Supporting Information

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SI Materials and Methods

Proteins. Murine P-, L-, and E-selectin-human IgM Fc chimeras and control murine CD45-human IgM Fc chimera were described previously (1). Murine recombinant E-selectin-IgG Fc, ICAM-1-IgG Fc, CXCL1, and TNF-α were from R&D Systems. The following mAbs to murine proteins were from BD Biosciences: rat anti-PSGL-1 (4RA10), hamster anti–ICAM-1 (3E2), rat anti–CD18 (β2 integrin; GAME46), phycoerythrin-labeled rat anti–Gr-1 (RB6-8C5), anti-CD45 (30-F11), anti–PSGL-1 (2PH1), FITC-labeled rat anti-CD18 (C71/16), anti-CD24 (M1/69), anti-CD44 (IM7), and anti–L-selectin (Mel-14). Goat anti-murine CD43 IgG was from Santa Cruz Biotechnology. FITC-labeled goat anti-human IgM and murine anti-goat IgG mAb were from Caltag. Rabbit antibodies to Syk or phospho-Syk were from Cell Signaling Technology. Murine anti-Tn antigen mAb Ca3638 was biotinylated as described (2). Rat anti-murine CD44 mAb KM114 (3) was a gift from Paul Kincade (Oklahoma City, OK). Rat anti-murine E-selectin mAb 9A9 and rat anti-murine P-selectin mAb 5H1 (4) were gifts from Barry Wolitzky (San Diego, CA). Rabbit anti–ESL-1 IgG (5) was a gift from Dietmar Vestweber (Muenster, Germany). FITC-conjugated streptavidin was from Jackson Immunoresearch.

Mice. PSGL-$1^{-/-}$ mice were generated as described (1). $CD44^{-/-}$ mice were purchased from Jackson Laboratory. $CD43^{-/-}$ mice (6) were provided by Paul Kubes (Calgary, AB, Canada). PSGL-$1^{-/-}$ mice were bred with $CD43^{-/-}$ or $CD44^{-/-}$ mice to generate double heterozygous mice; these mice were bred to yield PSGL-$1^{-/-}$/$CD43^{-/-}$ or PSGL-$1^{-/-}$/$CD44^{-/-}$ mice. $CD43^{-/-}$ mice were bred with PSGL-$1^{-/-}$/$CD44^{-/-}$ mice to generate PSGL-$1^{-/-}$/$CD44^{-/-}$/$CD43^{-/-}$ mice. EHC T-$syn^{-/-}$ mice were generated as described (2). EHC T-$syn^{-/-}$ mice were bred with $CD44^{-/-}$ mice to generate EHC T-$syn^{-/-}$/$CD44^{-/-}$ mice. E-$selectin^{-/-}$ mice (7) and P-, L-, E-$selectin^{-/-}$ mice (8) were provided by Klaus Ley (La Jolla, CA). Bone marrow leukocytes from C2GnT$1^{-/-}$ mice (9) were provided by Yuqing Huo (Minneapolis, MN). Chimeric mice were generated by bone marrow transplantation as described (10). WT or EHC T-$syn^{-/-}$ recipient mice were irradiated lethally (9.5 Gy). After irradiation, bone marrow cells (5×10^6 cells) from WT or EHC T-$syn^{-/-}$ donor mice were injected into recipients. Recipient mice were studied 8 weeks after transplantation.

Flow Cytometry. Flow cytometry of murine neutrophils was performed as described (1). Peripheral blood leukocytes were incubated with phycoerythrin- or FITC-labeled mAbs to CD18, CD24, CD44, CD45, L-selectin, or PSGL-1, with goat anti-CD43 IgG followed by FITC-labeled mouse anti-goat IgG mAb, or with biotinylated anti-Tn mAb followed by FITC-labeled streptavidin. Alternatively, they were incubated with murine P-, E-, or L-selectin-IgM or a control CD45-IgM followed by FITC-labeled goat anti-human IgM. In some experiments, E-selectin-IgM binding was measured in the presence of anti–E-selectin blocking mAb 9A9. Neutrophils were identified by light scatter.

