Markus Sperandio
Christian A. Gleissner
Klaus Ley

Glycosylation in immune cell trafficking

Authors' addresses
Markus Sperandio[1], *Christian A. Gleissner*[2], *Klaus Ley*[2]
[1]Walter Brendel Center of Experimental Medicine, Ludwig-Maximilians-Universität, Munich, Germany.
[2]Division of Inflammation Biology, La Jolla Institute for Allergy and Immunology, La Jolla, USA.

Correspondence to:
Markus Sperandio
Walter Brendel Center of Experimental Medicine
Ludwig-Maximilians Universität
Marchioninistr.15
81377 Muenchen
Germany
Tel.: +49 89 2180 76505
Fax: +49 89 2180 76503
e-mail: markus.sperandio@med.uni-muenchen.de

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Summary: Leukocyte recruitment encompasses cell adhesion and activation steps that enable circulating leukocytes to roll, arrest, and firmly adhere on the endothelial surface before they extravasate into distinct tissue locations. This complex sequence of events relies on adhesive interactions between surface structures on leukocytes and endothelial cells and also on signals generated during the cell–cell contacts. Cell surface glycans play a crucial role in leukocyte recruitment. Several glycosyltransferases such as α1,3 fucosyltransferases, α2,3 sialyltransferases, core 2 *N*-acetylglucosaminlytransferases, β1,4 galactosyltransferases, and polypeptide *N*-acetylgalactosaminyltransferases have been implicated in the generation of functional selectin ligands that mediate leukocyte rolling via binding to selectins. Recent evidence also suggests a role of α2,3 sialylated carbohydrate determinants in triggering chemokine-mediated leukocyte arrest and influencing β_1 integrin function. The recent discovery of galectin- and siglec-dependent processes further emphasizes the significant role of glycans for the successful recruitment of leukocytes into tissues. Advancing the knowledge on glycan function into appropriate pathology models is likely to suggest interesting new therapeutic strategies in the treatment of immune- and inflammation-mediated diseases.

Keywords: *glycan, glycosylation, selectin, leukocyte, inflammation*

Introduction

Over the last 20 years, much progress has been made in elucidating the molecular mechanisms governing trafficking of immune cells not only through the vascular system but also within different tissues. For exiting the vasculature, leukocytes follow a rather uniform cascade of events (1–3), which starts with the first contact (capture) of free flowing leukocytes to the vascular endothelium. In many tissues, this is followed by leukocyte rolling along the vessel wall. Both capture and rolling are mediated by specialized receptor-ligand pairs consisting of a member of the selectin family of adhesion molecules and specific carbohydrate determinants on selectin ligands (4, 5). During rolling, leukocytes are in intimate contact with the vascular endothelium. This enables endothelial-bound chemokines to interact with their respective chemokine receptors on the leukocyte surface. Upon binding to the receptor, chemokine receptor-mediated

signaling events trigger, together with signals derived from interactions between selectin ligands and selectins, the activation of leukocyte-expressed integrins (inside-out signaling) (6, 7). Activated integrins subsequently interact with endothelium-expressed ligands such as immunoglobulin superfamily members intercellular adhesion molecule 1 (ICAM-1) or vascular cell adhesion molecule 1 (VCAM-1), which leads to a reduction in leukocyte rolling velocity and eventually to firm leukocyte arrest on the endothelium. Following firm adhesion, leukocytes get prepared for extravasation into tissue, which includes leukocyte spreading and intravascular crawling along the endothelium until the right spot for exiting has been found (3).

In the context of leukocyte recruitment, protein glycosylation plays a major role in selectin–selectin ligand-mediated leukocyte capture and rolling. Recently, glycan modifications have also been found to be functionally relevant for the chemokine-dependent induction of firm leukocyte arrest (8) and integrin-mediated leukocyte adhesion (9), thus expanding the functional role of posttranslational glycosylation beyond leukocyte rolling. Here, we review the direct engagement of glycan structures in the regulation of leukocyte recruitment. The focus is on selectins and their ligands, but other glycosylation-dependent processes driven by galectins and siglecs will also be discussed.

Selectin–selectin ligand interactions

The contribution of posttranslational glycosylation on glycoprotein function relevant for adaptive and innate immunity has gained increasing interest in recent years (10) and selectin ligand–selectin interactions are among the best-studied carbohydrate–protein interactions known in biology. Selectins are C-type lectins characterized by recognizing and binding to specific carbohydrate determinants on selectin ligands in a Ca^{2+}-dependent fashion. Mammals express three selectins: P-selectin, E-selectin, and L-selectin (4). P-selectin is stored in Weibel–Palade bodies of endothelial cells and α-granules of platelets and can be mobilized to the cell surface within minutes after stimulation with different pro-inflammatory mediators. In contrast to P-selectin, E-selectin does not reside in intracellular storage pools but is newly synthesized, requiring regulation at the transcriptional and translational levels. L-selectin is the only selectin expressed on leukocytes and can be shed upon stimulation. Besides its role in mediating leukocyte rolling and neutrophil activation (11), L-selectin has been demonstrated to also regulate directed interstitial leukocyte migration (12).

Selectin ligands comprise a heterogeneous group of molecules characterized by carrying specific carbohydrate determinants, many of which include the tetrasaccharide sialyl Lewis X (sLe^x). Sialyl Lewis X binds all three selectins with low affinity (13) (*Fig. 1*). Some selectin ligands are relevant for mediating leukocyte capture and rolling during inflammation, and others are involved in the homing of lymphocytes into lymph nodes. Inflammatory selectin ligands are mostly presented on leukocytes; selectin ligands important for lymphocyte homing are located on the endothelial surface of high endothelial venules (HEV). This cell type-specific selectin ligand expression may not only explain the differences of inflammatory and homing selectin ligands at the protein level, but may also drive distinct posttranslational glycosylation patterns. Indeed, selectin binding affinity can be optimized for L-selectin by GlcNAc-6-sulfation, yielding 6-sulfo sialyl Lewis X, and for P-selectin by tyrosine O-sulfation of the underlying protein backbone (14–16) (*Fig. 1*). Many selectin ligands are sialomucins, which carry the selectin-binding carbohydrate moiety at their N-terminus away from the cell membrane. *Table 1* gives an overview on identified selectin ligands and their relevance under *in vivo* conditions.

P-selectin glycoprotein ligand-1 (PSGL-1)

PSGL-1 (gene name: *Selplg*) is a heavily glycosylated homodimeric sialomucin, which binds to P-, E-, and L-selectin under static and dynamic conditions (17, 18). Intravital microscopy studies in *Selplg*-deficient mice or in mice treated with PSGL-1 blocking monoclonal antibodies (mAbs) revealed that leukocyte-expressed PSGL-1 is the main and most relevant leukocyte rolling ligand during inflammation *in vivo* and indeed interacts with all three selectins (19–22). Structurally, PSGL-1 is a transmembrane protein with a short, highly conserved cytoplasmic tail containing an ezrin-radixin-moesin (ERM)-binding domain necessary to establish a link to the cytoskeleton and a Nef-associated factor-1 (Naf1) binding region important for signaling induced by P-selectin binding to PSGL-1 (23, 24). The transmembrane domain is followed by multiple decameric repeats, which are heavily decorated with O-glycans and help to locate the N-terminal selectin-binding domain away from the cell surface, which may facilitate binding to selectins. Because of its prominent role in mediating leukocyte rolling during inflammation and the dependence of this process on posttranslational glycosylation, the carbohydrate decoration of PSGL-1 has been extensively studied (5). Among modifications of PSGL-1 crucial for mediating leukocyte capture and rolling, α1,3 fucosylation, α2,3 sialylation,

