

P-selectin Glycoprotein Ligand-1 Mediates L-Selectin-dependent Leukocyte Rolling in Venules

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Abstract

Leukocyte rolling in postcapillary venules of inflamed tissues is reduced in L-selectin-deficient mice and mice treated with L-selectin blocking antibodies, but the glycoprotein ligand for L-selectin in inflamed venules is unknown. Here, we show that L-selectin-dependent rolling after P-selectin blockade is completely absent in P-selectin glycoprotein ligand-1 (PSGL-1)^{-/-} mice or wild-type mice treated with a PSGL-1 blocking monoclonal antibody. Immunohistochemistry and flow cytometry failed to show PSGL-1 expression on resting or inflamed endothelium or on platelets. To investigate whether leukocyte-expressed PSGL-1 is mediating L-selectin-dependent rolling, we reconstituted lethally irradiated wild-type mice with PSGL-1^{-/-} bone marrow cells. These chimeric mice showed no L-selectin-dependent rolling, suggesting that leukocyte-expressed PSGL-1 mediates L-selectin-dependent rolling. Frame-to-frame video analysis of L-selectin-dependent rolling in wild-type mice showed that the majority of observed L-selectin-dependent leukocyte rolling was between free flowing leukocytes and already adherent leukocytes or possibly leukocyte fragments, followed by E-selectin-dependent leukocyte rolling along the endothelium. Leukocyte rolling was significantly slower for leukocyte-endothelial than leukocyte-leukocyte interactions. We conclude that leukocyte-expressed PSGL-1 serves as the main L-selectin ligand in inflamed postcapillary venules. L-selectin binding to PSGL-1 initiates tethering events that enable L-selectin-independent leukocyte-endothelial interactions. These findings provide a molecular mechanism for the inflammatory defects seen in L-selectin-deficient mice.

Key words: PSGL-1 • L-selectin • inflammation • leukocyte rolling • intravital

Introduction

Leukocyte recruitment to sites of inflammation follows a multistep process (1) beginning with the initial capture of leukocytes to the vessel wall and leukocyte rolling along the endothelial layer. During rolling leukocytes gradually become activated (2), which eventually leads to firm adhesion and transmigration. Leukocyte capture and rolling, the initial steps of leukocyte recruitment, require the selectin

family of adhesion molecules to bind to their counterreceptors or selectin ligands (3, 4). L-selectin is constitutively expressed on most leukocytes and serves as a lymphocyte homing receptor with well-defined L-selectin ligands expressed on high endothelial venules (5).

L-selectin is also involved in the recruitment of leukocytes into sites of inflammation. This was originally shown as a reduction of leukocyte migration into inflamed skin in mice treated with the L-selectin blocking mAb MEL-14 (6) and confirmed by *in vivo* studies using mice deficient in L-selectin showing a significant reduction in the number of leukocytes recruited into inflamed peritoneum 24 to 48 h after intraperitoneal injection of thioglycollate (7). Leukocyte rolling in cremaster muscle venules of L-selectin-defi-

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cient mice or mice treated with a function blocking mAb against L-selectin was significantly decreased at 1 h or later after exteriorization of the cremaster muscle (8) or after prolonged stimulation with TNF- α (9) but not in freshly exteriorized venules (8). These findings suggested the existence of inducible endothelial ligand(s) for L-selectin.

In vitro studies on TNF- α stimulated human umbilical vein endothelial cells (HUVECs)* incubated with isolated leukocytes in a rotating assay showed that blocking of L-selectin led to a 50% reduction in leukocyte adhesion to the HUVEC monolayer (10). However, this study did not address if the reduction in leukocyte adhesion was due to a decrease in leukocyte rolling preceding the firm arrest of leukocytes. Bargatze et al. showed that rolling of isolated human neutrophils on HUVECs stimulated with IL-1 for 4 h was blocked by mAbs against L-selectin (11). Interestingly, a significant portion of neutrophils rolled on already adherent neutrophils and this rolling was mediated via L-selectin on the rolling cells (11). Alon et al. later introduced the term 'secondary tethering' for this phenomenon and demonstrated that human neutrophils perfused on P- or E-selectin accumulated significantly faster in the presence of L-selectin-dependent leukocyte-leukocyte interactions (12). These interleukocyte tethering events led to the characteristic formation of strings not observed after treatment with an L-selectin blocking mAb. However, such strings are not observed in vivo, and initial studies in TNF- α -treated postcapillary venules failed to identify secondary tethering as an important mechanism (13). In larger vessels with diameters $>45 \mu\text{m}$, but not in smaller venules (diameters $<45 \mu\text{m}$), the L-selectin blocking mAb MEL-14 significantly reduced leukocyte rolling (14), suggesting secondary tethering events.

