Detection of a Sulfotransferase (HEC-GlcNAc6ST) in High Endothelial Venules of Lymph Nodes and in High Endothelial Venule-Like Vessels within Ectopic Lymphoid Aggregates

Relationship to the MECA-79 Epitope

Annette Bistrup,* Durwin Tsay,* Priti Shenoy,[†]
Mark S. Singer,* Naveen Bangia,[†]
Sanjiv A. Luther,[‡] Jason G. Cyster,[‡]
Nancy H. Ruddle,[†] and Steven D. Rosen*

From the Departments of Anatomy* and Microbiology and Immunology,[‡] University of California, San Francisco, California; and the Department of Epidemiology and Public Health and Section of Immunobiology,[†] Yale University School of Medicine, New Haven, Connecticut

The interaction of L-selectin on lymphocytes with sulfated ligands on high endothelial venules (HEVs) of lymph nodes results in lymphocyte rolling and is essential for lymphocyte homing. The MECA-79 monoclonal antibody reports HEV-expressed ligands for L-selectin by recognizing a critical sulfation-dependent determinant on these ligands. HEC-GlcNAc6ST, a HEV-localized sulfotransferase, is essential for the elaboration of functional ligands within lymph nodes, as well as the generation of the MECA-79 epitope. Here, we use an antibody against murine HEC-GlcNAc6ST to study its expression in relationship to the MECA-79 epitope. In lymph nodes, the enzyme is expressed in the Golgi apparatus of high endothelial cells, in close correspondence with luminal staining by MECA-79. In lymph node HEVs of HEC-GlcNAc6ST-null mice, luminal staining by MECA-79 is almost abolished, whereas abluminal staining persists although reduced in intensity. HEV-like vessels in several examples of inflammation-associated lymphoid neogenesis, including nonobese diabetic mice, also exhibit concomitant expression of the sulfotransferase and luminal MECA-79 reactivity. The correlation extends to ectopic lymphoid aggregates within the pancreas of RIP-BLC mice, in which CXCL13 is expressed in islets. Analysis of the progeny of RIP-BLC by HEC-GlcNAc6ST-null mice establishes that the enzyme is responsible for the MECA-79 defined luminal ligands. *(Am J Pathol 2004, 164:1635–1644)*

Effective immune surveillance and the development of an immune response depend on the ability of lymphocytes to enter secondary lymphoid organs where foreign antigens are presented.[1] Lymphocytes also perform surveillance and effector functions in extralymphoid tissues. At these sites, excessive recruitment of lymphocytes can result in the development of chronic inflammatory lesions with pathological consequences to the surrounding tissues. In some cases, the inflammatory infiltrates resemble lymphoid organs, and the process is referred to as lymphoid organ neogenesis.[2,3] This ectopic formation of lymphoid structures can occur in several common autoimmune diseases, in response to certain infectious agents and in a variety of lymphomas. Priming of lymphocytes may occur in these organized lymphoid aggregates, which could perpetuate disease in autoimmune settings.

During normal lymphocyte recirculation into lymph nodes, lymphocyte trafficking from the blood occurs across specialized postcapillary venules called high endothelial venules (HEVs), which are distinguished by their plump endothelial cells.[1] Egress is initiated by transient interactions (tethering) between the lymphocytes and high endothelial cells (HECs) lining the blood vessel wall, leading to rolling of the lymphocytes along the vessel.[4] For lymph nodes, the primary adhesion molecule mediating this interaction is L-selectin on lymphocytes. As a C-type lectin, L-selectin interacts with specific carbohydrate-based HEV ligands.[5] In mucosal lymphoid organs such as Peyer's patches (PPs), the integrin $\alpha 4\beta 7$ initiates

rolling of effector or memory lymphocytes, while L-selectin predominates in this function for naïve lymphocytes.[1] The recruitment cascade is completed by the firm adherence and subsequent transmigration of the lymphocytes across the endothelial cell layer into the parenchyma of the organs.[1,4]

The HEV-expressed ligands for L-selectin thus far identified consist of a set of heavily O-glycosylated glycoproteins, which include GlyCAM-1 and CD34 in the mouse and podocalyxin and CD34 in the human.[5] Recognition of these ligands by L-selectin requires sialylation, fucosylation, and sulfation of their mucin-like domains.[6] The minimal recognition epitope appears to be comprised of a capping group known as 6-sulfo sialyl Lewis x (6-sulfo sLex) in which the C-6 position of GlcNAc within sialyl Lewis x (sLex) is esterified with sulfate. The key evidence implicating this structure is based on structural analysis of O-glycans of L-selectin ligands,[7–9] testing of chemically synthesized sulfated oligosaccharides as inhibitors of L-selectin binding[10] and tissue staining with carbohydrate-directed monoclonal antibodies.[11] The most complete structural analysis to date has demonstrated that 6-sulfo sLex can cap both a core 2 branch and an extended core 1 branch of biantennary O-glycans within GlyCAM-1.[8,12]

A parallel approach to defining L-selectin ligands has taken advantage of a monoclonal antibody, MECA-79.[13] This antibody stains HEVs in various secondary lymphoid organs of many species, including mouse and human.[14,15] MECA-79 recognizes a set of sialomucins, including the aforementioned L-selectin ligands, together with another recently identified sialomucin called endomucin.[16] The complex is referred to as peripheral node addressin (PNAd).[13] MECA-79 blocks the attachment of lymphocytes to peripheral lymph node (PN) HEVs *in vitro* and inhibits lymphocyte homing to PNs *in vivo* (mouse).[13] MECA-79 does not, however, inhibit *in vitro* lymphocyte attachment to PP HEVs or *in vivo* homing to this organ. Blocking studies indicate that MECA-79 competes with L-selectin for recognition of the sialomucin ligands.[17,18]

The utility of MECA-79 extends beyond the analysis of normal secondary lymphoid organs. Thus, the organized lymphoid aggregates that form in chronic inflammatory settings generally contain HEV-like vessels that express PNAd and/or MAdCAM-1.[3,19] MECA-79+ vessels, of a high-walled or flat phenotype, are also found in other inflammatory lesions where organized lymphoid structures are not present.[14,20] In several examples of chronic inflammation, the L-selectin/PNAd system has been confirmed to be functionally significant in lymphocyte recruitment.[21–24]