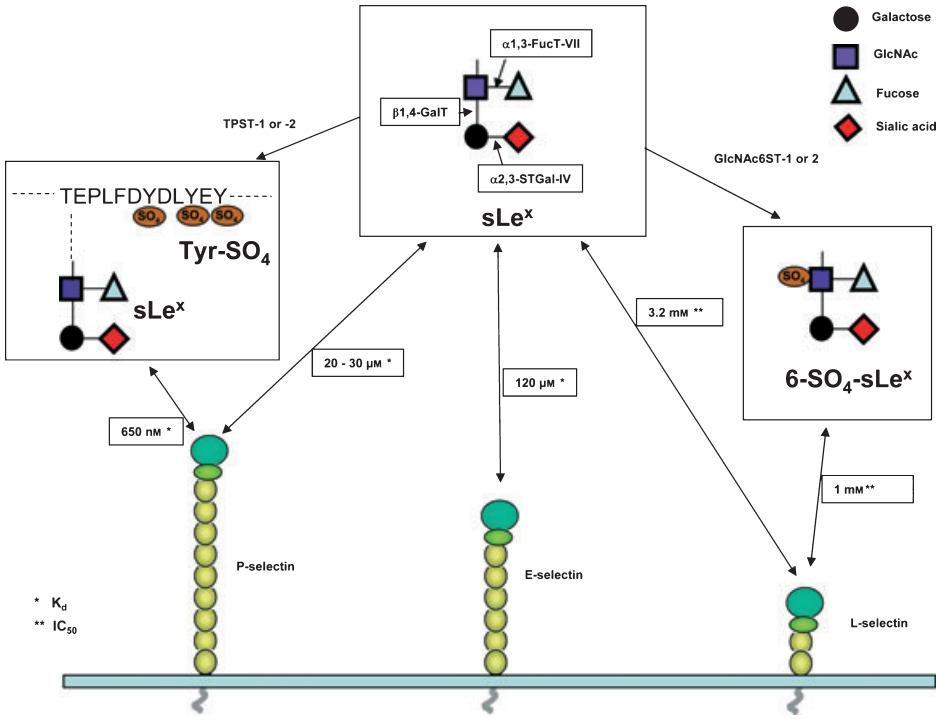

Fig. 1. Carbohydrate selectin ligands. Sialyl Lewis X (sLex) binds all three selectins with modest affinity (indicated in boxes as Kd or IC50) (14–16). Binding of P-selectin is improved by core 2 and adjacent Y-SO_4 (left) (15). Binding to L-selectin is enhanced by GlcNAc-6-sulfation (right) (16). The precise requirements for E-selectin binding beyond sLe^x are not known. Binding affinities of selectins to natural glycoprotein ligands like GlyCAM-1 are much higher, with apparent Kd in the nM range, suggesting that other determinants are involved or ligand clustering occurs.

β1,4 galactosylation, and modification by core 2 β1,6-N-acetylglucosaminyltransferase have been identified (25–28).

Although PSGL-1 is heavily glycosylated along the serine and threonine-rich decameric repeat region, the crucial core 2 decorated O-glycan important for binding to P- and L-selectin is located at threonine 57 near the N-terminus of human PSGL-1 (29). For optimal binding of PSGL-1 to P-selectin, sulfation of at least one tyrosine near the N-terminus of PSGL-1 is necessary (30). Three tyrosines for potential sulfation are located at residues 46, 48, and 51 in human PSGL-1 (31), while murine PSGL-1 contains two potential tyrosine sulfation sites near the N-terminus (32). One sulfated tyrosine is sufficient for P-selectin binding (31, 33). PSGL-1 appears to express multiple E-selectin binding sites that may reside below (C-terminal to) the P- and L-selectin binding sites (17), but the E-selectin binding sites have not been mapped. PSGL-1 is expressed on most leukocytes and was also found in chronically inflamed human prostate tissue, on activated human umbilical vein endothelial cells (HUVEC), and on endothelial cells from small intestine and mesenteric lymph nodes of mice with chronic ileitis (34–36). Most leukocyte-expressed PSGL-1 is concentrated at the tips of leukocyte microvilli and redistributes to the uropod upon activation of leukocytes (37, 38). PSGL-1 is a major signaling molecule and activates spleen tyrosine kinase (Syk) through the Rous sarcoma oncogene (Src) family kinase Gardner–Rasheed feline sarcoma viral (Fgr) oncogene homolog and immunoreceptor tyrosine-based activation motif (ITAM) adapters upon E-selectin binding (39, 40) and Naf1-activation upon P-selectin binding (24).

E-selectin ligand-1

E-selectin ligand-1 (ESL-1) is a 150 kDa glycoprotein, which was initially discovered by affinity purification methods from myeloid cells (41). Sequence analysis revealed that ESL-1 is identical to a membrane sialoglycoprotein of the Golgi apparatus termed MG-160, also known as cysteine-rich fibroblast growth-factor (FGF) receptor (42, 43). These proteins are products of a single gene locus called Golgi-complex-localized glycoprotein-1 (*Glg1*). In neutrophils, ESL-1 is mainly localized in the Golgi apparatus. However, immunogold labeling revealed that some ESL-1 is also expressed on microvilli of the cell surface (44), where it becomes available as an extracellular ESL. Of note, ESL-1 is only decorated by N-glycans, but not by O-glycans, which are considered to carry most of the selectin ligand activity on leukocytes (42). Although the role of ESL-1 as functional ESL has been elusive for many years, recent evidence indicates that ESL-1 cooperates with PSGL-1 and

Table 1. **(A) Inflammatory selectin ligands with *in vivo* relevance. (B) Selectin ligands involved in lymphocyte homing**

(A)*

PSGL-1
- Expressed on most leukocytes and on chronically inflamed endothelium
- Mediates P-, E-, and L-selectin-dependent leukocyte rolling *in vivo* (19, 20, 22)
- Binding to selectins dependent on core 2 branched, α1,3 fucosylated, and α2,3 sialylated glycans (25, 26, 28)
- Contains an ERM-binding domain and a Naf-1 binding region in its cytoplasmic domain (23, 24)

CD44
- Abundantly expressed on leukocytes, but also on erythrocytes, and in the brain
- Involved in E-selectin-dependent rolling *in vivo* (21, 46)
- Binding to E-selectin depends on specific N-glycan decorated glycoforms of CD44 (46)
- Mediates L- and P-selectin dependent rolling of different carcinoma cell lines (168, 169)
- Mediates leukocyte rolling via interacting with liver sinusoid-expressed hyaluronan (HA) in an LPS-mediated inflammatory liver disease model (49)
- Associates with β_1 integrin and influences β_1 integrin-mediated rolling and adhesion of T-cells (52)

ESL-1
- Expressed in leukocytes
- Mostly Golgi-localized, but some expression on the cell surface (21)
- Mediates E-selectin dependent leukocyte rolling and influences rolling velocity(21)

(B)

PNAd (GlyCAM-1, CD34, podocalyxin, and endomucin)
- Constitutively expressed in HEV of peripheral lymph nodes and recognized by mAb MECA 79 (62)
- Induced expression in different chronically inflamed venules (63)
- Redundant group of glycoproteins which provide a protein scaffold for presentation of appropriate carbohydrate determinants with binding affinity to L-selectin

MAdCAM-1
- Constitutively expressed in Peyer's patch HEV, intestinal lamina propria vessels, and mesenteric lymph node HEV
- Induced expression in different chronically inflamed tissues (71)
- Isolated MAdCAM mediates lymphocyte rolling (76)
- Interacts with $\alpha_4\beta_7$ integrin (67)
- L-selectin ligand activity regulated at the level of posttranslational carbohydrate sulfation (77)

*Other selectin ligands with unclear function in leukocyte recruitment *in vivo*: CD43, CD24, heparin derivatives, versican, nucleolin, glycosphingolipids.
LPS, lipopolysaccharide; ESL-1, E-selectin ligand-1; HEV, high endothelial venules; PNAd, peripheral node addressins; MAdCAM-1, mucosal addressin cell adhesion molecule-1.