Precipitation of Leukocyte Glycoproteins with E-Selectin. Glycoproteins were precipitated with E-selectin or streptavidin from lysates of surface-biotinylated bone marrow leukocytes as described (11), with some modifications. Bone marrow leukocytes were biotinylated with sulfo-NHS-biotin (Thermo Scientific) and washed with PBS solution. Surface-biotinylated cells were lysed with 1% Triton X-100, 125 mM NaCl, 50 mM Tris, pH 7.4, 2 mM $CaCl_2$, 50 μg/mL aprotinin, 50 μg/mL leupeptin, 50 μg/mL pepstatin, and 50 μg/mL bestatin. Some of the lysates were precleared with protein G agarose for 3 h at 4 °C and then incubated with monomeric avidin agarose (Thermo Scientific) for 3 h at 4 °C. After washing the beads, biotinylated surface proteins were eluted by boiling in SDS/PAGE buffer with β-mercaptoethanol. In some experiments, lysates were incubated with murine E-selectin IgM or CD45-IgM premixed with goat anti-human IgM mAb for 2 h at 4 °C in the presence or absence of 5 mM EDTA and then with protein G agarose for 3 h at 4 °C. After washing the beads, proteins bound to E-selectin IgM were eluted with 1% Triton, 125 mM NaCl, 50 mM Tris, pH 7.4, and 5 mM EDTA. In some experiments, eluted E-selectin–bound proteins were precipitated with monomeric avidin agarose for 3 h at 4 °C and then eluted by boiling in SDS/PAGE buffer with β-mercaptoethanol. Equivalent portions of lysate or precipitated proteins were analyzed by Western blotting (1, 12) with antibodies to CD44, PSGL-1, or ESL-1.

Flow Chamber Assay. Bone marrow leukocytes or peripheral blood leukocytes (10^6/mL in HBSS with 0.5% human serum albumin) were perfused over E-selectin at a wall shear stress of 1 dyn/cm^2 (1, 12). In some experiments, ICAM-1-Fc and CXCL1 were also immobilized. Cells were analyzed using video microscopy coupled to a digital analysis system.

E-Selectin–Mediated Syk Phosphorylation. Bone marrow leukocytes were incubated on immobilized E-selectin-IgM or CD45-IgM on a rotary shaker at 65 rpm for 5 min. Cell lysates were analyzed by Western blotting using antibodies against Syk or phospho-Syk (13).

Intravital Microscopy. Intravital video microscopy of anesthetized mice was performed as described (1, 12). The cremaster muscle was isolated and superfused with thermocontrolled (35 °C) bicarbonate-buffered saline solution 4 h after intrascrotal injection of TNF-α. Microvessel diameters, lengths, centerline velocities, and wall shear rates were measured offline and were comparable in mice from all genotypes. Mean leukocyte rolling velocities and rolling flux were analyzed offline. The number of adherent cells was divided by the surface area of the vessel wall seen in the field of view. Surface area was calculated for each vessel, using $S = \pi \times d \times l_v$, where d is the diameter and l_v is the length of the vessel (14).

Thioglycollate-Induced Peritonitis. Mice were injected i.p. with 1 mL of 4% thioglycollate. After 4 h, peritoneal cells were collected with 10 mL PBS solution containing 0.1% BSA and 5 mM EDTA, and the cells were analyzed by flow cytometry. Neutrophils were counted based on scatter properties and high expression of Gr-1 (15).

Competitive Neutrophil Recruitment Assay. Competitive recruitment of fluorescent-labeled neutrophils into the peritoneum after thioglycollate challenge was measured as described (13, 15).

Semiquantitative RT-PCR. Total RNA was isolated from peripheral blood leukocytes with TRIzol reagent (Invitrogen) and then treated with DNase I (Ambion). cDNA was synthesized from 2 μg total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and oligo dT primer. Ten sets of primers specific for different glycosyltransferases were used. These included *Gcnt1* (forward, 5′-GCT TGA TAG GAA CTT GGC AGC AC-3′; reverse, 5′-CAC CTT CTG GAT TTC TTC TGG GTC 3′), *Gcnt3* (forward, 5′-ACC TTC ACT CCA CAT CAC TCA CGG-3′; reverse, 5′-TTA TTC AGC AGA GCC TGG