The structural basis of the MECA-79 epitope and its relationship to the L-selectin recognition epitopes has been an issue of considerable interest. The reactivity of MECA-79 with multiple components first suggested that it recognizes a posttranslational modification that is shared by L-selectin ligands. Early studies with the metabolic inhibitor chlorate established that the binding of L-selectin and MECA-79 share a requirement for sulfation.[25] Recently, the minimal MECA-79 epitope was shown to consist of an extended core 1 structure modified with GlcNAc-6-sulfate[8]

Although MECA-79 reactivity is retained in the sialylated and/or fucosylated forms,[8] these modifications are not required for the MECA-79 epitope.[26] Thus, the MECA-79 epitope overlaps with a subregion of the 6-sulfo sLex determinant, thus explaining the function-blocking activity of the antibody.

Recent work from several groups has identified candidate enzymes for the biosynthesis of L-selectin ligands and the MECA-79 epitope in HEVs. Core 2 β1,6-*N*-acetylglucosaminyl-transferase-I (Core2GlcNAcT-I) and core 1 β1,3-*N*-acetylglucosaminyltransferase (Core1-β3GlcNAcT),[8,12,27] are involved in the formation of the core structures of O-glycans within these ligands. Core1-β3GlcNAcT is required for the generation of the MECA-79 epitope.[8,12] The α1,3-fucosyltransferases FucT-VII and to a lesser extent Fuc-TIV are responsible for fucosylation of ligands within lymph node HEVs.[26,28]

With respect to sulfation, one member of the GlcNAc-6-*O*-sulfotransferase (GlcNAc6ST) family, known variously as HEC-GlcNAc6ST, L-selectin ligand sulfotransferase (LSST), GST-3, or GlcNAc6ST-2 (gene name, *CHST4*) has received the most attention.[29–31] Transcripts corresponding to HEC-GlcNAc6ST are abundantly and preferentially expressed in HECs.[29,30] The cDNA encoding HEC-GlcNAc6ST, when co-transfected into CHO cells together with cDNAs encoding Core2GlcNAcT-I, FucT-VII, and CD34 confers L-selectin ligand activity onto these cells as measured in both equilibrium binding and flow chamber assays.[29,30] Moreover, the phenotype of mice genetically deficient in HEC-GlcNAc6ST has clearly established a role for this enzyme in the generation of L-selectin ligands *in vivo*.[32,33] In particular, lymphocyte homing to PNs is reduced by 50% in the HEC-GlcNAc6ST−/− mice compared with wild-type mice. This is reflected in a smaller size of the PN in the −/− mice, and a 60% decrease in the number of total lymphocytes within this organ. The residual homing to PNs in the −/− mice remains L-selectin-dependent, ruling out the induction of alternative, L-selectin-independent homing mechanisms in this lymphoid organ. The deficiency in homing to PNs in the −/− mice is reflected in a dramatic reduction of luminally disposed, MECA-79-reactive ligands for L-selectin.[32] Correspondingly, MECA-79 has no effect on homing to PNs in these mice, and does not perturb rolling on their HEVs.[33] However, MECA-79- and L-selectin-reactive ligands remain on the abluminal aspect of the PN HEVs in these mice. These ligands are also present in the HEVs of MN and PP in wild-type mice and correspond to those described by Streeter and colleagues[13] in PPs in the original characterization of the MECA-79 monoclonal antibody. The presence of HEC-GlcNAc6ST-independent, MECA-79-reactive L-selectin ligands in the lymph nodes and PPs of the HEC-GlcNAc6ST-null mice strongly implicates another GlcNAc-6-*O*-sulfotransferase in the synthesis of these ligands (see Discussion).

To further characterize the role of HEC-GlcNAc6ST, we have developed an antibody directed against a peptide predicted from its amino acid sequence. A preliminary description of this antibody has been presented by one of us in an analysis of LTβ-deficient and RIP-LTαβ trans-

genic mice.[19] In the current study, we exploit this antibody as a staining reagent to gain information on the relationship between expression of the HEC-GlcNAc6ST protein and the expression pattern of MECA-79-reactive ligands. We investigate HEVs in both secondary lymphoid organs and HEV-like vessels in several examples of ectopic lymphoid aggregates.

Materials and Methods

Mice

Mice genetically deficient in HEC-GlcNAc6ST have been described.[32] Null mice and wild-type controls used for immunofluorescence were 12- to 16-week-old males. Mice expressing B-lymphocyte chemokine (BLC) under the rat insulin promoter (RIP-BLC) have been described.[34] To generate RIP-BLC mice deficient in HEC-GlcNAc6ST, we bred HEC-GlcNAc6ST−/− mice to RIP-BLC+/− mice. The genotype of the resulting offspring was determined by polymerase chain reaction using the following primer sets: HEC-GlcNAc6ST; forward primer-1 5′-ttttacattgctccttggat-gggaatc-3′, forward primer-2 5′-gacatagcgttggctacccgt-gatatt-3′, reverse primer 5′-aggctgtgctgctggtgaaagtcat-3′; RIP-BLC; forward primer 5′-caacccctgactatcttccag-3′, reverse primer 5′-gagatgatagtggcttcaggcag-3′. The resulting HEC-GlcNAc6ST+/−/RIP-BLC+/− mice were bred to HEC-GlcNAc6ST−/− mice to generate HEC-GlcNAc6ST−/−/RIP-BLC+/−, which were subjected to the phenotypic analysis described in the present study. Sections of thymus from AKR/J mice, 19 weeks old (female) and from salivary glands (female) and lacrimal glands (male) of nonobese diabetic (NOD) mice, 30 weeks old (prediabetic) were provided by Sara Michie, Department of Pathology, Stanford University. Jeffrey Bluestone of University of California at San Francisco provided the pancreas from a diabetic 12-week-old NOD mouse (female) that had been treated with cyclophosphamide at 8 weeks. A 10-week-old Sprague-Dawley rat (female) was used for the Golgi localization experiment.

