Commentary

Endothelial Ligands for L-Selectin

From Lymphocyte Recirculation to Allograft Rejection

Steven D. Rosen

From the Department of Anatomy, Program in Immunology, and Cardiovascular Research Institute, University of California, San Francisco, California

The article by Toppila et al¹ in this issue of *The American Journal of Pathology* raises the provocative possibility that the adhesion molecule L-selectin may play a significant role in the recruitment of lymphocytes to human heart allografts during rejection. The case made by these authors relies strongly on current knowledge about the high-endothelial-venule-expressed ligands (HEV ligands) for L-selectin, which participate in the constitutive homing of lymphocytes to secondary lymphoid organs. The purpose of this Commentary is to summarize and update this rapidly evolving field.

L-Selectin and its HEV-Ligands in Normal Lymphocyte Recirculation

L-selectin is broadly distributed on leukocytes in the blood. Extensive studies have established its participation in many instances of leukocyte-endothelial cell and leukocyte-leukocyte interactions.²⁻⁶ The first established function for L-selectin was as a lymphocyte homing receptor mediating the interaction of blood-borne lymphocytes with the plump endothelial cells of HEV within peripheral lymph nodes.⁷⁻⁹ As a critical step in the constitutive process of lymphocyte recirculation, this adhesive interaction initiates the recruitment of blood-borne lymphocytes into lymph nodes, where sensitization to sequestered antigens may occur. Recruitment of lymphocytes across HEV occurs as a result of a complex cascade of adhesion and signaling steps^{10,11} in which L-selectin mediates the initial tethering and rolling of lymphocytes along the specialized high endothelial cells (HEC) of HEV.¹² Subsequently, chemokines such as secondary lymphoid tissue chemokine (SLC),¹³ perhaps acting in concert with signals transduced through L-selectin,¹⁴ rapidly trigger activation of LFA-1 ($\alpha\text{L}\beta\text{2}$) on the lymphocytes.¹² The lymphocytes arrest on the endothelium and finally migrate across the HEV to complete the recruitment cascade.

In the past several years, a great deal of attention has been devoted to the molecular identification of the HEV-expressed counterreceptors (usually termed ligands) for L-selectin. Consistent with the presence of a C-type lectin domain at the amino terminus of L-selectin, all of the ligands identified to date contain carbohydrate-based recognition determinants (see next section). In mouse lymph nodes, two such ligands have been identified as GlyCAM-1¹⁵ and CD34,¹⁶ both of which are sialomucins. CD34 is a type I transmembrane glycoprotein, whereas GlyCAM-1 is a secreted molecule that lacks a transmembrane domain. Additionally, MAdCAM-1, which contains a mucin domain in addition to Ig-like domains, can function as a ligand for L-selectin in HEV of mesenteric lymph nodes and Peyer's patches.^{17,18} In human, four glycoprotein ligands have been identified at the biochemical level,^{19,20} two of which have been molecularly defined as CD34²⁰ and podocalyxin.²¹ As in the mouse, all of these molecules are sialomucin-like in character.²⁰ Interestingly, CD34 and podocalyxin share the same overall structural organization (Figure 1), with considerable sequence homology in their cytoplasmic domains.²¹ An important feature shared by these ligands is that only certain glycoforms are reactive with L-selectin. In the cases of GlyCAM-1, MAdCAM-1, CD34, and podocalyxin, naturally occurring forms exist that lack the necessary posttranslational modifications for L-selectin binding.^{17,20-22} Thus, for example, although CD34 and podocalyxin are widely distributed on vascular endothelium, a limited number of vessels (eg, HEV) express glycoforms that are L-selectin reactive.^{21,23} A similar dichotomy exists for PSGL-1, a major leukocyte ligand for P- and L-selectin.²⁴

The original identification of GlyCAM-1 and CD34 as L-selectin ligands in extracts of mouse lymph nodes used a recombinant L-selectin/IgG chimera as an affinity re-

Supported by grants from the National Institutes of Health (R37GM23547 and RO1GM5741) and Roche Bioscience.

Accepted for publication August 18, 1999.

Address reprint requests to Steven D. Rosen, Department of Anatomy, University of California, San Francisco, CA 94143-0452. E-mail: sdr@itsa.ucsf.edu.

Peripheral node addressin (PNAd)

Figure 1. Model of lymph node HEV ligands for L-selectin. Four sialomucins are shown. GlyCAM-1, CD34, and Sgp200 have been identified in mouse lymph node. CD34, podocalyxin, and Sgp200 have been identified in human tonsils. All of these components are recognized by MECA-79. The complex, defined by purification with MECA-79, is referred as the peripheral node addressin (PNAd). The cDNA encoding Sgp200 (sulfated glycoprotein of 200 kd) has not been cloned as yet. The white circles denote the posttranslational modifications of these ligands including sialylation, fucosylation, and sulfation. CD34 and podocalyxin share the same overall structural organization, each having an amino-terminal mucin domain, a presumed globular domain with cysteines, a transmembrane domain, and a cytoplasmic tail. There is significant sequence homology in the cytoplasmic tails.

agent.^{15,16,25} A parallel immunological approach has relied on the MECA-79 mAb, which stains HEV in mouse lymph nodes and blocks both lymphocyte attachment to HEV *in vitro* and short-term homing of lymphocytes to lymph nodes *in vivo*.²⁶ An important convergence of these two strategies was the demonstration that the two probes react with same complex of glycoproteins from mouse and human lymphoid organs.^{21,27} The complex of four or more glycoproteins defined by reactivity with MECA-79 is given the name peripheral lymph node addressin (PNAd).^{19,28} More recent work has extended the range of potential HEV ligands for L-selectin to nonmucin components lacking the MECA-79 epitope.²⁹ Biochemical definition of these components has not been reported to date. Also, it should be noted that MECA-79-negative ligands for L-selectin, again as yet uncharacterized, are induced on flat-walled vessels in settings of acute inflammation.³⁰

A large number of parallels (Table 1) strongly argues that the HEV-associated, L-selectin-reactive components identified in lymph nodes constitute physiological ligands in lymphocyte homing. However, definitive proof of this function has not been achieved. In the case of Peyer's patch homing, antibody blockade experiments performed *in vivo* suggest that MAdCAM-1 is an important L-selectin ligand.³¹ With respect to lymph node homing,