Several studies identified the homodimeric sialomucin P-selectin glycoprotein ligand-1 (PSGL-1) as an L-selectin ligand mediating leukocyte-leukocyte interactions in vitro (15, 16). In contrast, L-selectin ligands mediating leukocyte capture and rolling in inflamed or cytokine-activated venules could only be identified at a functional but not at a molecular level (5, 8, 9). Studies on human microvascular endothelial cells stimulated with TNF- α suggested that L-selectin-dependent leukocyte rolling required sulfated L-selectin ligands (17, 18). In mice deficient in core2 β 1,6 N-acetyl glucosaminyltransferase I (19), a key enzyme in the posttranslational glycosylation of functional selectin ligands, L-selectin ligand activity was completely lost on inflamed postcapillary venules of the cremaster muscle while L-selectin ligand activity was unchanged in Peyer's patch high endothelial venules (20). In addition, fucosylation-dependent epitopes such as HECA452 have been identified on putative L-selectin ligands expressed on fucosyltransferase-VII transfected human vascular endothelial cells (18), suggesting a contribution of α 1,3 linked fucose to L-selectin ligand activity on inflamed endothelium. Cumulatively, these functional data provided strong evidence

*Abbreviations used in this paper: HUVEC, human umbilical vein endothelial cell; PSGL, P-selectin glycoprotein ligand-1.

for the existence of an inducible L-selectin ligand on inflamed endothelium exhibiting glycosylation requirements typical of selectin ligands.

The present study was undertaken to find ligand glycoproteins relevant for L-selectin-dependent rolling on inflamed venular endothelium. To this end, we investigated leukocyte rolling in unstimulated and TNF- α -treated cremaster muscle venules under conditions of isolated L-selectin-mediated rolling (8) achieved by blocking P- and E-selectin function. Unexpectedly, we found a complete absence of L-selectin-dependent leukocyte rolling in PSGL-1-deficient mice.

Materials and Methods

Animals. Mice lacking a functional gene encoding PSGL-1 were generated as described earlier (21) and maintained as a heterozygous breeding colony at the University of Virginia. The wild-type PSGL-1 allele was detected using PCR primers PC-I (5'-CCA TAT CTC TGC TCC TGT CTC-3') and PC-II (5'-TAC AGC CTG AAT CCT GGG AAG CTG-3') located adjacent to the deleted region, while the inserted *Neo*-gene of the mutant allele was detected using PKO-1 (5' CTT TTT GTC AAG ACC GAC CTG TC 3') and PKO-2 (5' AGA TCA TCC TGA TCG ACA AGA CC 3'). Heterozygous (+/-) and homozygous (+/+) littermates were used for control experiments. All PSGL-1^{-/-} mice and wild-type mice used in this study were at least 8 wk of age and appeared healthy. Mice were housed in a barrier facility under SPF conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Antibodies and Cytokines. Monoclonal blocking antibodies RB40.34 (rat IgG1, 30 $\mu\text{g}/\text{mouse}$; reference 22) against P-selectin and 4RA10 (rat IgG1, 30 $\mu\text{g}/\text{mouse}$; reference 23) against PSGL-1 were provided by Dr. D. Vestweber, University of Münster, Münster, Germany. E-selectin blocking mAb 9A9 (rat IgG1, 30 $\mu\text{g}/\text{mouse}$; reference 24) blocks E-selectin-dependent rolling in vivo and in vitro and was provided by Dr. B. Wolitzky (Hoffmann-La Roche, Inc., Nutley, NJ). The rat anti-mouse L-selectin blocking mAb MEL-14 (rat IgG2a; reference 25) and CD18 mAb GAME-46 were purified from hybridoma supernatant (American Type Culture Collection). MEL-14 was processed to F(ab')₂-fragments (Pierce Immunoglobulin F(ab')₂ Preparation Kit; Pierce Chemical Co.). In some experiments, recombinant murine TNF- α (R&D Systems) was injected intrascrotally at a dose of 500 ng per mouse in a volume of 0.3 ml of sterile normal saline more than 4 h before the intravital microscopy experiment was started.

Transfer of PSGL-1^{-/-} Bone Marrow into Wild-Type Mice. Approximately 2×10^6 unfractionated bone marrow cells from femora and tibiae of PSGL-1^{-/-} mice were injected into lethally irradiated wild-type littermates (2 doses of 600 rad each 4 h apart) via tail vein as described (26). After bone marrow transplantation, recipient mice were transferred back to individually ventilated cages with high-efficiency particulate air filters and received autoclaved water containing 5 mM sulfamethoxazole and 0.86 mM trimethoprim (Sigma-Aldrich). Recipient mice were used for experiments more than 4 wk after bone marrow reconstitution when white blood cell counts had fully recovered.

