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Critical functions of *N*-glycans in L-selectin-mediated lymphocyte homing and recruitment

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Lymphocyte homing is mediated by specific interaction between L-selectin on lymphocytes and the carbohydrate ligand 6-sulfo sialyl Lewis X on high endothelial venules. Here we generated mice lacking both core 1 extension and core 2 branching enzymes to assess the functions of *O*-glycan-borne L-selectin ligands *in vivo*. Mutant mice maintained robust lymphocyte homing, yet they lacked *O*-glycan L-selectin ligands. Biochemical analyses identified a class of *N*-glycans bearing the 6-sulfo sialyl Lewis X L-selectin ligand in high endothelial venules. These *N*-glycans supported the binding of L-selectin to high endothelial venules *in vitro* and contributed *in vivo* to *O*-glycan-independent lymphocyte homing in wild-type and mutant mice. Our results demonstrate the critical function of *N*-glycan-linked 6-sulfo sialyl Lewis X in L-selectin-dependent lymphocyte homing and recruitment.

Lymphocytes encounter foreign antigens in lymph nodes and Peyer's patches. A very small proportion of antigen-specific T lymphocytes and B lymphocytes trigger an acquired immune response after interacting with antigen-presenting cells and dendritic cells[1]. Antigen-presenting cells and dendritic cells enter lymph nodes through afferent lymphoid vessels, but lymphocytes enter lymph nodes through the specialized vasculature of high endothelial venules (HEVs)[2]. This process, called 'lymphocyte homing', is essential for immune surveillance and depends on specific interaction between the lymphocyte-homing receptor L-selectin and its ligands specifically expressed on HEVs[3,4]. L-selectin-dependent interactions mediate lymphocyte tethering and rolling that trigger chemokine-dependent activation, integrin-mediated firm attachment to the endothelium and eventual cell transmigration across HEVs[1,5,6]. Studies have shown that natural killer cells are also recruited to lymph nodes in an L-selectin-dependent way[7,8].

L-selectin on lymphocytes is a carbohydrate-binding protein that binds to ligands on HEVs in a calcium-dependent way. The function of those ligands depends entirely on their 'decoration' with the specific carbohydrate 6-sulfo sialyl Lewis X (sialyl-(2-3)-galactopyranosyl-(1-4)-*N*-acetylglucosamine-((1-3)-fucopyranosyl); sLe^X), whose structure is sialic acidα2→3Galβ1→4[Fucα1→3(sulfo→6)]GlcNAc[4]. The essential function of sialic acid in the formation of L-selectin ligands has been demonstrated by experiments showing that the treatment of lymph node slices with sialidase abrogates L-selectin-dependent binding of lymphocytes to HEVs[4]. The pivotal function of fucosylation of L-selectin ligands has been established by genetic studies of mice. Mice lacking fucosyltransferase VII (FucT-VII) or both FucT-VII and FucT-IV have 80% or more than 95%, respectively, less homing of lymphocytes to lymph nodes[9,10]. Mice lacking both FucT-IV and FucT-VII have fewer lymph node cells than do wild-type mice, indicating that these enzymes work together in the synthesis of L-selectin ligands. L-selectin ligands are unique in requiring the sulfation of carbohydrates for optimal function. Indeed, studies have shown that the synthesis of 6-sulfo sLe^X in L-selectin ligands requires two 6-sulfotransferases, specifically HEV-restricted *N*-acetylglucosamine-6-*O*-sulfotransferase-2 (GlcNAc6ST-2) and ubiquitously expressed GlcNAc6ST-1 (refs. 11,12). Mice lacking both sulfotransferases show impaired lymphocyte homing and contact hypersensitivity associated with complete loss of the 6-sulfate group in 6-sulfo sLe^X (ref. 11).

HEV-bound L-selectin ligands carrying 6-sulfo sLe^X include the glycoproteins GlyCAM-1, CD34, MAdCAM-1, podocalyxin-like protein, endomucin and endoglycan, all of which have mucin-like domains that function as scaffolding for *O*-linked oligosaccharides[4]. Both GlcNAc6ST-1 and GlcNAc6ST-2 can form 6-sulfo sLe^X on core 2–branched *O*-glycans, one of the most abundant *O*-linked oligosaccharides. 6-sulfo sLe^X on core 2–branched *O*-glycans in CD34 mediates more L-selectin-dependent adhesion than does unsulfated sLe^X on the same *O*-glycans[13].

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In HEVs, core 2 branches are formed by core 2 β1,6-*N*-acetylglucosaminyltransferase-1 (Core2GlcNAcT)[14]. However, mice lacking Core2GlcNAcT have only slightly less lymphocyte homing[15,16]. Studies of such mice have shown that 6-sulfo sLe^X on extended core 1 *O*-linked oligosaccharides (sialic acidα2→3Galβ1→4[Fucα1→3(sulfo→6)]GlcNAcβ1→3Galβ1→3GalNAcα→R) function as the remaining L-selectin ligands[17]. The same studies have shown that the antibody MECA-79 binds to 6-sulfo *N*-acetyllactosamine and its sialylated fucosylated form, 6-sulfo sLe^X, in extended core 1 oligosaccharide. MECA-79 specifically recognizes the lumenal surface of HEVs and inhibits lymphocyte adhesion to HEVs *in vivo* and *ex vivo*[18].

Extended core 1 structures are formed by the core 1 extension enzyme Core1-β3GlcNAcT (also called β3GlcNAcT-3)[17]. Expression of Core1-β3GlcNAcT together with either GlcNAc6ST-1 or GlcNAc6ST-2 leads to expression of the MECA-79-specific antigen in Chinese hamster ovary (CHO) cells. Mice lacking both GlcNAc6ST-1 and GlcNAc6ST-2 sulfotransferases show no expression of MECA-79-specific antigen in any secondary lymphoid organs[11,12]. However, it is not known if, among the various β3GlcNAcT enzymes, Core1-β3GlcNAcT is solely responsible for the synthesis of the MECA-79-specific antigen backbone.

To address that issue, we generated mice lacking Core1-β3GlcNAcT. Although expression of MECA-79-specific antigen was abolished in these mice, considerable lymphocyte-homing activity remained. We then hypothesized that elimination of both extended core 1 and core 2 branches would eliminate 6-sulfo sLe^X on *O*-linked oligosaccharides. To test our hypothesis, we generated mice lacking both Core1-β3GlcNAcT and Core2GlcNAcT (called 'double-knockout mice' here). Indeed, GlyCAM-1 from double-knockout mice contained no detectable sulfated *O*-linked oligosaccharide and did not support lymphocyte rolling. Intravital microscopy showed that lymphocyte rolling in double-knockout mice was lower because of higher velocity of rolling cells. This defect, however, resulted in only minimally lower lymphocyte numbers in peripheral and mesenteric lymph nodes, and contact hypersensitivity was only marginally compromised. Notably, enzymatic removal of *N*-glycans attached to HEVs and CD34 abolished the remaining L-selectin ligand activity. Similarly, treatment with *N*-glycan-specific lectins resulted in inhibition of lymphocyte homing in both wild-type and double-knockout mice. We conclude that 6-sulfo sLe^X on *N*-glycans has a critical function as an L-selectin ligand and is essential for lymphocyte trafficking in normal and disease conditions.