Table 1. Parallels between Properties of L-Selectin Ligand Candidates and Physiological Ligands in Lymph Node HEV

Property	References
Present in HEV	15, 16, 21
React with L-selectin	8, 15, 16, 21
React with MECA 79	20, 21, 25, 27, 28
Support tethering and rolling of lymphocytes under physiological flow	12, 20, 21, 84
Selectin binding requires sialic acid	20, 21, 25, 34
Selectin binding require fucosylation	35, 61, 64
Carbohydrate determinants presented in mucin domain	20, 21, 29, 85

All of the properties listed above have been demonstrated for GlyCAM-1, podocalyxin, and CD34. Many apply to Sgp200 as well.

null mice have been generated for GlyCAM-1³² and CD34³³ with no obvious consequences for lymphocyte interactions with HEV. Whether there is compensation in these mutant mice or whether one of the other ligand candidates or a complex of the molecules constitutes the physiological ligands in lymph node HEV is not clear at present.

Posttranslational Modifications of HEV-Ligands

Consistent with the function of L-selectin as a lectin-like receptor, its HEV ligands require carbohydrate-based posttranslational modifications for recognition. These requirements include sialylation, fucosylation, and carbohydrate sulfation.^{25,27,34–37} A detailed structural analysis of the O-linked chains of mouse GlyCAM-1 attempted to rationalize these requirements in terms of actual oligosaccharide structures.^{38–40} Two sulfation modifications were detected at equal levels: sulfation at C-6 of Gal and sulfation at C-6 of GlcNAc. These modifications were found, respectively, within two capping structures, 6'-sulfo sLe^x and 6-sulfo sLe^x (Table 2), but also occur in other structures. In the simplest O-linked chains (heptasaccharide), these capping groups branch from an internal trisaccharide known as core 2 (Table 2, Figure 2). The monosulfated heptasaccharide chains represent less than 25% of the O-linked oligosaccharides of GlyCAM-1. The remaining chains, whose structures have not been solved, are more complex, with additional monosaccharides and/or multiple sulfation modifications per chain.

Several studies have examined the contribution of individual sulfate esters to L-selectin binding using sulfated sLe^x derivatives or analogues thereof. There is consensus that the GlcNAc-6-sulfate modification confers enhanced binding to L-selectin as compared to the non-sulfated sLe^x.^{41–44} However, the contribution of the Gal-6-sulfate modification is controversial, with disparate reports of enhanced binding relative to sLe^x,^{43,45} no significant effect,^{42,46} and even reduced binding.⁴⁴ In competition assays, 6'- and 6-sulfo derivatives of lactose inhibit binding of L-selectin to GlyCAM-1.⁴⁷ In fact, 6',6-

Table 2. Nomenclature and Structure of Oligosaccharides

Name	Structure
sLe ^x (sialyl Lewis x)	Siaα2→3Galβ1→4[Fucα1→3]GlcNAc
6'-sulfo sLe ^x	Siaα2→3[SO ₃ →6]Galβ1→4[Fucα1→3]GlcNAc
6-sulfo sLe ^x	Siaα2→Galβ1→4[Fucα1→3][SO ₃ →6]GlcNAc
6',6-disulfo sLe ^x	Siaα2→3[SO ₃ →6]Galβ1→4[Fucα1→3][SO ₃ →6]GlcNAc
core 2	Galβ1→3[GlcNAcβ1→6]GalNAc
6'-sulfo lactose	[SO ₃ →6]Galβ1→4Glc
6-sulfo lactose	Galβ1→4[SO ₃ →6]Glc
6',6-disulfo lactose	[SO ₃ →6]Galβ1→4[SO ₃ →6]Glc

disulfo lactose is superior to sLe^x in these assays, illustrating that the relevant sulfation modifications on a lactose core can confer a significant degree of binding to L-selectin.

It should also be noted that L-selectin binds to sLe^x or its sulfated derivatives with relatively low affinity.⁴³ However, it is strongly suspected that the overall affinity of ligand binding to L-selectin is greatly amplified through the multivalent presentation of oligosaccharide determinants in a mucin domain.⁴⁸ Consistent with this view, Toppila et al⁴⁹ found that a tetravalent form of sLe^x is much more potent than monovalent sLe^x as an inhibitor of L-selectin-dependent adhesion of lymphocytes to HEV.

A series of mAbs have provided additional information on relevant carbohydrate epitopes in HEV of human lymphoid organs. Thus, in agreement with the structural analysis of GlyCAM-1, several mAbs with specificity for sLe^x-related structures stain HEV.^{29,46,50–52} Two of these (2F3 and HECA-452) were used in the study by Toppila et al.¹ Some of the sLe^x-reactive antibodies are capable of blocking *in vitro* attachment of lymphocytes to HEV; others are not.^{29,50,52} Recently, two additional antibodies were described, G72 and G152, that recognize 6-sulfo

sLe^x, one of the capping groups in GlyCAM-1.⁴⁶ Importantly, these antibodies strongly stain HEV in human lymph nodes and tonsils and block staining of lymph node HEV with an L-selectin/IgG chimera. With respect to the 6'-sulfo sLe^x and 6',6-disulfo sLe^x structures, a series of antibodies that react with chemically synthesized versions of these structures fail to stain HEV in human lymphoid organs.⁴⁶

Another mAb used by Toppila et al¹ is MECA-79, which, as reviewed above, has been useful for the biochemical identification of ligand molecules. One of the remarkable features of this mAb, in contrast to the others, is that it reacts with HEV across a wide range of species including mouse and human.⁵³ Structural characterization of the MECA-79 epitope, although incomplete at the present time, has established that it depends on sulfation,²⁷ in particular the GlcNAc-6-sulfate modification⁵⁴ (see next section). In contrast to the sLe^x-reactive mAbs, the MECA-79 epitope is independent of sialylation and fucosylation.^{27,35} Another series of mAbs, the JG series, was recently prepared against the MECA-79-reactive complex of glycoproteins isolated from human tonsils (PNAd).⁵⁵ These antibodies stain HEV in various organs, and some are function-blocking. Other than their sialic acid dependency, the epitopes of the JG antibodies are not structurally defined.