CD44 to provide the complete ESL activity on neutrophils (21). Intravital microscopic observations of E-selectin-dependent leukocyte rolling in tumor necrosis factor-α (TNF-α)-stimulated cremaster muscle venules revealed a significant increase in rolling velocities but no effect on the number of rolling cells after knock-down of ESL-1 by short hairpin (sh) RNA interference targeting *Glg1* (21). By contrast, in *Selplg*$^{-/-}$ mice, E-selectin-dependent leukocyte rolling velocities were unchanged compared with wildtype mice, while the number of rolling cells was significantly reduced. In *Cd44*$^{-/-}$ mice, the number of rolling leukocytes was similar to wildtype mice, but leukocyte rolling velocities were increased to a similar extent as seen in wildtype mice reconstituted with shESL-1-transduced bone marrow cells, implicating that ESL-1 and CD44 control leukocyte rolling velocities while PSGL-1 is mainly involved in leukocyte capture (21). These data are interpreted in terms of differential contributions to capture, rolling, and signaling, but the contribution of integrins to regulating rolling velocity was not explored in (21).

CD44

The hyaluronan (HA)-binding receptor CD44 belongs to the Link module superfamily and is abundantly expressed on leukocytes (45). Besides its binding activity to HA, immunopurified CD44 has recently been reported to bind to E-selectin under *in vitro* conditions, which was dependent on sialylated and fucosylated N-linked glycans on CD44 (46). Intravital microscopy observations in TNF-α-stimulated cremaster muscle venules showed that the loss of CD44 led to a significant increase in rolling velocity without affecting the number of rolling leukocytes (46). Based on these findings, Frenette and colleagues (21) suggested that CD44 together with PSGL-1 and ESL-1 accounts for all ESL activity on neutrophils under *in vivo* conditions with CD44 being involved in regulating E-selectin-dependent rolling velocity.

CD44 was initially identified as a leukocyte surface antigen and later shown to bind to HA (45). Various splice variants of CD44 with different glycosylation patterns exist. Mutagenesis studies and enzymatic treatment of CD44-transfected Chinese hamster ovary (CHO) cells revealed that increased sialylation of N-glycans on CD44 negatively regulates its binding to HA (47). Recent evidence also suggests that endothelial cell-expressed HA can increase its binding avidity to CD44 via interactions with serum-derived HA-associated protein (SHAP) (48). Interactions between neutrophil-expressed CD44 and sinusoidal endothelium-expressed HA play an important role in neutrophil sequestration in liver sinusoids following injection of lipopolysaccharide (LPS) (49). Interestingly, these interactions were critically dependent on a SHAP-induced increase in avidity of HA to bind to CD44, but did not involve changes in activity or expression of CD44 on neutrophils (49).

To study glycosylation-specific requirements of CD44 in binding to E-selectin, Zen *et al.* (50) used various breast carcinoma cell lines and identified a splice variant of CD44

(CD44v4) that was heavily decorated with sialyl Lewis X and bound to E-selectin. In addition, surface expression of CD44v4 in different breast carcinoma cell lines was positively correlated with *in vitro* transmigration and *in vivo* invasiveness. This may indicate that expression of distinct CD44 splice variants in leukocytes may positively affect binding of CD44 to E-selectin.

An interesting new approach to use the potential of CD44 to bind E-selectin was reported by Sackstein *et al.* (51). They treated isolated mesenchymal stromal cells (MSC) with the α1,3 fucosyltransferase Fut6. MSC show high expression of CD44 but lack α1,3-fucosylated glycostructures crucial for ESL activity on CD44 (51). Treatment with Fut6 led to the generation of functional ESLs on MSC. Fut6-treated MSC infusion into immunocompromised mice [non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice] led to specific infiltration of MSC into bone marrow via constitutively expressed E-selectin on bone marrow vessels (51).

Besides its role as HA receptor and ESL, Siegelman's group (52) demonstrated that CD44, via its cytoplasmic domain, associates with very late antigen-4 (VLA-4) ($\alpha_4\beta_1$ integrin). Association had a significant influence on rolling and firm adhesion of two T-cell lines, suggesting that CD44 regulates the activation state of VLA-4 (52). Additional studies will be necessary to clearly dissect the different functions of CD44 on leukocyte recruitment under *in vivo* conditions.

CD43 (Leukosialin)

The role of CD43 in leukocyte recruitment remains unclear. Although pro-adhesive properties as putative ESL have been documented (53–56), several reports also show anti-adhesive functions (54, 57). CD43 is a sialomucin and abundantly expressed on leukocytes. It contributes significantly to the overall negative electrical charge on the leukocyte surface via its numerous sialic acid residues (58). This may explain most of its anti-adhesive function. CD43 on activated T cells and neutrophils can be decorated with sialylated and core 2-modified O-glycans, yielding a 130 kDa glycoform of CD43 (53, 54, 59) that is specifically recognized by mAb 1B11 and binds to E-selectin (60). Intravital microscopic observations of leukocyte rolling and adhesion in inflamed cremaster muscle venules of *Cd43*$^{-/-}$ mice revealed a marked increase in the number of rolling and adherent cells in the absence of CD43 (57). These results were in part confirmed by Matsumoto *et al.* (54). Whether or not CD43 is involved in leukocyte extravasation during inflammation in an oyster-glycogen induced peritonitis model is controversial (54, 57, 61).

Peripheral node addressins

HEV of secondary lymphoid tissue are specialized venules, where naive and central memory lymphocytes roll, adhere, and enter lymph nodes and Peyer's patches. Leukocyte rolling in HEV of peripheral lymph nodes is critically dependent on leukocyte-expressed L-selectin and L-selectin ligands on HEV. These L-selectin ligands comprise a group of sialomucins termed peripheral node addressins (PNAds). PNAds are recognized by mAb MECA 79 (62) and include GlyCAM-1, CD34, sgp200, HEV-expressed podocalyxin, and a recently identified glycoprotein called endomucin (63). The role of the different PNAd members as functional L-selectin ligands is still unclear. Studies in *Glycam1*$^{-/-}$ and *Cd34*$^{-/-}$ mice demonstrate normal lymphocyte trafficking (64, 65) and there is growing evidence that the various PNAd members can be considered redundant. These glycoproteins provide a protein backbone as scaffold for the proper presentation of critical carbohydrate determinants such as 6-sulfo sialyl Lewis X, which supports binding to L-selectin. This 'redundant carrier' concept is supported by studies in glycosyltransfease deficient mice (see below), where the deletion of one enzyme (α1,3 fucosyltransferase Fut7) leads to structural changes in the carbohydrate decoration on the whole PNAd group, which results in a significant defect in lymphocyte rolling and lymphocyte homing to peripheral lymph nodes (25). Future studies using mice where several members of the PNAd group are deleted by genetic inactivation will help to better define possible specific functions for the various PNAd glycoprotein members in lymphocyte homing.

Mucosal addressin cell adhesion molecule-1

The mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is a multifunctional type l transmembrane adhesion molecule with two distal immunoglobulin-like domains, which are involved in α4β7 integrin binding, a mucin-like region that can display L-selectin-binding carbohydrate determinants, and a membrane-proximal immunoglobulin-like domain (66, 67). MAdCAM-1 is ubiquitously expressed during fetal development but disappears in most tissues after birth with the exception of small intestine, colon, Peyer's patches, and mesenteric lymph nodes, where MAdCAM-1 remains constitutively expressed (68, 69). MAdCAM-1 expression can be induced on endothelial cells throughout the intestinal tract by pro-inflammatory cytokines like TNF-α or interleukin-1β (IL-1β) (70). This is thought to play an important role in leukocyte recruitment during chronic intestinal inflammatory diseases such as ulcerative colitis or Crohn's disease (71).

Recently, MAdCAM-1 expression has also been detected in the chronically inflamed liver (72), in maternal vessels of the vascular zone during the critical period of initial placenta development (73), in inflamed brain tissue (74), and in the bone marrow (75). MAdCAM-1 isolated from young mice can support L-selectin-mediated leukocyte rolling (76). The L-selectin binding function of MAdCAM-1 is regulated at the level of carbohydrate modifications. Kobayashi *et al.* (77) investigated biopsy specimens from patients with ulcerative colitis and detected that active disease is accompanied by increased expression of the carbohydrate sulfotransferase GlcNAc6ST-1, which preferentially modifies MAdCAM-1 and may contribute to the generation of the L-selectin ligand 6-sulfo sialyl Lewis X on MAdCAM-1. Taken together, these findings suggest a role of MAdCAM-1 in lymphocyte homing to the gut. However, direct *in vivo* observations will require *Madcam1*-deficient mice, which have been generated recently (78).