RESULTS

Mice deficient in Core1-β3GlcNAcT

Core1-β3GlcNAcT is thought to be critical in L-selectin ligand synthesis (**Supplementary Fig. 1** online). To determine its physiological function, we eliminated Core1-β3GlcNAcT by mutagenesis of the gene encoding it. We replaced a genomic region containing most of exon 3 and all of exon 4 with cDNA encoding enhanced green fluorescent protein (EGFP) and a neomycin-resistance gene cassette. After homologous recombination, the resulting gene encoded the cytoplasmic, transmembrane and stem regions of Core1-β3GlcNAcT (amino acids 1–33) fused to EGFP and was driven by the promoter of the gene encoding Core1-β3GlcNAcT (**Supplementary Fig. 1**). The cytoplasmic, transmembrane and stem regions of Golgi-associated enzymes direct Golgi retention[19], and this fusion protein was indeed transported to the same Golgi compartment as the intact protein (data not shown). We injected targeted embryonic stem cells into C57BL/6 blastocysts, and crossed F_1 mice with chimeric mice to produce Core1-β3GlcNAcT-deficient mice, as determined by Southern blot analysis (**Supplementary Fig. 1**). Intercrosses of heterozygous progeny yielded litters of normal size with the expected mendelian transmission of alleles. Animals homozygous for the mutation lacked overt physical, neurological or reproductive defects.

Ablation of Core1-β3GlcNAcT resulted in complete loss of MECA-79-specific antigen in HEVs of peripheral lymph nodes (PLNs), mesenteric lymph nodes (MLNs), and Peyer's patches, as assessed by immunofluorescence staining and immunoblot analysis of proteins extracted from lymph nodes (**Fig. 1**). These results indicated that Core1-β3GlcNAcT is solely responsible for extension of core 1, which forms a backbone for the MECA-79-specific epitope with the structure Galβ1→4(sulfo→6)GlcNAcβ1→3Galβ1→3GalNAcα→R (**Supplementary Fig. 1**). The loss of MECA-79-specific antigen was accompanied by substantially higher *in vivo* rolling velocity of both T lymphocytes and B lymphocytes in the inguinal lymph nodes, as assessed by intravital microscopy (**Fig. 2a**). In these lymph nodes, paracortical HEVs can be divided into orders III–V. Order V is formed by merging capillaries[20]; orders I and II are flat walled and are located mainly in the medulla. Rolling and sticking of B lymphocytes was affected more severely than that of T lymphocytes (**Fig. 2b**). The lower sticking and altered rolling of T and B lymphocytes in these mice resembled the phenotype of Core2GlcNAcT-deficient mice[21]. In contrast, Core1-β3GlcNAcT-knockout mice and wild-type mice had similar total numbers of lymphocytes in secondary lymphoid organs, although Core1-β3GlcNAcT-knockout mice had fewer B lymphocytes in lymph nodes, which was compensated for by an increase in T lymphocytes (**Fig. 2c,d**). These results are consistent with published reports showing that reductions in rolling and sticking are more evident in B lymphocytes than in T lymphocytes when L-selectin ligands

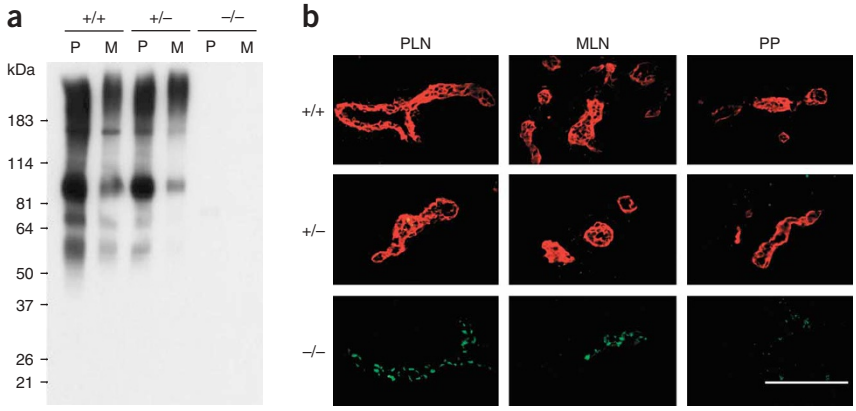

Figure 1 Core1-β3GlcNAcT-deficient mice have no MECA-79 antigen. (**a**) Immunoblot analysis with MECA-79 of detergent extracts of lymph node stroma of PLNs (P) and MLNs (M) from wild-type mice (+/+) and mice heterozygous (+/–) or homozygous (–/–) for Core1-β3GlcNAcT deficiency. Left margin, molecular size in kilodaltons (kDa). (**b**) Expression of MECA-79-binding antigen (red) and Core1-β3GlcNAcT–EGFP fusion protein (green) in frozen sections of PLNs, MLNs and Peyer's patches (PP) from wild-type mice and mice heterozygous or homozygous for Core1-β3GlcNAcT deficiency. Scale bar, 50 μm. Data are representative of two independent experiments.

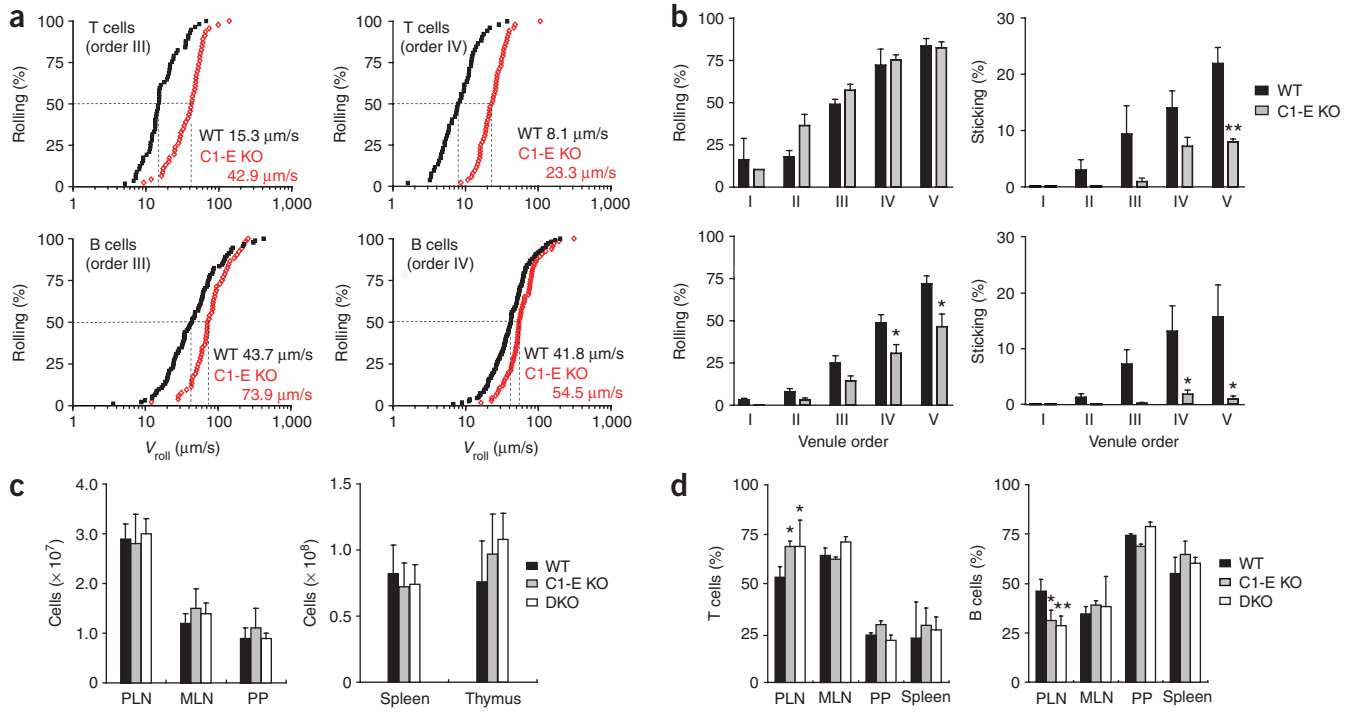

Figure 2 Lymphocyte trafficking in Core1-β3GlcNAcT-deficient mice. (**a**) Cumulative rolling velocity (V_{roll}) of T lymphocytes and B lymphocytes in order III and IV venules of inguinal lymph nodes from wild-type mice (WT) and Core1-β3GlcNAcT-deficient mice (C1-E KO), analyzed by intravital microscopy. Data represent percent cells rolling at or below a given velocity. Numbers in keys indicate median rolling velocity. (**b**) Rolling fraction (left) and sticking efficiency (right) of T lymphocytes (top) and B lymphocytes (bottom) in venule trees of PLNs from wild-type and Core1-β3GlcNAcT-deficient mice, analyzed by intravital microscopy. (**c**,**d**) Total lymphocytes (**c**) and relative frequency of T lymphocytes ($CD3^+$) and B lymphocytes ($CD19^+$; **d**; T + B = 100%). DKO, double-knockout. *, $P < 0.05$, and **, $P < 0.01$, compared with wild-type. Data (mean + s.e.m., **b**–**d**) are representative of two independent experiments with four to six mice for each genotype.

are decreased, most likely because B lymphocytes express approximately half the copies of L-selectin relative to those on the surfaces of T lymphocytes[12,21].