An intriguing feature of the aforementioned antibodies is the varied staining of HEV in different lymphoid organs.^{28,46,55} For example, although mouse Peyer's patch HEV clearly express functional apical ligands for L-selectin,¹⁸ staining of these vessels with MECA-79 is very weak and the reactivity is mostly abluminal.²⁶ In human, G72 and G152 reactivity of tonsillar and lymph node HEV is much stronger than that of appendix HEV.⁴⁶ Similar heterogeneity is observed for the JG antibodies.⁵⁵ These immunohistochemical findings indicate significant diversity in the carbohydrate-based epitopes expressed by different HEV.

R1 = H, R2 = SO₃ : 6-sulfo sLe^x

R1 = SO₃, R2 = H : 6'-sulfo sLe^x

R1, R2 = SO₃ : 6,6'-disulfo sLe^x

Figure 2. Sulfated O-linked chains of GlyCAM-1. Oligosaccharides bearing the 6'-sulfo sLe^x and 6-sulfo sLe^x tetrasaccharide capping groups (Table 2) are shown. They extend from the core 2 branch, indicated by a box. The monosulfated heptasaccharide structures, which occur in equal amounts, represent <25% of the O-linked chains in GlyCAM-1. The other structures, which have not been defined, are more complex. Some of these are larger than heptasaccharides and at least half contain more than one sulfate modification per chain. Among the more complex chains, the 6',6-disulfo sLe^x capping group is a candidate structure but has not yet been demonstrated.

Enzymes Involved in the Elaboration of Fucosylation and Sulfation Modifications of HEV-Ligands for L-Selectin

As reviewed in Toppila et al,¹ fucosyltransferase VII (FT-VII), an α 1,3 fucosyltransferase, has been directly implicated in the synthesis of the sLe^x-related ligands in lym-

Table 3. Carbohydrate Sulfotransferases Implicated in L-Selectin Ligand Biosynthesis

Nomenclature	Specificity	Tissue expression	References
KSGal6ST, CHST1	Gal-6-O	Broad, including HUVEC and probably HEC	56, 59, 60
GlcNAc6ST, CHST2	GlcNAc-6-O	Broad, including HUVEC and HEC	57–59
HEC-GlcNAc6ST, LSST	GlcNAc-6-O	Very few tissues but prominent in HEC	60, 61

All of these enzymes have been cloned in both mouse and human except KSGal6ST, which has been described only in human.

phoid organs of mouse. With respect to the carbohydrate sulfation of the ligands, the two relevant activities are GlcNAc-6-O- and Gal-6-O sulfotransferases. Three recently cloned enzymes with these specificities^{56–61} and belonging to the GST subfamily of carbohydrate sulfotransferases⁶² have been implicated in L-selectin ligand biosynthesis (Table 3). mRNA corresponding to each of these enzymes has been detected in lymph node and tonsillar HEV by *in situ* hybridization or reverse transcriptase-polymerase chain reaction.^{58,60,61} However, whereas the expression of HEC-GlcNAc6ST,⁶⁰ also termed L-selectin ligand sulfotransferase (LSST),⁶¹ is highly restricted to HEV, the other two enzymes are widely distributed. It is likely that HEC-GlcNAc6ST/LSST is responsible for the GlcNAc-6-O sulfotransferase activity, which has been shown to be highly enriched in isolated HEC from porcine lymph nodes.⁶³ All three of the cloned enzymes are capable of making the appropriate sulfation modification (Gal 6-sulfate or GlcNAc 6-sulfate) on actual L-selectin ligands (eg, GlyCAM-1, CD34, and MAdCAM-1) in transfected cells.^{60,61,64} Transfection of a cDNA for either of the two GlcNAc-6-O-sulfotransferases in combination with a FTVII cDNA leads to the elaboration of the 6-sulfo sLe^x epitope on the surface of transfected cells, as defined by the G72/G152 mAbs.^{54,60} These results establish that one of the key sulfated structures found in GlyCAM-1 and known to be present on HEV in human lymphoid organs can be generated by the two enzymes. In addition, stable transfection of the GlcNAc6ST cDNA (Table 2) into ECV304 cells (human bladder cancer cells) results in the generation of the MECA-79 epitope, thus establishing that the GlcNAc-6-sulfate modification is required for this structure.⁵⁴

Most importantly, the three enzymes can participate in the elaboration of functional L-selectin ligands when transfected along with a FTVII cDNA and a cDNA encoding a core-2 branching enzyme.^{54,60,61,64} Ligand activity is detected in both equilibrium binding assays with an L-selectin/IgM chimera as a probe and cell adhesion assays performed under physiological flow conditions. Interestingly, in the equilibrium assays, although each class of sulfotransferase (Gal-6-O or GlcNAc-6-O) is capable of conferring enhanced ligand activity, the greatest effect is produced by the combination of the two enzymes.⁶⁰ This result argues that optimal binding to L-selectin requires both the Gal-6-sulfate and GlcNAc-6-sulfate moieties, although the actual multisulfated structures underlying this apparent synergistic effect remain to be defined (Figure 2).

L-Selectin Ligands Induced on Endothelium at Sites of Chronic Inflammation

HEV-like vessels, possessing plump endothelial cells and other morphological features of HEV in secondary lymphoid organs, are induced in many settings of chronic inflammation.⁶⁵ These vessels occur in association with perivascular lymphocytes^{66,67} and can support *in vitro* lymphocyte attachment.^{68–71} Therefore, by analogy with the function of HEV in lymphoid organs, it is inferred that HEV-like vessels serve as a major portal of lymphocyte emigration from the blood into chronically inflamed tissues. Extensive studies performed in human and various animal models have demonstrated MECA-79 staining of HEV-like vessels in many examples of chronic inflammation (Table 4). In human, a wide variety of cutaneous lesions exhibit such staining.^{67,71} In several instances, staining with HECA 452^{50,72,73} or *in vitro* incorporation of ³⁵SO₄^{68,74} have been demonstrated for HEV-like vessels. Taken together, this evidence is strongly suggestive of the presence of L-selectin ligands. In a few cases, direct evidence for these ligands is available through either *in vitro* adhesion assays in which an L-selectin mAb is used to inhibit lymphocyte attachment to the HEV-like vessels.^{69–71} or direct staining of the HEV-like vessels with an L-selectin/IgG chimera.⁷⁵ For example, AKR mice exhibit a hyperplastic thymus which is associated with the presence of MECA-79+ HEV-like vessels in the medulla.⁷⁰ The MEL-14 mAb (anti-mouse L-selectin) and MECA-79 block *in vitro* lymphocyte attachment to these vessels by 68% and 60%, respectively. The residual adhesion in this system appears to be due largely the $\alpha 4\beta 7$ -MAdCAM-1 interaction. At cutaneous sites of inflammation, The VAP-1 system appears to complement the L-selectin system, with the latter responsible for about 60% of PBL adhesion, as judged by MECA-79 inhibition.⁷¹ *In vivo* confirmation that L-selectin is indeed a significant contributor to trafficking through HEV-like vessels is thus far available only in the AKR hyperplastic thymus model.⁷⁰ Injection of these animals with MEL-14 or with MECA-79 substantially decreases (70–80%) lymphocyte migration to the thymus.