Flow Cytometry. Flow cytometry was used to detect PSGL-1 expression on peripheral blood leukocytes and platelets of wild-type mice, PSGL-1-deficient mice, and lethally irradiated wild-

type mice transplanted with bone marrow from PSGL-1-deficient mice. In addition, L-selectin expression on leukocytes of PSGL-1-deficient mice was investigated. Whole blood was centrifuged and red blood cells were lysed with PharM-Lyse-10X solution (BD Biosciences), blood cells were suspended in PBS-1% BSA (Sigma-Aldrich) solution and incubated for 30 min on ice ($0.5 \mu\text{g}/10^6$ cells) in the dark with the anti PSGL-1 mAb 2PH-1 conjugated with PE to detect leukocyte- or platelet-expressed PSGL-1 or anti-L-selectin mAb MEL-14 conjugated with PE to detect leukocyte-expressed L-selectin. As a negative control, isotype matched control antibodies conjugated with PE were used (all BD Biosciences). After incubation, cells were washed twice in PBS-1% BSA and analyzed by flow cytometry. For platelet flow cytometry, whole carotid blood was centrifuged at 280 g for 8 min at room temperature. The plasma layer and upper portion of the cellular layer were removed and recentrifuged at 280 g for 3 min. Platelets from the platelet-rich plasma layer were then counted using a Hemavet 850 (CDC Technologies), divided into samples of 5×10^6 cells, and stained for 30 min at 4°C with optimal staining concentrations of PE rat anti-mouse PSGL-1 (2PH1) or rat IgG2a, FITC rat anti-mouse CD41 (MWRreg30), and APC rat anti-mouse GR-1 (RB6-8C5) or MAC-1 (M1/70) antibodies (BD Biosciences). Samples were centrifuged at 600 g for 15 min at room temperature and washed twice before analysis. (FACScan™ with CELLQuest™ software package; Becton Dickinson).

Intravital Microscopy. Mice were anesthetized (20) and prepared for intravital microscopy (27) as described. Venular diameter, venular vessel segment length, and leukocyte rolling velocity were measured using two digital image processing systems (28, 29). Venular centerline red blood cell velocity in the cremaster muscle preparation was measured using a dual photodiode and a digital on-line cross-correlation program (Circusoft Instrumentation), converted to mean blood flow velocity (30) and wall shear rate (31) and used to calculate rolling leukocyte flux fraction.

Frame-to-frame video-analysis of image sequences obtained during intravital experiments was used to assess interactions of rolling leukocytes with the vessel wall. Leukocyte-leukocyte interactions were defined as free flowing leukocytes interacting with already adherent or rolling leukocytes. Leukocyte-leukocyte interactions were also assumed for free flowing leukocytes when interactions were observed in close proximity ($<5 \mu\text{m}$) to the adherent leukocyte.

Immunohistochemistry. Wild-type mice were prepared for intravital microscopy as described above. More than 1 h after surgery, the P-selectin blocking mAb RB40.34 was injected and L-selectin-mediated rolling observed. The entire cremaster was then fixed in 4% paraformaldehyde overnight and transferred into 70% ethanol the next day, dehydrated through graded alcohol and xylene and embedded in paraffin. $4 \mu\text{m}$ tissue sections were deparaffinized, endogenous peroxidase activity was inactivated with 0.3% hydrogen peroxide, and sections were then washed in PBS with blocking solution (10% goat serum in PBS) for 20 min, washed, and incubated overnight at 4°C with anti-PSGL-1 mAb 4RA10, diluted 1:30. After washing, sections were incubated with a secondary isotype matched goat anti-rat IgG for 10 min, washed and incubated for 30 min with ABC reagent, containing a preformed avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain; Vector Laboratories). After washing, sections were placed in diaminobenzidine/hydrogen peroxide solution (DAB Peroxidase Substrate Kit; Vector Laboratories), counterstained with hematoxylin, 0.5% cupric acid and 1% lithium carbonate. After

dehydration, sections were enclosed in a xylene-based mounting medium (Cytoseal XYL; Stephens Scientific) for microscopic analysis, using an oil immersion objective (FLUAR Oil 100/1.3 numerical aperture).

Statistics. Sigma Stat 2.0 (SPSS Science) was used for statistical analysis. Leukocyte counts, vessel diameters, leukocyte rolling flux fractions, shear rates, and leukocyte rolling velocities between groups and treatments were compared with one-way ANOVA followed by a multiple pairwise comparison test (Dunn's test) or by Wilcoxon rank-sum test, as appropriate. Statistical significance was set at $P < 0.05$, indicated by *.

Online Supplemental Material. Flow cytometry results for PSGL-1 expression on platelets, frame-by-frame analysis of leukocyte rolling, and a video are available at <http://www.jem.org/cgi/content/full/jem.20021854/DC1>.