Generation of a HEC-GlcNAc6ST Antibody

Polyclonal rabbit anti-serum directed against murine HEC-GlcNAc6ST was prepared as described.[19] Briefly, rabbits were immunized with KLH-conjugates of three different peptides derived from the primary amino acid sequence of murine HEC-GlcNAc6ST (peptide 1, CHMS-VHRHLSQREESRR; peptide 2, KIICKSQVDIVKAIQTLPE; and peptide 3, RGKGMGQHAFHTNC). The resulting sera were tested for HEC-GlcNAc6ST reactivity by immunofluorescence staining of cryostat-cut sections of mouse PN, prepared as described below. The sera were diluted to 0.5 μg/ml in blocking solution [phosphate-buffered saline (PBS) containing 3% bovine serum albumin and 5% mouse serum] and applied to sections in the presence or absence of 10 μg/ml of each peptide. The desired anti-peptide antibody was purified by affinity chromatography on a column to which peptide 3 was coupled. The C-terminus of this peptide contains a cysteine residue that was bound to iodoacetyl-substituted agarose using the

SulfoLink kit (Pierce Chemical Co., Rockford, IL). The purified antibody was used in subsequent immunofluorescence studies. Comparison of the sequence of peptide 3 within HEC-GlcNAc6ST with corresponding regions of GlcNAc6ST-1, GlcNAc6ST-3, and GlcNAc6ST-4 revealed 23%, 40%, and 23% sequence identity, respectively.

Immunofluorescence

Tissues were dissected and immediately frozen in OCT (Tissue Tek). Ten-μm sections were prepared on poly-L-lysine-coated glass slides and allowed to dry for 1 hour. They were fixed in ice-cold acetone for 5 minutes followed by air-drying for 30 minutes. Sections were blocked 30 minutes in blocking solution: PBS containing 3% bovine serum albumin and 5% serum (mouse serum for mouse sections, rat serum for rat sections). All staining steps were performed in blocking solution. Primary antibodies were incubated on slides for 1 hour at room temperature and used at the following concentrations: purified rabbit anti-murine HEC-GlcNAc6ST (0.25 μg/ml), MECA-79 (1.0 μg/ml), MECA-367 (2.0 μg/ml, a gift from Dr. Eugene Butcher, Department of Pathology, Stanford University), and GM-130 (1.25 μg/ml; BD Biosciences). Anti-HEC-GlcNAc6ST was detected by biotinylated goat anti-rabbit IgG (1.3 μg/ml; Jackson Immuno-Research, West Grove, PA) followed by Cy2-conjugated streptavidin (1.8 μg/ml; Jackson Immuno-Research, Bar Harbor, ME). MECA-79 was detected by Cy3-conjugated goat anti-rat IgM (1.5 μg/ml, Jackson Immuno-Research). MECA-367 was detected by amino-methylcoumarin-conjugated goat anti-rat IgG (15 μg/ml, Jackson Immuno-Research). GM-130 was detected by Cy3-conjugated goat anti-mouse IgG (1.3 μg/ml, Jackson Immuno-Research). Sections were counterstained with Harris hematoxylin (Sigma, St. Louis, MO) and mounted with FluorSave Reagent (Calbiochem, La Jolla, CA).

In Vivo/ex Vivo Immunofluorescence

Unlabeled MECA-79 (50 μg per mouse in 100 μl of PBS) was injected into the tail vein of mice and allowed to circulate for 30 minutes. Mice were killed and frozen sections were prepared as described above. Sections were stained as above with Cy3-conjugated goat anti-rat IgM (1.5 μg/ml, Jackson Immuno-Research) followed by biotinylated MECA-79 (1.0 μg/ml) and Cy2-conjugated streptavidin (1.8 μg/ml, Jackson Immuno-Research). Finally, the sections were counterstained and mounted as above.

Enzyme-Linked Immunosorbent Assay

Peptide 3 was coated onto wells of a 96-well Immulon 2HB plate (Thermo Labsystems, Inc.), at 2 μg/ml in 15 mmol/L Na_2CO_3, 35 mmol/L $NaHCO_3$, pH 9.6, and incubated overnight at 4°C. The next day, wells were blocked with 3% bovine serum albumin in PBS/0.1% Tween 20 (blocking solution) for 2 hours at room temperature. Purified rabbit anti-murine HEC-GlcNAc6ST antibody was

reacted in a twofold-dilution series starting at 5 μg/ml in blocking solution for 2 hours at room temperature. The bound antibody was detected with biotinylated goat anti-rabbit IgG (1.3 μg/ml in blocking solution, Jackson Immuno-Research) followed by streptavidin-alkaline phosphatase (0.5 μg/ml in PBS; Caltag Laboratories, Burlingame, CA) and *p*-nitrophenyl phosphate substrate (5 mg, Pierce Biotechnology, Inc.) in 5 ml of 10% diethanolamine and 0.5 mmol/L MgCl$_2$, pH 9.8.

Western Blotting

cDNAs encoding murine HEC-GlcNAc6ST in pCDNA3.1/HISMyc (Invitrogen, Carlsbad, CA) or the vector control were transfected into COS cells by lipofectamine (Invitrogen)-mediated transfection. Three days after transfection, lysates were prepared from the transfected cells by lysis in PBS/2% Triton X-100. Aliquots (~10% of the total) of the lysates were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and transferred to Problott (Applied BioSystems, Foster City, CA). The membrane was blocked for 2 hours in blotto (5% dry milk in PBS/0.1% Tween 20). The membrane was incubated in blotto containing 1 μg/ml of purified HEC-GlcNAc6ST antibody for 1 hour at room temperature, followed by biotinylated goat anti-rabbit IgG (1 μg/ml, Jackson Immuno-Research) and streptavidin-horseradish peroxidase (Caltag Laboratories). The blot was developed with an enhance chemiluminescence (ECL) horseradish peroxidase substrate kit (Amersham, Arlington Heights, IL) followed by exposure to X-ray film.