L-Selectin Ligands Induced during Allograft Rejection

A hallmark of organ transplant rejection is the influx of lymphocytes into the graft.⁷⁶ Therefore, blocking lymphocyte recruitment is a promising approach for preventing

Table 4. Occurrence of MECA 79+ HEV-Like Vessels

Target organ	Disease process	Other features	Refs.
Synovium (human)	Rheumatoid arthritis	Stain with HECA-452	67, 73
Gut (human)	Crohn's disease	Stain with HECA-452	86, 87
Gut (human)	Ulcerative colitis		88
Skin (human)	Cutaneous sites of inflammation (e.g., allergic contact dermatitis, psoriasis, Lichen planus)		67, 71
Skin (human)	Cutaneous lymphomas (eg, Mycosis fungoides)		67
Lung (human)	Bronchiectosis	Chronic interstitial inflammation with defined lymphoid aggregates	94
Lung (human)	Chronic interstitial pneumonia	Present on relatively flat vessels found in thickened alveolar septac in association with leukocytes	94
Skin (sheep)	Delayed-type hypersensitivity reaction		89
Skin (pig)	Lesions induced by PHA injection		90
Pancreas (mouse)	NOD model of diabetes	Vessels support L-selectin-dependent lymphocyte binding	69
Salivary gland (mouse)	NOD model		91
Thymus (mouse)	Hyperplastic thymus of preleukemic AKR mice	Vessels support L-selectin-dependent lymphocyte binding	70
Pancreas (mouse)	Transgenic mice produced by expressing IL-10, IFN- γ , or SV40 T antigen under control of rat insulin promoter		92, 93
Pancreas (mouse)	Transgenic mice produced by expressing SV40 T antigen under control of rat insulin promoter	Vessels stain with L-selectin/Ig chimera, express GlyCAM-1	75

rejection, thus motivating investigation of the molecular mechanisms of lymphocyte trafficking in these systems. A pivotal study from the laboratory of R. Renkonen⁷⁷ provided the foundation for the present report by Toppila et al¹ in this issue of the *Journal*. Using a rat model of acute cardiac allograft rejection, these investigators demonstrated the induction of L-selectin ligands on flat-walled venules and capillaries within rejecting cardiac allografts. These vessels, defined by staining with sLe^x-related antibodies and an L-selectin/IgG chimera, support L-selectin-dependent binding of lymphocytes in *an vitro* adhesion assay. Transplantation studies in other animal models have also implicated the L-selectin pathway in lymphocyte recruitment and graft rejection.^{78,79}

Guided by these results, Toppila et al¹ have now addressed the question of whether L-selectin ligands are induced on vascular endothelium during rejection of human cardiac allografts. The approach was strictly histochemical, employing three mAbs (2F3, HECA-452, and MECA-79) that have been used to study L-selectin ligands in other systems. HECA-452 and 2F3 recognize sLe^x-related structures. HECA 452 does not block lymphocyte attachment to HEV.⁵⁰ Interestingly, it is inhibitory when tested on L-selectin ligands that are induced on TNF- α -treated HUVEC.⁸⁰ As reviewed above, MECA-79

recognizes a GlcNAc-6 sulfate-dependent within L-selectin ligands and is function-blocking, although its activity is varied at different anatomical sites.^{67,81}

Applying these reagents to endomyocardial biopsies taken from heart allografts, Toppila et al¹ observed a striking induction of these epitopes on intramuscular capillaries and venules in those individuals exhibiting histological signs of acute rejection. A correlation was established between the staining intensity on vessels (as well as the number of biopsies showing positive staining) and the severity of acute rejection. Moreover, in serial samples taken from three patients experiencing rejection, staining of vessels increased with rejection and subsided when immunosuppression therapy ameliorated the rejection episode. The availability of an antibody to FTVII allowed the investigators to demonstrate expression of this enzyme in activated vessels of the grafts, again in correspondence with histological parameters of rejection. This enzyme is likely to be pivotal in the synthesis of the sLe^x-related epitopes that were observed on the activated vessels.

Two distinguishing features of this study, in comparison to the anecdotal nature of previous investigations of inflammatory lesions in human patients, are that a large number of samples were analyzed (600 endomyocardial

biopsies, of which 91 showed signs of acute rejection) and the analysis was quantitative. Hence, the conclusions that were reached are supported by statistical tests.

A number of important issues remain to be addressed. As reviewed above, staining with the indicated mAbs is strongly predictive of L-selectin ligand activity. However, confirmation of this activity will require *in vitro* adhesion assays or staining with a soluble recombinant form of L-selectin. Use of mAbs in the adhesion assays will allow assessment of the possible contribution of adhesion pathways (P-selectin, E-selectin, VAP-1, $\alpha 4\beta 7$, $\alpha 4\beta 1$, etc.) other than the L-selectin pathway. Because histochemical staining with L-selectin/IgG chimeras has been limited by weak signals,⁷⁵ the use of high avidity IgM chimeras of L-selectin^{35,60,82} is likely to be beneficial. Alternatively, mild-periodate oxidation of tissue sections might be used to enhance staining reactions,⁸³ assuming that the L-selectin ligands are sialic acid-dependent.

The identity of the macromolecular ligands (CD34, podocalyxin, Sgp200, MAdCAM-1, or perhaps a unique protein scaffold) that are induced on the activated endomyocardial vessels remains to be determined. Without specific reagents that are function-blocking for individual components, it will be difficult to parse functions among what is likely to be a multiplicity of ligand candidates.

