Severe Impairment of Leukocyte Recruitment in ppGalNAcT-1–Deficient Mice

Helena Block, Klaus Ley and Alexander Zarbock

*J Immunol* 2012; 188:5674-5681; Prepublished online 27 April 2012; doi: 10.4049/jimmunol.1200392
http://www.jimmunol.org/content/188/11/5674

**References**

This article *cites 52 articles*, 30 of which you can access for free at:
http://www.jimmunol.org/content/188/11/5674.full#ref-list-1

**Why The JI? Submit online.**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Severe Impairment of Leukocyte Recruitment in ppGalNAcT-1–Deficient Mice

Helena Block,*‡ Klaus Ley,‡ and Alexander Zarbock*‡

P-selectin glycoprotein ligand-1 plays an important role in leukocyte recruitment. Its binding affinity to selectins is modulated by posttranslational modifications. The polypeptide N-acetylgalactosamine transferase-1 (ppGalNAcT-1) initiates core-type protein O-glycosylation. To address whether the glycosylation of P-selectin glycoprotein ligand-1 by ppGalNAcT-1 is important for leukocyte recruitment in vivo, we investigated leukocyte recruitment in untreated and TNF-α–treated cremaster muscles comparing ppGalNAcT-1–deficient mice (Galnt1−/−) and wild-type mice. In untreated and TNF-α–treated Galnt1−/− mice, leukocyte rolling, adhesion, and transmigration were significantly reduced, with markedly increased rolling velocity compared with control mice. L-selectin–dependent leukocyte rolling was completely abolished in Galnt1−/− mice compared with wild-type mice. Thioglycollate-induced peritonitis experiments with chimeric mice revealed that hematopoietic ppGalNAcT-1 is important for leukocyte recruitment. These data show that the loss of ppGalNAcT-1 led to reduced leukocyte rolling and recruitment and increased rolling velocity, suggesting a predominant role for ppGalNAcT-1 in attaching functionally relevant O-linked glycans to selectin ligands. The Journal of Immunology, 2012, 188: 5674–5681.

Leukocyte recruitment into inflamed tissue proceeds in a multistep adhesion cascade, beginning with leukocyte capturing and rolling (1, 2). During these steps, the leukocytes are in close contact with endothelial cells and are activated by different stimuli, which lead to integrin-dependent adhesion and transmigration (3). The first steps—capturing and rolling—are mediated by the family of selectins consisting of E-, P-, and L-selectin (4). Intravital microscopy studies demonstrated that selectins have both overlapping and unique functions (5). The overlapping function of selectins can be demonstrated by blocking or eliminating different selectins. Untreated Sele−/− mice have a normal inflammatory response in certain disease models (6), whereas blocking P-selectin in these mice severely impaired leukocyte rolling (7). By investigating the rolling velocity, it becomes obvious that the different selectins have characteristic properties, because they mediate leukocyte rolling at distinct velocities. In venules of the cremaster muscle, the L-selectin–dependent rolling (130 μm/s) is faster than the velocity on P-selectin (40 μm/s) (8). E-selectin mediates slow rolling, with a velocity of 3–7 μm/s (7, 9).

Most selectin ligands are glycoproteins, which require posttranslational modification for selectin binding (10). Selectins bind with low affinity to α1,3-fucosylated and α2,3-sialylated core 2 decorated O-glycans carrying the tetrasaccharide sialyl Lewis X (sLeX) (11). To achieve optimal selectin-binding capacity, several glycosyltransferases, including β1,4-galactosyltransferases I and IV (12, 13), α1,3-fucosyltransferases FucT-VII and -IV (14, 15), core 2 β1,6-N-acetylgalcosaminyltransferase (16, 17), and α2,3-sialyltransferase IV (18), are required. In addition, other modifications contribute to selectin ligand function. Sulfation of tyrosine residues at the N terminus of P-selectin glycoprotein ligand (PSGL)-1, the main selectin ligand on leukocytes, significantly modifies selectin binding (19).

