# Core 2 branching β1,6-*N*-acetylglucosaminyltransferase and high endothelial cell *N*-acetylglucosamine-6-sulfotransferase exert differential control over B- and T-lymphocyte homing to peripheral lymph nodes

Jean-Marc Gauguet, Steven D. Rosen, Jamey D. Marth, and Ulrich H. von Andrian

**Blood-borne lymphocyte trafficking to peripheral lymph nodes (PLNs) depends on the successful initiation of rolling interactions mediated by L-selectin binding to sialomucin ligands in high endothelial venules (HEVs). Biochemical analysis of purified L-selectin ligands has identified posttranslational modifications mediated by Core2GlcNAcT-I and high endothelial cell GlcNAc-6-sulfotransferase (HEC-GlcNAc6ST). Consequently, lymphocyte migration to PLNs of C2GlcNAcT-I$^{-/-}$ and HEC-GlcNAc6ST$^{-/-}$ mice was reduced; however, B-cell homing was more severely compromised than T-cell migration. Accordingly, intravital microscopy (IVM) of PLN HEVs revealed a defect in B-cell tethering and increased rolling velocity ($V_{roll}$) in C2GlcNAcT-I$^{-/-}$ mice that was more pronounced than it was for T cells. By contrast, B- and T-cell tethering was normal in HEC-GlcNAc6ST$^{-/-}$ HEVs, but $V_{roll}$ was accelerated, especially for B cells. The increased sensitivity of B cells to glycan deficiencies was caused by lower expression levels of L-selectin; L-selectin$^{+/-}$ T cells expressing L-selectin levels equivalent to those of B cells exhibited intravascular behavior similar to that of B cells. These results demonstrate distinct functions for C2GlcNAcT-I and HEC-GlcNAc6ST in the differential elaboration of HEV glycoproteins that set a threshold for the amount of L-selectin needed for lymphocyte homing. (Blood. 2004;104:4104-4112)**

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## Introduction

Lymphocyte entry into peripheral lymph nodes (PLNs) is mediated by a cascade of distinct molecular interactions in high endothelial venules (HEVs) involving tethering and rolling; chemokine signaling through G-protein–coupled receptors; integrin activation and subsequent firm arrest; and, finally, transmigration of adherent cells to the underlying tissue.[1,2] The first step of this process, rolling, is mediated by lymphocyte-expressed L-selectin and its carbohydrate ligands on high endothelial cells (HECs) in PLNs.[3] L-selectin, like E- and P-selectin, is characterized by high-bond association ($k_{on}$) and dissociation ($k_{off}$) rates that permit rolling at physiologic shear rates.[4,5] This interaction between L-selectin and its HEC ligands is essential for lymphocyte recruitment because L-selectin$^{-/-}$ lymphocytes fail to home to PLN,[6] and lymphocytes treated with an L-selectin function blocking antibody exhibit a substantial decrease in homing to PLN and rolling in HEVs.[7,8] Additionally, the copy number of L-selectin molecules on the lymphocyte surface determines the capacity of lymphocytes to traffic to PLNs; T cells express approximately twice as much L-selectin than B cells do, and T-cell homing is several-fold more efficient than B-cell homing.[9,10]

The glycoproteins that are decorated with L-selectin ligands in PLN HEVs are collectively referred to as peripheral node addressin (PNAd).[11] They include CD34,[12,13] Sgp200,[14,15] and the secreted glycoprotein, GlyCAM-1[16,17]; in humans, podocalyxin has been identified in tonsils.[18]

Biochemical analyses of purified CD34 and GlyCAM-1 have shown that these sialomucins are decorated with 2 sulfated isomers of sialyl Lewis$^X$ (sLe$^X$), 6′-sulfo sLe$^X$ and 6-sulfo sLe$^X$.[19,20] Although in vitro studies have yielded conflicting data on the function of 6′-sulfo sLe$^X$,[20,21] there is strong evidence for the physiologic importance of 6-sulfo sLe$^X$. The gene product of *CHST4* (carbohydrate sulfotransferase 4), HEC-GlcNAc6ST, also known as GlcNAc6ST-2, and LSST (L-selectin ligand sulfotransferase) is a GlcNAc-6-sulfotransferase that produces the 6-sulfo sLe$^x$ moiety and is highly and selectively expressed in HECs.[22] In vitro, HEC-GlcNAc6ST can decorate CD34 and GlyCAM-1 with 6-sulfo sLe$^X$, and this modification confers potent L-selectin ligand activity to both molecules.[23] Lymphocyte recruitment to PLNs in HEC-GlcNAc6ST$^{-/-}$ mice is reduced to approximately 50% of wild-type (WT) levels.[24] Interestingly, in vivo HEC-GlcNAc6ST deficiency has little effect on the frequency of L-selectin–mediated contact initiation (tethering), but it leads to greatly accelerated lymphocyte rolling velocity ($V_{roll}$) in vitro[25] and in vivo.[26]

Most L-selectin ligands in PLN HEVs are recognized by the monoclonal antibody (mAb) MECA-79,[11] which partially blocks lymphocyte homing to PLN and lymphocyte rolling in PLN

From the CBR Institute for Biomedical Research and the Department of Pathology, Harvard Medical School, Boston, MA; the Department of Anatomy, Program in Immunology, Cardiovascular Research Institute, University of California at San Francisco; and the Howard Hughes Medical Institute and the Department of Cellular and Molecular Medicine, University of California, San Diego.

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**Reprints:** Ulrich H. von Andrian, CBR Institute for Biomedical Research, Inc, 200 Longwood Ave, Boston, MA 02115; e-mail: uva@cbr.med.harvard.edu.

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HEVs.[11,26-28] Additionally, 6-sulfo sLe$^X$ is an essential component of the MECA-79 epitope[14,23] and HEC-GlcNAc6ST expression is required for luminal presentation of MECA-79–reactive glycans in HEVs.[23,24,26] Curiously, affinity-purified L-selectin ligands from PLN contain 6-sulfo sLe$^X$ on core 2 and core 1 O-glycan extensions, and both extensions support equivalent L-selectin–dependent lymphocyte interactions in flow chambers.[23,29] However, MECA-79 detects 6-sulfo sLe$^X$ only on core 1 O-glycans.[23] Therefore, although the biochemical structure of the MECA-79 epitope is now well understood, the physiologic role of sulfated and nonsulfated core 1 and core 2 extensions and their relative contributions to L-selectin ligand activity in PLN HEVs is still unclear.

Given the potent adhesion-blocking effect of MECA-79, the physiologic role of core 2 O-glycans (which are not detected by MECA-79) had been thought to be minor. Core 2 O-glycan extensions on HECs are generated by C2GlcNAcT-I (core 2 β-1,6-*N*-acetylglucosaminyltransferase-I),[30] and HEV-derived Gly-CAM-1 from C2GlcNAcT-I$^{-/-}$ mice is devoid of core 2 glycans.[23] An initial analysis of lymphocyte content and trafficking in secondary lymphoid organs (SLOs) of these animals did not detect a reproducible phenotype.[31] However, a more detailed recent analysis in the C57BL/6 background uncovered a significant defect in lymphocyte homing to PLNs of C2GlcNAcT-I$^{-/-}$ mice that was similar in magnitude to that in HEC-GlcNAc6ST$^{-/-}$ animals.[26,32] Combined deficiency in both enzymes had additive effects on homing, suggesting that C2GlcNAcT-I and HEC-GlcNAc6ST may have distinct functions.[26,32] However, whether and how the glycans generated by these 2 enzymes differentially affect the L-selectin–mediated adhesion and the extent to which their roles depend on L-selectin expression levels on lymphocytes has not been determined.