It is, however, presently feasible to obtain additional information about the potential sulfation modifications of the ligands expressed in the allografts. Although staining with MECA-79 suggests the presence of the GlcNAc-6-sulfate moieties, the newly described G72 and G152 mAbs are better characterized reagents with demonstrated specificity for 6-sulfo sLe^x. As reviewed above, this structure is a clearly validated recognition determinant for L-selectin (Table 1). Staining of the allograft samples with these antibodies, in conjunction with *in situ* hybridization assays for GlcNAc-6-O sulfotransferase transcripts (Table 3), could be very illuminating. In this regard, it is noteworthy that Hiraoka et al⁶¹ recently reported the induction of HEC-GlcNAc6ST/LSST transcripts in HEV-like vessels in the hyperplastic thymus of AKR mice. The presence of Gal-6-O sulfotransferases (eg, KSGal6ST) in vessels of the allografts should also be explored, as the modification conferred by this class of enzyme also enhances L-selectin ligand activity.⁶⁰ It has been suggested⁶⁰ that heterogeneity in L-selectin ligands within different vascular beds may be based on differential expression of the different classes of sulfotransferases.

As reviewed by Toppila et al,¹ a number of molecules other than L-selectin have been implicated in lymphocyte recruitment to rejecting allografts. In principle, components could act at later steps in an L-selectin-initiated cascade. Alternatively, other components could contribute to L-selectin-independent cascades. The example set by Toppila and coworkers provides a paradigm for the evaluation of other candidate molecules on a rigorous basis. Studies of this type may identify therapeutic targets for novel treatments of allograft rejection.

Acknowledgments

I want to express my gratitude to Annette Bistrup, Richard Bruehl, Stefan Hemmerich, Chris Sassetti, Mark Singer, and Kirsten Tangemann for their helpful comments on this manuscript.

References

1. Toppila S, Paavonen T, Nieminen MS, Häyry P, Renkonen R: Endothelial L-selectin ligands are likely to recruit lymphocytes into human rejecting heart transplants. *Am J Pathol* 1999, 155:1303-1310
2. McEver RP, Moore KL, Cummings RD: Leukocyte trafficking mediated by selectin-carbohydrate interactions. *J Biol Chem* 1995, 270:11025-11028
3. Tedder TF, Steeber DA, Chen A, Engel P: The selectins: vascular adhesion molecules. *FASEB J* 1995, 9:866-873
4. Lasky LA: Selectin-carbohydrate interactions and the initiation of the inflammatory response. *Annu Rev Biochem* 1995, 64:113-139
5. Vestweber D, Blanks JE: Mechanisms that regulate the function of the selectins and their ligands. *Physiol Rev* 1999, 79:181-213
6. Rosen SD: Selectins: Guide to the Extracellular Matrix and Adhesion Proteins, 2nd ed. Edited by Vale R, Kreis T. New York, Oxford University Press, 1999, pp 290-297
7. Gallatin W, Weissman I, Butcher E: A cell-surface molecule involved in organ-specific homing of lymphocytes. *Nature* 1983, 304:30-34
8. Geoffroy JS, Rosen SD: Demonstration that a lectin-like receptor (gp90^{MEL}) directly mediates adhesion of lymphocytes to high endothelial venules of lymph nodes. *J Cell Biol* 1989, 109:2463-2469
9. Arbonés ML, Ord DC, Ley K, Ratech H, Maynard-Curry C, Otten G, Capon DC, Tedder TF: Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity* 1994, 1:247-260
10. Springer TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994, 76:301-314
11. Butcher EC, Picker LJ: Lymphocyte homing and homeostasis. *Science* 1996, 272:60-66
12. Warnock RA, Askari S, Butcher EC, von Andrian UH: Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. *J Exp Med* 1998, 187:205-216
13. Gunn MD, Tangemann K, Tam C, Cyster JG, Rosen SD, Williams LT: A chemokine expressed in lymphoid high endothelial venules promotes adhesion and chemotaxis of naive T lymphocytes. *Proc Natl Acad Sci USA* 1998, 95:258-263
14. Hwang ST, Singer MS, Gliblin PA, Yednock TA, Bacon KB, Simon SI, Rosen SD: GlyCAM-1, a physiological ligand for L-selectin, activates $\beta 2$ integrins on naive peripheral lymphocytes. *J Exp Med* 1996, 184:1343-1348
15. Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel EJ, Fennie C, Gillett N, Watson SR, Rosen SD: An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell* 1992, 69:927-938
16. Baumhueter S, Singer MS, Henzel W, Hemmerich S, Renz M, Rosen SD, Lasky LA: Binding of L-selectin to the vascular sialomucin, CD34. *Science* 1993, 262:436-438
17. Berg EL, McEvoy LM, Berlin C, Bargatze RF, Butcher EC: L-selectin-mediated lymphocyte rolling on MAdCAM-1. *Nature* 1993, 366:695-698
18. Bargatze RF, Jutila MA, Butcher EC: Distinct roles of L-selectin and integrins $\alpha 4\beta 7$ and LFA-1 in lymphocyte homing to Peyer's patch-HEV *in situ*: the multistep model confirmed and refined. *Immunity* 1995, 3:99-108
19. Berg EL, Robinson MK, Warnock RA, Butcher EC: The human peripheral lymph node vascular addressin is a ligand for LECAM-1, the peripheral lymph node homing receptor. *J Cell Biol* 1991, 114:343-349
20. Puri KD, Finger EB, Gaudernack G, Springer TA: Sialomucin CD34 is the major L-selectin ligand in human tonsil high endothelial venules. *J Cell Biol* 1995, 131:261-270
21. Sassetti C, Tangemann K, Singer MS, Kershaw DB, Rosen SD: Identification of podocalyxin as an HEV ligand for L-selectin: parallels to CD34. *J Exp Med* 1998, 187:1965-1975