The biosynthesis of core-type O-glycan structures is initiated with the covalent linkage of N-acetylgalactosamine (GalNAc) to threonine or serine residues in the protein backbone (20). This enzymatic step is catalyzed by a polypeptide GalNAc transferase (ppGalNAcT) and is followed by the attachment of other saccharide linkages to this GalNAc by other enzymes in the Golgi apparatus (21, 22). More than a dozen ppGalNAcTs have been identified, and the isozymes differ with respect to expression patterns and substrate selectivity (21). Surprisingly, genetic elimination of ppGalNAcT-1 in the mouse did not abolish O-glycan formation in vivo (23, 24), indicating at least partially redundant functions of the different ppGalNAcT isoforms (24, 25). However, elimination of ppGalNAcT-1 results in a bleeding disorder caused by reduced plasma levels of different blood coagulation factors and impaired leukocyte recruitment and homing under normal and inflammatory conditions (26).

Recent work by Tenno et al. (26) provides evidence that O-glycosylation by ppGalNAcT-1, by adding galactosamine to serine or threonine residues of a protein backbone, is crucial for binding P-selectin and E-selectin IgM in vitro (26). Furthermore, leukocyte rolling on E-selectin and P-selectin in autoperfused flow chamber assays was significantly reduced in Galnt1−/− mice. Interestingly, they showed that, although ≥15 GalNAc transferases have been identified (27), the isoform Galnt1 plays a nonredundant role in the initiation of O-glycosylation of selectin ligands on leukocytes and is therefore essential for leukocyte recruitment into inflamed tissue.

Using Galnt1−/− mice and littermate control mice in different in vivo inflammation models, we found that the absence of ppGalNAcT-1 leads to impaired leukocyte rolling and recruitment and increased rolling velocity, suggesting a predominant role for
The Journal of Immunology 5675

with 1 ml sterile 4% thioglycollate (Sigma-Aldrich). After 4 or 8 h, mice were killed, and the peritoneum was rinsed with 10 ml PBS (containing 5 mM EDTA and 10 U/ml heparin) and massaged. The peritoneum was then opened, and the total number of leukocytes was determined. Neutrophils were detected by flow cytometry (FACS Canto; Becton Dickinson, Heidelberg, Germany) based on the expression of CD45 (clone 30-F11), GR-1 (clone RB6-8C5), and 7/4 (clone 7/4; all from BD Biosciences-Pharmingen, San Diego, CA).

Statistics

Statistical analysis was performed with SPSS (version 14.0; Chicago, IL). Differences between the groups were evaluated by one-way ANOVA, Student–Newman–Keuls test, and t test, where appropriate. Data are presented as mean ± SEM, and p < 0.05 was considered statistically significant.

Results

P-selectin–dependent leukocyte rolling in vivo is significantly impaired in Galnt1−/− mice

Leukocytes from Galnt1−/− mice have a reduced P-selectin–binding capacity in vitro (26). To investigate the functional consequences in vivo, leukocyte rolling was analyzed in 28 venules of five Galnt1−/− mice and compared with leukocyte rolling in 20 venules of four lipitmate controls. Hemodynamic parameters for both groups showed no significant differences in diameter, blood flow velocity, or wall shear rate (data not shown). Leukocyte rolling within the first hour after exteriorization of the cremaster muscle is mainly mediated by P-selectin expressed on the inflamed endothelium (28). In ppGalNacT-1−/− mice, the rolling flux fraction was 13% compared with 25% in control mice (p < 0.05) (Fig. 1A). Leukocyte rolling during that time was almost completely P-selectin dependent, because it was blocked by injecting an anti–P-selectin mAb (RB40.34) (Fig. 1A). Therefore, the lower leukocyte rolling flux fraction in Galnt1−/− mice can be attributed to an impairment in P-selectin–mediated rolling.