Glycosylation defects affecting leukocyte recruitment

Leukocyte and endothelial adhesion molecules carry sialylated glycostructures, which protect them from premature surface removal. Specific glycosylation patterns enable distinct functions during the intravascular events necessary for extravasation of leukocytes into tissue. Core 2 decorated O-glycans with the tetrasccharide sialyl Lewis X at their terminal end have been identified on selectin ligands and are crucial in mediating P-selectin-dependent leukocyte rolling along the endothelium. Additional posttranslational modifications targeting other adhesion molecules and chemokine receptors are beginning to be identified.

In general, posttranslational glycosylation of glycoproteins takes place along the endoplasmic reticulum (ER)/Golgi pathway and is dependent on a group of enzymes termed glycosyltransferases (see also the CAZY, Carbohydrate-Active enZYmes database under http://www.cazy.org/fam/acc_GT.html) (79). Glycosyltransferases involved in the synthesis of functional selectin ligands include polypeptide N-acetylgalactosaminyl transferase-1 (encoded by *Galnt1*) (80), core 2 β1,6-N-acetylglucosaminyltransferase-1 (*Gcnt1*) (28, 81), β1,4-galactosyltransferases (*B4gal1* and 4) (27, 82), α1,3-fucosyltransferases (*Fut4* and *7*) (83, 106), and α2,3-sialyltransferase (*St3gal4*) (26, 84) (*Table 2*). Furthermore, carbohydrate sulfation by GlcNAc6ST1 and 2 (encoded by *Glcnac6st1* and 2) yield 6-sulfo sialyl Lewis X, an important carbohydrate determinant on L-selectin ligands (85, 86). In addition, *St3gal4* has been implicated in regulating CXCR2-triggered firm leukocyte

Table 2. **Posttranslational glycosylation and its contribution to selectin ligand function and leukocyte recruitment**

Knockout Mouse	Observed defects in leukocyte recruitment
Galnt1$^{-/-}$	Reduced binding of P- and E-selectin to *Galnt1*$^{-/-}$ PMN (80) Reduced rolling of *Galnt1*$^{-/-}$ PMN on immobilized P- and E-selectin in a flow chamber assay (80) Reduced leukocyte recruitment into the inflamed peritoneal cavity (80) Reduced L-selectin ligand activity in peripheral node HEV and defective lymphocyte homing into peripheral node HEV (80)
Gcnt1$^{-/-}$	Reduced binding of all three selectins to *Gcnt1*$^{-/-}$ PMN (28) Reduced P-, E-, and L-selectin dependent rolling during inflammation(81, 92) Reduced B- and T-cell rolling with increased rolling velocity in peripheral lymph node HEV (90)
B4galt1$^{-/-}$	Reduced binding of *B4galt1*$^{-/-}$ PMN to P-selectin (27) Reduced leukocyte recruitment into inflamed peritoneal cavity and in a wound healing model (27, 99) *B4GALT1* mutation described to cause CDG IId in humans; no leukocyte recruitment defect noted in CDG IId patients (100)
Fut7$^{-/-}$	Reduced P- and E-selectin-dependent rolling during inflammation (106) Strongly reduced L-selectin dependent rolling in peripheral lymph node HEV (105)
Fut4$^{-/-}$	Increased E-selectin dependent rolling velocity (106)
Slc35c1$^{-/-}$	Dramatically reduced posttranslational fucosylation (116) Absent binding of selectins to *Slc35c1*$^{-/-}$ PMN (116) No E-, P-, or L-selectin-mediated rolling during inflammation (117) Defective L-selectin-dependent rolling in Peyer's patch HEV (116)
St3gal4$^{-/-}$	Increased E-selectin dependent rolling velocity (26) Strongly reduced L-selectin-dependent rolling during inflammation (84) Defective CXCR2-triggered firm leukocyte arrest during inflammation (8)
St6gal1$^{-/-}$	Reduced PMA-stimulated β1 integrin activation of a monocytoid cell line (9) Absent CD22 ligands and impaired B cell function (10)
Glcnac6st1$^{-/-}$	Reduced lymphocyte homing to peripheral lymph nodes and increased L-selectin-mediated rolling velocity of B and T cells in peripheral lymph node HEV (123)
Glcnac6st2$^{-/-}$	More severe phenotype than *Glcnac6st1*$^{-/-}$ mice Reduced lymphocyte homing, increase rolling velocity, and reduced leukocyte adhesion in peripheral lymph node HEV (125)

HEV, high endothelial venules; CDG IId, congenital deficiency of glycosylation IId; PMA, phorbol myristate acetate; PMN, polymorpho-nuclear leukocytes.

arrest, and *St6gal1* has been shown to influence β_1 integrin function (8, 9).

Polypeptide *N*-acetylgalactosaminyltransferase (Galnt1)

The biosynthesis of *O*-glycan structures (recently reviewed in 87) is initiated by the addition of galactosamine to serine or threonine residues of the protein backbone. This is catalysed by UDP-GalNAc:polypeptide GalNAcT (Galnt). At least 15 different isoenzymes of Galnt have been described in mammals (87), which have different substrate specificity, tissue and cell type expression (80). The loss of *Galnt3* has been found to be responsible for familial calcinosis, which can also be caused by mutations in the gene of fibroblast growth factor 23 (88), a glycoprotein modified by ppGalNAcT-3 (89). On the other hand, knockout mice lacking *Galnt4*, 5, and 13 did not show any obvious phenotype, suggesting at least some overlapping functions of Galnts.

In search for specific Galnts responsible for the initiation of *O*-glycan biosynthesis relevant for selectin ligands, Tenno *et al.* (80) recently generated *Galnt1*-deficient mice. These mice demonstrated a severe reduction in lymphocyte homing, which was more pronounced for B lymphocytes than for T lymphocytes, a finding which is reminiscent of that reported in *Gcnt1*$^{-/-}$ mice (90). Interestingly, the degree of defective lymphocyte homing was dependent on lymph node location and associated with a reduction in L-selectin ligand activity on lymph node HEV. Besides the homing defect, a significant decrease in the number of germinal centers and an increased number of apoptotic B-cells within the germinal centers of white pulp of the spleen and lymph nodes was noted in *Galnt1*$^{-/-}$ mice (80). This may help explain the marked decrease in the production of immunoglobulin (Ig) isotypes IgG1, IgG2a, IgG3 in the absence of *Galnt1*. Flow cytometric analysis demonstrated that P- and ESL activity on *Galnt1*$^{-/-}$ Gr-1^{+} neutrophils was significantly reduced, but not absent (80). Using an autoperfused flow chamber system, the authors also found a marked rolling defect of *Galnt1* deficient leukocytes on P- and E-selectin (80). In addition, leukocyte recruitment into the inflamed peritoneal cavity was impaired, suggesting a defect in the formation of functional selectin ligands which prevents an adequate innate immune response (80). Interestingly, neutrophil counts were not elevated in *Galnt1*$^{-/-}$ mice, which may be an indication that compensatory mechanisms exist that allow residual production of P- and ESLs. In addition to the observed immunological defects, a severe bleeding disorder was also found with reduced plasma concentrations of coagulation factors V, VII, VIII, IX, X, and XIII (80).

Core 2 decorated *O*-glycans significantly contribute to selectin ligand activity

The branching enzyme core 2 β1,6-N-acetylglucosaminyltransferase (Gcnt1) catalyses the addition of N-acetylglucosamine to N-acetylgalactosamine in β1,6 linkage, which initiates the core 2 extension, a prerequisite for the generation of core 2 decorated *O*-glycans carrying the tetrasaccharide sialyl Lewis X. In neutrophils, expression of sialyl Lewis X on mucin-type *O*-glycans is exclusively found on core 2 modified side branches (91), making this enzyme an interesting target to modulate selectin ligand activity on granulocytes. Three different Gcnts (*Gcnt1-3*) exist (91). Gcnt1 is considered the most critical isoenzyme in the synthesis of functional selectin ligands. Ellies *et al.* generated *Gcnt1*$^{-/-}$ mice and found that binding of recombinant murine P- and L-selectin to *Gcnt1*$^{-/-}$ neutrophils was almost completely lost, while recombinant murine E-selectin could still bind, although binding was reduced (28). Additional intravital microscopy studies in inflamed cremaster muscle venules of *Gcnt1*$^{-/-}$ mice revealed a significant reduction in P- and L-selectin dependent leukocyte rolling and a less pronounced reduction in E-selectin dependent rolling (81, 92). Interestingly, E-selectin-mediated leukocyte rolling velocity was normal, suggesting that the loss of *Gcnt1* mainly affects the initiation and not the maintenance of E-selectin-dependent rolling.

