Core 2 Branching β1,6-N-Acetylglicosaminytransferase and High Endothelial Venule-restricted Sulfotransferase Collaboratively Control Lymphocyte Homing* 

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L-selectin mediates lymphocyte homing by facilitating lymphocyte adhesion to carbohydrate ligands expressed on high endothelial venules (HEV) of the secondary lymphoid organs. Previous studies demonstrated that L-selectin ligand sulfotransferase (LSST) forms 6-sulfo sialyl Lewis x (sLeα) on both core 2 branch and MECA-79-positive extended core 1 O-glycans, but the chemical nature and roles of HEV ligands elaborated by LSST and core 2 β1,6-N-acetylglicosaminytransferase-1 (Core2GlcNAcT) have been undefined. In the present study, we have generated mutant mice with deficient LSST and show that inactivation of LSST gene alone leads to only partial impairment of lymphocyte homing to peripheral lymph nodes and moderate reduction in lymphocyte counts in the peripheral lymph nodes, despite the fact that L-selectin ligands that contain 6-sulfo sLeα are reduced at HEV. By contrast, LSST/Core2GlcNAcT double null mice exhibited a markedly reduced lymphocyte homing and reduced lymphocyte counts as a result of significantly decreased 6-sulfo sLeα on HEV L-selectin counterreceptors, relative to LSST- or Core2GlcNAcT-single null mice. Moreover, induction of LSST and Core2GlcNAcT transcripts was observed in HEV-like structure formed in the salivary gland of the non-obese diabetic mouse, which displays chronic inflammation. These results indicate that LSST and Core2GlcNAcT cooperatively synthesize HEV-specific L-selectin ligands required for lymphocyte homing and suggest that LSST and Core2GlcNAcT play a critical role in lymphocyte trafficking during chronic inflammation.

Lymphocyte recirculation through lymph nodes and Peyer’s patches is important for detection of foreign antigens by the immune system and subsequent processes that neutralize these molecules. Lymphocyte recirculation critically depends on interaction between the leukocyte adhesion molecule L-selectin and counterreceptors restricted on specialized postcapillary venules in secondary lymphoid organs. The counterreceptors on the luminal surface of HEV capture circulating lymphocytes via L-selectin-dependent adhesive interactions that lead, in turn, to lymphocyte tethering and rolling, chemokine-dependent activation, integrin-mediated firm attachment, and lymphocyte transmigration (1–4). L-selectin and its ligands are also implicated in lymphocyte recruitment in certain chronic inflammation. HEV-like microvasculature is induced on endothelium in association with insulitis characteristic of the non-obese diabetic (NOD) mouse and the rejection of heart transplants in rodents and humans (5–8). Similarly, HEV-like structure is observed in inflammatory bowel diseases, rheumatoid arthritis, lymphocytic thyroiditis, and the hyperplastic thymus of the AKR mouse (9–12). It has been suggested that recruitment of lymphocytes by induced L-selectin ligand may contribute to the pathogenesis of these diseases, which is characteristic of induced HEV-like microvasculature.

L-selectin present on leukocytes is a carbohydrate-binding protein characterized by dependence on Ca** for its activity. HEV-borne L-selectin counterreceptors include GlyCAM-1, CD34, podocalyxin, Sgp200, endoglycan, and MadCAM-1, all of which have muin-like domains that act as scaffolding for O-linked oligosaccharides (13). The function of these L-selectin counterreceptors entirely depends on their decoration with specific sialylated, fucosylated, and sulfated oligosaccharides, which contain 6-sulfo sialyl Lewis x (sLeα), NeuNAcα2→3Galβ1→4(sulfo−6)GlcNAcβ1→3Galβ1→3GlcNAcβ1→R (14–17). Indeed, recent studies demonstrate that mouse and human L-selectin ligand sulfotransferase (LSST), also known as HECL-GlcNAc6ST or GlcNAc6ST-2 (18) is capable of forming 6-sulfo sLeα on core 2-branched O-glycans (16, 19). More recent studies demonstrated that LSST together with core 1 extension enzyme (Core1-β1GlcNAcT) forms the MECA-79 epitope described as Galβ1→4(sulfo−6)GlcNAcβ1→3Galβ1→3GlcNAcβ1→R, which is a partial structure of 6-sulfo sLeα on extended core 1 O-glycans (20). The MECA-79 antibody also binds to 6-sulfo sLeα on extended core 1 and inhibits both in

1 The abbreviations used are: HEV, high endothelial venule; LSST, L-selectin ligand sulfotransferase; Core2GlcNAcT, β1,6-N-acetylglicosaminytransferase-1; Core1-β1GlcNAcT, core 1 extension β1,3-N-acetylglicosaminytransferase; sLeα, sialyl Lewis x; NOD, non-obese diabetic; EGF, enhanced green fluorescent protein; CMFDA, 5-chloromethyl fluorescein diacetate; GlcNAc6ST-1, GlcNAc-6-sulfotransferase-1; HPLC, high performance liquid chromatography; ES cells, embryonic stem cells.

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vivo and ex vivo lymphocyte attachment to HEV by neutralizing L-selectin ligands (20, 21). Significantly, 6-sulfo sLe^a on either extended core 1 (20)- or core 2-branched O-glycans provides greater L-selectin-dependent cell adhesion under shear force than does 6-sulfo sLe^a on N-glycans (16, 20, 22). Moreover, 6-sulfo sLe^a on bi-antennary O-glycans containing both core 2 branch and core 1 extension yield more efficient L-selectin-dependent cell adhesion than 6-sulfo sLe^a on core 2 branch or core 1 extension alone, indicating a synergistic effect of bivalent ligands on L-selectin-mediated adhesion (20).