To address these questions, we compared the capacity of B and T cells with defined L-selectin surface expression levels to home to SLOs and to tether and roll in PLN HEVs of C2GlcNAcT-I$^{-/-}$ and HEC-GlcNAc6ST$^{-/-}$ mice. We found that short-term B-cell trafficking to PLNs of C2GlcNAcT-I$^{-/-}$ mice was markedly reduced, whereas T-cell migration was only moderately decreased; in long-term studies, only B-cell homing was affected. Using IVM, we observed that B cells in WT mice tethered less frequently and rolled at higher velocities than did T cells; these differences became more exaggerated in C2GlcNAcT-I$^{-/-}$ mice. L-selectin$^{+/-}$ T cells, which expressed surface L-selectin levels similar to those of WT B cells, exhibited migratory and adhesive phenotypes similar to those of WT B cells in WT and C2GlcNAcT-I$^{-/-}$ mice. Similarly, in HEC-GlcNAc6ST$^{-/-}$ mice, B-cell homing to PLNs was also more severely compromised than that of T cells; however, T- and B-cell tethering was normal in HEC-GlcNAc6ST$^{-/-}$ HEV mice compared with WT mice, whereas $V_{roll}$ of both subsets was dramatically increased.

Our findings demonstrate that enzymatic modifications of L-selectin ligands in PLN HEVs by HEC-GlcNAc6ST and C2GlcNAcT-I control different qualities of L-selectin–dependent adhesion and that the absence of either activity raises the threshold amount of L-selectin necessary for efficient lymphocyte homing to PLNs.

## Materials and methods

### Mice

C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). C2GlcNAcT-I$^{-/-}$ and HEC-GlcNAc6ST$^{-/-}$ mice on a mixed C57Bl/6/129/Sv background were described previously.[24,31] Sex- and age-matched WT littermates were used as controls in all experiments. P-selectin glycoprotein ligand 1 (PSGL-1)$^{-/-}$ mice on a mixed background[33] were from Dr Roger McEver (University of Oklahoma, Oklahoma City). T green fluorescence protein (T-GFP) mice and L-selectin$^{-/-}$ T-GFP mice (T-GFPxL$^{-/-}$), which selectively express enhanced GFP (eGFP) in naive and central memory T cells, were characterized in our laboratory.[34,35] Mice were housed and bred in a specific pathogen-free/viral antibody–free animal facility. All experiments were performed in accordance with National Institutes of Health guidelines and were approved by the Committees on Animals of Harvard Medical School and the CBR Institute for Biomedical Research, Inc.

### Reagents

Monoclonal antibodies that included purified rat antimouse anti-CD90, anti-Ter119, anti-CD11b, anti-CD3ε, anti–Ly-6G (Gr-1), CyChrome (Cy)–labeled anti-B220, anti-CD4 fluorescein isothiocyanate (FITC), anti-CD19 phycoerythrin (PE), anti-CD8a Cy, and MEL-14 (anti-CD62L) PE were from BD PharMingen (San Diego, CA). Intravital dyes calcine AM and tetramethylrhodamine-5 and -6-isothiocyanate (TRITC) were from Molecular Probes (Eugene, OR).

### Homing assays

Lymphocytes from mesenteric lymph nodes (MLNs) and pooled PLNs (axillary, brachial, and inguinal) of T-GFP mice[34] or T-GFPxL$^{+/-}$ mice were stained with TRITC.[35] Labeled lymphocytes were injected into the tail veins of recipient mice, and lymphoid organs were harvested after 1 hour or 24 hours. Single-cell suspensions of mechanically dissociated organs were analyzed using flow cytometry to measure the number of homed donor cells. T cells were identified as GFP$^+$/TRITC$^+$ cells, whereas B cells were stained with CyChrome-conjugated anti-B220 and were identified as GFP$^-$/TRITC$^+$/B220$^+$. For homing experiments comparing T-GFP and T-GFPxL$^{+/-}$ lymphocytes, T-GFPxL$^{+/-}$ lymphocytes were stained with TRITC and were mixed with unstained lymphocytes from T-GFP mice. In subsequent experiments, T-GFP lymphocytes were stained instead to exclude possible effects of TRITC labeling. Homed B cells were identified by staining with Cy-B220. Only TRITC-stained adoptively transferred B cells could be distinguished from endogenous B cells; competitive homing experiments with L$^{+/+}$ and L$^{+/-}$ B cells were performed by pooling data where either L$^{+/+}$ or L$^{+/-}$ B cells were stained with TRITC.

### Purification of B cells

Splenocytes from C56BL/6 mice were incubated in a cocktail of rat immunoglobulin G (IgG) antibodies against CD90 (Thy1.1), Ter119, CD11b (Mac-1), CD3ε, and Gr-1. Antirat IgG magnetic beads (Miltenyi Biotec, Auburn, CA) were then used to negatively select B cells to over 95% purity (data not shown) using an LD purification column (Miltenyi Biotec). L-selectin levels and cell shape, measured by flow cytometry, were the same for purified B cells as for unpurified cells, indicating that the purification procedure had no discernible effect on B-cell phenotype (data not shown).

### Intravital microscopy

Intravital microscopy (IVM) of the murine inguinal lymph node was performed as described.[8,28] Briefly, young adult (6- to 12-week-old) female C2GlcNAcT-I$^{-/-}$, HEC-GlcNAc6ST$^{-/-}$, or WT mice were anesthetized by intraperitoneal injection of 10 mL/kg saline containing xylazine (1 mg/mL) and ketamine (5 mg/mL). An incision was made to expose a left abdominal skin flap, which was stretched over a glass slide immobilized on a Plexiglas microscope stage. The lymph node was covered in 0.9% saline, and surrounding fatty tissue was removed to expose the lymph node microvasculature. T-GFP, T-GFPxL$^{+/-}$, or calcein-labeled lymphocytes injected into a right femoral artery catheter were visualized in the PLN microvasculature by stroboscopic epifluorescence illumination using an intravital microscope (IV-500; Micron Instruments, Simi Valley, CA) and were recorded on an Hi8 video-cassette recorder (Sony, Tokyo, Japan) to permit off-line video analysis, as described previously.[8] Rolling fraction, sticking efficiency, and sticking fraction were measured as described previously.[36] Velocity analysis was performed using software for random line-length measurements.[37] The

velocities of 10 rolling and 20 noninteracting (fast) cells were measured to obtain fast cell velocity ($V_{fast}$) and rolling velocity ($V_{roll}$), as previously described.[36] $V_{roll}$ in order V venules was not analyzed because the large accumulation of adherent cells in these venules made accurate $V_{roll}$ measurements difficult.