To investigate whether PSGL-1 was necessary for P-selectin–dependent rolling in Galnt1−/− mice in vivo, ppGalNacT-1−/− deficient mice were treated with a blocking anti–PSGL-1 mAb (4RA10). Treating ppGalNacT-1 mice with a blocking anti–PSGL-1 mAb significantly reduced the number of rolling leukocytes (Fig. 1A). Injection of a blocking PSGL-1 mAb into control mice led to a significant reduction in rolling flux fraction, but a significant number of rolling leukocytes remained. Rolling in control mice treated with a blocking PSGL-1 mAb was completely blocked by additional injection of a blocking P-selectin mAb, confirming that rolling under these conditions was completely P-selectin dependent. These findings suggest that the P-selectin–dependent, PSGL-1–independent portion of rolling is particularly sensitive to the absence of ppGalNacT-1. It cannot be excluded that the used blocking Ab did not completely block all PSGL-1/P-selectin interactions under these conditions or that other transferases contribute to PSGL-1 activity. However, even when neither P-selectin nor PSGL-1 was blocked, the average rolling velocity of Galnt1−/− leukocytes was significantly increased compared with the rolling velocity of control leukocytes (61 ± 20 μm/s versus 38 ± 12 μm/s, respectively; p < 0.05) (Fig. 1B).

More than 1 h after cremaster exteriorization, leukocyte rolling is P-selectin and L-selectin dependent (28). To investigate L-selectin–dependent rolling, the P-selectin–blocking mAb RB40.34 was injected into Galnt1−/− and control mice 1 h after exteriorization of the cremaster muscle. After injecting the Ab, the rolling flux fraction in control mice was 15%, whereas leukocyte rolling was absent in Galnt1−/− mice (Fig. 1C). Leukocyte rolling in control mice after blocking P-selectin was L-selectin dependent, because the additional injection of F(ab′)2 fragments of the L-selectin–

MATERIALS AND METHODS

Animals and bone marrow chimeras

Eight-to-twelve-week-old Galnt1−/− mice (26) or littermate control mice were housed in a specific pathogen-free facility. The Animal Care and Use Committees of the University of Münster approved all animal experiments. Chimeric mice were generated by performing bone marrow transplantation, as described previously (28, 29). Briefly, unfractured bone marrow cells were cultured from genetic-deficient mice were injected i.v. into lethally irradiated mice (9.5 Gy). Experiments were performed 6–8 wk after bone marrow transplantation.

Abs and cytokines

The P-selectin mAb RB40.34 (rat IgG1; 30 μg/mouse) blocks P-selectin–dependent leukocyte rolling and recruitment in vivo (28). The rat anti-mouse E-selectin mAb 9A9 (rat IgG1; 30 μg/mouse) was previously shown to specifically block E-selectin function in vivo (7). The rat anti-mouse mAb to PSGL-1, 4RA10 (rat IgG1; 30 μg/mouse), blocks PSGL-1 in vitro and in vivo (30). The L-selectin mAb MEL14 (rat IgG2a) was purified from hybridoma supernatant (American Type Culture Collection, Manassas, VA). F(ab′)2 fragments of Mel-14 (30 μg/mouse) were prepared by pepsin digestion (Pierce Chemical, Rockford, IL). Optimal digestion (determined by SDS-PAGE) occurred at a reaction time of 5 h in a 37 °C shaker浴槽 at 100 rpm in a Bio-Rad Max Q10 (incubator 1000). F(ab′)2 fragments were purified (confirmed by SDS-PAGE) from undigested Ab and Fc fragments by passage over a protein A AffinityPak column (Pierce Chemical).