Morphometric Analysis

HEC-GlcNAc6ST−/−/RIP-BLC+/− and HEC-GlcNAc6ST+/+/RIP-BLC+/− mice (four mice in each group, 20 to 25 weeks of age) were killed and cryostat sections of pancreata were prepared as described above. Every 15th 10-μm section was counterstained with Harris hematoxylin (Sigma) and analyzed. The numbers of noninfiltrated and infiltrated islets per section were counted to yield the percentage of infiltrated islets. Infiltrate sizes were also measured using ImageJ 1.25t software (National Institutes of Health, Bethesda, MD) and the mean size of the lymphocytic aggregates on each section was determined. This procedure was applied to 24 to 60 sections per pancreas. Means and standard deviations were calculated. Student's *t*-test was applied to determine the statistical significance of the differences between the two types of mice.

Results

Expression of HEC-GlcNAc6ST in Secondary Lymphoid Organs

To obtain an antibody against HEC-GlcNAc6ST, we immunized a rabbit with a mixture of three peptide-KLH conjugates. The peptides consisted of residues derived from the predicted sequence of mouse HEC-GlcNAc6ST
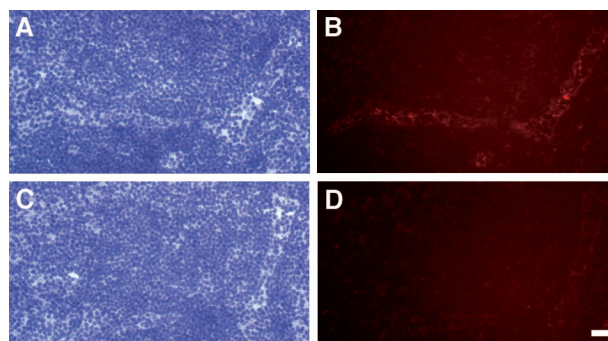

Figure 1. Staining of lymph nodes with an anti-HEC-GlcNAc6ST serum. An anti-HEC-GlcNAc6ST antiserum was produced by immunization of an individual rabbit with three peptides derived from the HEC-GlcNAc6ST sequence, as described in Materials and Methods. The serum was used to stain cryostat sections of mouse PN in the absence (**B**) or presence (**D**) of peptide 3 (RGKGMGQHAFHTNC). **A** and **C** show the bright-field images (hematoxylin stained) corresponding to **B** and **D**. Scale bar, 25 μm.

and were chosen in part because of conservation with the human orthologue of this sulfotransferase. The resulting serum stained HEVs in cryostat sections of mouse lymph node (Figure 1). As shown in Figure 1D, one of the three peptides (but not the other two, data not shown) substantially inhibited staining of HEVs in mouse PN sections. This peptide was therefore chosen as an immunoaffinity reagent for use with the serum. The purified antibody reacted specifically with the peptide by enzyme-linked immunosorbent assay and with recombinant HEC-GlcNAc6ST by Western blot (not shown).

Using this antibody and a two-color immunofluorescence protocol, we examined the relationship between expression of HEC-GlcNAc6ST protein and the presence of the MECA-79 epitope (Figure 2). As shown previously in our characterization of HEC-GlcNAc6ST-null mice,[32] the MECA-79 epitope was greatly diminished on the luminal surface of the HEVs in these mice (Cy3, red), whereas MECA-79 staining persisted in an abluminal pattern (Figure 2D). HEVs within PN of wild-type animals expressed HEC-GlcNAc6ST as detected by the peptide-specific antibody (Figure 2E; Cy2, green), whereas the HEVs of null mice were completely negative for the sulfotransferase (Figure 2F). The absence of staining in the null mice confirmed that the gene targeting had eliminated the expression of the encoded protein. Importantly, the expression of HEC-GlcNAc6ST was highly restricted to HEVs within lymph nodes, consistent with previous *in situ* hybridization findings.[29,30] A number of other tissues including kidney, pancreas, and heart in normal mice were devoid of staining (not shown), as predicted from mRNA expression.[30]

The staining pattern for the sulfotransferase in HEVs was in circular-, crescent-, or domed-shaped spots that were usually in the apical regions of the HEC (Figure 2E). Sulfation of carbohydrates occurs in the lumen of the Golgi apparatus.[35,36] To determine whether these spots co-localized with the Golgi apparatus, we performed two-color immunofluorescence with the HEC-GlcNAc6ST antibody and an antibody directed against a Golgi marker, GM-130 (Figure 3). We used sections of rat lymph node to permit the use of a mouse antibody as the primary
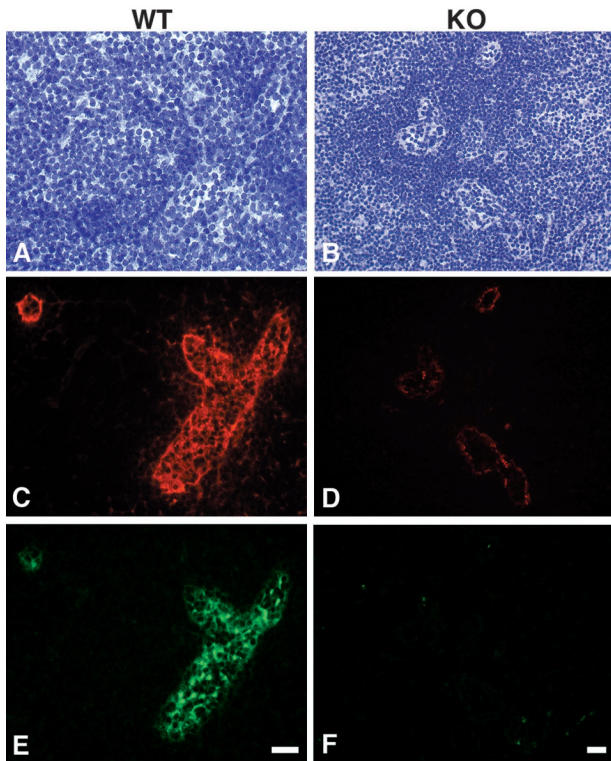
WT   KO