### Measurement of lymphoid organ cellularity

Lymphoid organs, including PLNs (pooled axillary, inguinal, and brachial), MLNs, and Peyer patches (PPs), were harvested from 8- to 10-week-old mice and dispersed into single-cell suspensions. Total cell counts were determined on a hemocytometer. Cell suspensions were stained with anti-CD4, anti-CD8, and anti-CD19 and were analyzed by flow cytometry to determine the percentages of T- and B-cell subsets in each organ.

### Statistical analysis

All data are reported as mean ± SEM unless otherwise noted. Homing experiments were analyzed using an unpaired Student *t* test, $V_{roll}$ data were compared by a nonparametric Mann-Whitney *U* test, and multiple values (Figure 4B) were analyzed using 1-way analysis of variance (ANOVA) with Bonferroni multiple comparison test. Significance was reached at $P$ less than .05.

## Results

### Differential effects of C2GlcNAcT-I deficiency on T- and B-cell homing to PLNs

We observed a subtle, but statistically significant, reduction in total lymphocyte numbers in C2GlcNAcT-I$^{-/-}$ PLNs compared with that for PLNs of WT littermates (Table 1). This phenotype was entirely caused by a reduction in the number of resident B cells, whereas the T-cell compartment was not altered in subset composition and size. Although there was a small reduction in circulating B-cell numbers in C2GlcNAcT-I$^{-/-}$ (90% of WT counts) and HEC-GlcNAc6ST$^{-/-}$ mice (70% of WT counts), B-cell numbers in C2GlcNAcT-I$^{-/-}$ and HEC-GlcNAc6ST$^{-/-}$ PLNs were reduced 2- and 5-fold, and other SLOs, such as spleen, MLNs, and PPs, contained B-cell numbers similar to those for the corresponding WT SLOs, indicating that the relative paucity of B cells in PLNs was not attributed to a global deficiency in B-cell numbers or trafficking.

**Table 1. Lymphocyte counts in WT, C2GlcNAcT-1$^{-/-}$, and HEC-GlcNAc6ST$^{-/-}$ organs**

| | Lymphocyte counts × $10^3$ ± SEM | | |
|---|---|---|---|
| | WT | C2$^{-/-}$ | HEC-GlcNAc6ST$^{-/-}$ |
| **PLN** | | | |
| Total | 11.98 ± 0.81 | 10.30 ± 1.46* | 6.68 ± 1.33† |
| CD4$^+$ | 5.58 ± 0.31 | 5.13 ± 0.74 | 3.87 ± 0.73* |
| CD8$^+$ | 3.56 ± 0.36 | 3.14 ± 0.55 | 1.96 ± 0.43* |
| CD19$^+$ | 2.47 ± 0.22 | 1.78 ± 0.20* | 0.47 ± 0.12‡ |
| **MLN** | | | |
| Total | 20.71 ± 2.74 | 24.52 ± 1.67 | 20.32 ± 1.30 |
| CD4$^+$ | 8.55 ± 1.03 | 9.74 ± 0.94 | 9.18 ± 0.33 |
| CD8$^+$ | 5.19 ± 0.89 | 6.27 ± 1.89 | 4.73 ± 0.16 |
| CD19$^+$ | 6.45 ± 0.99 | 8.48 ± 0.79 | 7.91 ± 1.98 |
| **PP** | | | |
| Total | 10.96 ± 0.85 | 9.76 ± 2.32 | 10.44 ± 4.20 |
| CD4$^+$ | 2.03 ± 0.28 | 1.62 ± 0.58 | 2.34 ± 1.13 |
| CD8$^+$ | 0.65 ± 0.16 | 0.77 ± 0.23 | 0.66 ± 0.30 |
| CD19$^+$ | 7.88 ± 0.70 | 6.65 ± 1.94 | 6.20 ± 1.75 |

*$P < .05$; †$P < .01$; ‡$P < .001$ versus WT (unpaired Student *t* test).
WT, n = 7 mice; C2GlcNAcT-1$^{-/-}$, n = 4 mice; HEC-GlcNAc6ST$^{-/-}$, n = 3 mice.

One possible explanation for this PLN-specific phenotype is a selective defect in B-cell homing to PLNs. To test this hypothesis, we examined the homing of T and B cells in WT and C2GlcNAcT-I$^{-/-}$ mice. Equal numbers of TRITC-labeled lymphocytes from T-GFP mice[34] were injected intravenously into WT and mutant recipients, and their accumulation in SLOs was assessed 1 and 24 hours later. Consistent with previous results,[9,10] T cells homed more efficiently to PLNs of WT recipients than did B cells, irrespective of the homing interval after transfer. T cells also homed better to MLNs, whereas both subsets homed equivalently well to the spleen and to PPs (Figure 1A-B). Importantly, the ratio of homed T cells to B cells was approximately 3-fold higher in PLNs of C2GlcNAcT-I$^{-/-}$ mice than in WT mice (Figure 1A-B). Comparing the absolute number of homed T and B cells at 1 hour after transfer, T cells exhibited 2-fold less homing to PLNs of C2GlcNAcT-I$^{-/-}$ mice than of WT mice (Figure 1C), whereas B-cell trafficking to C2GlcNAcT-I$^{-/-}$ PLNs was 6-fold (85%) lower (Figure 1E). B-cell homing to MLNs was also significantly lower after 1 hour in C2GlcNAcT-I$^{-/-}$ mice, whereas homing to PPs was not affected. At 24 hours after transfer, T-cell distribution in SLOs of C2GlcNAcT-I$^{-/-}$ mice was indistinguishable from that of WT SLOs, whereas B-cell homing to C2GlcNAcT-I$^{-/-}$ PLNs was still significantly compromised (Figure 1D, F).

### Intravital microscopy of C2GlcNAcT-I$^{-/-}$ PLNs reveals impaired lymphocyte adhesion in HEVs

Considering the defect in short-term B- and T-cell homing to PLNs and the importance of core 2–linked glycans in the generation of physiologic selectin ligands on leukocytes,[31] we postulated that C2GlcNAcT-I$^{-/-}$ HEVs may be defective in supporting L-selectin–mediated tethering, rolling, or both. To test this prediction, we used IVM of the inguinal LN.[28] In this model, 5 different venular branching orders can be distinguished; orders III to V are cortical HEV, whereas orders I and II are medullary-collecting venules with flat endothelia. The MECA-79 antigen is only found in venule orders III to V, which support the bulk of lymphocyte traffic into PLNs.[27,38]

Compared with WT PLNs, the rolling fraction of T cells was moderately decreased in C2GlcNAcT-I$^{-/-}$ PLNs; this difference was only significant in order III venules (Figure 2A). In contrast, T-cell sticking efficiency to the vessel wall was markedly decreased, particularly in venular orders IV and V of C2GlcNAcT-I$^{-/-}$ mice (Figure 2B).

In WT PLNs, B cells rolled less (10%-25%) frequently than T cells throughout the venular tree (Figure 2A, C), but this difference only reached statistical significance in orders III and IV ($P < .05$ and $P < .01$, respectively). In C2GlcNAcT-I$^{-/-}$ PLNs, the B-cell rolling fraction was even more reduced (35%-55%), especially in venular orders IV and V ($P < .01$). Accordingly, compared with WT PLNs, the sticking efficiency of B cells in order V venules of C2GlcNAcT-I$^{-/-}$ PLNs was also more severely compromised than that of T cells (Figure 2B, D). Thus, C2GlcNAcT-I deficiency has distinct effects on T and B cells in PLN HEVs. T-cell rolling fractions are only subtly reduced, whereas firm arrest is detectably compromised; in contrast, B-cell rolling fraction and sticking efficiency are more severely reduced.