Assessment of leukocyte rolling in HEV of secondary lymphoid tissues showed no obvious rolling defect in *Gcnt1*$^{-/-}$ Peyer's patch HEV, where rolling is mostly dependent on L-selectin and to a lesser degree on P-selectin and $\alpha_4\beta_7$-integrin (92). However, a subsequent study investigating leukocyte rolling in HEVs of peripheral lymph nodes found defective B-cell and T-cell rolling, which became evident in reduced numbers of rolling B and T cells and higher rolling velocities (90). Interestingly, the rolling impairment was slightly more pronounced in B cells than in T cells. Consistent with this, flow cytometric analysis revealed lower expression of L-selectin on B cells than on T cells, which appears to be of functional relevance for L-selectin-mediated interactions with their ligands on peripheral lymph node HEVs (90). Concerning the role of *Gcnt1* on L-selectin ligand activity on HEV, it seems that the contribution of *Gcnt1* is not essential for the synthesis of functional L-selectin ligands and hence lymphocyte homing in peripheral lymph nodes. An alternative pathway of selectin ligand synthesis may explain how the loss of Gcnt1 can be compensated for. Using *Gcnt1*$^{-/-}$ mice, Yeh *et al.* (93) identified 6-sulfo sialyl Lewis X on core 1-extended *O*-glycans.

In addition, their study revealed that core 1-decorated 6-sulfo sialyl Lewis X cooperates with core 2-decorated 6-sulfo sialyl Lewis X as L-selectin ligands on HEV.

Recently, Merzaban *et al.* (94) reported that activated $CD8^+$ T cells are able to support P-selectin-dependent rolling in the absence of *Gcnt1*. Expression analysis of *Gcnt2* and 3 in resting and activated $CD8^+$ T cells of $Gcnt1^{-/-}$ mice showed that *Gcnt3* (but not *Gcnt2*) is upregulated after stimulation, suggesting that *Gcnt3* may contribute to P-selectin ligand activity on activated $CD8^+$ T cells and can at least in part compensate for the loss of Gcnt1 (94). The first study translating the reported contribution of Gcnt1 in leukocyte recruitment into a disease-relevant model came from Huo and colleagues (95), who showed that the loss of *Gcnt1* in $Apoe^{-/-}$ mice reduces the development of atherosclerotic plaques.

β1,4 galactosyltransferases

The transfer of UDP-galactose to acceptor carbohydrates is widespread and can occur through several glycosidic linkages (96). For the synthesis of functional selectin ligands, β1,4 galactosyltransferase activity is required. Two β1,4 galactosyltransferases (encoded by *B4galt1* and *B4galt4*) have been implicated in the synthesis of functional selectin ligands as they can catalyse the addition of UDP-galactose to terminal N-acetylglucosamine in β1,4 linkage, which is part of the synthetic pathway to generate polylactosamine extensions of core 2 decorated *O*-glycans and the terminal capping group sialyl Lewis X (97). Asano *et al.* (98) used *B4galt1*-deficient mice to study the role of *B4galt1* on selectin ligand activity *in vitro* and *in vivo*. They found that binding of soluble P-selectin to neutrophils and monocytes was significantly impaired in the absence of *B4galt1* (27). In addition, $B4galt1^{-/-}$ mice showed elevated systemic neutrophil counts and a significant decrease in the number of neutrophils recruited into zymosan-treated ears. Both of these findings are indicative of a neutrophil recruitment defect in the absence of *B4galt1*, which is also supported by a subsequent study that showed reduced neutrophil infiltration in a wound healing model (99). Of note, analysis of *O*-glycan expression on isolated $B4galt1^{-/-}$ splenocytes by anion exchange chromatography revealed that decoration of *O*-glycans by core 2 side branches was dramatically reduced, leaving open the possibility that the observed reduction in P-selectin binding to $B4galt1^{-/-}$ neutrophils or monocytes is due to a reduced level of core 2 modified *O*-glycans (28). Lymphocyte homing to peripheral lymph nodes, which is dependent on HEV-expressed L-selectin ligands, was not significantly disturbed in the absence of *B4galt1* or *Gcnt1* (27, 90, 92).

The biological consequences of *B4GALT1* loss have also been reported in humans, (100). Hansske *et al.* (100) identified a patient with an insertion of a single nucleotide in the *B4GALT1* gene leading to premature translation stop and loss of the C-terminal 50 amino acids of the enzyme. The disorder was designated as congenital deficiency of glycosylation IId (CDG IId) (100). The clinical presentation consisted of mental retardation, Dandy-Walker malformation with progressive hydrocephalus, muscular hypotonia, transient cholestatic syndrome, and extensive coagulation abnormalities, However, impairment of immune functions have not been demonstrated so far (100, 101).

Another B4galt, *B4galt4*, has recently been implicated in the synthesis of 6-sulfo sialyl Lewis X on core 2 decorated *O*-glycans (82). As discussed below, 6-sulfo sialyl Lewis X is critical for functional L-selectin ligand activity on HEV of secondary lymphoid organs (102). Therefore, loss of *B4galt4* may cause a significant impairment in lymphocyte homing *in vivo*, but this has not been tested yet.

Fucosyltransferases are essential for functional selectin ligands *in vivo*

Fucosyltransferases catalyse the transfer of GDP-fucose to acceptor carbohydrates. The activated sugar nucleotide GDP-fucose can either be *de-novo* synthesized via GDP-mannose (90%) or provided from ingested food (salvage pathway, 10%) (103). Like other glycosyltransferase-dependent modifications, fucosylation of core 2 decorated *O*-glycans as well as N-glycans takes place in the Golgi apparatus. The fucosyltransferases (Fut) 7 and 4 are the only fucosyltransferases known to be expressed in human leukocytes and play a major role in the synthesis of leukocyte-expressed selectin ligands (103, 104). Fut7 and Fut4 are also expressed in high endothelial cells of secondary lymphoid organs, where they differ in their expression pattern. While Fut7 expression is mainly restricted to small postcapillary HEV, Fut4 is only found in larger venules (105). $Fut7^{-/-}$mice (83), $Fut4^{-/-}$ mice (106), and $Fut7^{-/-}$ $Fut4^{-/-}$ mice (25) have all been generated and were used to investigate the role of fucosylation on selectin ligand function. $Fut7^{-/-}$ mice show significantly elevated leukocyte counts. Intravital microscopy studies in $Fut7^{-/-}$ mice demonstrated that P- and E-selectin-dependent leukocyte rolling in inflamed venules of the ear and the cremaster muscle was markedly reduced. This was particularly true for P-selectin-mediated rolling, while the effect on E-selectin-dependent rolling was less pronounced (106). In addition to the significant reduction in leukocyte rolling during inflammation,

lymphocyte rolling in peripheral lymph node HEVs of *Fut7*$^{-/-}$ mice was strongly decreased (105). Interestingly, the dependence of L-selectin ligand activity on *Fut7* was particularly evident in MECA 79^{+} HEVs but less pronounced in MECA 79-negative venules (105). Consistent with the rolling defect in HEVs of *Fut7*$^{-/-}$ mice, lymphocyte homing assessed by injection of fluorescently-labeled wildtype lymphocytes into *Fut7*$^{-/-}$ mice was also significantly impaired (83).