Figure 2. Expression of HEC-GlcNAc6ST and the MECA-79 epitope in mouse lymph node. Serial cryostat sections were prepared from PNs of wild-type (**A, C, E**) or −/− (**B, D, F**) mice. Sections were stained by immunofluorescence with MECA-79 (**C, D**) or the anti-HEC-GlcNAc6ST antibody (**E, F**). The bright-field images (**A, B**) establish the presence of HEVs in both wild-type and −/− lymph nodes. Scale bar, 25 μm.

staining reagent. The expression of HEC-GlcNAc6ST in rat LN HEVs occurred in the same characteristic pattern as observed in mouse (Figure 3C). Dual immunofluorescence showed that positive staining for HEC-GlcNAc6ST was spatially contiguous with the Golgi marker, indicating
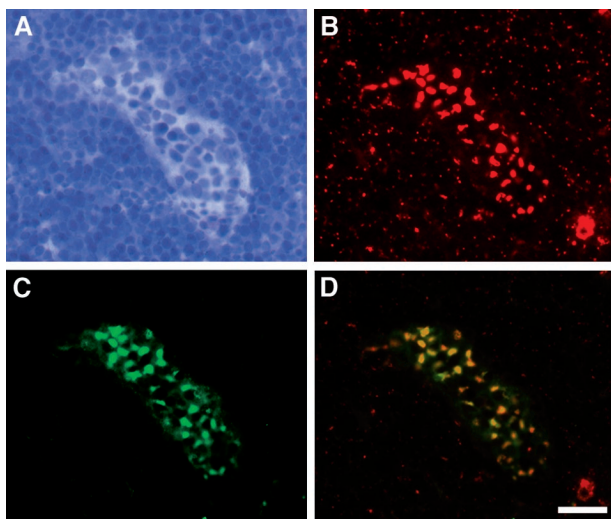

Figure 3. Golgi localization of HEC-GlcNAC6ST. A cryostat section from a rat lymph node was dual stained with an antibody for a Golgi marker (**B**; GM-130, Cy3, red) and the anti-HEC-GlcNAc6ST antibody (**C**; Cy2, green). **D:** Overlay of the two fluorescent images; yellow indicates overlap of the two stains. **A** is the bright-field image of the same field showing a single HEV. Scale bar, 25 μm.
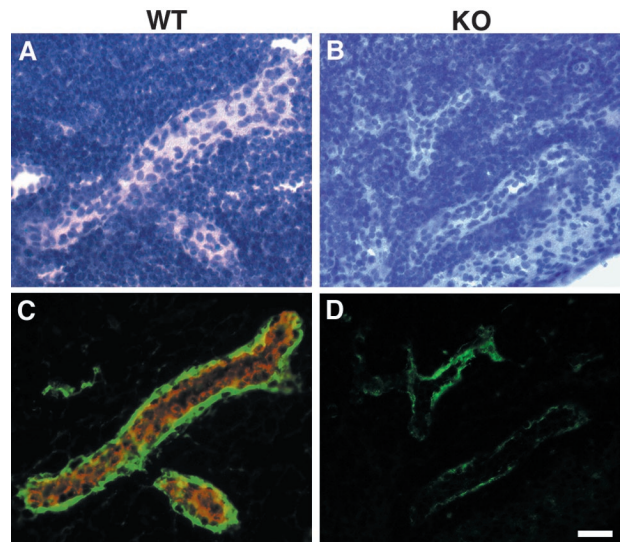
WT   KO

Figure 4. Contribution of HEC-GlcNAC6ST to the generation of luminal and abluminal MECA-79 epitopes. Cryostat sections of PN were prepared from wild-type (**A, C**) and −/− (**B, D**) mice that had received intravenous injections of unlabeled MECA-79. The sections were stained first with Cy3-conjugated secondary reagents to detect injected luminally bound MECA-79 and then with biotinylated MECA-79 followed by streptavidin-Cy2 to detect abluminal MECA-79 epitopes. **C** and **D:** Overlay of the two fluorescent images. **A** and **B** show the corresponding bright-field images. Scale bar, 25 μm.

a Golgi localization for the sulfotransferase within HECs (Figure 3, B and D).

To establish unambiguously that there was a reduction of luminal HEV MECA-79 epitope and to determine whether there was also an effect on abluminal staining in the HEC-GlcNAc6ST−/− mice, we used a dual *in vivo/ex vivo* staining approach. Unlabeled MECA-79 was injected into the tail vein of mice and allowed to circulate for 30 minutes. Cryostat sections of PNs were prepared and stained with Cy3-conjugated secondary reagents to reveal the injected MECA-79 that had bound to the blood-exposed (ie, luminal) regions of the HEVs. The sections were then treated sequentially with biotin-conjugated MECA-79 and streptavidin-Cy2 to detect the remaining epitopes that were not accessible from the blood. As shown in Figure 4, the HEVs of wild-type mice expressed abundant levels of luminal MECA-79 epitope, whereas luminal staining was barely detectable in the HEVs of −/− mice. Furthermore, although the *ex vivo* staining indicated the presence of abluminal MECA-79 epitopes in both wild-type and null mice, the staining intensity was consistently reduced in the HEVs of null mice. This result establishes that HEC-GlcNAc6ST also contributes to abluminal expression of the MECA-79 epitope.