### C2GlcNAcT-I–dependent glycans determine lymphocyte rolling velocity

Next, we sought to elucidate why T-cell sticking in C2GlcNAcT-I$^{-/-}$ HEVs was more severely reduced than might be expected

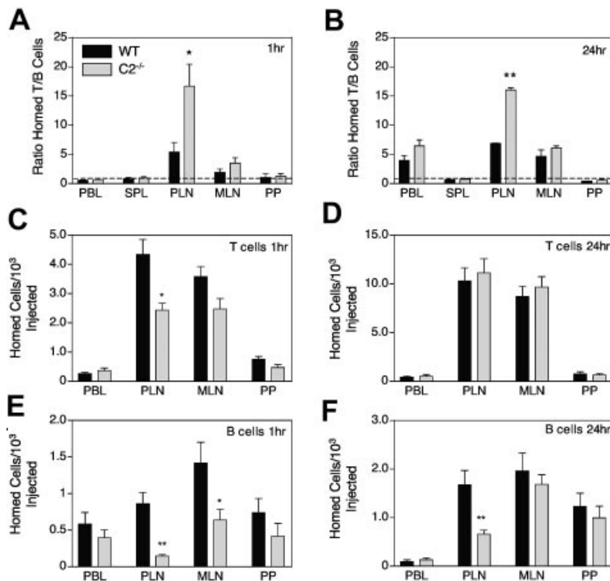



**Figure 1. Lymphocyte homing in C2GlcNAcT-I$^{-/-}$ lymphoid organs.** Naive donor lymphocytes from pooled MLNs and PLNs of T-GFP mice were TRITC labeled and injected into recipient mice. Lymphoid organs were harvested after 1 hour (A) and 24 hours (B), and the ratio of total homed T cells to B cells was measured by flow cytometry. The dashed line indicates a homing ratio of 1; that is, homing of both subsets was equivalent. Total numbers of homed T cells (C-D) and B cells (E-F) were measured after 1 hour (C, E) and 24 hours (D, F). Homed cell numbers were normalized to the number of injected cells. *$P < .05$ and **$P < .01$ versus WT. n = 5 to 7 mice per group. Error bars indicate SEM.

based on the relatively subtle reduction in rolling fraction. Rolling fraction is primarily a measure of the frequency at which free-flowing cells form an initial tether in HEV, but it does not reflect the quality of established rolling interactions.[39] The most sensitive parameter of selectin-dependent adhesiveness after tethering is $V_{roll}$, the velocity at which cells move downstream while they are in adhesive contact with the venular lining. Indeed, for T cells in C2GlcNAcT-I$^{-/-}$ orders III and IV venules, the median $V_{roll}$ was 2- and 4-fold higher, respectively, than it was in WT mice (Figure 3A-B; Table 2). B cells rolled 3- and 5-fold faster than T cells in WT orders III and IV HEVs, respectively, and B-cell $V_{roll}$ was significantly further increased in C2GlcNAcT-I$^{-/-}$ HEVs (Figure 3C-D). Irrespective of the branching order or genotype of individual HEV, the median $V_{roll}$ of B cells was always higher (2- to 5-fold, on average) than that of T cells.

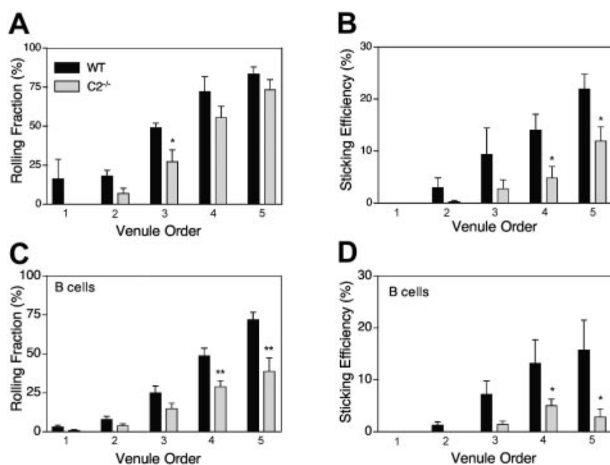



**Figure 2. Intravital microscopy of WT and C2GlcNAcT-I$^{-/-}$ PLN HEVs.** The rolling fraction of T cells (A) and B cells (C) was determined as the percentage of rolling cells in the total flux of cells in each HEV. Sticking efficiency of T cells (B) and B cells (D) was calculated as the percentage of adherent cells (30 seconds or longer) in the total lymphocyte flux through each HEV. *$P < .05$ and **$P < .01$ versus WT. For WT, n = 4 mice. For T cells, order I, n = 2 venules; order II, 5; order III, 6; order IV, 5; order V, 5. For B cells, order I, n = 5 venules; order II, 8; order III, 12; order IV, 9; order V, 9. C2GlcNAcT-I$^{-/-}$, n = 4 mice. For T cells, order I, n = 3 venules; order II, 6; order III, 9; order IV, 10; order V, 9. For B cells, order I, 5 venules; order II, 5; order III, 10; order IV, 13; order V, 8 venules.

### Impact of C2GlcNAcT-I deficiency on lymphocyte homing to PLNs is inversely correlated with L-selectin expression level

A possible explanation for the differential effect of C2GlcNAcT-I deficiency on B- versus T-cell trafficking to PLNs is the 50% lower expression level of L-selectin on B cells than on T cells.[10] To test this hypothesis, we crossed WT T-GFP mice ($L^{+/+}$) with L-selectin$^{-/-}$ ($L^{-/-}$) T-GFP mice to generate L-selectin heterozygous ($L^{+/-}$) T and B cells. Mean fluorescence intensity (MFI) of anti–L-selectin staining confirmed that WT T cells in T-GFP mice expressed twice as much surface L-selectin than WT B cells (Figure 4A). Consistent with previous reports,[10] $L^{+/-}$ T cells expressed half the amount of L-selectin as WT T cells, whereas $L^{+/-}$ B cells expressed approximately 70% of WT B-cell levels (Figure 4A). By contrast, there was no difference between WT and $L^{+/-}$ lymphocytes in expression levels of other relevant traffic molecules, such as LFA-1 (data not shown).

The ability of lymphocytes to home to PLNs in WT mice correlated with the amount of surface L-selectin[10]; compared with their $L^{+/+}$ counterparts, $L^{+/-}$ B- and T-cell homing to PLNs was reduced by 80% and 40%, respectively (Figure 4Bi-ii). Comparing $L^{+/+}$ and $L^{+/-}$ T-cell homing to WT PLNs and C2GlcNAcT-I$^{-/-}$ PLNs, there was a 40% decrease in $L^{+/+}$ homing, and a 70% reduction in $L^{+/-}$ T-cell homing (Figure 4Bi). Thus, reducing L-selectin expression on $L^{+/-}$ T cells to levels that are physiologically found on B cells resulted in a homing phenotype that was strikingly similar to that of WT B cells. Similarly, $L^{+/-}$ B cells exhibited an even more severe defect in homing to WT PLNs relative to WT B cells, and $L^{+/-}$ B-cell homing to C2GlcNAcT-I$^{-/-}$ PLNs became almost undetectable (Figure 4Bii). These findings indicate that C2GlcNAcT-I–dependent HEV glycans critically determine the amount of L-selectin needed for efficient lymphocyte homing to PLNs.