Selectin engagement assay and Western blotting

Bone marrow-derived neutrophils were suspended in PBS (1 mM CaCl2/ MgCl2) and were stimulated on E-selectin–coated dishes under shear, as described previously (19). Neutrophils were lysed with RIPA buffer (29). Lysates were boiled with sample buffer or incubated with Sepharose A/G beads and anti-Syk Ab (both from Santa Cruz Biotechnology) or PSGL-1 (4RA10). Immunoblots were immunoblotted using Abs against phosphotyrosine (4G10; Millipore), Syk (Santa Cruz Biotechnology), or PSGL-1 (4RA10). Immunoblots were developed using GE Healthcare’s ECL system.

Intravital microscopy

Mice were anesthetized using i.p. injection of ketamine hydrochloride (125 mg/kg; Sanoﬁ Winthrop Pharmaceuticals) and xylazine (12.5 mg/kg; TranqulVed, Phoenix Scientiﬁc). In the trauma model, the cremaster muscle was prepared for intravital imaging, as described previously (9, 29). To investigate short-term inflammation, mice received an intracutaneous injection of 500 ng TNF-α (R&D Systems, Wiesbaden-Nordenstadt, Germany) in 0.3 ml saline 2 h before cremaster muscle exteriorization. To investigate E-selectin–mediated slow rolling in vivo, some animals also received tail vein injections of 4 μg pertussis toxin (PTx; Sigma-Aldrich, Taufkirchen, Germany), suspended in 0.3 ml saline 5 min before TNF-α injection. Intravital microscopy was performed on an upright microscope (Axioskop; Zeiss, Goettingen, Germany) with a 40× 0.75 NA saline-immersion objective. Leukocyte rolling velocity, leukocyte rolling flux fraction (defined as the number of rolling leukocytes divided by the total number of leukocytes passing through the same vessel) (28), and leukocyte adhesion were determined by transillumination intravital microscopy, whereas leukocyte extravasation was investigated by reflected light oblique transillumination microscopy, as described previously (31, 32). Recorded images were analyzed off-line using Image J and AxioVision (Carl Zeiss) software. Postcapillary venules with a diameter of 20–40 μm were investigated. Blood flow centerline velocity was measured using a dual photodiode sensor system (Circusoft Instrumentation, Hockessin, DE). Emigrated cells were determined in an area extending 75 μm from each side of a vessel over a distance of 100 μm vessel length (representing 1.5 × 106 μm2 tissue area). The microcirculation was recorded using digital cameras (Sensicam QE, Cooke, Romulus, MI or Axiocam, Zeiss, Oberkochen, Germany).

Peritonitis model

Peritoneal recruitment of leukocytes was induced using thioglycollate, according to previously published methods (9, 29). Mice were injected i.p.
FIGURE 1. P-selectin–dependent leukocyte rolling in vivo is severely impaired in Galnt1−/− mice. (A) Leukocyte rolling flux fraction in untreated cremaster venules. Effect of blocking mAbs on leukocyte rolling flux fraction (mean ± SEM) in venules of the cremaster muscle of ppGalNAcT-1–deficient mice (open bars) and control mice (filled bars). Mice were left untreated (n = 4) or were injected with a blocking PSGL-1 mAb (n = 4; 4RA10) or a blocking P-selectin mAb (n = 4; RB40.34) or both (n = 4). (B) Leukocyte rolling velocities in venules of untreated Galnt1−/− mice (n = 4) and littermate control mice (n = 4). Data are presented as cumulative graphs of leukocyte rolling velocities measured during the first hour after exteriorization of the cremaster muscle. Velocity distribution for untreated Galnt1−/− mice (○) and littermate control mice (●). Dashed lines indicate mean rolling velocities. (C) Leukocyte rolling flux fraction in untreated cremaster venules of ppGalNAcT-1–deficient mice (n = 4; open bars) and control mice (n = 4; filled bars) was measured 90 min after exteriorization following injection of a blocking P-selectin mAb (RB40.34) to investigate L-selectin–dependent rolling. (D) Bone marrow–derived leukocytes of control or Galnt1−/− mice were lysed and immunoblotted against PSGL-1 (4RB12). *p < 0.05.

blocking Ab MEL-14 completely abolished rolling in this setting (data not shown).