Results

L-selectin-mediated Rolling in Venules with Trauma-induced Inflammation. We compared leukocyte rolling in 80 venules of seven PSGL-1^{-/-} mice with rolling in 70 venules of nine littermate controls. Vessel diameter, blood flow velocity, and wall shear rate showed no significant difference among the groups (Table I). Leukocyte rolling in untreated venules is initially P-selectin-dependent (8, 32), followed by a second phase beginning 1 h after exteriorization which is both L-selectin and P-selectin-dependent (8, 33). To investigate L-selectin-dependent rolling, the P-selectin blocking mAb RB40.34 was injected into wild-type mice and leukocyte rolling flux assessed at more than 60 min after exteriorization. This led to a considerable decrease in rolling flux fraction from 27 to 5% at more than 1 h after exteriorization (Fig. 1 A). The residual rolling was almost entirely dependent on L-selectin, because rolling was blocked by injection of the L-selectin blocking antibody F(ab')₂ MEL-14 (Fig. 1 A). L-selectin-dependent rolling is not equally distributed throughout the venular tree but observed only in some postcapillary venules which were used for analysis. Analysis of the recorded videotapes did not show any evidence of accumulated platelets in postcapillary cremaster muscle venules of wild-type mice where L-selectin-mediated rolling was observed.

Consistent with a previous report (34), the leukocyte rolling flux fraction in PSGL-1^{-/-} mice during the first hour after surgery was much lower than in wild-type mice (Fig. 1 A). Injection of the P-selectin blocking mAb RB40.34 more than one hour after surgery eliminated leukocyte rolling in PSGL-1^{-/-} mice. To further investigate the role of PSGL-1 as L-selectin ligand we injected the PSGL-1 blocking mAb 4RA10 into wild-type mice in which P-selectin had been blocked and found an almost complete loss of L-selectin-dependent leukocyte rolling (Fig. 1 A). These results demonstrate that PSGL-1 is required for L-selectin-dependent rolling in trauma-induced inflammation. The reduced L-selectin-mediated rolling in PSGL-1-deficient mice was not due to low expression of leukocyte L-selectin, because flow cytometry revealed equal expression of L-selectin on both wild-type and PSGL-1-deficient leukocytes (21).

Table I. Hemodynamic and Microvascular Parameters in Trauma- and TNF- α -induced Inflammation

Mouse genotype	Mice	Venules	Diameter	Centerline velocity	Wall shear rate	Systemic leukocyte counts
	<i>n</i>	<i>n</i>	μm	$\mu\text{m/s}$	s^{-1}	$\text{cells}/\mu\text{l}$
Trauma						
PSGL-1 ^{-/-}	7	80	31 ± 1	2,900 ± 300	900 ± 300	8,600 ± 900*
Wild-type	9	70	32 ± 1	2,900 ± 300	1,000 ± 100	5,800 ± 500*
Chimera	3	37	32 ± 3	3,200 ± 700	1,000 ± 200	7,200 ± 300
TNF-α						
PSGL-1 ^{-/-}	4	37	29 ± 2	1,800 ± 200	690 ± 110	3,000 ± 300
Wild-type	4	54	29 ± 1	1,800 ± 100	630 ± 50	2,200 ± 200
Chimera	2	16	31 ± 1	1,900 ± 200	700 ± 80	5,300 ± 600

Diameter, centerline velocity, and wall shear rate are presented as mean \pm SEM of all investigated venules. Significant difference ($P < 0.05$) in systemic leukocyte counts is indicated by *.

L-selectin-mediated Rolling in TNF- α -induced Inflammation. Intrascrotal injection of TNF- α induces the expression of E-selectin and augments expression of P-selectin (35). Four or more hours after TNF- α injection, significant L-selectin-mediated rolling can be observed (9). We investigated L-selectin-dependent rolling in 37 venules of four (4–6 h) TNF- α treated PSGL-1^{-/-} mice and compared it to rolling in 54 venules of four TNF- α -treated littermate control mice. Vessel diameter, blood flow velocity, and wall shear rate of the different treatment groups were similar (Table I). Consistent with previous studies, leukocyte counts were reduced in both PSGL-1-deficient mice and wild-type mice due to prolonged treatment with TNF- α (Table I).

Injection of anti-P-selectin mAb RB40.34 and anti-E-selectin mAb 9A9 into TNF- α treated wild-type mice more

than 4 h after TNF- α injection led to a significant drop in leukocyte rolling flux fraction from 13 to 6% (Fig. 1 B). The residual rolling was L-selectin-dependent as it could be blocked with the additional injection of the anti-L-selectin F(ab')₂ MEL-14 (Fig. 1 B). By contrast, no leukocyte rolling was seen after injecting anti-P-selectin mAb RB40.34 and anti-E-selectin mAb 9A9 into (4–6 h) TNF- α treated PSGL-1^{-/-} mice (Fig. 1 B), showing that L-selectin-mediated rolling is also PSGL-1 dependent in this model.

