Commentary

Endothelial Ligands for L-Selectin
From Lymphocyte Recirculation to Allograft Rejection

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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 steps10,11 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 (αLβ2) 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-
null mice have been generated for GlyCAM-1, podocalyxin, and CD34. Many apply to Sgp200 as well.

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. A detailed structural analysis of the O-linked chains of mouse GlyCAM-1 attempted to rationalize these requirements in terms of actual oligosaccharide structures. 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\(^\text{a}\) and 6-sulfo sLe\(^\text{a}\) (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\(^\text{a}\) 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\(^\text{a}\). However, the contribution of the Gal-6-sulfate modification is controversial, with disparate reports of enhanced binding relative to sLe\(^\text{a}\) and no significant effect and even reduced binding. In competition assays, 6'- and 6-sulfo derivatives of lactose inhibit binding of L-selectin to GlyCAM-1. In fact, 6',6-
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. 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. Consistent with this view, Toppila et al found that a tetravalent form of sLex is a more potent than monovalent sLex 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. Two of these (2F3 and HECA-452) were used in the study by Toppila et al. Some of the sLe^x-reactive antibodies are capable of blocking in vitro attachment of lymphocytes to HEV; others are not. Recently, two additional antibodies were described, G72 and G152, that recognize 6-sulfo sLe^x, one of the capping groups in GlyCAM-1.

Another mAb used by Toppila et al 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. Structural characterization of the MECA-79 epitope, although incomplete at the present time, has established that it depends on sulfation, in particular the GlcNAc-6-sulfate modification (see next section). In contrast to the sLe^x-reactive mAbs, the MECA-79 epitope is independent of sialylation and fucosylation.

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

| Table 2. Nomenclature and Structure of Oligosaccharides |

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>sLe^x (sialyl Lewis x)</td>
<td>Siaα2→3Galβ1→4[Fucα1→3]GlcNAc</td>
</tr>
<tr>
<td>6'-sulfosLe^x</td>
<td>Siaα2→3[SO3→6]Galβ1→4[Fucα1→3]GlcNAc</td>
</tr>
<tr>
<td>6-sulfo sLe^x</td>
<td>Siaα2→3[SO3→6]Galβ1→4[Fucα1→3][SO3→6]GlcNAc</td>
</tr>
<tr>
<td>6,6'-disulfo sLe^x</td>
<td>Galβ1→3[GlcNAcβ1→4][SO3→6]Glc</td>
</tr>
</tbody>
</table>

**R1 = H, R2 = SO_3^{-} : 6-sulfo sLe^x**

**R1 = SO_3^{-}, R2 = H : 6'-sulfo sLe^x**

**R1, R2 = SO_3^{-} : 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, an α1,3 fucosyltransferase (FT-VII), has been directly implicated in the synthesis of the sLe^x-related ligands in lymphoid organs.
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 sulfo-transferases. Three recently cloned enzymes with these specificities 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. However, whereas the expression of HEC-GlcNAc6ST, also termed L-selectin ligand sulfotransferase (LSST), 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. All three of the cloned enzymes are capable of making the appropriate sulfate modification (Gal-6-sulfate or GlcNAc-6-sulfate) on actual L-selectin ligands (eg, GlyCAM-1, CD34, and MadCAM-1) in transfected cells. Transfection of a cDNA for either of the two GlcNAc-6-O-sulfotransferases in combination with a FTVII cDNA leads to the elaboration of functional L-selectin ligands when transfected along with a FTVII cDNA and a cDNA encoding a core-2 branching enzyme. 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. 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).

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

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Specificity</th>
<th>Tissue expression</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSGal6ST, CHST1</td>
<td>Gal-6-O</td>
<td>Broad, including HUVEC and probably HEC</td>
<td>56, 59, 60</td>
</tr>
<tr>
<td>GlcNAc6ST, CHST2</td>
<td>GlcNAc-6-O</td>
<td>Broad, including HUVEC and HEC</td>
<td>57–59</td>
</tr>
<tr>
<td>HEC-GlcNAc6ST, LSST</td>
<td>GlcNAc-6-O</td>
<td>Very few tissues but prominent in HEC</td>
<td>60, 61</td>
</tr>
</tbody>
</table>

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

### 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. These vessels occur in association with perivascular lymphocytes and can support lymphocyte attachment. 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.

### L-Selectin Ligands Induced during Allograft Rejection

A hallmark of organ transplant rejection is the influx of lymphocytes into the graft. Therefore, blocking lymphocyte recruitment is a promising approach for preventing...
rejection, thus motivating investigation of the molecular mechanisms of lymphocyte trafficking in these systems. A pivotal study from the laboratory of R. Renkonen\textsuperscript{77} provided the foundation for the present report by Toppila et al.\textsuperscript{1} 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 sLex-related antibodies and an L-selectin/IgG chimera, support L-selectin-dependent binding of lymphocytes in an \textit{in vitro} adhesion assay. Transplantation studies in other animal models have also implicated the L-selectin pathway in lymphocyte recruitment and graft rejection.\textsuperscript{78,79}

Guided by these results, Toppila et al.\textsuperscript{1} have now addressed the question of whether L-selectin ligands are induced on vascular endothelium during rejection of human cardiac allografts. These vessels, defined by staining with sLe\textsuperscript{x}-related antibodies and an L-selectin/IgG chimera, support L-selectin-dependent binding of lymphocytes in an \textit{in vitro} adhesion assay. Transplantation studies in other animal models have also implicated the L-selectin pathway in lymphocyte recruitment and graft rejection.\textsuperscript{78,79}

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

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

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

Applying these reagents to endomyocardial biopsies taken from heart allografts, Toppila et al.\textsuperscript{1} 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\textsuperscript{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, α4β7, α4β1, etc.) other than the L-selectin pathway. Because histochemical staining with L-selectin/IgG chimeras has been limited by weak signals, the use of high avidity IgM chimeras of L-selectin is likely to be beneficial. Alternatively, mild-periodate oxidation of tissue sections might be used to enhance staining reactions, 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 endomycocardial 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 sLeα. 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 immunohistochemical assays for GlcNAc-6-O sulfotransferase transcripts (Table 3), could be very illuminating. In this regard, it is noteworthy that Hiraoka et al 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 (e.g., 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. It has been suggested 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., 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.

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References