22. Dowbenko D, Kikuta A, Fennie C, Gillett N, Lasky LA: Glycosylation-dependent cell adhesion molecule 1 (GlyCAM 1) mucin is expressed by lactating mammary gland epithelial cells and is present in milk. *J Clin Invest* 1993, 92:952-960
23. Baumhueter S, Dybdal N, Kyle C, Lasky LA: Global vascular expression of murine CD34, a sialomucin-like endothelial ligand for L-selectin. *Blood* 1994, 84:2554-2565
24. Alon R, Rossiter H, Wang X, Springer TA, Kupper TS: Distinct cell surface ligands mediate T lymphocyte attachment and rolling on P and E selectin under physiological flow. *J Cell Biol* 1994, 127:1485-1495
25. Imai Y, Singer MS, Fennie C, Lasky LA, Rosen SD: Identification of a carbohydrate-based endothelial ligand for a lymphocyte homing receptor. *J Cell Biol* 1991, 113:1213-1221
26. Streeter P, Berg E, Rouse T, Bargatze R, Butcher E: A tissue-specific endothelial cell molecule involved in lymphocyte homing. *Nature* 1988, 331:41-43
27. Hemmerich S, Butcher EC, Rosen SD: Sulfation-dependent recognition of HEV-ligands by L-selectin and MECA 79, an adhesion-blocking mAb. *J Exp Med* 1994, 180:2219-2226
28. Streeter PR, Rouse BTN, Butcher EC: Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. *J Cell Biol* 1988, 107:1853-1862
29. Clark RA, Fuhlbrigge RC, Springer TA: L-Selectin ligands that are O-glycoprotease resistant, and distinct from MECA-79 antigen are sufficient for tethering, and rolling of lymphocytes on human high endothelial venules. *J Cell Biol* 1998, 140:721-731
30. Ley K, Tedder TF: Leukocyte interactions with vascular endothelium. New insights into selectin-mediated attachment and rolling. *J Immunol* 1995, 155:525-528
31. Bargatze RF, Jutila MA, Butcher EC: Distinct roles of L-selectin and integrins $\alpha 4\beta 7$ and LFA-1 in lymphocyte interactions with Peyer's Patch HEV in situ: the multi-step model confirmed and refined. *Immunology* 1995, 3:99-108
32. Kansas GS: Selectins and their ligands: current concepts and controversies. *Blood* 1996, 88:3259-3287
33. Suzuki A, Andrew DP, Gonzalo JA, Fukumoto M, Spellberg J, Hashiyama M, Takimoto H, Gerwin N, Webb I, Molineux G, Amakawa R, Tada Y, Wakeham A, Brown J, McNiece I, Ley K, Butcher EC, Suda T, Gutierrez-Ramos JC, Mak TW: CD34-deficient mice have reduced eosinophil accumulation after allergen exposure, and show a novel crossreactive 90-kD protein. *Blood* 1996, 87:3550-3562
34. Rosen SD, Singer MS, Yednock TA, Stoolman LM: Involvement of sialic acid on endothelial cells in organ-specific lymphocyte recirculation. *Science* 1985, 228:1005-1007
35. Maly P, Thall AD, Petryniak B, Rogers CE, Mith PL, Marks RM, Kelly RJ, Gersten KM, Cheng G, Saunders TL, Camper SA, Camphausen RT, Sullivan FX, Isogai Y, Hindsgaul O, von Andrian UH, Lowe JB: The Fuc-TVII $\alpha(1,3)$ fucosyltransferase controls lymphocyte homing, and blood leukocyte emigration through an essential role in L-, E- and P-selectin ligand biosynthesis. *Cell* 1996, 86:643-653
36. Imai Y, Lasky LA, Rosen SD: Sulphation requirement for GlyCAM-1, an endothelial ligand for L-selectin. *Nature* 1993, 361:555-557
37. Shailubhai K, Streeter P, Smith CE, Jacob GS: Sulfation and sialylation requirements for a glycoform of CD34, a major endothelial ligand for L-selectin in porcine peripheral lymph nodes. *Glycobiology* 1997, 7:305-314
38. Hemmerich S, Bertozzi CR, Leffler H, Rosen SD: Identification of the sulfated monosaccharides of GlyCAM-1, an endothelial derived ligand for L-selectin. *Biochemistry* 1994, 33:4820-4829
39. Hemmerich S, Rosen SD: 6'-sulfated, sialyl Lewis X is a major capping group of GlyCAM-1. *Biochemistry* 1994, 33:4830-4835
40. Hemmerich S, Leffler H, Rosen SD: Structure of the O-glycans in GlyCAM-1, an endothelial-derived ligand for L-selectin. *J Biol Chem* 1995, 270:12035-12047
41. Scudder PR, Shailubhai K, Duffin KL, Streeter PR, Jacob GS: Enzymatic synthesis of a 6-sulfated sialyl-Lewis x which is an inhibitor of L-selectin binding to peripheral addressin. *Glycobiology* 1994, 4:929-933
42. Saunders WJ, Katsumoto TR, Bertozzi CR, Rosen SD, Kiessling LL: Selectin-carbohydrate interactions: an investigation into the relevant modifications of the Lewis x trisaccharides. *Biochemistry* 1996, 35:14862-14867
43. Koenig A, Jain R, Vig R, Norgard-Sumnicht KE, Matta KL, Varki A: Selectin inhibition: synthesis and evaluation of novel sialylated, sulfated and fucosylated oligosaccharides, including the major capping group of GlyCAM-1. *Glycobiology* 1997, 7:79-93