Inactivation of both Core1-β3GlcNAcT and Core2GlcNAcT

The results reported above indicated that L-selectin ligands remained accessible after abrogation of extended core 1–based mucin-type *O*-glycans, including the MECA-79-specific antigen. Published studies have shown that the L-selectin ligand 6-sulfo sLe^X is present in HEVs as a capping structure on both extended core 1 and core 2 branches[17,22]. To assess the function of L-selectin ligands on such mucin-type *O*-linked oligosaccharides, we crossed Core1-β3GlcNAcT-deficient mice with Core2GlcNAcT-deficient mice, generating double-knockout mice. Double-knockout mice, like Core1-β3GlcNAcT-deficient mice, had no MECA-79-specific antigen on HEVs from PLNs, MLNs or Peyer's patches (data not shown). In contrast,

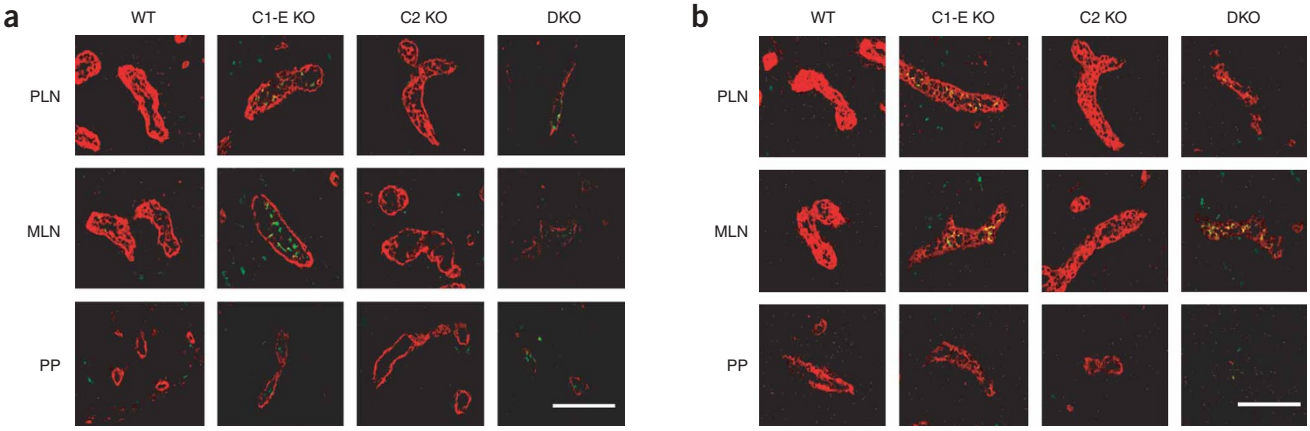

Figure 3 Expression of L-selectin and E-selectin ligands. Frozen sections of PLNs, MLNs and Peyer's patches from wild-type, Core1-β3GlcNAcT-deficient, Core2GlcNAcT knockout (C2 KO) and double-knockout mice were stained with L-selectin–IgM (**a**) or E-selectin–IgM (**b**). Green fluorescence is from the Core1-β3GlcNAcT–EGFP chimeric protein. Data are representative of ten independent experiments.