GTC ACC-3′), *Gcnt4* (forward, 5′-GCC GCT GTT CTT GCT GTT TTG-3′; reverse, 5′-AGT CAC TTG TCA TCG CCA CGA C-3′), *B3gnt1* (forward, 5′-TGC CTA CGT GGT GCC CTG GA-3′; reverse, 5′-AAC CCT GCC ACG TGC AGC TC-3′), *B3gnt2* (forward, 5′-GCC GAG CTG GAG GTG TCC CT 3′; reverse, 5′-CTCGGC CCC AAG ACT CCC GA-3′), *B3gnt3* (forward, 5′-CCA CTG CCG CGA CTT CCC AG-3′; reverse, 5′-AGG GCG GCC ACG GTA AAA CG-3′), *B3gnt4* (forward, 5′-GCT CCA AGG CCC ACC AGC AG-3′; reverse, 5′-CCA GCT TCA GCT GCC GAC CC-3′), *B3gnt5* (forward, 5′-ATC AGG CCC GCC CCT CTC AG-3′; reverse, 5′-CAT CCA GGA GCG GCG TTG GT-3′), *B3gnt6* (forward, 5′-TGC TGC TGG CCG TGA AGT CG-3′; reverse, 5′-GCC CGG TGA AGA GGT GGT GC-3′), and *B3gnt7* (forward, 5′-CAG CGC AGT GTG ACC CCT GG-3′; reverse, 5′-TGA GGG TCA GGT TGA AGG AGC TGT-3′). For all amplifications, 30 cycles of PCR were carried out using an iCycler instrument (Bio-Rad). The quantity of RNA was confirmed by agarose gel electrophoresis with reference to the internal control, *GAPDH*.

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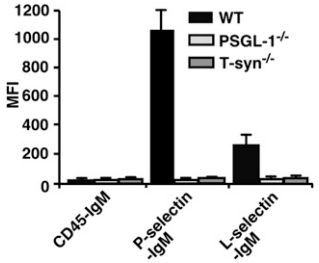

Fig. S1. P- and L-selectin-IgMs do not bind to PSGL-1$^{-/-}$ and T-syn$^{-/-}$ neutrophils. Flow cytometric analysis of binding of selectin-IgMs to leukocytes of the indicated genotype. The cells were incubated with P- or L-selectin-IgM or control CD45-IgM. Neutrophils were gated by light scatter. Binding was detected with FITC-conjugated goat anti-human IgM antibodies and is represented as mean fluorescence intensity (MFI). The data represent the mean ± SEM from at least three experiments.

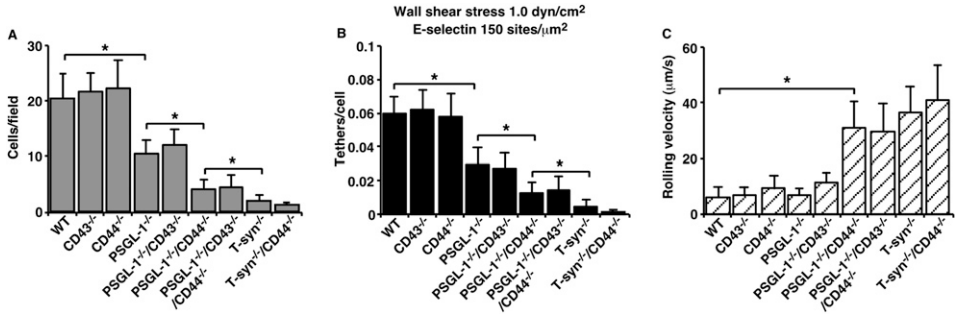

Fig. S2. T-syn$^{-/-}$ neutrophils from bone marrow have markedly impaired rolling on E-selectin in vitro. Bone marrow leukocytes of the indicated genotype were perfused over E-selectin immobilized at 150 sites/μm^2. (*A*) Number of neutrophils rolling per field of view. (*B*) Number of neutrophils that tethered to E-selectin during the first 30 s was divided by the number of neutrophils delivered across the field of view in the focal plane. (*C*) Velocities of neutrophils rolling on E-selectin. The data represent the mean ± SEM from five experiments. *$P < 0.01$.