HEVs of mesenteric lymph nodes (MNs) show a mosaic expression pattern for PNAd and MAdCAM-1 (a ligand for $\alpha 4\beta 7$).[13] We performed triple staining on MNs for PNAd (MECA-79), HEC-GlcNAc6ST, and MAdCAM-1 (MECA-367) (Figure 5). We found many HEVs that were mosaic, particularly the large vessels. In most MN HEVs, MECA-79 stained the luminal (and abluminal) aspects, irrespective of the presence of MAdCAM-1. The presence of HEC-GlcNAc6ST in the characteristic Golgi pattern correlated with luminal MECA-79 epitope. Conversely, wherever
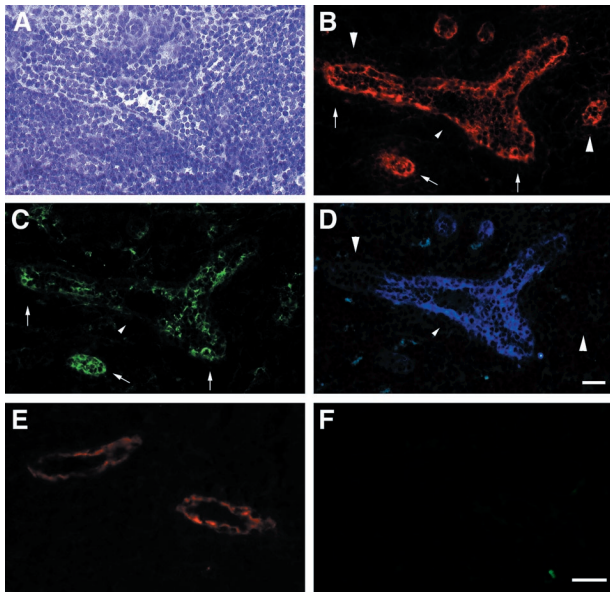
Figure 5. Expression of HEC-GlcNAc6ST in MNs and PPs. Cryostat sections from MNs of wild-type mice were stained simultaneously for MECA-79 (**B**; Cy3, red), HEC-GlcNAc6ST (**C**; Cy2, green), and MAdCAM-1 (**D**; aminomethylcoumarin, blue). **Large arrowheads** in **B** and **D** indicate vessels or segments of vessels that are MAdCAM-1-negative but contain luminal MECA-79 epitopes. **Arrows** in **B** and **C** indicate the correspondence between expression of HEC-GlcNAc6ST and luminal MECA-79 epitopes. **Small arrowheads** in **B**, **C**, and **D** indicate a segment of a vessel that is positive for MAdCAM-1, negative for HEC-GlcNAc6ST, and in which MECA-79 is confined to the luminal aspect. In **E** and **F**, a section of PP was dual stained for MECA-79 (**E**; Cy3, red) and HEC-GlcNAc6ST (**F**; Cy2, green). Scale bar, 25 μm.

MECA-79 was confined to the abluminal aspects of the HEVs, HEC-GlcNAc6ST was absent. In some of these segments, MAdCAM-1 was clearly evident.

Because we had previously shown that in the PP, the MECA-79 staining pattern was identical between wild-type and HEC-GlcNAc6ST−/− mice and was confined to the abluminal aspect of HEVs,[32] we wanted to determine whether HEC-GlcNAc6ST was expressed in PP HEVs. Our previous *in situ* hybridization analysis had failed to detect transcripts for HEC-GlcNAc6ST in wild-type PP (K Tangemann and SD Rosen, unpublished data). As shown in Figure 5F, the PPs of wild-type mice did not express HEC-GlcNAc6ST at the protein level. Consistent with previous observations,[13,32] only abluminal MECA-79 staining was present (Figure 5E). However, as expected, staining with MECA-367 demonstrated strong expression of MAdCAM-1 on HEVs in a pericellular pattern (not shown).

Expression of HEC-GlcNAc6ST in HEV-Like Vessels

As noted above, the MECA-79 epitope is observed on the activated vascular endothelium in many inflammatory sites.[14,15,20] We wanted to know whether HEC-GlcNAc6ST expression was induced on such activated endothelium. We first examined AKR mice, which progressively develop hyperplasia of the thymic medulla with age.[22] Infiltrated T and B cells become segregated as in lymph nodes, with HEV-like vessels confined to

non-B-cell regions. Hiraoka and colleagues[30] demonstrated the presence of HEC-GlcNAc6ST mRNA in MECA-79^{+}, HEV-like vessels in this model. As shown in Figure 6, such vessels also expressed HEC-GlcNAc6ST protein in a pattern similar to that seen in lymph nodes.

NOD mice spontaneously develop autoimmune diabetes with a pathology similar to that seen in human type I insulin-dependent diabetes mellitus.[21,37] In these mice, mononuclear islet infiltrates develop starting at 3 weeks of age and progressively acquire characteristics of secondary lymphoid organs. Similar infiltrates also develop in the salivary and lacrimal glands of these mice, with consequent progressive destruction of these glands.[23] Consistent with the observations made in these previous reports, we confirmed the presence of MECA-79^{+} HEV-like vessels in the inflamed regions of pancreas, lacrimal glands, and salivary glands (Figure 6). The high-walled vessels with strong pericellular MECA-79 staining were also strongly positive for HEC-GlcNAc6ST in the characteristic Golgi pattern found for lymph node HEVs.

In the RIP-BLC transgenic mouse, BLC (a B-cell chemokine also known as CXCL13) is expressed ectopically in the pancreas under the control of the rat insulin promoter.[34,38] The result is the formation of lymph node-like aggregates with segregated B-cell and T-cell areas and HEV-like structures. These are similar to aggregates that form in the inflammatory settings of the NOD pancreas and salivary glands and the RIPLT$\alpha\beta$ mouse,[19] with one notable difference that B cells are the predominant infiltrating cells. As shown in Figure 7, the presence of luminal MECA-79 epitopes again correlated with HEC-GlcNAc6ST expression. To establish that this relationship was causal, we crossed the RIP-BLC transgene onto a HEC-GlcNAc6ST-null background. In the RIP-BLC+/HEC-GlcNAc6ST-null mice, MECA-79 staining of the HEV-like vessels disappeared from the luminal aspect but was retained abluminally. With respect to the degree of infiltration, there was a trend toward reduced size and frequency of pancreatic infiltrates on the HEC-GlcNAc6ST-null background (28% and 22% reduction, respectively) but these differences did not reach statistical significance. As in the RIP-BLC mice, the lymphoid aggregates formed on the null background showed a segregation of B and T cells, with the predominance of the former (not shown).