This interpretation was further supported by IVM analysis of $L^{+/-}$ T cells in WT and C2GlcNAcT-I$^{-/-}$ PLN HEVs. $L^{+/-}$ T cells

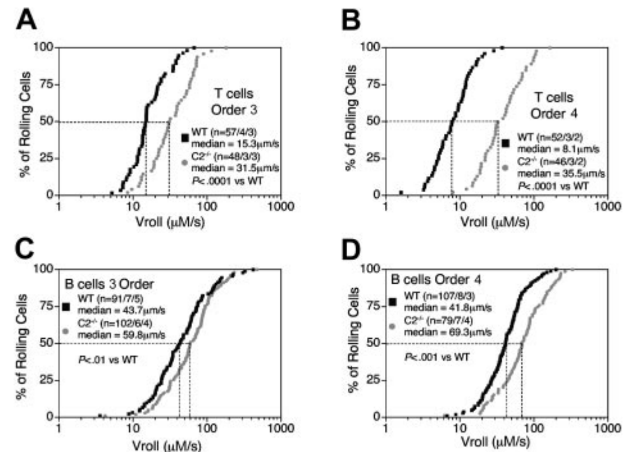



**Figure 3. Cumulative lymphocyte rolling velocities in PLN HEVs of WT and C2GlcNAcT-I$^{-/-}$ mice.** Percentages of (A-B) T cells and (C-D) B cells that rolled in order III (A, C) or order IV (B, D) HEVs at or below a given velocity are graphed as a function of $V_{roll}$. n = total number of cells/venules/mice analyzed per group.

**Table 2. L-selectin MFI and median $V_{roll}$**

| | L-selectin MF1 | Order III $V_{roll}$, μm/s | | | Order IV $V_{roll}$, μm/s | | |
|---|---|---|---|---|---|---|---|
| | | WT | $C2^{-/-}$ | HEC-GlcNAc6$ST^{-/-}$ | WT | $C2^{-/-}$ | HEC-GlcNAc6$ST^{-/-}$ |
| $L^{+/+}$ T cells | 855 | 15.3 | 31.5 | 41.9 | 8.1 | 35.5 | 42.5 |
| $L^{+/-}$ T cells | 433 | 26.6 | 56.5 | 85.5 | 9.7 | 46.8 | 82.1 |
| $L^{+/+}$ B cells | 426 | 43.7 | 59.8 | 123.1 | 41.8 | 69.3 | 86.9 |

exhibited significant defects in rolling fraction (Figure 4C), sticking efficiency (Figure 4D), and $V_{roll}$ (Figure 4E-F; Table 2) in C2GlcNAcT-$I^{-/-}$ PLNs that was strikingly similar to the adhesive behavior of WT B cells (Figure 2C-D). Thus, C2GlcNAcT-I modification of PLN HEV glycans specifies how much L-selectin is needed on a circulating lymphocyte for efficient tethering (which determines the rolling fraction) and subsequent slow rolling in PLN HEVs.

### HEC-GlcNAc6ST–dependent HEV glycans also lower the threshold requirement for L-selectin levels, but their function is distinct from that of C2GlcNAcT-I–dependent glycans

HEC-GlcNAc6$ST^{-/-}$ mice have a 50% reduction in total lymphocyte homing to PLNs.[24,32] However, recent IVM experiments have shown that the rolling fraction of unsorted lymphocytes was not different from that of WT HEVs, whereas $V_{roll}$ was increased and the sticking fraction was markedly decreased in HEC-GlcNAc6$ST^{-/-}$ PLN venules.[26] To examine the role of HEC-GlcNAc6ST–dependent glycans in more detail, we compared the trafficking of T and B cells in HEC-GlcNAc6$ST^{-/-}$ mice. Similar to the C2GlcNAcT-$I^{-/-}$ PLNs, there was, on average, a 16-fold difference between T- and B-cell homing to HEC-GlcNAc6$ST^{-/-}$ PLNs compared with a 5-fold difference in WT PLNs (Figure 5A). Although the T- and B-cell homing ratios in mutant recipients were higher (in 5 of 6 independent experiments), this difference did not reach statistical significance ($P = .25$). Compared with WT PLNs, the absolute numbers of homed T and B cells in HEC-GlcNAc6$ST^{-/-}$ PLNs were reduced by 35% (Figure 5B) and 65%, respectively (Figure 5C). Thus, HEC-GlcNAc6ST deficiency, like C2GlcNAcT-I deficiency, preferentially affects B-cell homing. This finding was reflected in a dramatic reduction in resident B cells in HEC-GlcNAc6$ST^{-/-}$ PLNs, which was significantly more severe than that of CD4 or CD8 T cells (Table 1).

As predicted by earlier studies with mixed lymphocytes,[26] the frequency of T-cell rolling in PLN venules was not affected in HEC-GlcNAc6$ST^{-/-}$ mice (Figure 5D), whereas B-cell rolling was slightly lower, but this difference was only statistically significant in order V HEVs (Figure 5F). However, $V_{roll}$ of T cells, and especially of B cells, in HEC-GlcNAc6$ST^{-/-}$ PLNs was markedly faster than in WT PLNs (Figure 6; Table 2). This finding helps explains why B and T cells fail to undergo firm adhesion in venular orders IV and V (Figure 5E, G), despite their near normal rolling