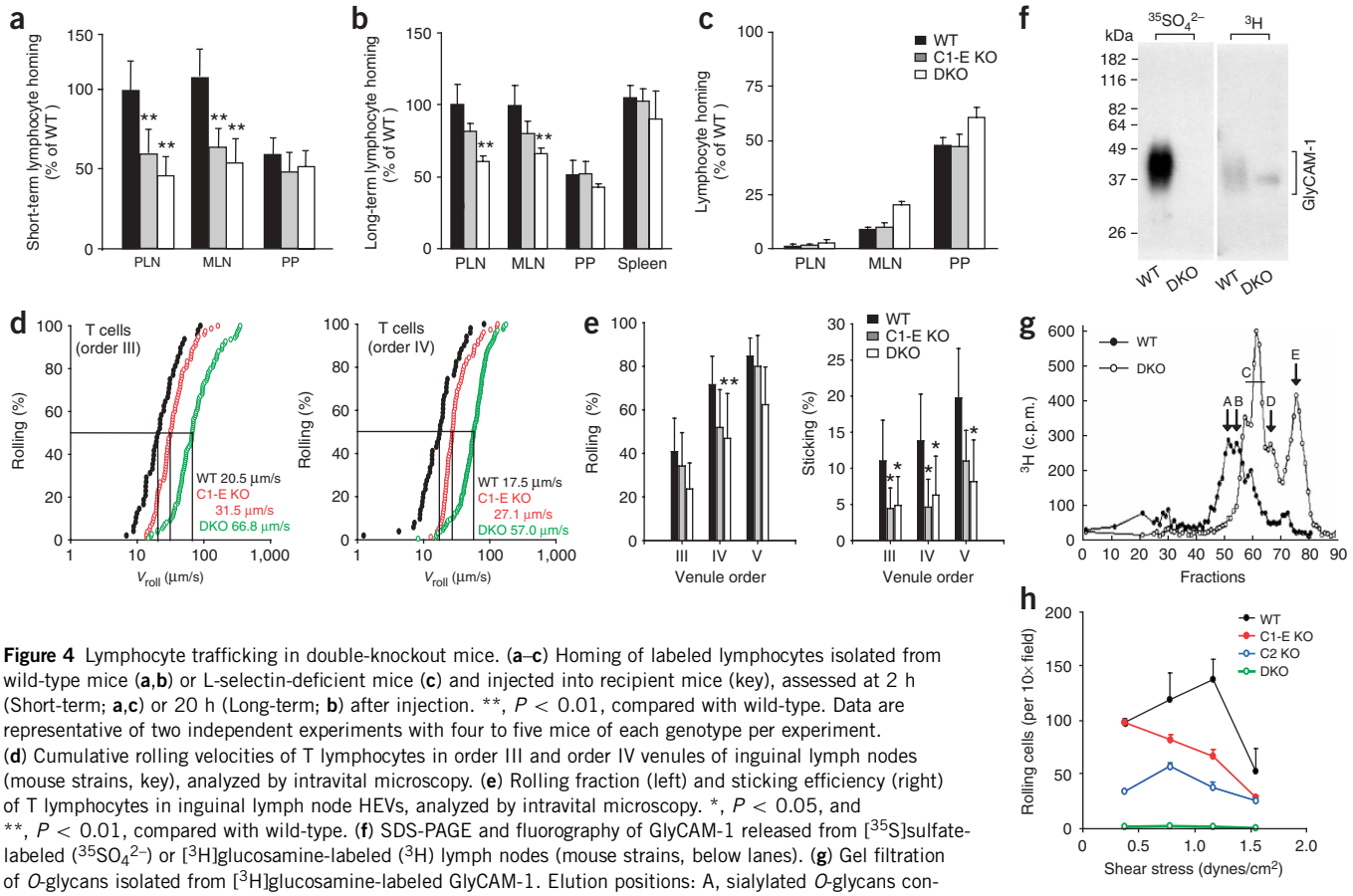

Figure 4 Lymphocyte trafficking in double-knockout mice. (**a**–**c**) Homing of labeled lymphocytes isolated from wild-type mice (**a**,**b**) or L-selectin-deficient mice (**c**) and injected into recipient mice (key), assessed at 2 h (Short-term; **a**,**c**) or 20 h (Long-term; **b**) after injection. **, $P < 0.01$, compared with wild-type. Data are representative of two independent experiments with four to five mice of each genotype per experiment. (**d**) Cumulative rolling velocities of T lymphocytes in order III and order IV venules of inguinal lymph nodes (mouse strains, key), analyzed by intravital microscopy. (**e**) Rolling fraction (left) and sticking efficiency (right) of T lymphocytes in inguinal lymph node HEVs, analyzed by intravital microscopy. *, $P < 0.05$, and **, $P < 0.01$, compared with wild-type. (**f**) SDS-PAGE and fluorography of GlyCAM-1 released from [^{35}S]sulfate-labeled ($^{35}SO_4^{2-}$) or [^{3}H]glucosamine-labeled (^{3}H) lymph nodes (mouse strains, below lanes). (**g**) Gel filtration of *O*-glycans isolated from [^{3}H]glucosamine-labeled GlyCAM-1. Elution positions: A, sialylated *O*-glycans containing both core 1 extensions and core 2 branches; B, sialylated *O*-glycans containing core 2 branches or core 1 extensions; C, sialylated Galβ1→3GalNAcOH; D, Galβ1→3GalNAcOH; E, GalNAcOH. (**h**) Lymphocyte rolling on GlyCAM-1 (mice, key). Data (mean + s.e.m., **a**–**c**,**e**) are representative of two independent experiments with four to five (**a**,**b**), five (**d**,**e**) or four (**f**–**h**) mice of each genotype per experiment.

HEVs of Core2GlcNAcT-deficient and wild-type mice had robust expression of MECA-79-specific antigen, as shown before[15,16].

Binding of a fusion protein of L-selectin and immunoglobulin M (L-selectin–IgM) to HEVs was moderately lower in Core1-β3GlcNAcT-deficient mice and slightly lower in Core2GlcNAcT-deficient mice (**Fig. 3a**). Binding of the L-selectin–IgM chimeric protein was even less but was still detectable in double-knockout mice. In contrast, binding of P-selectin–IgM to HEVs was not detected in any of the mice (data not shown). These results indicated that small quantities of L-selectin ligands were still present even after abrogation of both Core1-β3GlcNAcT and Core2GlcNAcT. Binding of E-selectin–IgM was lower and followed the same incremental pattern as L-selectin–IgM in the mutant mice, consistent with a published report[23] showing that E-selectin also binds to 6-sulfo sLe^x (**Fig. 3b**). As E-selectin apparently has higher binding affinity than L-selectin, we also used E-selectin–IgM thereafter to detect L-selectin ligands of low abundance.

The homing of lymphocytes to PLNs in double-knockout mice was 45% and 65% that of wild-type mice in 2-hour and 20-hour assays, respectively (**Fig. 4a**,**b**). There was almost no homing of L-selectin-deficient lymphocytes to PLNs, indicating that the lymphocyte homing remaining in the double-knockout mice also depended on L-selectin-mediated adhesion (**Fig. 4c**). As predicted, lymphocyte homing in wild-type but not Core1-β3GlcNAcT-deficient mice was inhibited by MECA-79 (**Supplementary Fig. 2** online). Analysis by intravital microscopy showed that the rolling velocity of T lymphocytes in venule orders III and IV was three times higher in double-knockout mice than in wild-type mice and this was accompanied by less rolling and sticking of cells (**Fig. 4d**,**e**). We suspect that sticking of B lymphocytes would be more severely compromised than that of T lymphocytes, as the number of lymph node–resident B lymphocytes was much lower in double-knockout mice (**Fig. 2d**). Despite the much lower lymphocyte rolling and homing to PLNs and MLNs in double-knockout mice, we found no changes in the total numbers of lymphocytes recovered from secondary lymphoid tissues (**Fig. 2c**). Overall, these results indicated that L-selectin ligands remaining in double-knockout mice supported lymphocyte trafficking that was sufficiently robust to maintain a population of recirculating lymphocytes in PLNs and MLNs essentially similar to that in wild-type mice.

L-selectin ligands in double-knockout mice

The results reported above could be explained if *O*-linked oligosaccharides other than extended core 1 and core 2 branched structures carried 6-sulfo sLe^X. Alternatively, glycans other than *O*-linked oligosaccharides could function as L-selectin ligands in double-knockout mice. To determine which was the case, we immunoprecipitated the mouse glycoprotein GlyCAM-1, which lacks *N*-glycans[24], from lymph nodes after metabolic labeling with [^{35}S]sulfate or [^{3}H]glucosamine. [^{35}S]sulfate-labeled GlyCAM-1 was immunoprecipitated from lymph node culture medium from wild-type but not from double-knockout mice. In contrast, [^{3}H]glucosamine-labeled GlyCAM-1 was immunoprecipitated from lymph node culture media of both wild-type and

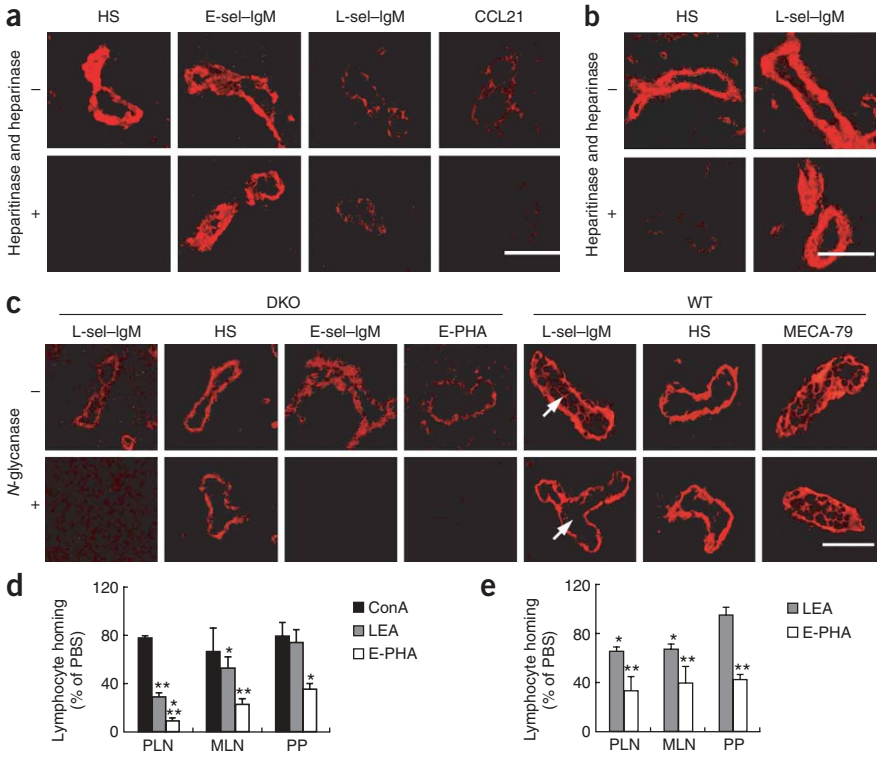

Figure 5 *N*-glycan-based L-selectin ligands in double-knockout and wild-type mice. (**a**,**b**) Double-knockout HEVs (**a**) and wild-type HEVs (**b**) stained with E-selectin–IgM (E-sel–IgM), L-selectin–IgM (L-sel–IgM), antibody 10E4 specific to heparan sulfate (HS), or antibody to CCL21 at 2 h after digestion by heparinase and heparitinase (**a**) or after injection of heparinase and heparitinase through a tail vein (**b**). (**c**) HEVs treated with *N*-glycanase and stained with L-selectin–IgM, E-selectin–IgM, E-PHA, antibody 10E4 or MECA-79. Arrows indicate slight decrease in L-selectin–IgM staining after *N*-glycanase treatment of wild-type mice. Scale bars (**a**–**c**), 50 μm. (**d**,**e**) Flow cytometry of CMRA-stained lymph nodes. Mice were injected intravenously with concanavalin A (ConA), tomato lectin (LEA) or E-PHA; 1 h later, $CMRA^+$ lymphocytes were injected into the tail veins of double-knockout mice (**d**) or wild-type mice (**e**) and lymph nodes were recovered for analysis 1 h later. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, compared with PBS control. Data are representative of ten independent experiments (**a**–**c**) or are representative of two independent experiments with four mice of each genotype in each experiment (**d**,**e**; mean + s.e.m.).