Fut4$^{-/-}$ mice present with a much milder phenotype. These mice appear healthy and the leukocyte counts are within the normal range (106). Leukocyte rolling as assessed in postcapillary venules of an inflamed ear model revealed a significant increase in rolling velocities in inflamed venules of *Fut4*$^{-/-}$ mice compared to control mice, which was demonstrated to be due to defective ESLs (106). In *Fut7*$^{-/-}$ *Fut4*$^{-/-}$ mice, E-, P-, and L-selectin dependent rolling is completely lost, demonstrating the importance of α1,3 fucosylation in the generation of selectin ligands (25).

Besides the leukocyte recruitment defects observed in the absence of *Fut7* and *Fut4*, another enzyme called FX (GDP-4-keto-deoxymannose 3,5-epimerase-4-reductase encoded by the *Tsta3* gene), which is involved in the de-novo-synthesis of GDP-fucose, significantly contributes to posttranslational fucosylation of selectin ligands (107). *Tsta3*$^{-/-}$ mice lack FX and are embryonic lethal with some occasional survivors. These survivors die at weaning unless fed a high fucose diet, which restores fucosylation and hence, selectin ligand activity (107). This is reminiscent to the defect identified in Leukocyte adhesion deficiency II (LAD II) patients and *Slc35c1*-deficient mice (see below), where a supplemental diet with fucose also restores selectin ligand function (108).

LAD II caused by defective GDP-fucose transporter Slc35c1

In 1992, two unrelated boys from consanguineous marriages presented with neutrophilia, recurrent bacterial infections without pus formation, and chronic gingivitis (109, 110). Neutrophils from these patients did not express sialyl Lewis X and did not roll when injected into inflamed venules of the rabbit mesentery (111). Subsequent studies in patients suffering from this disease termed Leukocyte Adhesion Deficiency II (LAD II) or alternatively Congenital Deficiency of Glycosylation-IIc (CDG-IIc) revealed a defect in fucose metabolism caused by a single amino acid exchange, which affects the nucleotide sugar transporter solute carrier family 35 member C1 (*SLC35C1*) (112–114). This transporter transfers GDP-fucose into the Golgi apparatus, where it becomes available for posttranslational fucosylation, a crucial step in the synthesis of functional selectin ligands. Besides the two original reports on the genetic defect, Helmus and colleagues (115) later identified a patient with a novel mutation leading to the expression of a truncated form of the fucose transporter, which is unable to localize to the Golgi compartment.

Recently, Hellbusch *et al.* (116, 117) generated a mouse model of LAD II (*Slc35c1*$^{-/-}$ mice) (116). *Slc35c1*$^{-/-}$ mice showed marked growth retardation, reduced postnatal survival, dilated pulmonary alveoli, and recurrent infections. Similar to the observations in LAD II patients, intravital microscopy studies in *Slc35c1*$^{-/-}$ mice demonstrated an almost complete absence of P-, E-, and L-selectin-mediated leukocyte rolling during inflammation (117). In addition, L-selectin-dependent lymphocyte homing into lymph nodes and Peyer's patches was dramatically reduced (116, 117). Interestingly, lymphocyte homing to the spleen was unaffected in the absence of *Slc35c1*. This suggests that fucose-independent mechanisms of lymphocyte homing into the spleen of *Slc35c1*$^{-/-}$ mice may explain the reported normal antibody response in LAD II patients (118, 119).

Carbohydrate sulfation, a crucial modification in mediating lymphocyte homing

Successful lymphocyte homing into peripheral lymph nodes is crucially dependent on L-selectin mediated lymphocyte rolling along HEV. For L-selectin-dependent rolling to occur, lymphocyte-expressed L-selectin interacts with L-selectin ligands expressed on HEVs. These ligands are collectively termed peripheral node addressins (PNAds) and are presented on sialomucins including GlyCAM-1, CD34, sp200, HEV-expressed podocalyxin, and endomucin (63). The monoclonal antibody MECA 79 recognizes PNAds, blocks lymphocyte rolling on HEVs (62, 120) and is therefore considered a reporter of L-selectin ligand activity. Subsequent studies aimed at identifying the carbohydrate determinant on PNAd that allows optimal binding to L-selectin revealed that sialyl Lewis X is not sufficient to mediate lymphocyte rolling. Rosen and colleagues showed that sulfation of GlcNAc at C6 position leads to the generation of 6-sulfo sialyl Lewis X and significantly enhances binding of GlyCAM-1 to L-selectin (121).

GlcNAc-6-O-sulfation is catalyzed by GlcNAc-6-O-sulfotransferases. In humans, five GlcNAc6STs (GlcNAc6ST1-5) exist, four of which have orthologs in mice (102, 122). Recent studies using *Glcnac6st1*$^{-/-}$ mice (123), *Glcnac6st2*$^{-/-}$

mice (124), and *Glcnac6st1*$^{-/-}$ *Glcnac6st2*$^{-/-}$ mice (85, 86) were instrumental in dissecting the specific functions of different GlcNAc6STs in lymphocyte rolling on HEV. Loss of both *Glcnac6st1* and 2 almost completely abolished lymphocyte rolling on HEVs and, therefore, homing to peripheral lymph nodes. In addition, MECA 79 staining was completely lost on HEV (85, 86). This illustrates the functional importance of GlcNAc-6-O-sulfation for L-selectin ligand activity on peripheral lymph node HEVs and shows that *Glcnac6st1* and *Glcnac6st2* account for most if not all of GlcNAc-6-sulfation during the synthesis of 6-sulfo sialyl Lewis X. Of note, the observed residual leukocyte rolling observed in *Glcnac6st1*$^{-/-}$ *Glcnac6st2*$^{-/-}$ mice was completely blocked by systemic injection of L-selectin blocking mAb MEL-14, suggesting that sulfation-independent L-selectin ligands on HEV exist. Alternatively, PSGL-1, a functional L-selectin ligand on leukocytes (22), may also contribute to GlcNAc-6-sulfation-independent rolling on HEV.

Analysis of *Glcnac6st1*$^{-/-}$ and *Glcnac6st2*$^{-/-}$ single knockout mice demonstrated overlapping as well as distinct functions (85, 86). Although *Glcnac6st1* is expressed ubiquitously, mice deficient in *Glcnac6st1* showed a milder phenotype than *Glcnac6st2*$^{-/-}$ mice with a moderate reduction (30%) in lymphocyte homing to peripheral lymph nodes, mesenteric lymph nodes, and Peyer's patches (123). Interestingly, observation of lymphocyte rolling in peripheral lymph node HEV of *Glcnac6st1*$^{-/-}$ mice showed a similar number of rolling cells compared to control mice. However, analysis of rolling velocities in *Glcnac6st1*$^{-/-}$ mice revealed higher velocities of rolling B and T cells than in control mice (85). This is reminiscent to the alterations in rolling velocities of B and T cells in HEV of *Gcnt1*$^{-/-}$ mice, which may suggest that *Glcnac6st1* leads to GlcNAc-6-sulfation of sialyl Lewis X presented on core 2 decorated side branches. This is indirectly supported by the observation that loss of *Glcnac6st2* predominantly affects GlcNAc-6-O-sulfation of extended core 1 structures recognized by mAb MECA 79 (93).

Glcnac6st2 is constitutively expressed by HEV of lymphoid tissues. In addition, expression of *Glcnac6st2* can be induced in tertiary lymphoid structures of chronically inflamed tissues (102). Investigation of lymphocyte homing to peripheral lymph nodes showed a more severe reduction in *Glcnac6st2* deficient mice than in *Glcnac6st1* deficient mice. However, similar to *Glcnac6st1*, the number of rolling leukocytes in HEV of peripheral lymph nodes was not affected in the absence of *Glcnac6st2*. Analysis of lymphocyte rolling velocities in peripheral lymph node HEV revealed a marked increase accompanied by a reduction in adhesion (125).

