hematoxylin and eosin.

L- and E-Selectin/IgM Chimera Staining

Axillary and brachial lymph nodes from 10-week-old HEC-GlcNAC6ST −/− and +/+ mice were dissected, embedded in O.C.T. (TissueTek/Fisher Scientific, Pittsburgh, PA), and frozen in 2-methylbutane cooled in liquid nitrogen. Ten micrometer sections were cut, picked up onto Superfrost Plus slides (Fisher Scientific), air dried for 10 min, and fixed in 1% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.3) for 20 min on ice. After washing with Dulbecco's PBS (PBS), the tissue was incubated with 5% normal goat serum in PBS (block) for 10 min at 4°C. L-selectin/IgM, secreted into OptiMEM (GIBCO-BRL, Grand Island, NY) after transfection of COS7 cells with a plasmid encoding the chimera (Bistrup et al., 1999), was concentrated 6-fold and then diluted 1/4 in block. E-selectin/IgM, a gift from Drs. Randy Knibbs and Lloyd Stoolman (University of Michigan), was diluted 1/16 in block. Both selectin chimeras were incubated with the tissue for 30 min at 4°C. After washing in PBS, the tissue was incubated with a 1/500 dilution of biotinylated goat anti-human IgM (Caltag Laboratories, S. San Francisco, CA) in block for 30 min at RT. After washing in PBS, the tissue was incubated with a 1/500 dilution of streptavidin-HRP (Caltag) in PBS for 30 min at RT. Chromogen development was with NovaRed (Vector Laboratories, Burlingame, CA). Sialidase treatment (Vibrio cholera) was performed as previously described (Rosen et al., 1985).

Antibody Staining of Lymph Nodes and Peyer's Patches

Same procedure as above except primary antibody incubation was for 1 hr at RT. All antibodies were diluted in block. MECA 367 (rat IgG2a; gift from Dr. Eugene Butcher, Stanford University) was used at 5 μg/ml, MECA 79 (rat IgM; ascites, custom prepared by Caltag) was used at 1 μg/ml, and anti-CD31 (rat IgG2a; BD Pharmingen, San Diego, CA) was used at 1 μg/ml. Control rat IgM (Caltag) and rat IgG2a (BD Pharmingen) were used at the equivalent concentrations. Goat anti-SLC (R&D Systems, Minneapolis, MN) and control goat IgG (Zymed, S. San Francisco, CA) were used at 1 μg/ml in TNB Block (NEN, Boston, MA) with 2% pig serum (T-block). After washing in PBS, 1/500 dilution in block of biotinylated goat anti-rat IgG (Caltag) was added to the wells with rat IgG2a, 1/500 dilution in block of biotinylated mouse anti-rat IgM (Caltag) was added to the wells with rat IgM, and 1/500 dilution in T-block of biotinylated swine anti-goat (Caltag) was added to the wells with goat IgG. In all cases it was followed by 30 min incubation at RT. After washing, streptavidin-HRP addition and chromogen development were performed as above.

In Vitro Adherence Assay

Axillary, brachial, and inguinal lymph nodes from 10-week-old mice were dissected, embedded in O.C.T., and frozen in 2-methylbutane cooled in liquid nitrogen. The assay was performed as previously described (Rosen et al., 1989). In brief, 10 μm sections were cut, picked up onto 3-well epoxy-coated slides (Carlson Scientific, Peotone, IL), air dried, and fixed in 1% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.3) for 20 min on ice. Sections were overlaid with 100 μl/well of a lymphocyte suspension (6×10^6/ml), in PBS containing 1% BSA, prepared from MN of 10-week-old CD1 mice (Charles River Laboratories, Wilmington, MA). The shear was provided by a gyratory shaker.

In Vivo Homing

Lymphocyte homing in vivo was determined based on published procedures (Maly et al., 1996). Briefly, mouse MN lymphocytes were labeled with 5 μM 5-chloromethylfluorescein diacetate (CMFDA, Cell-Tracker; Molecular Probes, Eugene, OR) and injected (1.7×10^7 cells in 200 μl PBS) into tail veins of recipients (8–14 weeks of age). For the MEL-14 experiments, the labeled lymphocytes were preincubated with the MEL-14 mAb (17 μg per 1.7×10^7 cells in 200 μl PBS, followed by one wash in PBS) or in PBS 15 min prior to injection. One hour after injection, mice were sacrificed and PN (axillary and brachial nodes), MN, PP, and spleen were dissected out. Lymphocyte suspensions from these organs were subjected to flow cytometry (FACScan, Becton-Dickinson) (500,000 cells per organ) to determine the fractional content of fluorescent cells. The data were normalized for each animal (fractional value of CMFDA positive cells divided by the mean of the fractional values for all of the +/+ mice within the experiment). This normalization procedure allowed pooling of data from four separate experiments. To determine the relative homing of L-selectin +/+ and L-selectin −/− lymphocytes, we isolated splenocytes (Arbonés et al., 1994) from L-selectin null mice (Xu et al., 1996) and wild-type mice and labeled them with CMFDA and CMTMR dyes, respectively. The cells were mixed in an equal number (34 million cells total per animal) and injected intravenously into HEC-GlcNAc6ST −/− mice. The relative homing of the two populations into each lymphoid organ was determined (see legend for Table 1).

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