L-selectin-dependent Rolling in Inflamed Venules Is Mediated by PSGL-1 Derived from Bone Marrow-derived Cells. To address the question whether L-selectin-dependent rolling is mediated by a yet unidentified expression of PSGL-1 on endothelial cells, we conducted immunohistochemistry of the cremaster muscle one hour after surgery using the

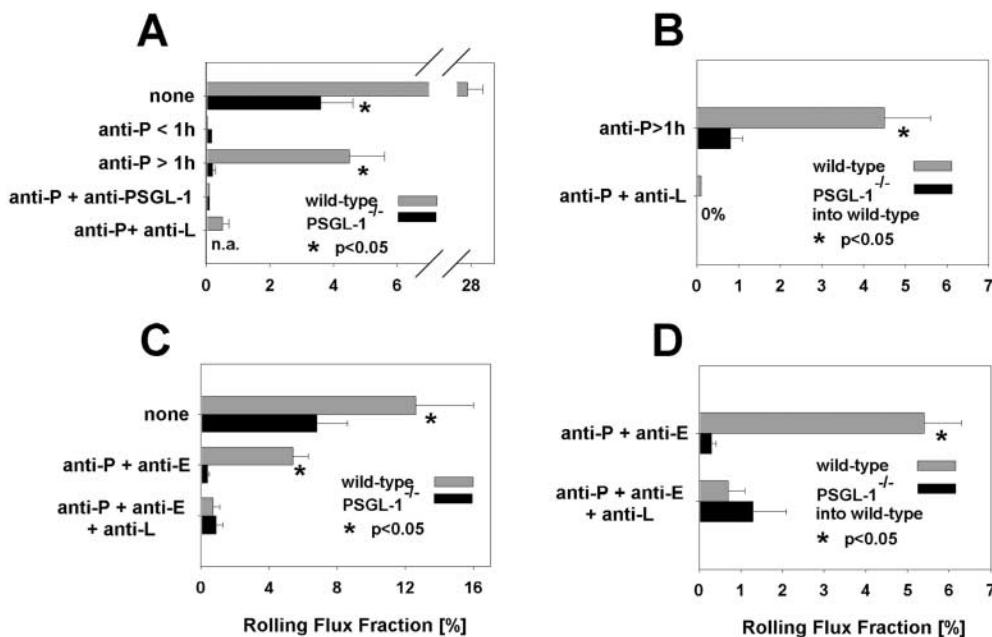


Figure 1. Leukocyte rolling flux fraction (mean \pm SEM) in (A) untreated cremaster muscle venules of PSGL-1 deficient mice (black bars, $n = 7$) and wild-type mice (gray bars, $n = 9$), (C) in 4–6 h TNF- α treated cremaster muscle venules of PSGL-1-deficient ($n = 4$) and wild-type mice ($n = 4$), or in (B) untreated or (D) TNF- α -treated wild-type mice reconstituted with bone marrow from PSGL-1-deficient mice (black bars, $n = 3$ and $n = 2$, respectively). anti-P, P-selectin blocking mAb RB40.34; anti-PSGL-1, PSGL-1 blocking mAb 4RA10; anti-L, L-selectin blocking F(ab')₂ of mAb MEL-14, anti-E, E-selectin blocking mAb 9A9. < 1 h means within 1 h of exteriorization, all other data at > 1 h after exteriorization. Significant differences ($P < 0.05$) in leukocyte rolling flux fraction between PSGL-1^{-/-} and wild-type group are indicated by *.

PSGL-1 mAb 4RA10. We could not detect any expression of PSGL-1 on venular endothelium throughout the cremaster muscle (unpublished data). To formally demonstrate the importance of PSGL-1 expressed on bone marrow-derived cells, we transferred PSGL-1^{-/-} bone marrow cells into lethally irradiated control littermates ($n = 3$). Leukocytes of chimeric mice failed to stain with phycoerythrin-labeled anti-PSGL-1 mAb 2PH-1 using flow cytometry (unpublished data). Hemodynamic parameters and leukocyte counts in chimeric mice during intravital experiments were similar compared with PSGL-1^{-/-} mice and wild-type mice (Table I). L-selectin-dependent leukocyte rolling was assessed more than 1 h after surgery and after injection of P-selectin blocking mAb RB40.34. As shown in Fig. 1 C, L-selectin-dependent rolling was sharply reduced in the chimeric mice, which demonstrates that PSGL-1 expressed on bone marrow-derived cells mediates L-selectin-dependent rolling in this model. Similar results were obtained in cremaster venules of bone marrow-transplanted mice treated with TNF- α for 4 h (Fig. 1 D), ruling out the possibility that TNF- α treatment induces functionally significant amounts of PSGL-1 in endothelial cells. Since in one study (23) PSGL-1 expression has also been reported on platelets, we measured PSGL-1 by flow cytometry and found that PSGL-1 was not detectable on CD41⁺ blood platelets (online supplemental Fig. S1).