Discussion

In this study, we used a polyclonal anti-peptide antibody to study the expression of HEC-GlcNAc6ST, a member of the GlcNAc6ST subfamily of sulfotransferases comprised of four members in mouse and five in human.[31,35] Confirming previous *in situ* hybridization and Northern blot analyses,[29,30] we demonstrated that this sulfotransferase was indeed highly restricted in its expression at the protein level. Specific staining was observed only for HEVs in lymph nodes and HEV-like vessels in several instances of lymphoid neogenesis. In further agreement with previous gene expression analyses, staining was absent in a panel of normal tissues. In contrast, the other members of the GlcNAc6ST family are predicted to have a wider tissue
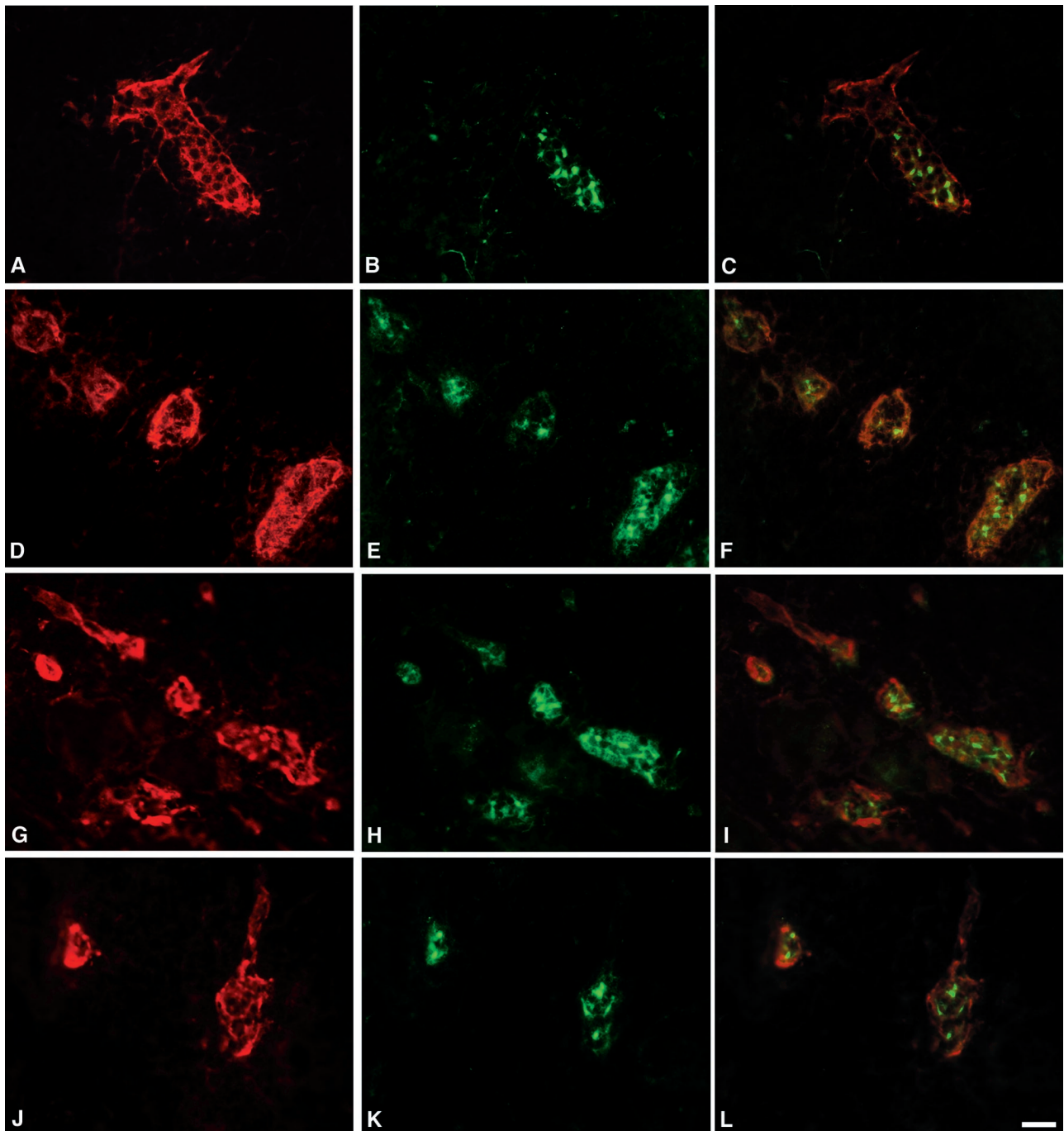
Figure 6. Expression of HEC-GlcNAc6ST in HEV-like vessels at sites of inflammation. Cryostat sections from hyperplastic AKR thymus (**A–C**), NOD pancreas (**D–F**), NOD lacrimal glands (**G–I**), and NOD salivary glands (**J–L**) were dual stained for MECA-79 (**A**, **D**, **G**, **J**; Cy3, red) and HEC-GlcNAc6ST (**B**, **E**, **H**, **K**; Cy2, green). **C**, **F**, **I**, and **L** are the respective overlays. Scale bar, 25 μm.

distribution based on Northern analysis[31,35] and the National Center for Biotechnology Information-expressed sequence tag (EST) database. As predicted for a carbohydrate-modifying sulfotransferase, staining for HEC-GlcNAc6ST co-localized with the Golgi compartment. HEC-GlcNAc6ST was not detected in the HEVs of PPs.