double-knockout mice in the same conditions (**Fig. 4f**). GlyCAM-1 from double-knockout mice was of lower molecular weight and seemed more homogenous than wild-type GlyCAM-1 (**Fig. 4f**), consistent with the finding that GlyCAM-1 from double-knockout mice contained simpler *O*-linked oligosaccharides because of the loss of extended core 1 and core 2 branches (**Fig. 4g**). These results indicated that GlyCAM-1 from double-knockout mice lacked 6-sulfo sLe^X in *O*-linked oligosaccharides. Consistent with that finding, GlyCAM-1 from double-knockout mice did not support lymphocyte rolling (**Fig. 4h**). GlyCAM-1 from single-knockout mice supported lymphocyte rolling, although to a lesser extent than GlyCAM-1 from wild-type mice. These results collectively indicated that L-selectin ligands are absent from mucin-type *O*-linked oligosaccharides synthesized in HEVs of double-knockout mice.

Those unexpected findings prompted us to search for glycans functioning as L-selectin ligands in the absence of L-selectin ligands on mucin-type *O*-glycans. Studies have reported that the binding of L-selectin–IgM to acutely inflamed lung endothelial cells is lower after the removal of heparan sulfate by heparitinase[25]. Treatment of frozen sections of PLNs with heparitinase (**Fig. 5a**) or injection of heparitinase through the tail vein (**Fig. 5b**) did not reduce the binding of L-selectin–IgM or E-selectin–IgM but did reduce the chemokine CCL21 on HEVs, which was associated with heparan sulfate loss. These results indicated that heparan sulfate has a negligible function as an HEV L-selectin ligand in both wild-type and double-knockout mice.

We next sought to determine if *N*-glycans function as L-selectin ligands. *N*-glycans constitute chief carbohydrates attached to proteins, and their innermost *N*-acetylglucosamine residue is linked to an asparagine residue of proteins[26]. Unexpectedly, treatment of PLN sections from double-knockout mice with *N*-glycanase resulted in complete loss of the binding of E-selectin–IgM and L-selectin–IgM to HEVs without a reduction in the binding of heparan sulfate (**Fig. 5c**). Treatment of wild-type mice with *N*-glycanase had only a small effect on HEV L-selectin ligands. However, treatment with *N*-glycanase removed almost all L-selectin ligands of wild-type PLN sections that remained after treatment with *O*-sialoglycopeptidase, which cleaves mucin-type glycoproteins (**Supplementary Fig. 3** online). Moreover, pretreatment with tomato lectin, which specifically 'decorates' the surfaces of endothelial cells[27] and binds to complex *N*-glycans (**Supplementary Fig. 4** online), resulted in significantly less homing of lymphocytes to PLNs in wild-type mice (35%), although this was less severe than that in double-knockout mice (**Fig. 5d**,**e**). Erythroagglutinating phytohemagglutinin (E-PHA), which binds sialylated *N*-glycans[28], affected lymphocyte homing more severely in double-knockout mice, although more E-PHA was needed to inhibit lymphocyte homing in wild-type mice (**Fig. 5e**). Complex *O*-glycans present in wild-type mice may have hindered the binding of E-PHA to *N*-glycans carrying L-selectin ligands. In contrast, concanavalin A, which binds high-mannose *N*-glycans devoid of 6-sulfo sLe^X, did not alter lymphocyte homing, although the staining pattern of concanavalin A, E-PHA and tomato lectin was similar for PLNs from wild-type and double-knockout mice (**Supplementary Fig. 4**). These results collectively indicated that L-selectin ligands on *N*-glycans represent almost all the remaining L-selectin ligands in double-knockout mice and a substantial portion of the L-selectin ligands in wild-type mice.

The results reported above suggested that *N*-glycans in HEVs contain 6-sulfo sLe^X functioning as an L-selectin ligand. As there has been no report to our knowledge on the presence of 6-sulfo sLe^X in *N*-glycans, we isolated *N*-glycans from PLN and MLN stroma from double-knockout and wild-type mice and analyzed them by mass spectrometry. We found a series of sulfated *N*-glycans, some of which yielded the molecular fragment ion of 6-sulfo sLe^X by tandem mass spectrometry (mass/charge = 1,093; **Fig. 6a–c**). We also confirmed the presence of 6-sulfo sLe^X by sequential exoglycosidase digestion of