Gene inactivation of LSST through homologous recombination results in significant reduction in lymphocyte homing and loss of MECA-79 at the luminal side of HEV. LSST null (LSST^a) mice, however, still exhibit slightly over 50% of lymphocyte homing to the peripheral lymph nodes relative to wild-type mice (23; our results shown below). These observations thus indicate that 6-sulfo sLe^a on extended core 1 O-glycans is almost absent in LSST null mice, but 6-sulfo sLe^a on other structures remaining after inactivation of LSST must play a role as L-selectin ligands. One of those ligands is likely to be 6-sulfo sLe^a on core 2-branched O-glycans (16, 20). The formation of 6-sulfo sLe^a on core 2-branched O-glycans requires core 2 β1,6-N-acetylglucosaminyltransferase-I (Core2GlcNAcT) (24). In Core2GlcNAcT null (Core2GlcNAcT^-/-) mice previously published, L-, P-, or E-selectin ligand on neutrophils was significantly diminished. Concomitantly, neutrophil recruitment to inflamed peritoneum in Core2GlcNAcT^-/- mice was diminished to 20% of levels seen in wild-type mice (25). However, the same studies showed that Core2GlcNAcT^-/- mice exhibited only partial reduction in lymphocyte homing and lymphocyte counts in the peripheral lymph nodes. Analysis of O-glycans remaining after Core2GlcNAcT inactivation demonstrated that no core 2-branched O-glycans was detected in GlyCAM-1 isolated from HEV and that the majority of O-glycans contains core 1 extension in HEV of Core2GlcNAcT^-/- mice (20). These results suggest that almost all of the 6-sulfo sLe^a on L-selectin counterreceptors reside either on core 2-branch or extended core 1 O-glycans. However, precise structures of these O-glycans and their roles in lymphocyte homing have remained undefined.

To determine the roles of LSST and Core2GlcNAcT in L-selectin ligand biosynthesis at HEV, we first generated mutant mice deficient in LSST and then generated double knockout mice deficient in both LSST and Core2GlcNAcT by cross-breeding LSST^-/- mice with Core2GlcNAcT^-/- mice, which had been previously established (25). Double knockout mice deficient in both LSST and Core2GlcNAcT exhibited significantly reduced lymphocyte homing activity and lymphocyte counts in the peripheral lymph nodes, compared with single knockout LSST^-/- or Core2GlcNAcT^-/- mice, or to wild-type mice. In addition, LSST and Core2GlcNAcT transcripts were detected in HEV-like microvascular endothelium formed in the salivary gland of NOD mice. These results together with structural analysis of HEV-derived O-glycans ligands demonstrate that LSST and Core2GlcNAcT cooperatively synthesize L-selectin ligands and together play dominant roles in lymphocyte homing and suggest that LSST and Core2GlcNAcT play a critical role in lymphocyte trafficking during chronic inflammation.

EXPERIMENTAL PROCEDURES

Generation of Targeted ES Cells—A genomic fragment of ~25 kb containing mouse LSST gene was isolated from a mouse genomic library as a BAC clone (Research Genetics) and subcloned into pBluescript II SK- (Stratagene). To disrupt the enzymatic function of mouse LSST, and to create a fusion protein of enhanced green fluorescent protein (EGFP) with the N terminus of LSST, including the short cytoplasmic domain, transmembrane domain, and stem region, a 1-kb NdeI and BglII segment of the LSST gene encoding the catalytic domain was replaced with a 2-kb fragment carrying EGFP-F and the G418 resistance selection (PGKneo) cassette. The inserted EGFP-F segment carrying EGFP coding sequences plus the Rho farnesylation signal, and an SV40 polyA signal was prepared by amplification of pEGFP-F vector (Clontech), in which the multicloning sites had been deleted by PCR using 5'-TCATATGTCGCTCAACAGACCTTATGTTGACAGCCAGGCGAGGAGG-3' (5'-primer) and 5'-CGGGATCCGCTTAAAGATTACCATGTAGGTCTG-3' (3'-primer). The EGFP-F and PGKneo cassette was flanked by 1 kb of mouse genomic DNA at its 5' region and by 4.5 kb of mouse genomic DNA at its 3' region. The targeting vector was linearized and electroporated into R1 ES cells. ES cells were positively selected for G418 resistance and negatively selected by expression of the diphtheria toxin gene, inserted into the targeting vector. Targeting events were identified by PCR amplification with EGFP-specific primer and MECA-79-specific primer (21). LSST sequence was found in the LSST sequence contained in the targeting vector (see Fig. 1A). ES cells positively screened by PCR were confirmed by Southern blot analysis using XbaI digestion and the genomic probe A in the 5' region of homologous recombination (20 kb WT, 10.5 kb mutant) and EcoRI digestion and genomic probe B in the 3' arm of the LSST genome using the targeting vector (30 kb WT, 5.5 kb mutant).

Generation of LSST^a Mice and LSST^-/-/Core2GlcNAcT^-/- Mice—Chimeric males were produced by injection of LSST^-/- ES cells into blastocysts and were bred with C57BL/6 females to produce F1 heterozygotes. Germ line transmission was confirmed by PCR, and LSST^-/- progeny were backcrossed onto C57BL/6 wild-type mice. Heterozygous males and females were mated to produce wild-type, heterozygous, and homozygous mutant animals. For PCR analysis to genotype the mice, genomic DNA was purified from mouse tail and used as template. F1M and R1M primer pairs were used for detection of the mutated allele. For detection of the wild-type allele, F1W and R1W were used (see Fig. 1A). In some analyses, the following PCR primers were used for simultaneous detection of wild-type and mutant alleles in the same PCR reaction: 5'-AAGAAGGAGCTGCTGTAGTCTC-3' (2W) was used as the 5'-primer and 5'-TCCCACTATCAAAAGGCTGCTGA-3' (2R2W) and R1M as 3'-primers. LSST^-/- mice were back-crossed with C57BL/6 for a total of three generations. C57BL/6 Core2GlcNAcT^-/- and C57BL/6 LSST^-/- mice were then bred to generate a C57BL/6 LSST^-/-/Core2GlcNAcT^-/- strain.