Posttranslational tyrosine sulfation and selectin ligand function

Several reports have demonstrated that sulfation of critical tyrosines at the N-terminus of PSGL-1 improves binding of PSGL-1 to P- and L-selectin (30, 31). Tyrosine O-sulfation occurs in the trans-Golgi network and is catalysed by two known tyrosyl protein sulfotransferases (*Tpst1* and 2) which transfer a sulfuryl group from the sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to the hydroxyl group of peptidyl-tyrosine (30). Tpst1 and 2 are ubiquitously expressed, although a thorough investigation of tissue distribution has been hampered by the lack of isotype-specific antibodies to Tpsts. Recent studies on the enzymatic activity of *Tpst1* and 2 demonstrated that both Tpsts are indistinguishable in their efficiency of transferring sulfate to critical tyrosine residues on PSGL-1 (126, 127), implying that both enzymes equally contribute to the generation of functional PSGL-1. *Tpst1*$^{-/-}$ and *Tpst2*$^{-/-}$ mice have been generated and characterized (128, 129). These mice are viable, appear healthy, and heterozygous breeders yield litters with Mendelian distribution at birth. In accordance with the assumed overlapping function of *Tpst1* and 2, no defect in innate immunity has been reported for either *Tpst1*$^{-/-}$ or *Tpst2*$^{-/-}$ mice. However, *Tpst1*$^{-/-}$mice show reduced body weight and an increased rate of postimplantation fetal death, which points to distinct functions of Tpst-1 (128). Other defects are observed in *Tpst2*$^{-/-}$mice. Loss of this sulfotransferase leads to male infertility due to a marked reduction in sperm motility in viscous media and a defect in fertilizing zona-pellucida-intact eggs (129). Recently, *Tpst1*$^{-/-}$ *Tpst2*$^{-/-}$ double deficient mice have been generated and reported (130). These mice were born at the expected frequency but most of them developed early postnatal pulmonary insufficiency due to an inability to properly expand their lungs after birth, which led to hypoxia, pulmonary hypertension, right-heart failure, and early postnatal death. Analysis of sulfotyrosine distribution using various fetal tissues showed complete absence of sulfotyrosines in the double knockout mouse, suggesting that *Tpst1* and 2 are the only Tpst in the mouse (130). Additional studies in *Tpst1*$^{-/-}$ *Tpst2*$^{-/-}$ mice are currently under way focusing on the role of sulfotyrosines in leukocyte recruitment (Dr. Kevin L. Moore, personal communication).

Sialylation affects leukocyte recruitment at different steps

Sialic acids are a group of negatively charged sugars, which usually appear at the terminal end of carbohydrate structures

of glycoconjugates (131). Due to their exposed position, sialic acids are involved in many biological processes ranging from cell adhesion, cell communication, host–pathogen interactions, and metastasis to barrier function and immune modulation (131, 132). In mammals, 20 sialyltransferases have been cloned and grouped into four families (*St3gal, St6gal, St6galnac, St8Sia*) based on linkage and acceptor substrate (132). Six different α2,3-sialyltransferases (*St3gal1-6*) exist in humans and mice. *St3gal4* and 6, and to a lesser degree, *St3gal3*, are able to utilize Galβ1,4GlcNAc on core 2 decorated O-glycans and contribute to the synthesis of sialyl Lewis X on core 2 branched O-glycans (133, 134). To investigate the potential role of sialylation on selectin ligand function, mice deficient in *St3gal4* have been generated (135). Although *in vitro* binding assays showed reduced binding of all three selectins to *St3gal4*$^{-/-}$ leukocytes, intravital microscopy in inflamed cremaster muscle venules revealed normal P-selectin-dependent rolling and only a mild defect in E-selectin-mediated leukocyte rolling manifested by moderately elevated rolling velocities (26). Interestingly, L-selectin-mediated rolling on inflamed cremaster muscle venules, which requires leukocyte-expressed PSGL-1 (22), was completely absent (84). This was in sharp contrast to normal L-selectin dependent rolling on Peyer's patch HEV, suggesting that expression of other St3gal family members in high endothelial cells of Peyer's patches compensate for the loss of *St3gal4* (84). *St3gal6* is one likely candidate.

In contrast to *St3gal3*, 4, and 6, *St3gal1* prefers Galβ1, 3GalNac structures on O-glycans (core 1 O-glycans) as acceptor substrate, which suggests that *St3gal1* does not contribute to the carbohydrate determinants relevant for selectin ligand function (132). Interestingly, studies in *St3gal1* deficient mice revealed that the loss of *St3gal1* caused a strong upregulation of core 2 decorated O-glycans with enhanced binding of selectins to selectin ligands in an *in vitro* assay. This suggests that both *St3gal1* and *Gcnt1* compete for the same acceptor substrate (136).

Recently, two groups (137, 138) independently demonstrated a role of posttranslational glycosylation on the function of chemokine receptors. Ludwig and colleagues (137) showed that N-glycosylation of human CXCR2 protected the receptor from proteolytic cleavage and disappearance from the neutrophil surface without interfering with chemokine-induced signaling via CXCR2. Bannert *et al.* (138) generated a canine thymocyte cell line stably transfected with wildtype CC chemokine receptor 5 (CCR5) or CCR5 mutants. Mutations of CCR5 were introduced by exchanging serine or threonine residues for alanine at putative O-glycosylation sites located at the N-terminus of CCR5 (138). Using various CCR5 mutated cell lines, the authors found that CCR5 binding to the chemokines CC chemokine ligand 3 (CCL3) [macrophage inflammatory protein (MIP-1α)] and CCL4 (MIP-1β) was strongly dependent on a sialic acid-carrying O-glycan linked to serine 6 near the N-terminus of CCR5 (138). These findings were the first identifying a role of posttranslational glycosylation on chemokine receptor function, which extended the role of glycosylation from selectin-dependent leukocyte capture and rolling to chemokine receptor-triggered firm leukocyte arrest.

A subsequent study provided strong evidence that glycosylation also plays an important role in chemokine-triggered firm leukocyte arrest *in vivo* (8). Using intravital microscopy in wildtype mice and *St3gal4*$^{-/-}$ mice, Sperandio and co-workers (8) found that systemic injection of CXCL1 led to a strong CXCR2-dependent induction of firm leukocyte arrest in exteriorized cremaster muscle venules of wildtype mice, while leukocytes were unable to adhere to the venular wall after CXCL1 injection in *St3gal4*$^{-/-}$ mice, suggesting that sialylation by sialyltransferase *St3gal4* is critical for CXCR2-induced firm leukocyte adhesion during inflammation *in vivo*. Of note, the same study revealed no role of *ST3Gal4* on CCR7-dependent dendritic cell migration, suggesting that sialylation by *St3gal4* is not a generally required modification for chemokine receptor-triggered leukocyte arrest *in vivo* (8).

Besides the *St3gal* family of sialyltransferases, α2,6 sialyltransferase *St6gal1* has gained increasing attention in modulating innate and adaptive immunity (9, 139–141). The protein encoded by *St6gal1* transfers sialic acid residues to the terminus of N- and potentially O-glycans and is essential for the synthesis of terminal Siaα2,6Galβ1,4GlcNAc (Sia6LacNAc) residues on N-glycans, which are ligands for the B cell-expressed lectin CD22 (Siglec-2) (10). CD22 contains three immunoreceptor tyrosine-based inhibitory motifs (ITIMs) which recruit the Src-homolgy-2-domain-containing protein tyrosine phosphatase 1 (SHP1) and attenuate B-cell receptor signaling (142). In *St6gal1*-deficient mice, CD22 ligands are absent and B-cell receptor-mediated signaling, B-cell proliferation, and IgM production are significantly altered, which is accompanied by co-localization of CD22 with the B-cell receptor. Woodard-Grice *et al.* (9) recently discovered a role of *St6gal1* in β_1 integrin activation during differentiation of monocytes to macrophages. Stimulation of the monocyte-like cell line U937 with phorbol myristate acetate (PMA) led to increased binding of U937-expressed $\alpha_4\beta_1$ integrin to its ligand VCAM-1. This was accompanied by hyposialylation of β_1 integrin but not α_4 integrin (9). Intriguingly, hyposialylation of β_1 integrin was dependent on BACE [β-site amyloid-precursor-protein (APP)-cleaving enzyme 1]-mediated proteolytic shedding of *St6gal1* (9), suggesting that *St6gal1* controls β_1 integrin activity and

therefore may influence the recruitment of monocytes during the inflammatory response.