To verify that PSGL-1 was modified by the ppGalNAcT-1 enzyme, we investigated PSGL-1 from bone marrow–isolated neutrophils by Western blot. PSGL-1 from Galnt1−/− neutrophils showed an increased mobility compared with neutrophils from littermate control mice (Fig. 1D). The calculated molecular masses were 110 and 207 kDa for the Galnt1−/− PSGL-1 monomer and dimer and 120 and 220 kDa for the control PSGL-1 monomer and dimer, respectively.

From these data, we conclude that ppGalNAcT-1 modifies PSGL-1 and that the absence of this modification partially blocks P-selectin–dependent rolling and completely blocks L-selectin–dependent rolling.

Short-term (2-h) TNF-α–induced inflammation

Two hours after TNF-α injection, leukocyte rolling is mediated by P-selectin and E-selectin expressed on inflamed endothelial cells of the cremaster muscle (9, 33). Galnt1−/− mice had a reduced rolling flux fraction on E- and P-selectin compared with control mice 2 h after TNF-α injection (Fig. 2A). To investigate whether the decreased rolling flux fraction is caused by reduced E- or P-selectin binding, we injected blocking Abs. The blockade of E-selectin did not alter the rolling flux fraction in untreated control mice or in Galnt1−/− mice. Blocking P-selectin reduced rolling in wild-type mice slightly, but it suppressed rolling significantly in Galnt1−/− mice. This finding suggests that E-selectin ligands are also affected by the absence of ppGalNAcT-1. As expected, blocking both E- and P-selectin significantly reduced leukocyte rolling in wild-type and Galnt1−/− mice (Fig. 2A). In this model, rolling is mediated by P- and E-selectin, and rolling velocity is further reduced by engagement of the β2-integrins LFA-1 and Mac-1 (33).

In the next step, we investigated the rolling velocity of leukocytes in mice pretreated with TNF-α. The mean rolling velocity of Galnt1−/− leukocytes was significantly elevated in comparison with control leukocytes (Fig. 2B). To investigate the role of Galnt1 in P-selectin–dependent rolling, a blocking E-selectin Ab was injected (Fig. 2C). In this setting, the rolling velocity in Galnt1−/− mice was also significantly increased. Conversely, we blocked P-selectin and studied E-selectin–dependent rolling. This experiment was done in the presence of PTx to eliminate chemokine signaling. E-selectin–mediated leukocyte rolling velocity was also significantly increased in Galnt1−/− mice compared with control mice (Fig. 2D), suggesting that at least some E-selectin ligands require modification by ppGalNAcT-1.

Leukocyte rolling velocities are also affected by the activation of β2-integrins. To investigate whether the signal-transduction cascade is disturbed after E-selectin binding to PSGL-1, we performed an E-selectin–engagement assay to demonstrate intact downstream signaling. We already showed that Syk is phosphorylated after stimulating neutrophils with E-selectin (9). Therefore, we checked Syk phosphorylation in Galnt1−/− and control mice. We demonstrated that downstream signaling is not affected in Galnt1−/− mice compared with control mice after E-selectin engagement (Fig. 2E).

Integrin-mediated adhesion, the step that follows capturing and rolling, is required for leukocyte recruitment into the inflamed tissue (3). Leukocyte adhesion in the cremaster muscle is dependent on E-selectin and G protein-coupled receptor-mediated integrin activation after TNF-α application (31, 34). To investigate whether ppGalNAcT-1–deficient mice show a reduced number of adherent leukocytes under inflammatory conditions, TNF-α was injected intrascrotally 2 h before the experiments, and the number of adherent cells in postcapillary venules of the cremaster muscle was determined. The number of adherent leukocytes in Galnt1−/− mice was significantly reduced compared with control mice (Fig. 3A). Adherent leukocytes eventually transmigrate to enter the inflamed tissue. Therefore, we investigated the number of extravasated leukocytes by using reflected-light oblique transillumination microscopy (31, 32). Galnt1−/− mice had a significantly reduced number of extravasated leukocytes compared with control mice (Fig. 3B). Representative reflected-light oblique transillumination microscopic images of PTx-pretreated control mice and p
Galnt1−/− mice 2 h after TNF-α application are shown in Fig. 3C and 3D, respectively. These data suggest that the reduced rolling and increased rolling velocity lead to a significant reduction in the number of adherent and transmigrated leukocytes in Galnt1−/− mice.