Detection of LSST-GFP Chimeric Protein and MECA-79 Antibody and Binding of L-selectin-IgM Chimeric Protein to HEV—The extracellular domain of human L-selectin was amplified by PCR and linked to the DNA sequence encoding for the CH2, CH3, and CH4 domains of human IgM in pcDNA1.1 as described for the mouse L-selectin IgM chimera (21). Binding of L-selectin-IgM was detected by goat biotinylated anti-human IgM antibodies followed by Texas red-conjugated avidin. MECA-79 antigen was detected using MECA-79 antibody (BD Pharmingen (21)), followed by rhodamine-conjugated goat anti-rat IgM. The expression of LSST in HEV was detected by fluorescence derived from LSST-GFP chimeric protein. Preparation of GlyCAM-1—GlyCAM-1 was prepared from sera of wild-type and LSST^-/- mice as described previously (27). The samples were subjected to SDS-PAGE, blotted to a nitrocellulose membrane and reacted with anti-GlyCAM-1 antibodies that had been prepared as described previously (27), or MECA-79 antibody followed by goat anti-rabbit immunoglobulins and goat anti-rat IgM antibodies, respectively. The bound antibodies were visualized by ECL (Amersham Biosciences).

Structural Analysis of Oligosaccharides Attached to GlyCAM-1—Axillary, cervical, and mesenteric lymph nodes from wild-type, LSST^a, Core2GlcNAcT^a, or LSST^-/-/Core2GlcNAcT^-/- mice were metabolically labeled with 0.5 mM [3H]galactose (20). GlyCAM-1 was purified from the condition medium using a column of anti-GlyCAM-1 antibodies attached to UltraLink Biosupport Medium (Pierce), as described previously (27).

O-Glycans from the purified GlyCAM-1 were isolated and separated by QAE-Sephadex A-25 column chromatography in 10 mM pyridine-acetate buffer (pH 5.5) before and after removal of sialic acid by mild acid hydrolysis as described previously (20, 28). Monosulfated, disulfated, and trisulfated O-glycans, eluted with 30 mM (plus 300 mM NaCl), and 250 mM (plus 300 mM NaCl), respectively, were separately applied to a Bio-Gel P-4 gel filtration column as described previously (16, 20).

Fractionated O-glycans were then sequentially treated with α1,3-fucosidase and Jack bean β-galactosidase followed by β-N-acetylgalactosaminidase B, which did not cleave non-sulfated N-acetylgalactosamine. After these digestions, the majority of oligosaccharides should yield (sulfo-6)GlcNAcβ1-6Galβ1-3GalNAcOH from sulfated core 2 branch O-glycans and (sulfo-6)GlcNAcβ1-3Galβ1-3GalNAcOH from sulfated, extended core 1 O-glycans, respectively. These two com-
phocytes were initially introduced into the flow chamber at a wall shear
Western blotting analysis using the anti-GlyCAM-1 antibodies. Lym-
adjusted to provide equivalent amounts of GlyCAM-1 as assessed by
WT
wild-type (WT) and LSST mutant mice. Using PCR primer sets of F1W/R1W and F1M/R1M (shown in
DF-A
the cytoplasmic, transmembrane, and stem regions of LSST fused with EGFP under control of the LSST promoter. Diphtheria toxin (DT-A) was
Core1-
were also digested by hexosaminidase A, which cleaves both non-sul-
–388)
N-acetylglucosamine (30). Standard oligosaccharides
Core2GlcNAcT
were also separated by SDS-polyacrylamide gel electrophoresis, and subjected to Western analysis using anti-GlyCAM-1 antibodies or the MECA-79
Core2GlcNAcT
from Wild-type, LSST
H9004
Mice —
lysins of 7- to 8-week-old wild-type, LSST
H9004
, and LSST
H9004
Core2GlcNAcT
, and LSST
H9004
Core2GlcNAcT
mice. After 1 h, animals were sacrificed, and peripheral
GlyCAM-1
samples were carried out with the MECA-79 monoclonal antibody (left panel). The right panel shows the ratio of MECA-79 over GlyCAM-1, obtained by densitometric analysis of the left panel.

Lympocyte Homing—Lympocyte homing in vivo was assayed as described previously (28, 31). Briefly, 2.5 × 10
5
chloromethyl fluores-
–3GlcNAcT or Core2GlcNAcT-I or both.

Measurement of L-selectin-mediated Rolling on GlyCAM-1 Samples from Wild-type, LSST
H9004
, Core2GlcNAcT
, and LSST
H9004
Core2GlcNAcT
Mice—GlyCAM-1 was prepared from sera of wild-type, LSST
H9004
, Core2GlcNAcT
, and LSST
H9004
Core2GlcNAcT
mice, as described above, and captured on polystyrene plates, which had been coated with anti-
GlyCAM-1 antibodies (20, 31). The amount of GlyCAM-1 from wild-
type, LSST
H9004
Core2GlcNAcT
, and LSST
H9004
Core2GlcNAcT
mice was adjusted to provide equivalent amounts of GlyCAM-1 as assessed by Western blotting analysis using the anti-GlyCAM-1 antibodies. Lympocytes were initially introduced into the flow chamber at a wall shear
stress of 5 dynes/cm
2
for 15 s, followed by the termination of flow, allowing cells to adhere under static conditions. Flow rate was then reinitiated at various shear forces. Image analysis was performed and analyzed as described (20, 32). At the same time, rolling velocities for individual cells (between 120 and 200 rolling cells per experiment) were determined for the results obtained on GlyCAM-1 isolated from wild-type and LSST
H9004
mice as described previously (16).