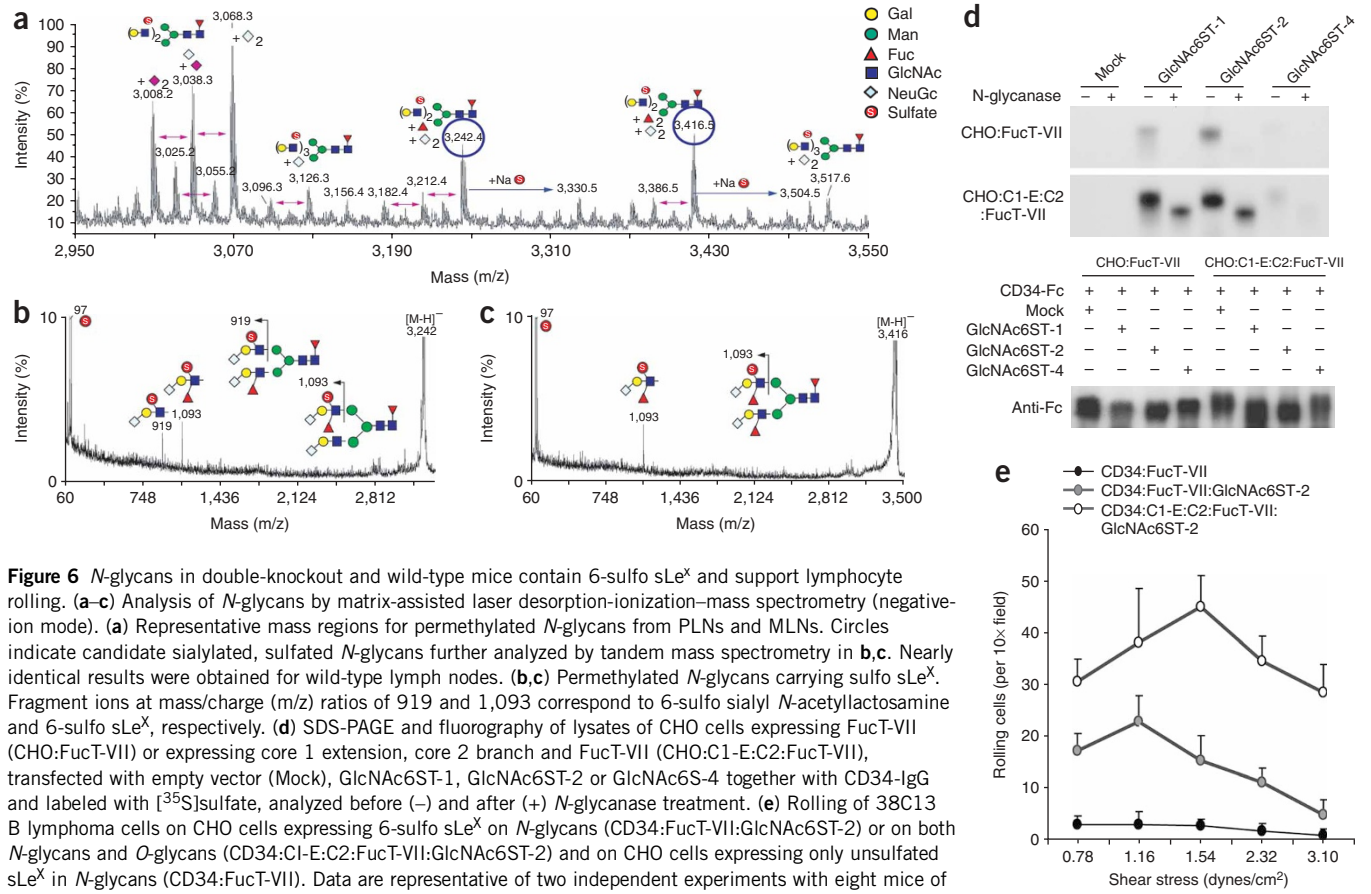

Figure 6 *N*-glycans in double-knockout and wild-type mice contain 6-sulfo sLe^x and support lymphocyte rolling. (**a**–**c**) Analysis of *N*-glycans by matrix-assisted laser desorption-ionization–mass spectrometry (negative-ion mode). (**a**) Representative mass regions for permethylated *N*-glycans from PLNs and MLNs. Circles indicate candidate sialylated, sulfated *N*-glycans further analyzed by tandem mass spectrometry in **b**,**c**. Nearly identical results were obtained for wild-type lymph nodes. (**b**,**c**) Permethylated *N*-glycans carrying sulfo sLe^X. Fragment ions at mass/charge (m/z) ratios of 919 and 1,093 correspond to 6-sulfo sialyl *N*-acetyllactosamine and 6-sulfo sLe^X, respectively. (**d**) SDS-PAGE and fluorography of lysates of CHO cells expressing FucT-VII (CHO:FucT-VII) or expressing core 1 extension, core 2 branch and FucT-VII (CHO:C1-E:C2:FucT-VII), transfected with empty vector (Mock), GlcNAc6ST-1, GlcNAc6ST-2 or GlcNAc6S-4 together with CD34-IgG and labeled with [^{35}S]sulfate, analyzed before (–) and after (+) *N*-glycanase treatment. (**e**) Rolling of 38C13 B lymphoma cells on CHO cells expressing 6-sulfo sLe^x on *N*-glycans (CD34:FucT-VII:GlcNAc6ST-2) or on both *N*-glycans and *O*-glycans (CD34:CI-E:C2:FucT-VII:GlcNAc6ST-2) and on CHO cells expressing only unsulfated sLe^X in *N*-glycans (CD34:FucT-VII). Data are representative of two independent experiments with eight mice of each genotype (**a**–**c**) or are representative of two independent experiments (**d**,**e**; mean + s.e.m. in **e**).

[^{3}H]glucosamine-labeled glycopeptides isolated from lymph nodes (**Supplementary Methods** online) by a published procedure[11,17]. Given the mass spectrometry data (**Supplementary Fig. 5** and **Supplementary Table 1** online), some lymph node *N*-glycans apparently contained two 6-sulfo sLe^X moieties in one *N*-glycan. Such bivalent *N*-glycans may provide more efficient L-selectin ligand activity than do monovalent *N*-glycans, as has been shown for bivalent *O*-linked oligosaccharides[17].

To determine whether 6-sulfo sLe^X in *N*-glycans supports lymphocyte rolling, we used CHO cells transfected with various combinations of specific glycosyltransferases[13,17]. CHO cells lack core 2 branch, core 1 extension, α1,3-linked fucose and 6-sulfo *N*-acetylglucosamine enzymes[13,14,17]. CHO cells transfected with GlcNAc6ST-1 or GlcNAc6ST-2 synthesized 6-sulfo sLe^X in *N*-glycans, as *N*-glycanase removed about a half of the [^{35}S]sulfate incorporated into CHO cells expressing core 1 extension and core 2 branching enzymes (**Fig. 6d**). Because GlcNAc6ST-2 is more abundant than GlcNAc6ST-1 in HEVs[11], we stably transfected GlcNAc6ST-2 cDNA together with CD34 cDNA and FucT-VII cDNA into CHO cells to evaluate the ability of cell surface *N*-glycans carrying 6-sulfo sLe^X to support lymphocyte rolling. We confirmed the presence of 6-sulfo sLe^X on *N*-glycans by mass spectrometry (data not shown). To obtain CHO cells carrying 6-sulfo sLe^X also in *O*-glycans, we additionally transfected Core2GlcNAcT cDNA and Core1-β3GlcNAcT cDNA. 'CD34–FucT-VII' CHO cells, devoid of 6-sulfo sLe^X, did not support lymphocyte rolling. There was lymphocyte rolling on 'CD34–FucT-VII–GlcNAc6ST-2' CHO cells, which produced 6-sulfo sLe^X only in *N*-glycans, although it was not as robust as the rolling on 'C1-E–C2–CD34–FucT-VII–GlcNAc6ST-2' CHO cells, which expressed 6-sulfo sLe^X in both *N*-glycans and *O*-glycans (**Fig. 6e**).

N-glycan L-selectin ligands in inflammation

To determine which ligands carry 6-sulfo sLe^X on *N*-glycans, we first used E-selectin–IgM to detect 6-sulfo sLe^x. Among a series of glycoproteins recognized by E-selectin–IgM, we identified the main protein (molecular mass, about 100,000) as CD34 using an antibody (**Fig. 7a**,**b**) that specifically recognizes mouse CD34 (**Supplementary Fig. 6** online). Moreover E-selectin–IgM and L-selectin–IgM bound to immunoprecipitated CD34 but not to *N*-glycanase-treated CD34 from double-knockout mice (**Fig. 7c**,**d**). After the same treatment, CD34 from wild-type mice bound E-selectin, L-selectin and MECA-79 (**Fig. 7c–e**). These results established CD34 as a chief ligand for L-selectin and showed that CD34 contains L-selectin ligands in both *N*-glycans and *O*-glycans in wild-type mice but only in *N*-glycans in double-knockout mice.

All of the measurements reported above indicated that 6-sulfo sLe^X on *N*-glycans functions as an efficient L-selectin ligand. However, L-selectin ligands on *N*- and *O*-linked oligosaccharides may differ qualitatively. After sensitization and challenge with dinitrofluorobenzene (DNFB), double-knockout mice had 37% less ear swelling and had less CD3$^+$ T lymphocyte infiltration than wild-type mice had (**Fig. 7f**,**g** and **Supplementary Fig. 6**). The homing of lymphocytes to draining lymph nodes and lymphocyte number were also 37% less than wild-type (**Fig. 7h**), suggesting