ppGalNAcT-1 deficiency does not affect chemokine-induced arrest

To test whether ppGalNAcT-1 is involved in chemokine-induced arrest, we conducted intravital microscopy of the untreated cremaster muscle (9). In this model, neutrophils roll in cremaster venules as the result of P-selectin expression on the endothelium but rarely adhere. Injection of 600 ng of recombinant murine CXCL1, which binds CXCR2, induced the same number of adherent leukocytes in venules of Galnt1−/− mice (n=4; ○) and littermate control mice (n=4; ⌧) 2 h after TNF-α application. Velocity distribution for untreated Galnt1+/+ mice and littermate control mice. Cumulative velocity distribution for Galnt1−/− mice (n=4; ○) and littermate control mice (n=4; ⌧) with E-selectin–blocking mAb 9A9 (C) or P-selectin–blocking mAb RB40.34 and PTx (4 μg/mouse) to block G0-coupled signaling (D). Dashed lines indicate mean rolling velocities. (E) Control and Galnt1−/− bone marrow cells were stimulated with E-selectin under shear or were left untreated and subjected to immunoprecipitation with a Syk Ab, followed by immunoblotting with a general phosphotyrosine Ab (4G10) or total Syk Ab. *p<0.05.

Discussion

In this study, we used the mouse cremaster muscle and a peritonitis model to investigate the role of ppGalNAcT-1 in the biosynthesis and physiological function of selectin ligands in vivo. We found that P-, E-, and L-selectin–dependent rolling is significantly reduced in cremaster muscle venules of Galnt1−/− mice. In addition, we show that Galnt1−/− mice have a reduced number of adherent and transmigrated leukocytes after TNF-α application. This novel finding shows that ppGalNAcT-1 in hematopoietic cells has a prominent role for leukocyte recruitment.

ppGalNAcT-1 deficiency in leukocytes impairs leukocyte recruitment

To test leukocyte recruitment in a second model of acute inflammation and to differentiate between hematopoietic and non-hematopoietic ppGalNAcT-1 in leukocyte recruitment, we generated bone marrow chimeras and investigated leukocyte recruitment into the peritoneal cavity 4 h (Fig. 5A) or 8 h (Fig. 5B) after thioglycollate injection. Analysis of both time points revealed that control mice or Galnt1−/− mice that received bone marrow from Galnt1−/− mice showed significantly reduced recruitment of leukocytes into the peritoneal cavity (Fig. 5), suggesting that ppGalNAcT-1 in hematopoietic cells has a prominent role for leukocyte recruitment.
The size difference in PSGL-1 monomer and dimer expressed on leukocytes of Galnt1<sup>−/−</sup> and control mice is not consistent with a loss of O-glycosylation, suggesting that the targeted enzyme is only partially involved in O-glycosylation of PSGL-1. Other transferases might contribute to O-glycosylation as well. Consistent with our findings, flow chamber experiments showed that neutrophils from Galnt1<sup>−/−</sup> mice had a reduced, but not abolished, binding capacity to P-selectin and E-selectin under flow conditions (26).