In Situ Hybridization and Immunohistochemistry—The immunohisto-
Core1-
–3GlcNAcT, Core2GlcNAcT, LSST, and FucT-VII were
Core2GlcNAcT
mice as described previously (16, 20). Digoxigenin-labeled antisense and
sense RNA probes were prepared by in vitro transcription from pGEM-
3Zf (+) (Promega) containing a partial cDNA sequence of mouse Core1-
β3GlcNAcT (nucleotides +705 to +854), Core2GlcNAcT (nucleotides +991 to +1140), LSST (nucleotides +556 to +744), or FucT-VII (nucleotides +2196 to +2497). Hybridized probes were detected by alkaline phosphatase-conjugated anti-digoxigenin antibody, and no specific signals were found in control experiments using sense probes.

Immunohistochemistry with the MECA-79 antibody was performed by the indirect immunoperoxidase method. Control experiments were
done by omitting primary antibody from the procedure, and in this case no specific staining was seen. Counterstaining was carried out with
hematoxylin.

RESULTS

Disruption of the Mouse LSST Locus—The gene encoding LSST was incorporated into a targeting vector by replacing a portion of the LSST gene with cDNAs encoding EGFP and the neomycin resistance enzyme. After homologous recombination, the resultant gene encodes the cytoplasmic, transmembrane, and stem regions of LSST (amino acid residues 1–32), which are fused with EGFP and driven by the LSST promoter (Fig. 1A). It
has been shown that the cytoplasmic, transmembrane, and stem regions of Golgi-associated enzymes specify Golgi retention (33, 34), and thus the fused protein is expected to be transported to the same Golgi compartment as the intact LSST.

Targeted ES cell clones were injected into C57BL/6 blastocysts, and F1 crosses of chimeric mice produced LSST/H9004 as determined by PCR of genomic DNA (Fig. 1B). Intercrosses of heterozygous progeny yielded litters of normal size with Mendelian transmission of the null allele.

L-selectin Ligand Expression in HEV of LSST/H9004 Mice—LSST/H9004 mice did not show a detectable anomaly in the abundance or anatomy of HEV in the secondary lymphoid organs, compared with genetically matched wild-type mice. However, the binding of L-selectin-IgM chimeric protein was almost entirely lost on the luminal side of HEV derived from peripheral and mesenteric lymph nodes under the conditions where strong binding was observed in HEV of wild-type mice (Fig. 1, C and F). Only a residual binding was detected on the intraluminal surface of the bottom layer of high endothelial cells in LSST/H9004 mouse HEV (Fig. 1F). However, a more prominent binding of L-selectin-IgM chimeric protein persisted at the bottom layer of high endothelial cells in HEV even after inactivation of LSST (Fig. 1, F–H). This layer is called the abluminal lining hereafter. The abluminal expression of L-selectin ligand is also present on Peyer’s patches of wild-type mice (Fig. 1E). Consistent with these findings, MECA-79 antigen is drastically reduced in GlyCAM-1 isolated from LSST/H9004 mice, and only 34% of MECA-79 antigen was detected compared with GlyCAM-1 from wild-type mice (Fig. 1I). These results combined strongly suggest that probably the majority of 6-sulfo sLex on the luminal region of HEV is dependent on LSST, whereas the majority of L-selectin ligand, including 6-sulfo sLe^x in the abluminal lining of lymph nodes and Peyer’s patches, is synthesized by a sulfotransferase other than LSST.

GlyCAM-1 Derived from LSST/H9004 Mice Still Contains Significant Amounts of 6-Sulfo sLe^x—The above results suggest that levels of L-selectin ligand in peripheral and mesenteric lymph nodes should be significantly reduced, and only a small fraction of GlyCAM-1 O-glycans should contain 6-sulfo sLe^x. Because no structural information was available on GlyCAM-1 from LSST/H9004 mice in the previous report (23), we analyzed O-glycans attached to GlyCAM-1 isolated from peripheral and mesenteric lymph nodes cultured in the presence of [3H]galactose as described previously (20). Mucin-type O-glycans prepared from the GlyCAM-1 samples of wild-type mice contained a significant amount of sulfated O-glycans, which were bound and eluted from a QAE-Sephadex column, whereas appreciable loss of sulfated O-glycans was noted for GlyCAM-1 from LSST/H9004 mice (Fig. 2B).

These oligosaccharides were further analyzed using gel filtration and HPLC as described previously (16, 20). 6-Sulfo sLe^x was reduced in all of GlyCAM-1 O-glycans isolated from LSST/H9004 mice than wild-type mice (Fig. 2C). In particular, 6-sulfo sLe^x in extended core 1 O-glycans (shown in orange in Fig. 3)
Loss of L-selectin Ligands and 6-Sulfo sLe\(^\text{x}\) in Double Knockout Mice Deficient in Both LSST and Core2GlcNAcT—To cross-breed the mice, both LSST null mice and Core2GlcNAcT null mice were separately back-crossed with C57BL/6. Cross-breed of C57BL/6-LSST\(^{-/-}\) and C57BL/6-Core2GlcNAcT\(^{-/-}\) produced LSST\(^{-/-}\)/Core2GlcNAcT\(^{-/-}\) mice with Mendelian transmission of the null allele (Fig. 4A). Double knockout mice exhibited no anomalies in gross morphology, were fertile, and were not susceptible to endogenous microbes in a regular pathogen-free environment.

Examination of lymph nodes showed that binding of L-selectin-IgM chimera to HEV was decreased in Core2GlcNAcT\(^{-/-}\) mice, whereas no appreciable loss of MECA-79 antigen was observed (Fig. 4B). On the other hand, LSST\(^{-/-}\)/Core2GlcNAcT\(^{-/-}\) mice exhibited substantially decreased binding of L-selectin-IgM chimera to HEV and negligible amount of MECA-79 antigen at the luminal side of HEV (Fig. 4B). This loss in L-selectin ligand and MECA-79 antigen at the luminal side of the lymph nodes was associated with expression of LSST-GFP protein. These results establish that LSST and Core2GlcNAcT are primarily responsible for L-selectin ligand expression on the luminal side of HEV.