P-selectin-dependent Rolling Facilitates L-selectin-mediated Rolling in Trauma-induced Inflammation. As demonstrated above, leukocyte-expressed PSGL-1 mediates L-selectin-dependent rolling in inflamed cremaster muscle venules. This led us to hypothesize that leukocyte-endothelial interactions mediated via P-selectin during early time-points (within the first hour after exteriorization of the cremaster muscle) may help to deliver leukocytes and possibly leukocyte fragments to the venular wall that support L-selectin-mediated rolling more than 1 h after surgery. To test this, we pretreated wild-type mice with the P-selectin blocking mAb Rb40.34 1 h before surgery aiming to block leukocyte rolling completely during the first hour after exteriorization of the cremaster muscle and hence prevent most leukocytes from interacting with the venular endothelium. Untreated mice were not included in our analysis, because we wished to focus on the L-selectin-dependent portion of leukocyte rolling. As shown in Fig. 2 A, L-selectin-dependent leukocyte rolling was dramatically reduced to 1% when P-selectin was blocked before surgery. This contrasts with a rolling flux fraction of 5% when P-selectin was blocked acutely during intravital microscopy. No statistically significant effect was seen after blocking CD18 before surgery (Fig. 2 A), suggesting that leukocyte rolling through P-selectin is required to support L-selectin-dependent rolling later, and that leukocyte adhesion is not prominent in this model. L-selectin-dependent rolling was also reduced in TNF- α -treated mice when rolling was inhibited by blocking E- and P-selectin or when firm adhesion was inhibited by blocking CD18 integrins before injection of TNF- α (Fig. 2 B), suggesting that both rolling and firmly adherent leukocytes contribute to later L-selectin-dependent rolling.

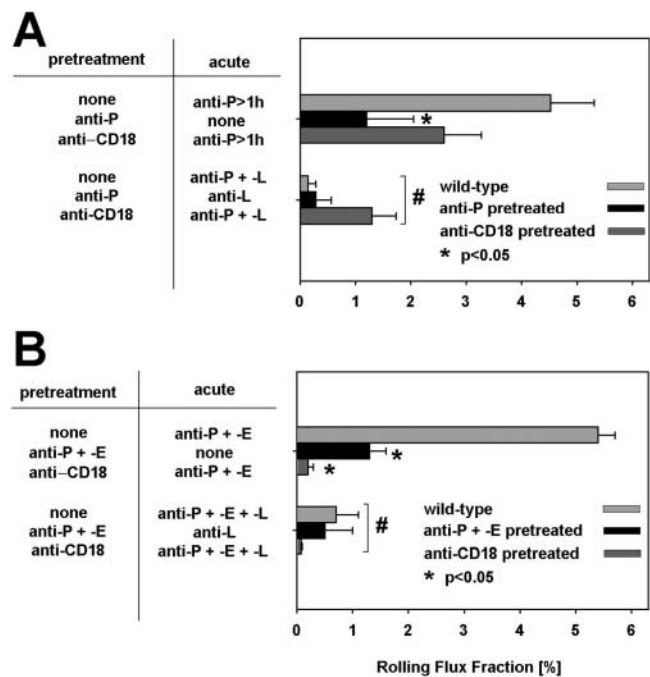


Figure 2. L-selectin-dependent leukocyte rolling flux fraction (mean \pm SEM) at >60 min after exteriorization of (A) the untreated (trauma) or (B) TNF- α -treated cremaster muscle in wild-type mice in which P-selectin (anti-P, black bars, $n = 3$), both P- and E-selectin (anti-P/E, black bars, $n = 4$) or CD18 (dark gray bars, $n = 3$ for trauma, $n = 2$ for TNF- α) was blocked before exteriorization of the cremaster muscle (pretreated). For comparison, L-selectin-dependent rolling in both models is shown (light gray bars, data from Fig. 1). Significant differences between different pretreatments ($P < 0.05$) indicated by *. To demonstrate that rolling is L-selectin-dependent in all cases, anti-L, L-selectin blocking F(ab')₂ of mAb MEL-14 was added (lower group of bars in each panel), resulting in significantly reduced rolling flux fractions (indicated by #) compared with mice in which only P-selectin (A) or P- and E-selectin were blocked (B).

Leukocyte Rolling Velocities for L-Selectin-dependent Rolling. Leukocyte rolling velocities were measured more than 1 h after surgery and after injection of P-selectin blocking mAb RB40.34 (Fig. 3 A). This rolling was L-selectin dependent, because it was completely abolished by injecting F(ab')₂ fragments of MEL-14. In wild-type mice, leukocyte rolling velocities ($n = 127$) distributed broadly with an average rolling velocity of $64 \pm 4 \mu\text{m/s}$ (Fig. 3 A). This is consistent with an earlier report on L-selectin-dependent rolling velocities (33). In PSGL-1-deficient mice, L-selectin-dependent rolling was abolished so that leukocyte rolling velocities of only 11 leukocytes could be measured, which averaged $40 \pm 13 \mu\text{m/s}$ (Fig. 3 B). This rolling was blocked by injection of mAb 9A9 to E-selectin. Analysis of E-selectin-dependent leukocyte rolling velocities in wild-type mice treated with blocking antibodies against P-selectin (RB40.34) and against L-selectin (F(ab')₂ MEL-14) revealed a similar rolling velocity in the few rolling cells left ($23 \pm 5 \mu\text{m/s}$, $n = 15$; Fig. 3 C).