Glycolipids as potential selectin ligands

Glycolipids carrying the tetrasaccharide sialyl Lewis X have long been discussed as potential ESLs (143). Sialylated lactosylceramides isolated from leukocytes of patients with chronic myelogenous leukemia showed binding affinity to E-selectin-transfected COS cells in a static binding assays (144). Subsequently, rolling of E-selectin transfected CHO-cells on immobilized glycolipids was demonstrated in a flow chamber assay (145). These studies provided evidence that ESL activity on leukocytes may not only be dependent on carbohydrate determinants linked to glycoproteins, but also on glycolipids decorated with sialyl Lewis X. In mice, this has been challenged recently, as it had been demonstrated that ESL activity is independent of glycolipids but resides entirely on the glycoproteins PSGL-1, CD44, and ESL-1 (21). On the other hand, Nimrichter *et al.* (146) isolated glycolipids from human neutrophils and found a sialylated glycosphingolipid with 5N-acetyllactosamine repeats carrying two to three fucose residues that showed binding affinity to E-selectin, but not to P-selectin, suggesting that sialylated glycosphingolipids may indeed be relevant ESLs in humans. This is supported by investigations showing that treatment with a panel of proteases completely abolishes E-selectin binding to murine neutrophils, while E-selectin binding to human neutrophils is still substantial (146).

Siglecs and their role in leukocyte recruitment

Sialic acid-binding Ig-like lectins (siglecs) belong to the immunoglobulin-like lectins (I-type lectins) and are characterized by recognizing and binding to terminal sialic acid residues on glycoconjugates (147–149). Structurally, siglecs are single-pass type I transmembrane proteins with a V-set-immunoglobulin domain at their N-terminus, which contains a conserved arginine for sialic acid binding. This is followed by a variable number of C2-set immunoglobulin domains linked to the transmembrane domain (147). Most of the siglecs possess a cytoplasmic domain containing one or more ITIMs. Due to their sequence similarities and evolutionary conservation, two groups of siglecs can be distinguished. The first group includes sialoadhesin (Sn or siglec-1), CD22 (siglec-2), and siglec-15. The second group comprises the CD33-related siglecs (CD33, siglecs-5 through -11 in humans and CD33, siglec-E, F-, -G, and -H in mice) (147). Most siglecs are expressed on cells of the hematopoietic or immune system with a high degree of cell specificity for the Sn (expressed on macrophages) and CD22 (expressed on B-cells) containing group, which is less pronounced for the CD33-related siglecs.

A role in mediating direct adhesive cell–cell contacts and therefore in contributing to leukocyte recruitment has been suggested for siglec-1. As shown by flow cytometric analysis, recombinant siglec-1 binds strongly to granulocytes and somewhat weaker to natural killer (NK) cells, monocytes, B cells, and a subset of $CD8^+$ T cells (149). High expression of siglec-1, which does not have any known signaling domain, was found at local cell contact points in hematopoietic and lymphoid tissues (150). Besides siglec-1, CD22 on a subset of B cells was shown to bind to α2,6-linked sialic acid ligands. This interaction plays a role in B-cell homing into the bone marrow (151). In general, direct siglec-mediated cell–cell contacts seem to be tightly controlled by masking sialic acid binding sites via interactions *in cis* (i.e. on the same cell) which prevents binding of siglecs to sialic acid ligands *in trans* (i.e. cell – cell). This view is supported by the fact that most siglecs (except siglec-1) do not extend beyond the cellular surface layer (glycocalyx) and therefore are exposed to many sialic acid residues they can bind to in their immediate vicinity (149). Future studies in siglec deficient mice are warranted and will help better define their contribution to leukocyte recruitment in the *in vivo* situation.

Galectins

Galectins are a class of β galactose-binding proteins, which are characterized by a approximately 130 amino acid conserved peptide element in their carbohydrate recognition domain (CRD) (152). They can occur as monomers (one CRD, e.g. galectin-5) or dimers (two CRDs, e.g. galectin-1). Some of the latter have a tandem repeat connecting the two CRDs (e.g. galectin-9). Galectin-3 has a unique structure as it is a monomer with an additional non-CRD domain. Galectins are secreted via non-canonical pathways and affect the immune response by various mechanisms (153). Currently, the galectin family comprises 15 known members (153).

Most data regarding leukocyte trafficking have been obtained for galectin-3, expression of which has been demonstrated not only in epithelial and endothelial cells but also in inflammatory cells like monocytes, macrophages, dendritic cells, neutrophils, and mast cells (154). Expression in lymphocytes can be induced upon activation (154). Galectin-3 binds to extracellular matrix proteins like laminin, elastin, fibronectin, and others (154).

Galectin-3 is involved in mediating rolling and adhesion of eosinophils on VCAM-1 under flow *in vitro* (155). Also, eosinophils can roll on immobilized galectin-3, which requires α4

integrin (155). Eosinophil-expressed galectin-3 may also interact with immobilized galectin-3 and thereby modulate eosinophil trafficking (155). A plausible explanation for these findings is that galectin-3 may associate with α4β1 integrin on the eosinophil surface and enhance α4β1 binding to VCAM-1. In a model of airway inflammation and bronchial hyperresponsiveness, galectin-3 has been shown to be involved in eosinophil recruitment (156). *In vivo*, galectin-3 may have chemotactic activity for monocytes, because it increases their accumulation in a subcutaneous air pouch model (157). Conversely, lack of *Lgals3*, the gene coding for galectin-3, resulted in impaired neutrophil recruitment to the peritoneal cavity after thioglycollate injection (158, 159). Furthermore, galectin-3 interaction with β2 integrins has been demonstrated to be a specific pathomechanism of neutrophil recruitment in streptococcal pneumonia, which is not found in pneumonia induced by other bacteria like *E.coli* (160). In summary, galectin-3 seems to promote eosinophil, neutrophil, and monocyte adhesion and recruitment, and thereby enhance inflammation.

Galectins 8 and 9 promote adhesion of several human leukocyte cell lines and primary leukocytes under static conditions, an effect that was inhibited by antibodies against α4 and β1, but not β2 integrin (161, 162). Galectin-9 also promotes adhesion of eosinophils to fibroblast *in vitro* (163).

In contrast to galectins 3, 8, and 9, galectin-1 seems to inhibit rather than promote leukocyte recruitment. Thus, neutrophil adhesion and transmigration through an endothelial cell line was inhibited by galectin-1 (164). These findings were confirmed *in vivo* in a model of interleukin-1β-induced peritonitis (164). *Lgals1*$^{-/-}$ mice show enhanced leukocyte adhesion and recruitment (165). Galectin-1 also inhibits L-selectin-dependent neutrophil rolling and adhesion under flow conditions (165) and lymphocyte adhesion to fibronectin and laminin (166). Furthermore, adhesion to HUVEC under flow has been reported to be reduced by galectin-1 in a dose-dependent manner (167).

Conclusion

The navigation of immune cells from the vasculature into different tissue compartments requires a complex network of adhesion and guidance molecules including selectins, integrins, chemokines, and chemokine receptors. Proper post-translational glycosylation of some of these molecules is instrumental for their function, defining another hierarchical level in the regulation of immune cell trafficking.

Selectin ligands are the most prominent example of how glycan modifications influence the function of the underlying scaffold molecule. P- and L-selectin ligands are based on core 2 decorated *O*-glycans modified by α1,3 fucosylation and α2,3 sialylation. Glycosylation requirements for optimal ESL function beyond α1,3 fucosylation are less clear, at least as far as the role of ESLs in slow leukocyte rolling is concerned. Slow rolling may involve selectin ligands like ESL-1 and CD44, which carry *N*-glycans.

With the recent availability of an increasing number of glycosyltransferase-deficient mice combined with advances in bioimaging tools, our understanding of the influence of glycosylation on immune cell trafficking just begins to expand beyond selectin-dependent leukocyte rolling. Recent reports have revealed the importance of sialylation on chemokine receptor triggered neutrophil arrest (8) and α4β1 integrin function (9). In addition, leukocyte recruitment is also influenced by galectins and siglecs. These findings open new research areas and may offer interesting new drug targets aiming to modify immune cell trafficking under various pathological conditions ranging from acute and chronic inflammatory conditions to autoimmune diseases and cancer.

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