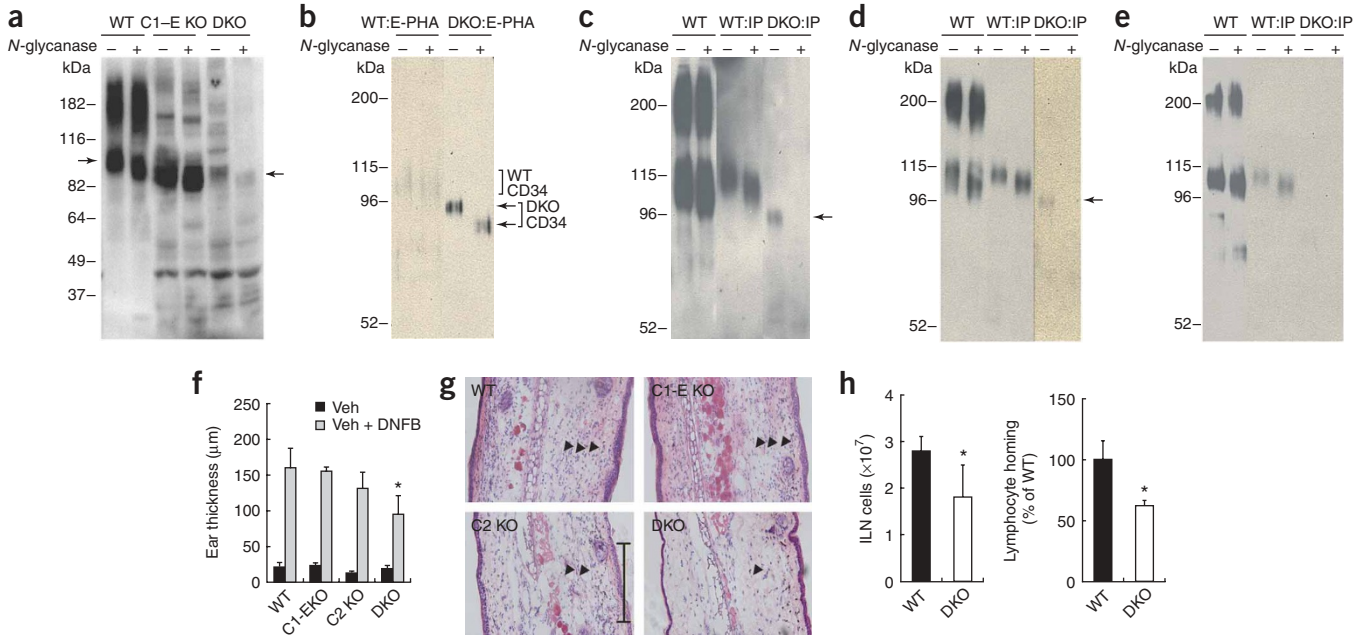

Figure 7 *N*-glycan-based L-selectin ligands and contact hypersensitivity. (**a**) Binding of E-selectin–IgM to lymph node stroma (mouse genotypes, above lanes) before (–) and after (+) *N*-glycanase treatment. Arrows indicate migration of CD34. (**b**) Detection of E-PHA-bound proteins with the CD34-specific antibody CD34-MB. (**c**–**e**) Immunoblot analysis of CD34 immunoprecipitated (IP) from wild-type and double-knockout mice, left untreated or treated with *N*-glycanase, and analyzed with E-selectin–IgM (**c**), L-selectin–IgM (**d**) or MECA-79 (**e**). Left two lanes in **c**–**e** are blots of wild-type PLNs without immunoprecipitation. Arrows indicate migration of CD34 in the double-knockout mouse. (**f**) Ear swelling in mice (genotype, horizontal axis) given initial sensitization of the back skin by DNFB, followed by ear sensitization initiated 5 d later by DNFB challenge or vehicle (Veh) alone, then analysis 24 h later. *, $P < 0.05$, compared with wild-type. (**g**) Hematoxylin-and-eosin staining of ear sections in **f**. Arrowheads indicate recruited leukocytes. Scale bar, 200 μm. (**h**) Lymphocyte recovery from inguinal lymph nodes (left) and trafficking of CMRA-labeled lymphocytes to the inguinal lymph nodes (right) 5 d after sensitization of the back skin with DNFB; lymphocyte homing was assayed after 1 h. * $P < 0.05$, compared with wild-type. Data are representative of two independent experiments (**a**–**e**) or are representative of two independent experiments with four mice for each genotype in each experiment (**f**–**h**; mean + s.e.m., **f**,**h**).

that less recruitment of lymphocytes to draining lymph nodes caused the lower contact hypersensitivity of double-knockout mice. In contrast, Core1-β3GlcNAcT or Core2GlcNAcT single-knockout mice had almost the same ear swelling results that wild-type mice had (**Fig. 7f**). These results collectively indicated that L-selectin ligands on *N*-glycans mostly compensate for the loss of L-selectin ligands on *O*-glycans in lymphocyte recruitment during inflammatory response.

DISCUSSION

Functional L-selectin ligands, 6-sulfo sLe^X molecules, are present as capping structures on underlying backbone glycans. We have shown here that inactivation of Core1-β3GlcNAcT abolished MECA-79-binding but unexpectedly resulted in only slightly less lymphocyte trafficking. MECA-79 inhibits lymphocyte homing *in vivo* and has therapeutic effect in a sheep asthma model[29]. Our results suggest that the considerable effect of MECA-79 on lymphocyte homing *in vivo* and *ex vivo* is partly due to MECA-79-mediated steric blockade of 6-sulfo sLe^X expressed on various underlying glycans, including core 2 branches and *N*-glycans. In Core1-β3GlcNAcT-deficient mice, 6-sulfo sLe^X present on core 2 branches may compensate for loss of 6-sulfo sLe^X on extended core 1 structures. However, as a second unexpected finding, we found here that deletion of both Core1-β3GlcNAcT and Core2GlcNAcT did not result in substantial loss of L-selectin ligands, despite the fact that 6-sulfo sLe^X was absent from *O*-glycans, as shown specifically for GlyCAM-1, a prototypic ligand regulating lymphocyte homing. Those findings initiated our search for unidentified sLe^X L-selectin ligands in HEVs.

Although heparan sulfate had a function as an L-selectin ligand on lung endothelial cells during acute inflammation, such was not the case for HEV-borne L-selectin ligands. In contrast, treatment of PLN sections with *N*-glycanase resulted in complete loss of L-selectin–IgM binding, and lymphocyte homing in both wild-type and double-knockout mice was inhibited by preinjection of *N*-glycan-specific tomato lectin and E-PHA lectins. We also found that *N*-glycans derived from lymph node stroma contain 6-sulfo sLe^X as capping structures. CHO cells expressing 6-sulfo sLe^X only on *N*-glycans supported shear stress–dependent lymphocyte rolling, as seen for L-selectin-dependent lymphocyte rolling[30]. Our results indicate that deletion of Core1-β3GlcNAcT and Core2GlcNAcT is compensated for not by other mucin-type *O*-linked oligosaccharides but by *N*-glycan-based L-selectin ligands.

One E-selectin ligand, ESL-1, contains only *N*-glycans[31]. It has also been reported that L-selectin ligands are not completely removed by treatment of human tonsil tissue with *O*-sialoglycopeptidase[32]. Our studies here have shown that *N*-glycanase removed almost all HEV L-selectin ligands that remained after digestion with *O*-sialoglycopeptidase. Similar to results reported before[32], *O*-sialoglycopeptidase did not remove all MECA-79-binding antigens without damaging HEVs; thus, L-selectin ligands remaining after the sequential digestion probably resided on *O*-linked oligosaccharides. Those findings are consistent with our conclusion that 6-sulfo sLe^X on *N*-glycans functions as an L-selectin ligand as well as 6-sulfo sLe^X in *O*-glycans.

L-selectin ligands have mucin-like domains as scaffolds to which *O*-linked oligosaccharides are attached. Because mouse GlyCAM-1, the first scaffold protein identified, contains exclusively *O*-linked oligosaccharides, it was assumed after its identification that mucin-type *O*-linked oligosaccharides function efficiently as L-selectin ligands because they can present ligands as a cluster. However, in the dimeric mucin-like molecule PSGL-1, one particular sLe^X on a core 2 branch near sulfated tyrosine residues in the N-terminal region has a critical function as a P-selectin[33] and L-selectin[34,35] ligand. Similarly, *N*-glycosylation sites are near the CD34 N-terminal domain[36]. Notably, most other L-selectin ligands, such as podocalyxin-like protein[37], endomucin[38], endoglycan[39] and MAdCAM-1 (ref. 40), contain putative *N*-glycosylation sites. Such *N*-glycans probably provide L-selectin ligands, as we have shown here for CD34.