Most selectin ligand activity is mediated by α2,3-sialylated and α-1,3-fucosylated core 2–decorated O-glycans carrying the sLe<sup>α</sup> motif as capping group (10). Several studies documented the contribution of enzymes involved in O-glycan formation to leukocyte recruitment in inflammation. It was shown that the formation of the core 1 and core 2 extensions, the formation of the sLe<sup>α</sup> cap, and the sulfation of a tyrosine of PSGL-1 as main selectin ligand are indispensable for its full function (11). Taken together, our findings confirm and extend previous in vitro findings to physiologically relevant in vivo models. Tenno et al. (26) demonstrated that, under noninflammatory conditions, the number of ppGalNAcT-1–deficient leukocytes rolling on P-selectin or E-selectin was significantly reduced. We extended these findings by using different inflammation animal models, confirming the predominant role of ppGalNAcT-1 in attaching functionally relevant O-linked glycans to selectin ligands under inflammatory conditions. The loss of ppGalNAcT-1 had a severe impact on neutrophil recruitment in the thioglycollate-induced peritonitis model (26). To differentiate between hematopoietic and nonhematopoietic ppGalNAcT-1 in leukocyte recruitment, we generated bone marrow chimeras and investigated leukocyte recruitment into the peritoneal cavity after thioglycollate injection. These experiments revealed that hematopoietic ppGalNAcT-1 is important for leukocyte recruitment. These data demonstrate that, under inflammatory conditions, the loss of ppGalNAcT-1 led to reduced leukocyte rolling and recruitment and increased rolling velocity. The increased leukocyte rolling velocity seen in the absence of ppGalNAcT-1 suggests an increased off-rate of the P- and E-selectin–PSGL-1 bonds in Galnt1<sup>−/−</sup> mice.

Interestingly, rolling on L-selectin is completely abolished in Galnt1<sup>−/−</sup> mice in contrast to control mice. These findings are in accordance with the results of Tenno et al. (26), who showed an impaired lymphocyte homing in Galnt1<sup>−/−</sup> mice. As published recently, mice deficient for core 2 β1,6-N-acetylgalcosaminyltransferase (Gent1), which initiates the core 2 extension, also exhibited reduced L-selectin binding (16). Absence of the sialyltransferase St3GalIV, which contributes to the sLe<sup>α</sup> cap, resulted in complete abolishment of L-selectin–dependent rolling in the cremaster muscle (36). Taken together, these data suggest that binding of L-selectin to its ligand might be exclusively dependent on O-glycans. However, at least in the thioglycollate-induced peritonitis model, the L-selectin defect does not result in reduced recruitment. L-selectin was also reported to bind heparan sulfate proteoglycans on endothelial cells (37, 38), which are not affected by the absence of ppGalNAcT-1. This component of L-selectin–dependent rolling is not obvious in the cremaster muscle model, but it may be present in the peritonitis model. Alternatively, L-selectin–dependent interactions may be irrelevant in thioglycollate-induced peritonitis.

Requirements for E-selectin–dependent rolling in vivo are not well understood. Therefore, we addressed whether E-selectin ligands require modification by ppGalNAcT-1. It is well established that E-selectin ligands must be modified by fucosyl-
transfase VII or IV or another fucosyltransferase to be functional (15). Transfection of fucosyltransferase VII into different cell lines conferred the ability to bind to E-selectin in flow chamber experiments (39, 40), indicating that the glycoprotein requirements for E-selectin binding may be more relaxed than for P-selectin. The three glycoproteins PSGL-1, CD44, and ESL-1 on murine leukocytes are the most physiologically relevant E-selectin ligands (41). Elimination of PSGL-1 impairs E-selectin–mediated tethering in vitro and in vivo (42). ESL-1 was also shown to be an E-selectin ligand in vivo (43). ESL-1 is decorated with N-linked, carbohydrates (44). Therefore, ESL-1 glycosylation should not be affected in ppGalNAcT-1–deficient mice. The third physiological E-selectin ligand in the murine system is CD44 (45). CD44 expresses both N- and O-glycans (46), but it uses N-glycans to interact with E-selectin (45, 47, 48). Therefore, CD44 binding could account for the less severe phenotype observed for E-selectin–dependent rolling. This suggests that loss of ppGalNAcT-1 is unlikely to influence the function of CD44 in leukocyte recruitment. Our data suggest that PSGL-1 is especially impaired in *Galnt1<sup>−/−</sup>* mice.