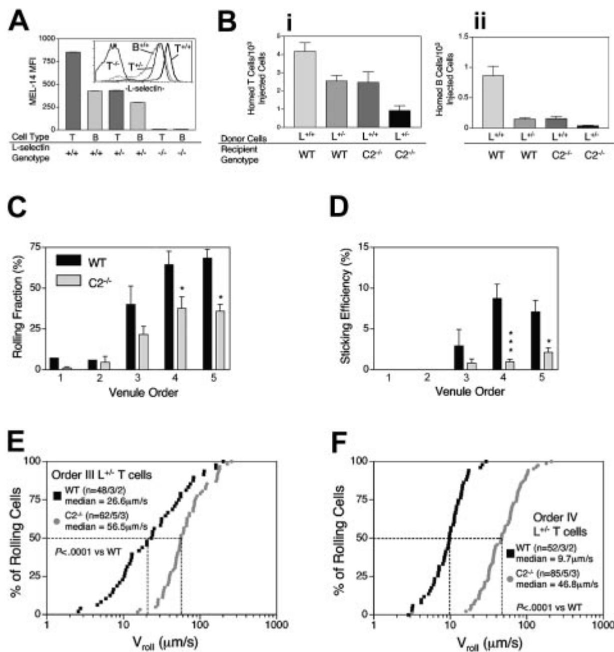



**Figure 4. Effect of reducing L-selectin levels on lymphocyte trafficking to PLNs.** (A) Expression of L-selectin on WT, $L^{+/-}$ (L-selectin$^{+/-}$), and $L^{-/-}$ T and B cells. n = 3 mice per group. (inset) Histogram depicting L-selectin mean fluorescence intensity (MFI) of WT T cells ($T^{+/+}$, thick solid line), WT B cells ($B^{+/+}$, solid grey line), $L^{+/-}$ ($T^{+/-}$), and $L^{-/-}$ ($T^{-/-}$) T cells (solid lines). (B) One-hour homing of T cells (i) and B cells (ii) to WT and C2GlcNAcT-$I^{-/-}$ PLNs. For T cells, 1-way ANOVA with Bonferroni multiple comparison test revealed statistical significance ($P < .05$) between $L^{+/+}$ cells in WT mice and $L^{+/-}$ cells in $C2^{-/-}$ mice. For B cells, 1-way ANOVA with Bonferroni multiple comparison test revealed statistical significance between $L^{+/+}$ cells in WT mice ($P < .05$), $L^{+/+}$ cells in WT mice and $L^{+/+}$ cells in $C2^{-/-}$ mice ($P < .01$), and $L^{+/+}$ cells in WT mice and $L^{+/-}$ cells in $C2^{-/-}$ mice ($P < .01$). When separate 2-group comparisons were performed using multiple unpaired 2-tailed Student *t* test, $L^{+/-}$, T- and B-cell homing to WT PLNs was significantly different from that of all other groups ($P < .05$), except for $L^{+/+}$ homing to $C2^{-/-}$ PLNs. n = 5 mice per group. Rolling fraction (C) and sticking efficiency (D) of $L^{+/-}$ T cells in WT and C2GlcNAcT-$I^{-/-}$ PLN venules. WT: n = 2 mice (order I, n = 1 venule; order II, 1; order III, 3; order IV, 6; order V, 5). C2GlcNAcT-$I^{-/-}$: n = 4 mice (order I, 3 venules; order II, 4; order III, 8; order IV, 9; order V, 2). *$P < .05$, and ***$P < .0001$ compared with WT. (E-F) Rolling velocities of $L^{+/-}$ T cells in order III (E) and order IV (F) venules WT and C2GlcNAcT-$I^{-/-}$ PLN.

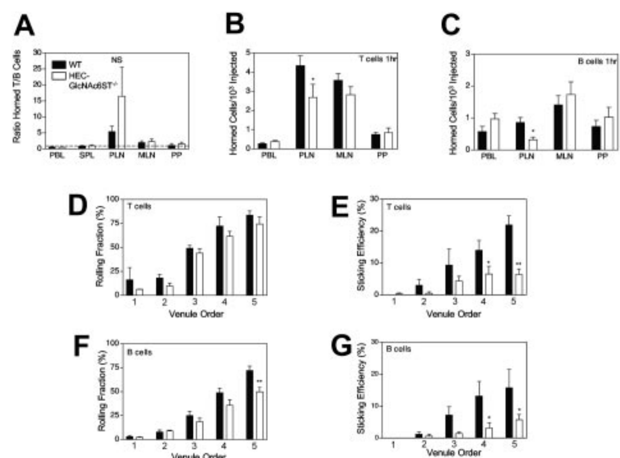



**Figure 5. Differential T- and B-cell trafficking in HEC-GlcNAc6$ST^{-/-}$ PLNs.** (A) The ratio of homed T and B cells to WT and HEC-GlcNAc6ST lymphoid organs. One-hour homing of (B) T cells and (C) B cells to WT and HEC-GlcNAc6$ST^{-/-}$ lymphoid organs. n = 5 to 6 mice per group. (D-E) T-cell and (F-G) B-cell rolling fraction (D, F) and sticking efficiency (E, G) in WT and HEC-GlcNAc6$ST^{-/-}$ PLN venules. NS indicates not significant. *$P < .05$ and **$P < .01$ compared with WT. WT for T cells: n = 4 mice (order I, 2 venules; order II, 5; order III, 6; order IV, 5; order V, 5); for B cells: n = 4 mice (order I, 5 venules; order II, 8; order III, 12; order IV, 9; order V, 9 venules). HEC-GlcNAc6ST for T cells: n = 2 mice (order I, 2 venules; order II, 3; order III, 5; order IV, 7; order V, 5); for B cells: n = 3 mice (order I, n = 3 venules; order II, 5; order III, 10; order IV, 13; order V, 11).

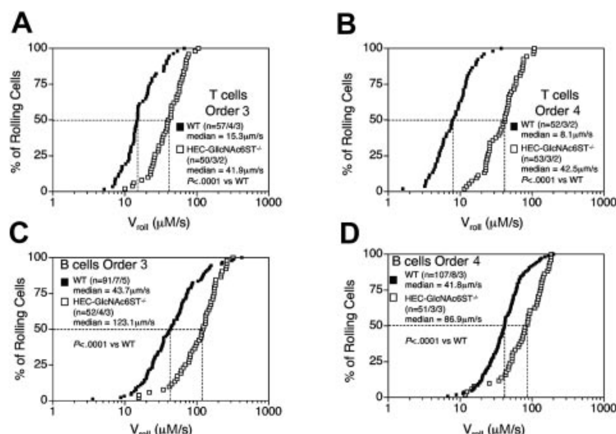



**Figure 6. Cumulative lymphocyte rolling velocities in PLN venules of WT and HEC-GlcNAc6ST$^{-/-}$ mice.** $V_{roll}$ of $L^{+/-}$ T cells in order 3 (A) and order 4 (B) PLN venules rolling velocity in order 3 and order 4 venules. B-cell rolling velocity in order 3 (C) and order 4 (D) venules. n = total number of cells/venules/mice analyzed per group.

fractions (Figure 5D, F). Consistent with these findings, $L^{+/-}$ T cells rolled at a normal frequency in HEC-GlcNAc6ST$^{-/-}$ HEVs, but their $V_{roll}$ was even more dramatically increased than that of $L^{+/+}$ T cells, and their ability to undergo firm arrest was as compromised as that of B cells (Supplemental Figure 1 on the *Blood* website; Table 2).

Together, these findings clearly demonstrate that the dependence of lymphocytes on C2GlcNAcT-I– and HEC-GlcNAc6ST–derived glycans for PLN homing is inversely correlated with L-selectin levels. In addition, each enzyme makes subtly distinct contributions to different phases of L-selectin engagement with HEV glycans. C2GlcNAcT-I generates glycans involved in the tethering and established rolling phase, whereas HEC-GlcNAc6ST–dependent sulfoconjugates have little or no impact on tethering but are indispensable for subsequent slowing of the tethered cells.

## Discussion

Here we demonstrate that the trafficking of T and B cells to PLNs exhibits different levels of sensitivity to changes in carbohydrate modification on PLN HEVs. Although it is well known that these 2 lymphocyte populations home differently to SLOs,[9,10] differential susceptibility of T and B cells to distinct HEV glycan modifications had not been established previously. We show that the physiologic approximately 6-fold difference in short-term homing efficiency of T cells compared with B cells in WT PLNs was increased to as much as approximately 16-fold in C2GlcNAcT-I$^{-/-}$ and HEC-GlcNAc6ST$^{-/-}$ PLNs. A selective defect in B-cell homing to PLNs in the absence of C2GlcNAcT-I or HEC-GlcNAc6ST was apparent in longer-term (24-hour) homing studies. This trafficking defect was also manifest in a decreased steady state number of resident B cells in C2GlcNAcT-I$^{-/-}$ and HEC-GlcNAc6ST$^{-/-}$ PLNs. In perfect agreement with these observations, our IVM analysis revealed that L-selectin–dependent tethering and rolling of B cells in PLN HEVs was always more severely affected than that of T cells, resulting in a disproportionate reduction in B-cell sticking.