44. Galustian C, Lubineau A, le Narvor C, Kiso M, Brown G, Feizi T: L-selectin interactions with novel mono- and multisulfated Lewis x sequences in comparison with the potent ligand 3'-sulfated Lewis x. *J Biol Chem* 1999, 274:18213-18217
45. Tsuboi S, Isogai Y, Hada N, King JK, Hindsgaul O, Fukuda M: 6'-sulfo sialyl Lex but not 6-sulfo sialyl Lex expressed on the cell surface supports L-selectin-mediated adhesion. *J Biol Chem* 1996, 271:27213-27216
46. Mitsuoka C, Sawada-Kasugai M, Ando-Furui K, Izawa M, Nakanishi H, Nakamura S, Ishida H, Kiso M, Kannagi R: Identification of a major carbohydrate capping group of the L-selectin ligand on high endothelial venules in human lymph nodes as 6-sulfo sialyl Lewis X. *J Biol Chem* 1998, 273:11225-11233
47. Bertozzi CR, Fukuda S, Rosen SD: Sulfated disaccharide inhibitors of L-selectin; deriving structural leads from a physiological selectin ligand. *Biochemistry* 1995, 34:14271-14278
48. Nicholson MW, Barclay AN, Singer MS, Rosen SD, van der Merwe PA: Affinity and kinetic analysis of L-selectin (CD62L) binding to GlyCAM-1. *J Biol Chem* 1998, 273:763-770
49. Toppila S, Lauronen J, Mattila P, Turunen JP, Penttilä L, Paavonen T, Renkonen O, Renkonen R: L-selectin ligands in rat high endothelium: multivalent sialyl Lewis x glycans are high-affinity inhibitors of lymphocyte adhesion. *Eur J Immunol* 1997, 27:1360-1365
50. Duijvestijn AM, Horst E, Pals ST, Rouse BN, Steere AC, Picker LJ, Meijer CJLM, Butcher EC: High endothelial differentiation in human lymphoid and inflammatory tissues defined by monoclonal antibody HECA-452. *Am J Pathol* 1988, 130:147-155
51. Paavonen T, Renkonen R: Selective expression of sialyl-Lewis X and Lewis A epitopes, putative ligands for L-selectin, on peripheral lymph-node high endothelial venules. *Am J Pathol* 1992, 141:1259-1264
52. Mitsuoka C, Kawakami-Kimura N, Kasugai-Sawada M, Hiraiwa N, Toda K, Ishida H, Kiso M, Hasegawa A, Kannagi R: Sulfated sialyl Lewis x, the putative L-selectin ligand, detected on endothelial cells of high endothelial venules by a distinct set of anti-sialyl Lewis x antibodies. *Biochem Biophys Res Commun* 1997, 230:546-551
53. Girard JP, Baekkevold ES, Amalric F: Sulfation in high endothelial venules: cloning and expression of the human PAPS synthetase. *FASEB J* 1998, 12:603-612
54. Kimura N, Mitsuoka C, Kanamori A, Hiraiwa N, Uchimura K, Muramatsu T, Tamatani T, Kansas GS, Kannagi R: Reconstitution of functional L-selectin ligands on a cultured human endothelial cell line by cotransfection of $\alpha 1\rightarrow 3$ fucosyltransferase VII and newly cloned GlcNAc β 6-sulfotransferase cDNA. *Proc Natl Acad Sci USA* 1999, 96:4530-4535
55. Berg EL, Mallowney AT, Andrew DP, Goldberg JE, Butcher EC: Complexity and differential expression of carbohydrate epitopes associated with L-selectin recognition of high endothelial venules. *Am J Pathol* 1998, 152:469-477
56. Fukuta M, Inazawa J, Torii T, Tsuzuki K, Shimada E, Habuchi O: Molecular cloning and characterization of human keratan sulfate Gal-6-sulfotransferase. *J Biol Chem* 1997, 272:32321-32328
57. Uchimura K, Muramatsu H, Kaname T, Ogawa H, Yamakawa T, Fan QW, Mitsuoka C, Kannagi R, Habuchi O, Yokoyama I, Yamamura K, Ozaki T, Nakagawara A, Kadomatsu K, Muramatsu T: Human N-acetylglucosamine-6-O-sulfotransferase involved in the biosynthesis of 6-sulfo sialyl Lewis X: molecular cloning, chromosomal mapping, and expression in various organs and tumor cells. *J Biochem (Tokyo)* 1998, 124:670-678
58. Uchimura K, Muramatsu H, Kadomatsu K, Fan QW, Kurosawa N, Mitsuoka C, Kannagi R, Habuchi O, Muramatsu T: Molecular cloning and characterization of an N-acetylglucosamine-6-O-sulfotransferase. *J Biol Chem* 1998, 273:22577-22583
59. Li X, Tedder TF: CHST1, and CHST2 sulfotransferases expressed by human vascular endothelial cells: cDNA cloning, expression, and chromosomal localization. *Genomics* 1999, 55:345-347
60. Bistrup A, Bhakta S, Lee JK, Belov YC, Gunn MD, Zuo F-R, Huang C-C, Kannagi R, Rosen SD, Hemmerich S: Sulfotransferases of two specificities function in the reconstitution of high-endothelial-cell ligands for L-selectin. *J Cell Biol* 1999, 145:899-910
61. Hiraoka N, Petryniak B, Nakayama J, Tsuboi S, Suzuki M, Yeh J-C,