Leukocyte-Leukocyte Interactions Account for Most of the L-Selectin-dependent Rolling in Inflamed Postcapillary Venules. To investigate whether leukocyte-leukocyte interactions contribute to L-selectin-dependent rolling, frame-to-frame

video-analysis of leukocyte rolling was conducted under conditions where rolling is mostly dependent on L-selectin in wild-type mice. A total of 127 leukocytes were investigated in wild-type mice. Most of the rolling leukocytes (85%) interacted near already adherent leukocytes. In some cases, interactions were initiated in the absence of visible leukocyte–leukocyte interactions, suggesting that leukocyte fragments deposited on the surface of endothelial cells may be responsible for initiating leukocyte rolling. Scanning electron microscopy of microvessels failed to reveal a clear picture of leukocyte fragments as initiating events (unpublished data). However, in support of the idea that such fragments may be generated during rolling, we found that sev-

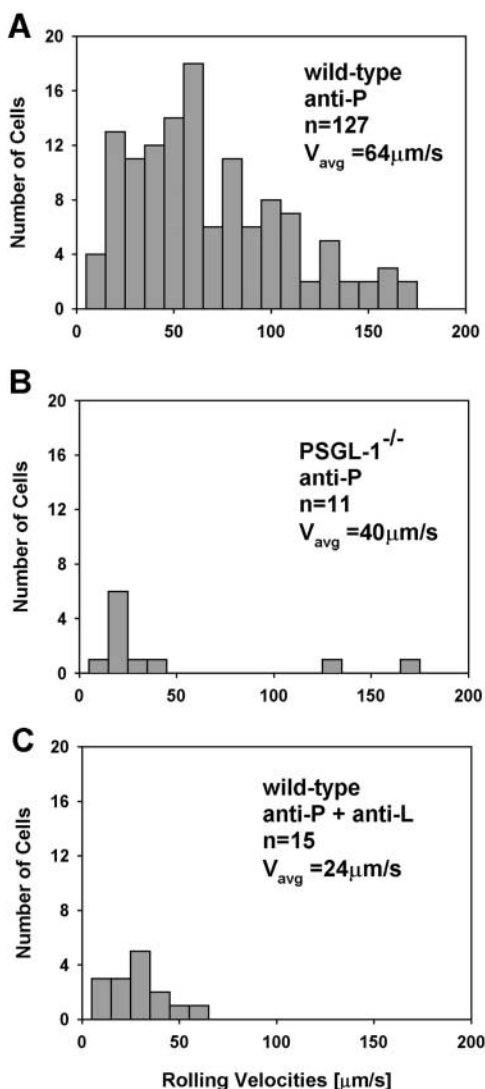


Figure 3. Leukocyte rolling velocity distribution in untreated cremaster muscle venules of (A) wild-type mice treated with P-selectin blocking mAb RB40.34 (anti-P), (B) PSGL-1^{-/-} mice treated with anti-P, and (C) wild-type mice treated with anti-P and anti-L (L-selectin blocking F(ab')₂ of mAb MEL-14) >60 min after exteriorization. Rolling was completely blocked by adding L- and E-selectin mAb in panel A, or E-selectin mAb in panels B and C. Absolute cell numbers are shown for leukocyte velocity groups with bin sizes of 10 $\mu\text{m/s}$.

eral micrometer-long tethers were pulled from some rolling cells (Fig. 4). Although we did not directly observe these tethers detach from the leukocyte, such a mechanism is plausible based on in vitro experiments (36) conducted under conditions of superior optical resolution not achievable by intravital microscopy.

Frame-to-frame video-analysis revealed intermittent leukocyte interaction while moving from one adherent leukocyte to the next (supplemental Fig. S2, A and B). In some instances, leukocyte–leukocyte interactions were followed by direct contact of the rolling leukocyte with the endothelium, followed by a dramatic decrease in rolling velocity. Leukocyte rolling in wild-type mice treated with blocking mAbs against P- and L-selectin also showed rare, E-selectin-dependent leukocyte–endothelial interactions (supplemental Fig. S2 C), while leukocyte–leukocyte interactions were absent.

Discussion

This study identifies PSGL-1 as the L-selectin ligand responsible for L-selectin-dependent leukocyte rolling in inflamed venules of the cremaster muscle. We show that L-selectin-mediated rolling is absent in PSGL-1^{-/-} mice, in mice where PSGL-1 is blocked by mAb 4RA10, or in mice transplanted with PSGL-1^{-/-} bone marrow, thus providing evidence that PSGL-1 expressed by leukocytes functions as the L-selectin ligand in inflamed postcapillary venules. L-selectin ligand activity in inflamed postcapillary venules is of physiological relevance and is responsible for significant leukocyte accumulation in mice deficient in E- and P-selectin (9).