T cells exhibited markedly increased sensitivity to C2GlcNAcT-I and HEC-GlcNAc6ST deficiencies when their expression of L-selectin was reduced to levels found on WT B cells. Thus, modifications of HEV glycoproteins by C2GlcNAcT-I and HEC-GlcNAc6ST are particularly essential for the recruitment of lymphocyte subsets with low levels of L-selectin. Interestingly, though the magnitude of the homing defect was similar in both mutant mouse strains, our IVM observations suggest distinct functions for each enzyme. C2GlcNAcT-I activity was critical for initial tethering of lymphocytes with low levels of L-selectin (B and $L^{+/-}$ T cells) and for slow rolling of all lymphocytes in HEV. In contrast, HEC-GlcNAc6ST deficiency had little or no effect on lymphocyte tethering but had an even greater impact on $V_{roll}$ than C2GlcNAcT-I–dependent glycans. These findings are consistent with other IVM observations,[26] but in vitro studies using parallel-plate flow chambers suggest that HEC-GlcNAc6ST–dependent twin 6-sulfo sLe$^{X}$ on core 1 and core 2 branches on the same O-glycans is the most effective substrate for lymphocyte tethering.[23,25] Although it remains theoretically possible that another sulfotransferase generates ligands for L-selectin tethering, our in vivo data strongly suggest that glycans that depend on HEC-GlcNAc6ST sulfation are not required for the initiation of B- or T-cell rolling in the complex physiologic milieu of HEV.

A distinguishing characteristic between lymphocyte subsets with respect to homing receptor expression is the approximately 2-fold higher level of L-selectin on T cells compared with B cells.[10] In addition, the 2 populations also respond to different chemokine signals that trigger integrin-mediated arrest in PLN HEVs. Although naive T cells rely almost exclusively on CCL21/CCL19 signaling through CCR7,[40-43] B cells use both CCL21/CCL19-CCR7 and CXCL12/CXCR4 signaling, and probably even CXCL13/CXCR5 signaling.[44,45] Thus, differences between T- and B-cell trafficking could be a consequence of differential L-selectin expression or differential usage of chemokine receptors, or both. However, T cells that are genetically deficient in one *L-selectin* allele express the same amount of L-selectin and home to PLNs at least as efficiently as B cells do[10] (Figure 4B). If one assumes that heterozygosity for L-selectin does not affect T-cell responses to chemokines, it must be concluded that B cells cannot use their broader repertoire of chemokine receptors to compensate for their relative paucity of L-selectin during homing to PLNs. Therefore, the level of surface-expressed L-selectin is an essential determinant of subset-specific lymphocyte recruitment to PLNs.

How might L-selectin engagement with HEV-expressed ligands account for this dose-response relationship? Three distinct consequences of L-selectin binding to HEV must be considered. The first is lymphocyte tethering, which determines the frequency at which free-flowing cells form a mechanically stable transient bond in a vessel. The ability of L-selectin to initiate tethering under physiologically high-shear conditions in HEV is aided by its conspicuous clustering on the tips of microvilli[39,46] and its rapid bond association rate ($k_{on}$), a hallmark of selectin-mediated interactions.[47,48] Thus, a plausible explanation for the reduced rolling fractions in C2GlcNAcT-I$^{-/-}$ HEVs may be the absence of luminal core 2 O-glycans with preferential accessibility to microvillous-expressed L-selectin on passing cells. Ligand accessibility may be determined by several nonexclusive factors, including ligand density, which is well known to influence tethering efficiency[47,48]; a specialized spatial orientation; or prominent presentation within the dense luminal glycocalyx. Consistent with this idea, it has been shown that compared with WT PLN HEVs, C2GlcNAcT-I$^{-/-}$ PLN HEVs exhibited reduced staining with an L-selectin IgM chimera.[31]

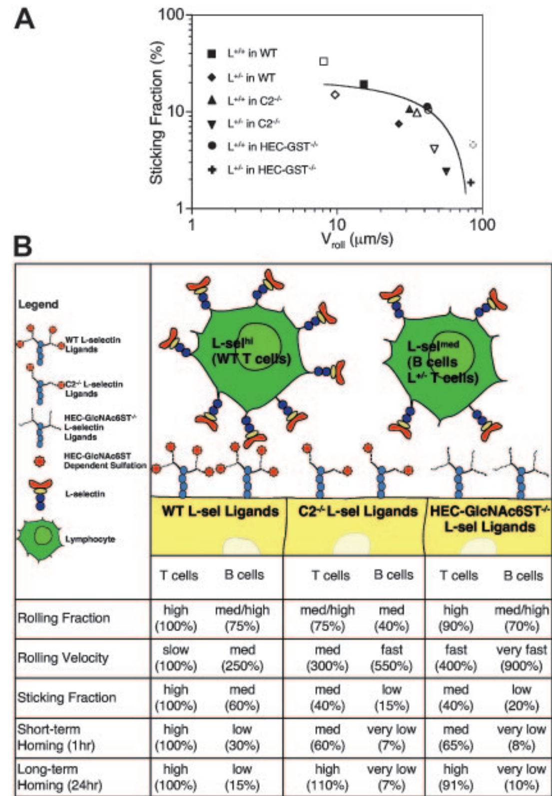
The second step in L-selectin–dependent interactions is established rolling, the strength of which is reflected in $V_{roll}$. The tensile strength and dissociation rate constant ($k_{off}$) of L-selectin bonds are critical determinants of the quality of rolling.[5,48,49] In vitro analysis

of L-selectin engagement with GlyCAM-1 has also shown that lymphocytes roll more slowly on sulfated than on unsulfated GlyCAM-1.[25] Our current and previous IVM data further indicate that carbohydrate ligand biochemistry and, in particular, sulfation determine the tensile strength and kinetics of bond formation and dissociation, as evidenced by the marked increase in $V_{roll}$ in C2GlcNAcT-I$^{-/-}$ and HEC-GlcNAc6ST$^{-/-}$ HEVs.[26] Interestingly, though core 2 O-glycans contribute to tethering, sulfation by HEC-GlcNAc6ST is apparently of minor importance for this initial step, even for B cells, because rolling fractions were near normal in HEC-GlcNAc6ST$^{-/-}$ HEVs. In contrast, a recent study of mice that were doubly deficient in C2GlcNAcT-I and HEC-GlcNAc6ST found that lymphocyte homing to PLNs was more severely affected than in singly deficient animals.[32] Thus, C2GlcNAcT-I and HEC-GlcNAc6ST probably synergize in the production of at least partially distinct glycans and together mediate slow rolling in HEVs. It is likely that this involves HEC-GlcNAc6ST–mediated sulfation of core 1 and core 2 O-glycans.