- Izawa D, Tanaka T, Miyasaka M, Lowe JB, Fukuda M: A novel, high endothelial venule-specific sulfotransferase expresses 6-sulfo sialyl Lewis x, an L-selectin ligand displayed by CD34. *Immunity* 1999, 11:79–89
62. Rosen SD, Bistrup A, Hemmerich S: Carbohydrate sulfotransferases. *Oligosaccharides in Chemistry and Biology*. Edited by Ernst B, Sinay P, Hart G. Weinheim, Germany, Wiley-VCH, in press
63. Bowman KG, Hemmerich S, Bakhta S, Singer MS, Bistrup A, Rosen SD, Bertozzi CR: Identification of an N-acetylglucosamine-6-O-sulfotransferase activity specific to lymphoid tissue: an enzyme with a possible role in lymphocyte homing. *Chem Biol* 1998, 5:447–460
64. Tangemann K, Bistrup A, Hemmerich S, Rosen SD: Sulfation of an HEV-expressed ligand for L-selectin: effects on tethering and rolling of lymphocytes. *J Exp Med*, in press
65. Girard J-P, Springer TA: High endothelial venules: specialized endothelium for lymphocyte migration. *Immunol Today* 1995, 16:449–457
66. Iguchi T, Ziff M: Electron microscopic study of rheumatoid synovial vasculature. Intimate relationship between tall endothelium and lymphoid aggregation. *J Clin Invest* 1986, 77:355–361
67. Michie SA, Streeter PR, Bolt PA, Butcher EC, Picker LJ: The human peripheral lymph node vascular addressin. *Am J Pathol* 1993, 143: 1688–1698
68. Freemont A: Functional and biosynthetic changes in endothelial cells of vessels in chronically inflamed tissues: evidence for endothelial control of lymphocyte entry into diseased tissues. *J Pathol* 1988, 155:225–230
69. Hänninen A, Taylor C, Streeter PR, Stark LS, Sarte JM, Shizuru JA, Simell O, Michie SA: Vascular addressins are induced on islet vessels during insulinitis in nonobese diabetic mice and are involved in lymphoid binding to islet endothelium. *J Clin Invest* 1993, 92:2509–2515
70. Michie SA, Streeter PR, Butcher EC, Rouse RV: L-selectin and $\alpha 4 \beta 7$ integrin homing receptor pathways mediate peripheral lymphocyte traffic to AKR mouse hyperplastic thymus. *Am J Pathol* 1995, 147: 412–421
71. Arvilommi AM, Salmi M, Kalimo K, Jalkanen S: Lymphocyte binding to vascular endothelium in inflamed skin revisited: a central role for vascular adhesion protein-1 (VAP-1). *Eur J Immunol* 1996, 26:825–833
72. Kabel PJ, Voorbij HA, de Haan-Meulman M, Pals ST, Drexhage HA: High endothelial venules present in lymphoid cell accumulations in thyroids affected by autoimmune disease: a study in men and BB rats of functional activity and development. *J Clin Endocrinol Metab* 1989, 68:744–751
73. van Dinther-Janssen AC, Pals ST, Scheper R, Breedveld F, Meijer CJ: Dendritic cells and high endothelial venules in the rheumatoid synovial membrane. *J Rheumatol* 1990, 17:11–17
74. Higuchi Y, Herrera P, Muniesa P, Huarte J, Belin D, Ohashi P, Aichele P, Orci L, Vassalli JD, Vassalli P: Expression of a tumor necrosis factor α transgene in murine pancreatic β cells results in severe and permanent insulinitis without evolution towards diabetes. *J Exp Med* 1992, 176:1719–1731
75. Onrust SV, Hartl PM, Rosen SD, Hanahan D: Modulation of L-selectin ligand expression during an immune response accompanying tumorigenesis in transgenic mice. *J Clin Invest* 1996, 97:54–64
76. Hayry P, von Willebrand E, Parthenais E, Nemlander A, Soots A, Lautenschlager I, Alföldy P, Renkonen R: The inflammatory mechanisms of allograft rejection. *Immunol Rev* 1984, 77:85–142
77. Turunen JP, Majuri ML, Seppo A, Tiisala S, Paavonen T, Miyasaka M, Lemstrom K, Penttilä L, Renkonen O, Renkonen R: De novo expression of endothelial sialyl Lewis(a), and sialyl Lewis(x) during cardiac transplant rejection: superior capacity of a tetravalent sialyl Lewis(x) oligosaccharide in inhibiting L-selectin-dependent lymphocyte adhesion. *J Exp Med* 1995, 182:1133–1141
78. Turunen JP, Paavonen T, Majuri M-L, Tiisala S, Mattila P, Mennander A, Gahmberg CG, Häyry P, Tamatani T, Miyasaka M, Renkonen R: Sialyl Lewis x and L-selectin-dependent site specific lymphocyte extravasation into renal transplants during acute rejection. *Eur J Immunol* 1994, 24:1130–1136
79. Tang ML, Hale LP, Steeber DA, Tedder TF: L-selectin is involved in lymphocyte migration to sites of inflammation in the skin: delayed rejection of allografts in L-selectin-deficient mice. *J Immunol* 1997, 158:5191–5199
80. Tu L, Delahunty MD, Ding H, Luscinskas FW, Tedder TF: The cutaneous lymphocyte antigen is an essential component of the L-selectin ligand induced on human vascular endothelial cells. *J Exp Med* 1999, 189:241–252
81. von Andrian UH: Intravital microscopy of the peripheral lymph node microcirculation in mice. *Microcirculation* 1996, 3:287–300
82. Giuffrè L, Cordey AS, Monai N, Tardy Y, Schapira M, Spertini O: Monocyte adhesion to activated aortic endothelium: role of L-selectin and heparan sulfate proteoglycans. *J Cell Biol* 1997, 136:945–956
83. Norgard KE, Han H, Powell L, Kriegler M, Varki A, Varki NM: Enhanced interaction of L-selectin with the high endothelial venule ligand via selectively oxidized sialic acids. *Proc Natl Acad Sci USA* 1993, 90:1068–1072
84. Dvir O, Shimron F, Chen C, Singer MS, Rosen SD, Alon R: GlyCAM-1 supports leukocyte rolling in flow: evidence for a greater dynamic stability of L-selectin rolling of lymphocytes than of neutrophils. *Cell Adhes Commun* 1998, 6:349–370
85. Norgard KE, Moore KL, Diaz S, Stults NL, Ushiyama S, McEver RP, Cummings RD, Varki A: Characterization of a specific ligand for P-selectin on myeloid cells. A minor glycoprotein with sialylated O-linked oligosaccharides. *J Biol Chem* 1993, 268:12764–12774
86. Salmi M, Granfors K, MacDermott R, Jalkanen S: Aberrant binding of lamina propria lymphocytes to vascular endothelium in inflammatory bowel diseases. *Gastroenterology* 1994, 106:596–605
87. Duijvestijn A, Kerkhove M, Bargatzte R, Butcher E: Lymphoid tissue- and inflammation-specific endothelial cell differentiation defined by monoclonal antibodies. *J Immunol* 1987, 138:713–719
88. Salmi M, Jalkanen S: Regulation of L-selectin expression on cultured bone marrow leukocytes and their precursors. *Eur J Immunol* 1992, 22:835–843
89. Mackay CR, Marston W, Dudler E: Altered patterns of T cell migration through lymph nodes and skin following antigen challenge. *Eur J Immunol* 1992, 22:2205–2210
90. Whyte A, Haskard DO, Binns RM: Infiltrating $\gamma\delta$ T-cells, and selectin endothelial ligands in the cutaneous phytohemagglutinin-induced inflammatory reaction. *Vet Immunol Immunopathol* 1994, 41:31–40
91. Faveeuw C, Gagnerault MC, Lepault F: Expression of homing and adhesion molecules in infiltrated islets of Langerhans and salivary glands of nonobese diabetic mice. *J Immunol* 1994, 152:5969–5978
92. Wogensen L, Huang X, Sarvetnick N: Leukocyte extravasation into the pancreatic tissue in transgenic mice expressing interleukin 10 in the islets of Langerhans. *J Exp Med* 1993, 178:175–185
93. Lee MS, Sarvetnick N: Induction of vascular addressins and adhesion molecules in the pancreas of IFN- γ transgenic mice. *J Immunol* 1994, 152:4597–4603