To study the relationship of HEC-GlcNAc6ST expression to functional ligands for L-selectin, we used MECA-79 as a reporter for a major class of L-selectin ligands. Two patterns of MECA-79 staining were discerned: luminal staining, which was correlated with the expression of HEC-GlcNAc6ST; and abluminal staining, which did not require the expression of this sulfotransferase though it was reduced in its absence. The unambiguous assignment of luminal and abluminal aspects of HEVs was accomplished by a two-stage protocol in which intravascular administration of MECA-79 was followed by staining of the cryostat sections with a biotinylated form of MECA-79. In the absence of HEC-GlcNAc6ST, as occurs in the lymph nodes of the null
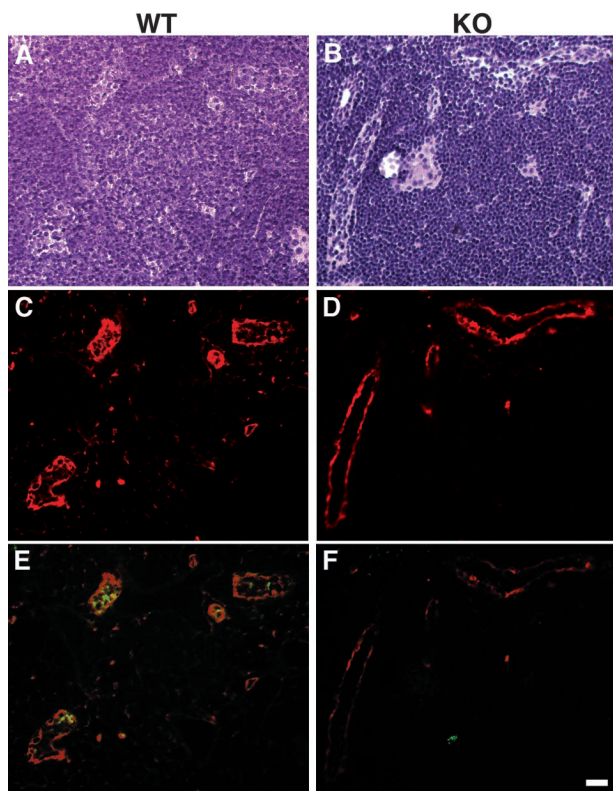
WT   **KO**

Figure 7. Contribution of HEC-GlcNAc6ST to luminal MECA-79 epitopes and lymphoid aggregates in the pancreas of RIP-BLC mice. Cryostat sections from infiltrated pancreas of RIP-BLC/HEC-GlcNAc6ST+/+ (**A**, **C**, and **E**) and RIP-BLC/HEC-GlcNAc6ST−/− (**B**, **D**, and **F**) mice were dual stained for MECA-79 (Cy3, red) and HEC-GlcNAc6ST (Cy2, green). Shown are the bright-field images (**A**, **B**), the MECA-79 staining (**C**, **D**), and the merged MECA-79 and anti-HEC-GlcNAc6ST staining (**E**, **F**). Scale bar, 25 μm.

mouse as well and naturally in PP HEVs, abluminal staining was clearly present whereas luminal MECA-79 epitopes were present at very low to undetectable levels. Despite this paucity of luminal MECA-79 staining, a proportion of functional L-selectin ligands persists on venules within the lymph nodes of HEC-GlcNAc6ST-null mice[32,33] and in the HEVs of normal PPs.[39,40] Whether these ligands carry essential sulfation modifications is unknown. With respect to the abluminal ligands that are found in the absence of HEC-GlcNAc6ST, another enzyme in the same subfamily, GlcNAc6ST-1, is the prime candidate as the relevant sulfotransferase. This enzyme is detected at the mRNA level in lymph node HEVs[41] and can contribute to the elaboration of L-selectin ligand activity, the MECA-79 epitope, and the 6-sulfo sLex determinant in transfected cells.[42,43] The function of the abluminal ligands is not understood at this time.

MECA-79 staining of HEV-like vessels in inflammatory sites has been confirmed in several instances to reflect L-selectin ligand activity: MECA-79 reactivity has been correlated with positive staining by an L-selectin/IgG chimera,[44] L-selectin-dependent attachment of lymphocytes in an *in vitro* adhesion assay,[21,22,45] and L-selectin-dependent homing of lymphocytes to the inflamed organ.[22,23] In the homing studies, short-term lymphocyte accumulation was inhibited by intravenous administration of MECA-79.[22,23] Previously, the presence of HEC-

GlcNAc6ST protein in HEV-like vessels has been noted within the inflamed pancreas of RIP-LTαβ mice, in which both the LTα and LTβ are expressed under the control of the rat insulin promoter, generating expression of the LTαβ complex.[19] The HEC-GlcNAc6ST-positive vessels exhibit both abluminal and luminal MECA-79 staining. Here, we have extended the correlation between the expression of HEC-GlcNAc6ST and strong luminal staining by MECA-79 to several other examples of lymphoid neogenesis, including two spontaneous models, ie, the AKR thymoma and the NOD mouse. Moreover, by crossing the RIP-BLC transgene in the context of the HEC-GlcNAc6ST-null background, we have established that this sulfotransferase is required for the presence of abundant luminal epitopes. In the absence of HEC-GlcNAc6ST, there was a trend to a reduction in the frequency (22% reduction) and size of the infiltrates (28% reduction). We previously noted a similar level of reduction (≈20%) in the cellularity of MNs in HEC-GlcNAc6ST-null mice.[32] In common with the HEVs of mesenteric nodes, the HEV-like vessels in the infiltrates of RIP-BLC mice are mosaic, expressing both PNAd and MAdCAM-1, sometimes simultaneously.[34] The adhesion mechanisms induced to compensate for the absence of HEC-GlcNAc6ST may be similar at the two sites. It should be noted that the HEV-like vessels in NOD pancreas and AKR thymus are also chimeric for PNAd and MAdCAM-1.[21,22]

As mentioned in the Introduction, a number of studies have reported MECA-79+ vessels in inflammatory lesions in the human, frequently but not always, in the context of organized lymphoid tissue.[14,15] The first reports of MECA-79+ vessels in human inflammatory lesions were anecdotal, involving small numbers of samples. Subsequent studies by Renkonen and co-workers[20,46–49] of several inflammatory conditions (rejecting heart and kidney allografts, peribronchial specimens from asthmatics, thyroiditis, and psoriasis) have demonstrated the consistent induction of MECA-79+ vessels in association with inflammatory infiltrates in large numbers of independent specimens. Similarly, Salmi and colleagues[50] reported that this epitope is present on a subpopulation of synovial vessels in the majority of chronic arthritis patients. It remains to be determined whether the MECA-79+ vessels within these various lesions express HEC-GlcNAc6ST, in analogy with our findings in ectopic lymphoid aggregates in mouse.

Acknowledgments

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