A third potential mechanism by which L-selectin can influence leukocyte trafficking is outside-in signaling. It has been suggested that surface cross-linking of L-selectin by antibodies or physiologic ligands induces integrin activation and contributes to cellular arrest and accumulation of rolling granulocytes at sites of inflammation.[50] Surface ligation of lymphocyte L-selectin by GlyCAM-1 can also induce integrin activation, at least in vitro.[51,52] However, IVM studies indicate that inhibiting integrins on rolling lymphocytes does not alter the rolling fraction or $V_{roll}$ in PLN HEVs.[8,53] In addition, though granulocytes use L-selectin to roll in HEVs, they do not stick, indicating that putative signals from L-selectin cross-linking are insufficient for integrin activation in this setting.[8] Thus, it seems unlikely that the pronounced defect in sticking efficiency in HEV of C2GlcNAcT-I$^{-/-}$ and HEC-GlcNAc6ST$^{-/-}$ mice was primarily caused by defective ligand-induced L-selectin signaling, but a minor contribution cannot be excluded.

Given the considerations outlined above, our data argue for $V_{roll}$ as the most critical criterion regarding whether a rolling lymphocyte will undergo integrin-mediated arrest in vivo. Indeed, a comparison between our data on median $V_{roll}$ and data on sticking fraction from all experimental groups revealed that a T cell's ability to stick in HEVs of orders III and IV decreased precipitously at levels greater than a $V_{roll}$ of approximately 50 μm/s (Figure 7A). If we assume that the axial length of the contact area formed between a rolling T cell and the HEV surface is approximately 5 μm, then any given surface molecule (eg, chemokine receptor or integrin) on a cell rolling at 50 μm/s will have an average contact time of 100 milliseconds. Our data predict that shorter contact intervals (caused by higher $V_{roll}$) than this estimated threshold duration will be insufficient for successful completion of the complex intracellular and extracellular molecular events that must occur to mediate T-cell sticking in PLN HEVs.

The essential role of glycosylation and posttranslational modifications, including fucosylation, sialylation, and sulfation, in generating SLO L-selectin ligands that can mediate lymphocyte homing has been known for a long time.[15] However, when we consider the effect of different levels of L-selectin expression on lymphocyte trafficking, the role of glycan modification becomes more complex. As summarized in Figure 7B, our data indicate that the impact of different glycans on leukocyte behavior in HEV depends not only on the kinetics of bond formation and dissociation but also on the density of L-selectin on the interacting cell. These findings are consistent with a previous IVM study in PPs that found that microspheres coated




| | WT L-sel Ligands | | C2-/- L-sel Ligands | | HEC-GlcNAc6ST-/- L-sel Ligands | |
|---|---|---|---|---|---|---|
| | T cells | B cells | T cells | B cells | T cells | B cells |
| Rolling Fraction | high (100%) | med/high (75%) | med/high (75%) | med (40%) | high (90%) | med/high (70%) |
| Rolling Velocity | slow (100%) | med (250%) | med (300%) | fast (550%) | fast (400%) | very fast (900%) |
| Sticking Fraction | high (100%) | med (60%) | med (40%) | low (15%) | med (40%) | low (20%) |
| Short-term Homing (1hr) | high (100%) | low (30%) | med (60%) | very low (7%) | med (65%) | very low (8%) |
| Long-term Homing (24hr) | high (100%) | low (15%) | high (110%) | very low (7%) | high (91%) | very low (10%) |

**Figure 7. Glycan modification established a threshold for L-selectin–dependent lymphocyte adhesion.** (A) Relationship between T-cell sticking fraction and $V_{roll}$ velocity. Closed and open symbols correspond to order III and IV vessels, respectively. (B) Schematic diagram summarizing the contribution of glycan modifications and L-selectin–dependent lymphocyte interaction with PLN HEVs.

with a low density of L-selectin on HEVs rolled well in WT HEVs but did so poorly in C2GlcNAcT-I$^{-/-}$ HEVs.[54] By contrast, microspheres coated with a higher density of L-selectin rolled equivalently well in HEVs of WT and C2GlcNAcT-I$^{-/-}$ PPs.

One caveat to the interpretation of our findings in C2GlcNAcT-I$^{-/-}$ PLN HEVs is the so-called secondary tethering phenomenon, whereby free-flowing leukocytes can use L-selectin to bind to P-selectin glycoprotein ligand (PSGL)–1 on leukocytes that already adhere to the vessel wall.[55-58] Because the selectin ligand activity of PSGL-1 strictly depends on modification by C2GlcNAcT-I,[31] we asked whether the homing phenotype of C2GlcNAcT-I$^{-/-}$ mice could have been caused by defective secondary tethering in HEVs. However, there was no significant difference in B- or T-cell homing to PLNs of WT or PSGL-1$^{-/-}$ mice (data not shown), indicating that the defect in lymphocyte recruitment to C2GlcNAcT-I$^{-/-}$ mice was caused by defective L-selectin ligands on endothelial cells, not on leukocytes.

PLN L-selectin engagement with HEV ligands represents the critical first step in the multistep cascade for lymphocyte recruitment to these organs.[2] In other SLOs, notably MLNs and PPs, $\alpha_4\beta_7$ integrins are involved in lymphocyte rolling on HEV-expressed MAdCAM-1.[59] Although L-selectin can tether to MAdCAM-1, studies in L-selectin knockout mice have shown that the role of L-selectin in homing to PPs and MLNs is less critical than in PLNs.[6] Because the $\alpha_4\beta_7$–MAdCAM-1 pathway is thought to be independent of core 2 glycans or GlcNAc sulfation, it is not unexpected that B and T cells homed normally to PPs and MLNs in C2GlcNAcT-I$^{-/-}$ and HEC-GlcNAc6ST$^{-/-}$ mice.

The physiologic significance of these observations is suggested by the differential effect of C2GlcNAcT-I and HEC-GlcNAc6ST expression on the recruitment of L-selectin$^{high}$ compared with

L-selectin$^{low/med}$ cells. This could constitute a mechanism by which endothelial cells fine-tune their function as gatekeepers of tissues. For example, in the presence of suitable chemoattractants, L-selectin$^{high}$ granulocytes and naive T cells are effectively recruited to nonlymphoid tissues by interaction with L-selectin ligands in flat-walled venules that are not thought to express HEC-GlcNAc6ST.[60-62] Our results predict that L-selectin$^{low}$ B cells would be unable to access such sites. On the other hand, it has been shown that during lymphoid neogenesis in chronically inflamed tissues, the expression of C2GlcNAcT-I and HEC-GlcNAc6ST is induced when normally flat-walled venules transform into HEVs.[32,63] This should allow effective recruitment of B cells and other L-selectin$^{low}$ lymphocytes, such as effector memory T cells, that begin to reexpress L-selectin during their slow conversion to L-selectin$^{+}$ central memory cells.[64]

In conclusion, this study illustrates that endothelial glycan modifications by C2GlcNAcT-I and HEC-GlcNAc6ST differentially control the composition of lymphocytes (and perhaps other leukocytes) recruited to PLNs by exerting distinct effects on L-selectin$^{high}$ and L-selectin$^{low}$ T cells and B cells, respectively. In future studies, it will be informative to evaluate whether the homing defect of T cells, but especially B cells, in C2GlcNAcT-I$^{-/-}$ and HEC-GlcNAc6ST$^{-/-}$ mice results in a significant alteration in T- or B-cell immunologic function.

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