Structural analysis of GlyCAM-1 was carried out to define the chemical nature of the sulfated oligosaccharides. Core2GlcNAcT\(^{-/-}\) mice contained much less sulfated O-glycans in GlyCAM-1, which were retained and eluted from a QAE-Sephadex column, than wild-type mice (Fig. 2B). Furthermore, a dramatic reduction in sulfated O-glycans was observed for GlyCAM-1 derived from LSST\(^{-/-}\)/Core2GlcNAcT\(^{-/-}\) mice. The sulfated oligosaccharide fractions isolated by QAE-Sephadex column chromatography were then subjected to Bio-Gel P-4 gel filtration, sequentially digested with specific glycosidases, and analyzed by Bio-Gel P-4 gel filtration and HPLC using an NH\(_2\)-bonded column as described in previous studies (16, 20). These structural analyses of O-glycans derived from wild-type, LSST\(^{-/-}\), Core2GlcNAcT\(^{-/-}\), and LSST\(^{-/-}\)/Core2GlcNAcT\(^{-/-}\) mice revealed the oligosaccharide structures shown in Fig. 5A. In GlyCAM-1 from wild-type mice, the majority of 6-sulfo sLe\(^\text{x}\) is contained in core 2-branched O-glycans (Fig. 5A, Structure 1), extended core 1 O-glycans (Structure 2), monosulfated bi-antennary O-glycans (Structure 3 and 4), and disulfated bi-antennary O-glycans (Structure 5) (Fig. 5A). In GlyCAM-1 from LSST\(^{-/-}\) mice, MECA-79-positive Structure 2 is dramatically decreased and MECA-79-positive Structures 4 and 5 are also decreased. These results are consistent with the above findings that LSST\(^{-/-}\) mice express a negligible amount of luminal MECA-79 antigen. In GlyCAM-1 derived from Core2GlcNAcT\(^{-/-}\) mice, Structures 1, 3, 4, and 5 containing core 2 branch are absent, whereas MECA-79-positive Structure 2, which contains core 1 extension alone, increases (Fig. 5, A and B).

In GlyCAM-1 derived from LSST\(^{-/-}\)/Core2GlcNAcT\(^{-/-}\) double null mice, the amount of oligosaccharides containing 6-sulfo sLe\(^\text{x}\) in extended core 1 (Structures 3–5) is further decreased and only a small amount of Structure 2 remains, when intraluminal MECA-79 antigen is almost absent (Fig. 4B). These results suggest that the majority of MECA-79 antigen in wild-type mice is carried by Structures 3–5. At the same time, the amount of non-sulfated sLe\(^\text{x}\) in LSST\(^{-/-}\)/Core2GlcNAcT\(^{-/-}\) mice is increased to 18.7% of total O-glycans compared from 11% of those in Core2GlcNAcT\(^{-/-}\) mice (data not shown). These results indicate that the loss of 6-sulfo sLe\(^\text{x}\) is compensated for by the increase of non-sulfated sLe\(^\text{x}\) in LSST\(^{-/-}\)/Core2GlcNAcT\(^{-/-}\) mice. These results also indicate that LSST is mainly responsible for 6-sulfation at extended core 1 O-glycans, whereas a sulfotransferase other than LSST contributes to 6-sulfation at core 2-branch in addition to LSST.

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**Fig. 3. Structure and biosynthesis of L-selectin ligand oligosaccharides.** Core 1 O-glycan can be extended by Core1-β3GlcNAcT, sulfated by LSST, then galactosylated to form the minimum epitope for the MECA-79 antibody (shown in the orange circle). The galactosylated oligosaccharide is further sialylated and fucosylated to form 6-sulfo sLe\(^\text{x}\) in extended core 1 (Core 2-branch). Core 1 oligosaccharide can be converted to core 2 O-glycan by Core2GlcNAcT, sulfated by LSST, galactosylated, sialylated, and fucosylated to form 6-sulfo sLe\(^\text{x}\) in core 2-branched O-glycans (shown in the magenta circle at left). Core 1-β3GlcNAcT can act on core 2-branched O-glycans, leading to biantennary O-glycans containing both core 2 branch and core 1 extension. By contrast, Core2GlcNAcT cannot act on extended core 1 O-glycans (20). One or both of these branches can be modified to contain 6-sulfo sLe\(^\text{x}\) (middle). All of these processed O-glycans function as L-selectin ligands. These schematic structures correspond to Structure 1 (left), Structure 2 (right), and Structures 3–5 (middle) shown in Fig. 5. In addition to LSST, GlcNAcSST1 likely forms 6-sulfo N-acetylglucosamine in HEV. After inactivation of LSST (−LSST), the majority of 6-sulfate in extended core 1 is abolished. After further inactivation of Core1-β3GlcNAcT (−Core2GlcNAcT), the majority of O-glycans is either core 1 O-glycans (bottom left) or extended core 1 O-glycans containing sLe\(^\text{x}\) (bottom right). β4GalIT, β1,4-galactosyltransferase (52); ST3Gal, α2,3-sialyltransferase (53); FucT-VII, fucosyltransferase VII.