After losing core 1 extensions and core 2 branches, mucin-like ligands still bear Galβ1→3GalNAcα1→(Thr or Ser) and its sialylated forms and thus maintain a rod-like shape, as the hydrogen bond between the innermost *N*-acetylgalactosamine and hydroxyl group of amino acids allows the peptide backbone to extend[41]. The superiority of mucin-type glycoproteins as a selectin ligand scaffolds may be due to this rod-like shape[42,43], which presents selectin ligands extended from the plasma membrane. In support of that conclusion, expression of CD34 and 6-sulfo sLe^x together on COS-1 cells supports much more L-selectin-mediated rolling in a higher shear stress than does expression of 6-sulfo sLe^x alone[23]. Extended forms of L-selectin and P-selectin demonstrate enhanced adhesion under shear flow[44]. After lymphocyte rolling, integrins on lymphocytes are immediately activated, and this activation converts bent integrins to extended conformations, allowing them to bind the adhesion molecule ICAM-1 on endothelial cells[45,46]. These findings suggest that interactions of extended molecules are critical in lymphocyte tethering, rolling and firm attachment to HEVs.

Mice lacking both Core1-β3GlcNAcT and Core2GlcNAcT had approximately 55% less lymphocyte homing, but tissue-resident lymphocyte numbers were not lower than those in wild-type mice. Similarly, published studies have shown that the reduction in lymphocyte number is less than the reduction in lymphocyte homing in mice lacking both GlcNAc6ST-1 and GlcNAc6ST-2 sulfotransferases[11,12]. Also similarly, in the absence of *O*-glycan-based L-selectin ligands, the decrease in contact hypersensitivity is not as severe as that seen in mice lacking GlcNAc6ST-1 and GlcNAc6ST-2 sulfotransferases[11]. These results suggest that 6-sulfo sLe^X on *N*-glycans is critical in presenting L-selectin ligands during inflammatory responses.

The 6-sulfo sLe^x on *N*-glycans may also have a function in low-order HEVs, which lack MECA-79 antigen[20], and in the L-selectin-mediated fast rolling of lymphocytes on non-HEV vessels[47]. Similarly, 6-sulfo sLe^x on unstimulated skin-homing lymphocytes, detected by HECA-452 and G152 antibodies[48], may represent 6-sulfo sLe^x on *N*-glycans, as HECA-452 binds to both sLe^x and 6-sulfo sLe^x on *N*-glycans as well[49]. L-selectin on lymphocytes and its ligands on HEVs have also been linked to the pathogenesis of certain chronic inflammatory diseases[1,4]. These include lymphoid thyroiditis, ulcerative colitis and infection with *Helicobacter pylori*[49,50]. Further studies are needed to determine how the synthesis of 6-sulfo sLe^x on *N*-glycans is regulated in physiological and pathological conditions.

METHODS

Mice. The generation of core1-β3GlcNAcT-deficient mice and double-knockout mice is described in the **Supplementary Methods**. Mice were treated according to guidelines of the National Institutes of Health, and experiments were approved by the Animal Research Committee of the Burnham Institute for Medical Research.

***N*-glycanase, heparitinase and *O*-sialoglycopeptidase treatment.** Frozen lymph node sections were fixed with acetone, washed with PBS and treated for 2 h at 37 °C with 100 mU/ml of *N*-glycosidase F (Calbiochem) in 10 mM HEPES-NaOH, pH 7.4, 0.1% (vol/vol) Triton X-100 and complete protease inhibitor (Roche). For heparitinase treatment, acetone-fixed sections were incubated for 2 h at 37 °C with 25 mU/ml of heparitinase I and II and heparinase (Seikagaku) in PBS containing 1 mg/ml of BSA, 1 mM $CaCl_2$ and protease inhibitor. In separate experiments, lymph node sections were prepared 2 h after injection of heparitinase I and II (40 mU each) through a tail vein, followed by incubation with E-selectin–IgM, L-selectin–IgM and specific antibodies and lectins. PLN frozen sections were digested with *O*-sialoglycopeptidase as described[32].

Metabolic labeling and immunoblot analysis. Mouse axillary, brachial and cervical lymph nodes and MLNs were depleted of lymphocytes by being gently squeezed. Lymph node stroma was metabolically labeled in organ culture with [^{3}H]glucosamine or [^{35}S]sulfate, and GlyCAM-1 was immunoprecipitated from culture medium as described[11]. Unlabeled lymph node stroma was directly made soluble in 1% (vol/vol) Triton X-100 in 10 mM Tris-HCl buffer, pH 8.0, and was separated by SDS-PAGE before and after *N*-glycanase treatment. After blotting onto an Immobilon-P membrane (Millipore), the blot was incubated with MECA-79 (BD Pharmingen) followed by a secondary horseradish peroxidase–conjugated antibody. From the solubilized stroma, CD34 was immunoprecipitated with rabbit antibody to CD34 (CD34-M), which was produced using a synthetic peptide (RGAQENGTGATSRNGH SA) corresponding to the C-terminal region of mouse CD34. The lysate was also incubated with *N*-glycan-specific E-PHA–agarose (EY Laboratories), and E-PHA-bound glycoproteins were eluted and blotted in the same conditions. Immunoblot analysis for E-selectin–IgM and L-selectin–IgM was done as for MECA-79 blotting except that 1.5 mM $CaCl_2$ was included in the buffers.

Lymphocyte homing assay. Lymphocytes from wild-type and L-selectin-deficient mice were labeled with the dyes Cell Tracker Green CMFDA and Orange CMRA (Molecular Probes), respectively, and were injected into 7- to 8-week-old wild-type and mutant mice[11]. The fractional content of fluorescent cells that homed to different organs was analyzed as described[11]. In certain experiments, concanavalin A (200 μg), tomato lectin (300 μg), E-PHA (200 μg for double-knockout and 400 μg for wild-type) or PBS (as a control) were injected 1 h before the injection of CMRA-labeled lymphocytes of wild-type mice. After 1 h, mice were killed and analyzed as described above. Lectin concentrations that would not cause overt reactions in the mice were used.

Intravital microscopy. Intravital microscopy of inguinal lymph nodes of was done as described[20,21]. Lymphocytes from PLNs and MLNs derived from transgenic mice expressing fluorescent proteins in T cells, or calcein-labeled splenic B lymphocytes from C57BL/6 mice, were injected through a catheter in the right femoral artery. Cells in the lymph node vasculature were visualized by stroboscopic epifluorescence illumination with intravital microscopy and were recorded as described[20,21].

Lymphocyte rolling assay. GlyCAM-1 was purified from serum obtained from mice of each genotype[51]. After dilution to achieve almost equivalent concentrations, GlyCAM-1 was applied to polystyrene plates coated with antibody to GlyCAM-1 (ref. 11). Then, 38C13 B lymphoma cells[51] were added to the flow chamber, followed by analysis as described[17]. Similarly, CHO cells expressing the following were plated as monolayers for lymphocyte rolling assays: CD34, FucT-VII and GlcNAc6ST-2; CD34, FucT-VII, GlcNAc6ST-2, Core1-β3GlcNAcT and Core2GlcNAcT; or CD34 and FucT-VII. CHO transfectants were prepared as described[52,53].

Contact hypersensitivity. Contact hypersensitivity responses were measured as described[11]. Mice were sensitized by the application of DNFB (Sigma) to the skin of the lower back on days 0 and 1. On day 5, the right ear was treated

with DNFB and the left ear was treated with vehicle. Swelling was measured with a thickness gauge before and 24 h after treatment. Homing of lymphocytes to the draining inguinal lymph nodes of wild-type and double knockout mice was assayed separately as described above 5 d after the initial sensitization.

Statistical analysis. Statistical significance was evaluated by the one-tailed Student's *t*-test.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

J.M. and X.B. did most of the experiments; B.P. did the rolling assays; P.S. and J.-M.G. did intravital microscopy; S.-Y.Y. did mass spectrometry; H.K. and H.S. assisted with the lymphocyte homing assay; K.O. and J.D.M. did embryonic stem cell culture; J.M., X.B., K.-H.K., U.H.v.A. and J.B.L. contributed to the preparation of the manuscript; and M.F. conceptualized the work, organized all experiments and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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