As published recently, E-selectin engagement leads to the activation of LFA-1 via a Syk-dependent pathway (9, 29, 31, 49, 50). This pathway, together with chemokine-induced signaling, induces leukocyte adhesion and extravasation in the cremaster muscle after TNF-α application and neutrophil recruitment into the peritoneal cavity after thioglycollate injection (31, 34). In *Galnt1<sup>−/−</sup>* mice, leukocyte recruitment was severely impaired in both the cremaster muscle and peritonitis models. In contrast to the role of α2,3-sialyltransferase IV in chemokine-induced arrest (51), *Galnt1<sup>−/−</sup>* mice did not exhibit altered chemokine-induced arrest. Furthermore, *Galnt1<sup>−/−</sup>* leukocytes exhibit an unaltered Syk phosphorylation after stimulation with E-selectin compared with control leukocytes, suggesting that both pathways are intact in *Galnt1<sup>−/−</sup>* leukocytes. We conclude that both P- and E-selectin–dependent rolling is impaired in the absence of ppGalNAcT-1 under physiological flow conditions. This might be due to a reduced affinity of P- and E-selectin for their respective ligands or a reduction in the total number of glycan ligands available on the cell surface to get in contact with their interaction partners on the endothelium. As a result of the impaired selectin–ligand interaction, leukocyte adhesion and transmigration are reduced. Interestingly, the ratio of extravasated leukocytes to intravascular leukocytes is very similar for both *Galnt1<sup>−/−</sup>* mice and control mice. This suggests that the rate of transmigration is not specifically affected by the absence of ppGalNAcT-1. The defect in neutrophil migration can most likely be attributed to the dramatic decrease in neutrophil rolling flux.

These data are in concordance with a recently published study dealing with T-synthease–deficient mice. The T-synthease is responsible for the addition of galactose to GalNAcc1-Thr/Ser (Tn Ag) to form the core 1 backbone (52). In *T-syn<sup>−/−</sup>* mice, E-selectin failed to bind PSGL-1, indicating that PSGL-1 uses core 1–derived O-glycans as E-selectin ligands. However, CD44 and ESL-1 bind normally to *T-syn<sup>−/−</sup>* neutrophils, confirming...
their N-glycan-mediated binding (52). In contrast to Galnt1−/− mice, T-syn−/− mice also exhibited impaired Syk phosphorylation after E-selectin engagement (50). These partially divergent results suggest that other O-glycosylated ligands, which are not affected by ppGalNAcT-1, participate in downstream signaling after E-selectin engagement.

The present study identifies some of the mechanisms by which the absence of ppGalNAcT-1 impairs leukocyte recruitment to sites of inflammation. We conclusively show that P-selectin–dependent rolling via PSGL-1 is severely impaired in Galnt1−/− mice and that rolling through other P-selectin ligands is completely absent in Galnt1−/− mice. Furthermore, we demonstrate that E-selectin–dependent rolling under physiological flow conditions is impaired in Galnt1−/− mice. It is possible that other defects exist in Galnt1−/− mice that might interfere with normal signaling, adhesion, or transmigration of neutrophils. However, the severe functional deficits in leukocyte rolling appear to be sufficient to explain the severe impairment of leukocyte recruitment seen in Galnt1−/− mice.

Acknowledgments
We thank Dr. Jamey D. Marth for sharing the ppGalNAcT-1−/− deficient mice, Dr. Barry Wolitzky (Hoffman-La Roche, Nutley, NJ) for providing the murine E-selectin blocking Ab 9A9, and Dr. Dietmar Vestweber (Max-Planck Institute for Molecular Biomedicine) for providing the RB4.04 and 4RA10 Abs.

Disclosures
The authors have no financial conflicts of interest.

References