Almost absent (see after inactivation of LSST, −LSST in Fig. 3). This finding is consistent with the above finding that the MECA-79 epitope was dramatically diminished in GlyCAM-1 from LSST\(^{-/-}\) mice. Most of the remaining 6-sulfo sLe\(^\text{x}\) on mucin-type O-glycans in HEV was found in core 2-branch (shown in magenta in Fig. 3, see below for detailed structural analysis). Core 2 branch in HEV is synthesized exclusively by Core2GlcNAcT (20). These results thus prompted us to generate double knockout mice deficient in both LSST and Core2GlcNAcT to determine the in vivo roles of 6-sulfo sLe\(^\text{x}\) as L-selectin ligands.
Defective Lymphocyte Homing in LSST\(^{Δ}/Core2GlcNAcT\(^{Δ}\) Mice—To determine the role of LSST and Core2GlcNAcT in L-selectin ligand activity at HEV, a lymphocyte homing assay was carried out on wild-type, LSST\(^{Δ}\), Core2GlcNAcT\(^{Δ}\), or LSST\(^{Δ}/Core2GlcNAcT\(^{Δ}\) mice. Homing of wild-type lymphocytes to the peripheral and mesenteric lymph nodes of LSST\(^{Δ}\) or Core2GlcNAcT\(^{Δ}\)-recipient mice was decreased to 55 and 85%, respectively, relative to homing in wild-type-recipient mice (Fig. 6A). The number of lymphocytes in the peripheral lymph nodes was decreased to 60% in LSST\(^{Δ}\) mice and 78% in Core2GlcNAcT\(^{Δ}\) mice (Fig. 6B). By inactivating both LSST and Core2GlcNAcT, a dramatic decrease in lymphocyte homing to peripheral lymph nodes and mesenteric lymph nodes was observed (Fig. 6A). The remaining lymphocyte homing activity in the peripheral lymph nodes of LSST\(^{Δ}/Core2GlcNAcT\(^{Δ}\) mice still depended on L-selectin-mediated adhesion, because anti-L-selectin antibody treatment of lymphocytes completely abolished lymphocyte homing (Fig. 6A). This complete inhibition is equivalent to that observed in mice deficient in L-selectin (35) or mice deficient in both FucT-VII and FucT-IV (31). Lymphocyte numbers in the peripheral lymph nodes of double knockout mice were decreased to 40% relative to that of wild-type mice, whereas only a small decrease was observed in lymphocyte numbers in mesenteric lymph nodes of the same mutant mice (Fig. 6B). These results in combination suggest that the efficiency of lymphocyte homing is in proportion to the amount of 6-sulfo sLe\(^{α}\), regardless of whether 6-sulfo sLe\(^{α}\) is present on extended core 1 or core 2 branch structure.

Loss of 6-Sulfo sLe\(^{α}\) Leads to Increase in the Velocity of Rolling Lymphocytes—To further determine the roles of L-selectin ligands directed by LSST and Core2GlcNAcT, wild-type lymphocytes were rolled over plates that had been coated with GlyCAM-1 prepared from the sera of wild-type, LSST\(^{Δ}\), Core2GlcNAcT\(^{Δ}\), and LSST\(^{Δ}/Core2GlcNAcT\(^{Δ}\). Surprisingly, GlyCAM-1 from LSST\(^{Δ}\) mice exhibited only a marginal decrease in L-selectin ligand activity compared with wild-type mice (Fig. 6C). By contrast, rolling on GlyCAM-1 from Core2GlcNAcT\(^{Δ}\) mice was decreased significantly compared with that seen in wild-type GlyCAM-1. GlyCAM-1 from double knockout LSST\(^{Δ}/Core2GlcNAcT\(^{Δ}\) mice supported the least amount of lymphocyte rolling (Fig. 6C).

The above results suggest, however, that the role of 6-sulfo sLe\(^{α}\) was not precisely measured by examining the number of tethering and rolling lymphocytes, because only a marginal decrease was observed on GlyCAM-1 derived from LSST\(^{Δ}\) mice. We thus measured the velocity of rolling lymphocytes, because we previously found that the effect of 6-sulfation on L-selectin ligand is measured best by examining the velocity of rolling lymphocytes (16). GlyCAM-1 from wild-type mice yielded an average velocity of 18 \(\mu\)m/s at a shear stress of 2.32 dyne/cm\(^{2}\), whereas GlyCAM-1 from LSST\(^{Δ}\) yielded 31 \(\mu\)m/s at the same shear stress (Fig. 6D). These results indicate that 6-sulfation on sLe\(^{α}\) dramatically decreases the velocity of rolling lymphocytes. By contrast, the number of rolling lymphocytes on LSST\(^{Δ}\) GlyCAM-1 did not decrease at the same shear force and decreased only by 35\% under weaker shear forces (Fig. 6C). These results suggest that 6-sulfation facilitates lymphocyte homing mainly by decreasing the velocity of rolling lymphocytes.

Coordinate Expression of LSST, Core2GlcNAcT, Core1-β3GlcNAcT, and FucT-VII in HEV-like Microvasculature of NOD Mice—L-selectin ligand-positive HEV is constitutively present in secondary lymphoid organs. By contrast, conversion of flat walled vascular endothelium to a HEV-like morphology is observed in association with certain inflammatory and pre-neoplastic conditions. A well characterized such example, the NOD mouse, displays HEV-like structures in pancreatic islets and inflammatory salivary glands. In these organs, recruitment of lymphocytes through L-selectin ligand leads to β-cell...
destruction and sialoadenitis of salivary glands (5, 6). In situ hybridization shows that LSST and Core2GlcNAcT are transcribed in HEV-like MECA-79-positive structures in salivary glands of NOD mice (Fig. 7). These transcripts colocalize in adjacent sections with transcripts for Core1-
3GlcNAcT, FucT-VII, and Core2GlcNAcT, although the expression level of Core2GlcNAcT transcripts is apparently lower than that of the others (Fig. 7). These results imply that LSST, Core1-
3GlcNAcT, FucT-VII, and to a lesser extent Core2GlcNAcT contribute to induction of L-selectin ligands in HEV-like structures present in inflamed salivary glands of the NOD mouse.