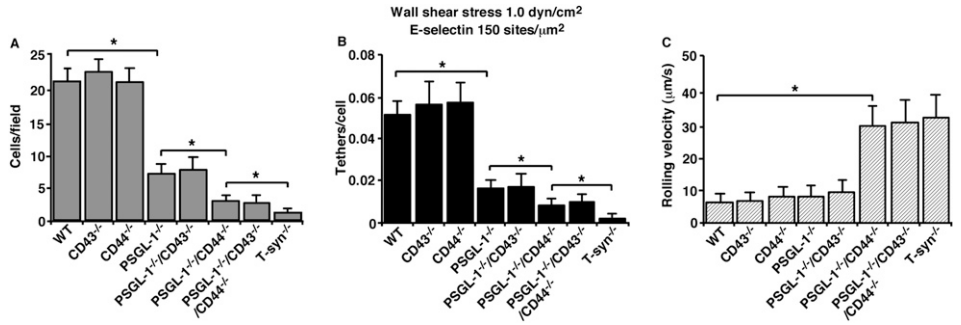

Fig. S3. T-syn$^{-/-}$ neutrophils from peripheral blood have markedly impaired rolling on E-selectin in vitro. Peripheral blood leukocytes of the indicated genotype were perfused over E-selectin immobilized at 150 sites/μm^2. (*A*) Number of neutrophils rolling per field of view. (*B*) Number of neutrophils that tethered to E-selectin during the first 30 s was divided by the number of neutrophils delivered across the field of view in the focal plane. (*C*) Velocities of neutrophils rolling on E-selectin. The data represent the mean ± SEM from five experiments. *$P < 0.01$.

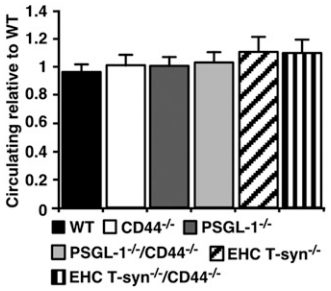

Fig. S4. Equal numbers of injected fluorescent-labeled WT and T-syn$^{-/-}$ neutrophils are recovered from blood. WT mice were injected i.p. with thioglycollate. After 2 h, they were injected i.v. with a 1:1 mixture of PKH67-labeled WT leukocytes and PKH26-labeled leukocytes of the indicated genotype. After 2 h, blood was collected, and the number of neutrophils labeled with each dye was measured by flow cytometry. Results are plotted as the ratio of neutrophils from the indicated genotype to WT neutrophils. The data represent the mean ± SEM from five experiments. Fig. 6 in the main text presents the ratios of labeled cells collected from the peritoneum at the same time.

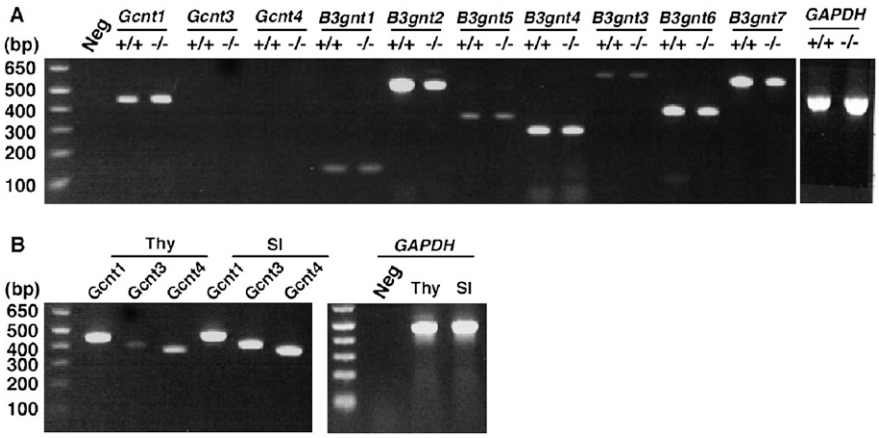

Fig. S5. RT-PCR amplification of mRNA for C2GnTs and β3GlcNAcTs. Total RNA was isolated from peripheral blood leukocytes of WT ($^{+/+}$) or EHC T-syn$^{-/-}$ ($^{-/-}$) mice (*A*) or from thymus (Thy) or small intestine (SI) from WT mice (*B*). cDNAs were synthesized with RT and amplified by PCR using primers specific for the indicated gene. PCR amplification without prior RT was used as negative control (Neg). RT-PCR of the constitutively expressed *GAPDH* gene was used as internal control. The genes *Gcnt1*, *Gcnt3*, and *Gcnt4* encode C2GnT1, C2GnT2, and C2GnT3, respectively. The genes *B3gnt1-7* encode β3GlcNAcT1-7, respectively. The data are representative of three experiments.

Table S1. Leukocyte counts in peripheral blood from WT and T-syn$^{-/-}$ mice

Blood cell type	WT (n = 10)	EHC T-syn$^{-/-}$ (n = 12)
Total leukocytes (per μL)	6,100 ± 500	15,700 ± 1,000
Neutrophils	800 ± 80	7,200 ± 600
Lymphocytes	4,800 ± 120	6,200 ± 600
Monocytes	200 ± 10	500 ± 50
Eosinophils	60 ± 5	100 ± 10

Data are presented as mean ± SEM.