**DISCUSSION**

Intense interest in the analysis of oligosaccharides elaborated by HEV has been shown in an attempt to understand the role of L-selectin-mediated adhesion in lymphocyte homing. Attempts to answer this question by ablating counterreceptors such as GlyCAM-1 and CD34 have not been useful in defining these oligosaccharides due to the number of highly glycosylated HEV proteins, which carry the same functional oligosaccharides (3, 36). By contrast, deletion of FucT-VII or fucosyltransferase IV (FucT-IV) or their double mutant has yielded useful information indicating an absolute requirement of \( 1,3 \)-linked fucose for L-selectin ligand activity (26, 31). To define oligosaccharide elements other than fucose, we have inactivated a key sulfotransferase (LSST) that forms 6-sulfo N-acetylglucosamine in the context of 6-sulfo sLex, a major ligand for L-selectin. LSST\(^\Delta\) mice, however, exhibited substantial residual L-selectin ligand activity at HEV, despite the fact that the LSST\(^\Delta\) mice lost almost all luminal expression of 6-sulfo sLex in extended core 1 \( O \)-glycans. These results agree in principle with the previous report on mutant mice deficient in LSST (23). Considering that 6-sulfo sLex and possibly sLe\(^\alpha\) on core 2-branched \( O \)-glycans most likely contribute to the remaining L-selectin ligand activity at HEV, we have generated LSST\(^\Delta\) /Core2GlcNAcT\(^\Delta\) mice by cross-breeding LSST\(^\Delta\) mice with Core2GlcNAcT\(^\Delta\) mice. These studies reveal that LSST and Core2GlcNAcT, which form 6-sulfo N-acetylglucosamine and core 2-branch, respectively, control L-selectin ligand synthesis in a cooperative manner.

The present study demonstrates that a large portion of L-selectin ligand, 6-sulfo sLe\(^\alpha\) on HEV, is capped on core 2-branched \( O \)-glycans. Deletion of Core2GlcNAcT resulted in a significant loss of lymphocyte homing activity and an appreciable decrease in lymphocyte numbers in peripheral lymph...
nodes. These results are, however, slightly different from those obtained in the previous report (25). In the present study, we made efforts to obtain a similar genetic background for LSST\(^{a}\) and Core2GlcNAcT\(^{a}\) mice by cross-breeding Core2GlcNAcT\(^{a}\) and LSST\(^{a}\)/H9004 mice with C57BL/6. Those resultant Core2-GlcNAcT\(^{a}\) and LSST\(^{a}\)/H9004 mice were then cross-bred to generate

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**Fig. 6.** Lymphocyte homing and selectin ligand expression in wild-type LSST\(^{a}\), Core2GlcNAcT\(^{a}\), and LSST\(^{a}\)/Core2GlcNAcT\(^{a}\) mice. 

A, CMFDA-labeled lymphocytes from mesenteric lymph nodes and spleens of wild-type mice were injected into the tail vein of different mouse strains. Mice were sacrificed 1 h later, and fluorescent lymphocytes in lymphocyte suspensions from lymphoid organs were quantitated by flow cytometry. Residual lymphocyte homing to peripheral lymph nodes in LSST\(^{a}\)/Core2GlcNAcT\(^{a}\) mice was completely abolished by anti-L-selectin antibody (MEL-14) treatment of lymphocytes before the lymphocyte injection. At least four recipient mice were tested for each experiment. B, lymphocytes recovered from different lymphoid organs of 7-week-old mice were quantitated. C and D, lymphocyte rolling using a flow chamber coated with GlyCAM-1 isolated from different strains. The number of adhering lymphocytes is shown for all four mouse strains (C), whereas the average rolling velocity of lymphocytes is shown for wild-type and LSST\(^{a}\)/recipient mice (D). In A–D, two independent experiments yielded almost identical results and results from one experiment are shown.

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**Fig. 7.** Co-localized expression of MECA-79 antigen, LSST, Core2-GlcNAcT, Core1-\(\beta3\)GlcNAcT, and FucT-VII in a HEV-like vessel in the salivary gland of NOD mice. HEV-like vessels were found in the salivary glands of 55-week-old NOD mice, which developed sialoadenitis accompanying marked lymphocyte infiltration. Serial sections of the representative site were subjected to immunochemistry using the MECA-79 antibody and in situ hybridization using antisense probes for mouse LSST, Core2GlcNAcT, FucT-VII, and Core1-\(\beta3\)GlcNAcT. Bar, 10 \(\mu\)m.
LSST^5/Core2GlcNAcT^5 double knockout mice. For lymphocyte homing assay, lymphocytes from mesenteric lymph nodes and spleen were used in the present study, whereas mesenteric lymphocytes were used in the previous study (25). This difference may cause a different homing profile. In this aspect, it is noteworthy that the previous studies showed a decrement in both lymphocyte homing activity and lymphocyte numbers in the secondary lymphoid organs for Core2GlcNAcT null mice, although it was judged to be statistically insignificant (25).

Recently, it has been shown that 6-sulfo sLe^x on bi-antennary O-glycans is a better L-selectin ligand than 6-sulfo sLe^x or O-glycans containing core 1 extension or core 2-branch alone (20). Core2GlcNAcT^5 mice entirely lack core 2-branch in HEV L-selectin ligands and such a synergistic effect cannot be achieved in Core2GlcNAcT^4 mice. This may explain why Core2GlcNAcT^4 mice exhibit a reduced lymphocyte homing activity relative to wild-type mice.

Stamaa et al. (37) reported the presence of 6-sulfo sLe^x on extended core 1 in CD34 isolated from human tonsil. However, the method employed in their study cannot differentiate 6-sulfo sLe^x in extended core 1 O-glycan from 6-sulfo sLe^x in core 2-branched O-glycan, because no tandem mass spectrometry analysis was performed. Further studies are thus necessary to determine if extended core 1 O-glycans (in particular, Structure 4 in Fig. 5) are present with significant quantity in human tonsil HEV.

Although LSST is concluded to play a critical role in the biosynthesis of 6-sulfo sLe^x at the luminal side of HEV, inactivation of LSST did not lead to loss of L-selectin ligand at the abluminal side of HEV. The abaluminal expression of L-selectin ligand and MECA-79 antigen, which constitutes a portion of L-selectin ligand, has been previously reported (21), but has not been studied further. The majority of L-selectin ligand and MECA-79 antigen in the peripheral lymph nodes is strongly expressed on the luminal side of HEV, which has obscured the abaluminal L-selectin ligand of the peripheral lymph nodes. However, the abaluminal side of Peyer’s patches in wild-type mice is also decorated by MECA-79 antigen and L-selectin ligands, which are retained in LSST^4 mice. In Peyer’s patches, it has been shown that L-selectin plays a limited but definite role in the tethering and rolling of lymphocytes, presumably by adhering to L-selectin ligands at the luminal sides of HEV (38, 39). However, this MECA-79-negative L-selectin ligand appears to be relatively weak, because the binding of L-selectin-IgM chimeric protein was not observed at the luminal side of Peyer’s patches in both wild-type and LSST^4 mice (Fig. 1, E and H). Moreover, L-selectin-mediated lymphocyte homing in Peyer’s patches can be compensated with relative ease by other adhesion mechanisms, because anti-L-selectin antibody treatment had a minimum effect on the lymphocyte homing to Peyer’s patches (Fig. 6A). It is not apparent if this limited role of L-selectin ligand activity on the luminal side of Peyer’s patches is supplemented by the presence of abaluminal L-selectin ligands.

Our studies also demonstrate that a small amount of 6-sulfo sLe^x is present on extended core 1 O-glycans attached to GlyCAM-1 isolated from HEV of LSST^5/Core2GlcNAcT^5 mice (Fig. 5B). This finding indicates that there must be another sulfotransferase that adds a 6-sulfate group in the context of extended core 1, thus forming the MECA-79 epitope as well. It is also possible that 6-sulfo sLe^x in the abaluminal side of HEV after inactivation of LSST may contribute to some of the remaining 6-sulfo sLe^x detected on GlyCAM-1 released from HEV. GlcNAc-6-O-sulfotransferase-1 (GlcNAc6ST-1) was shown to form the MECA-79 epitope (40, 41) and thus 6-sulfo sLe^x on extended core 1 O-glycans. Because GlcNAc6ST-1 is also expressed in HEV (16, 42), these combined results strongly suggest that GlcNAc6ST-1 also contributes to formation of 6-sulfo sLe^x on HEV in addition to LSST.

The same results also demonstrate that a significant portion of lymphocyte homing to the peripheral lymph nodes remains after inactivation of both LSST and Core2GlcNAcT. This remaining L-selectin ligand activity is apparently more than that expected from residual 6-sulfo sLe^x present in LSST^5/Core2GlcNAcT^5 mice. As shown recently, non-sulfated sLe^x on extended core 1 O-glycans serves as an L-selectin ligand, although it is not as good a ligand as sLe^x on core 2-branched O-glycans (43–45). Because LSST^5/Core2GlcNAcT^5 mice contain non-sulfated sLe^x in 18.7% of total O-glycans, it is likely that non-sulfated sLe^x on extended core 1 O-glycans contributes to this remaining L-selectin ligand. It is also possible that this remaining L-selectin ligand activity may be derived from L-selectin counterreceptors resistant to O-glycoproteinase (46), which cleaves the majority of glycoproteins that contain mucin type O-glycans. This class of MECA-79-negative L-selectin ligand might include heparan sulfate (47, 48) and may play a role in lymphocyte homing.

The transcripts of Core2GlcNAcT and Core-β3GlcNAcT are expressed in HEV, but they were also detected in wide variety of cells, including colon, lymphocytes, and neutrophils (20, 43, 49, 50). Similarly, the transcripts of Fus-T-VII are expressed in neutrophils, in addition to HEV (43, 51). In salivary glands of NOD mice, LSST, Core2GlcNAcT, Core-β3GlcNAcT, and Fus-T-VII are expressed in HEV-like microvasculature, which is decorated by MECA-79 antigen. Similarly, it was shown that LSST and Fus-T-VII transcripts were expressed in MECA-79-positive HEV-like microvasculature in hyperplastic thymus of the AKR mouse (16). These observations suggest that LSST, Fus-T-VII, and in some instances Core-β3GlcNAcT and Core2GlcNAcT are up-regulated in the formation of HEV and HEV-like structures, and coordinated regulation of these enzymes directs synthesis of L-selectin ligands. The observations on inflamed salivary glands of NOD mice, hyperplastic thymus of AKR mice and HEV in lymph nodes of wild-type mice also suggest that among these enzymes, LSST is most restricted to HEV, and that the up-regulation of LSST may be critical for induction of L-selectin counterreceptors on HEV-like microvasculature in chronic inflammation.

In conclusion, our observations demonstrate that Core2GlcNAcT and HEV-restricted LSST collaboratively control synthesis of L-selectin ligands in HEV. Expression of LSST and Core2GlcNAcT was also observed in HEV-like vasculature formed in inflamed salivary glands of NOD mice. These findings as a whole indicate that LSST and Core2GlcNAcT together provide a major contribution to synthesis of HEV-borne L-selectin ligands, to lymphocyte homing to secondary lymphoid organs, and to lymphocyte trafficking associated with transmigration of flat wall vasculature into HEV-like vessels under pathological conditions.

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Core 2 Branching β1,6-N-Acetylglosaminyltransferase and High Endothelial Venule-restricted Sulfotransferase Collaboratively Control Lymphocyte Homing
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