In most cases, L-selectin-dependent rolling occurred on already adherent leukocytes as a continuous series of secondary tethering or capture events. Individual secondary tethering events have been observed in vitro (11, 12, 15) and in vivo (14). Here, we demonstrate that series of such tethering events support L-selectin-dependent rolling. In addition, these tethering events can initiate rolling on E-selectin, similar to what was previously demonstrated in vitro

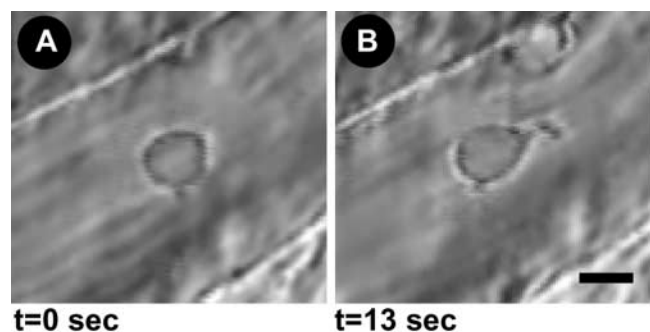


Figure 4. Some leukocytes rolling slowly along the endothelium (A) showed transient adhesion, during which time (here, 13 s) they pulled visible tethers of considerable length (B, arrow). Scale bar 5 μm . Supplemental video clip at <http://www.jem.org/cgi/content/full/jem.20021854/DC1>.

(12). In a minority of cases, L-selectin and PSGL-1-dependent rolling was seen without an obvious adherent leukocyte being present. At least three possible mechanisms may explain how PSGL-1 mediates L-selectin-dependent rolling in these cases: (a) PSGL-1 is deposited on inflamed venular endothelium on the surface of residual leukocyte tethers left behind by rolling leukocytes (36). (b) PSGL-1 is presented to free flowing leukocytes on the surface of leukocyte-derived microparticles bound to the endothelium. (c) Free flowing or rolling leukocytes interact with the uropods of partially transmigrated leukocytes. Schmidtke et al. conducted experiments using an *in vitro* flow chamber system where neutrophils were perfused over a layer of immobilized platelets (36). The results demonstrated that rolling leukocytes form membrane tethers of different length while rolling over the immobilized platelet layer. In some instances, these membrane tethers were dislodged from the leukocyte and remained attached to the platelet layer. The authors suggested that the endothelium-deposited leukocyte tethers may facilitate leukocyte capture to the endothelium which may be followed by rolling along the endothelium (36). We found evidence of tether formation on rolling leukocytes under *in vivo* conditions. It is possible that these tethers detach from the rolling leukocyte and remain bound to the endothelium, where they may present PSGL-1 to promote L-selectin-dependent capture and rolling events.

In cases where leukocyte-leukocyte interactions cannot be demonstrated to be responsible for L-selectin dependent rolling, the uropods of transmigrating cells may also provide a rich source of PSGL-1. Activation of neutrophils induces redistribution of PSGL-1 to the uropods of polarized (37) and migrating neutrophils (38). This is a very attractive possibility, suggesting that PSGL-1 redistribution during transmigration may initiate L-selectin-dependent interactions. Further investigations will be required to positively identify neutrophil tethers, microparticles, or uropods that support L-selectin- and PSGL-1-dependent rolling *in vivo*.

Two previous studies investigated the role of leukocyte-leukocyte interactions for leukocyte recruitment during inflammation *in vivo* (13, 14). Kunkel et al. observed leukocyte rolling and adhesion in (1–2.5 h) TNF- α stimulated cremaster muscle venules and found that leukocyte accumulation occurs in clusters whose formation was dependent on the expression of E-selectin (13). Leukocytes recruited into these clusters entered the cluster in the majority of cases while already rolling along the endothelium. The contribution of free flowing leukocytes entering the cluster by leukocyte-leukocyte interactions was very low (1% of the total number of recruited leukocytes; reference 13). Eriksson et al. (14) demonstrated that secondary capture or tethering events were responsible for a significant fraction of newly initiated leukocyte rolling events, especially in larger venules. Secondary capture accounted for 36% of all observed capture events during trauma-induced rolling, and for 25% in TNF- α and IL-1 β -induced rolling. All secondary capture was L-selectin-dependent, but the

role of PSGL-1 was not addressed in that study (14). The present study demonstrates a requirement for PSGL-1 and extends the previous findings to smaller venules with diameters from 10 to 40 μm .

L-selectin-dependent leukocyte rolling was not uniformly distributed, and most of the cremaster muscle venules did not show any L-selectin-dependent rolling. This may be due to a local variation in endothelial adhesion molecule expression. The most likely candidate would be E-selectin, as leukocyte-endothelial interactions observed during L-selectin-dependent rolling were sensitive to treatment with the E-selectin blocking mAb 9A9. Immunohistological studies on the distribution of endothelial adhesion molecules in the cremaster muscle microcirculation found a patchy expression of E-selectin in the unstimulated cremaster muscle vasculature (35), consistent with the functional data presented here. This interpretation is also consistent with the significant time delay seen between tissue preparation and the onset of L-selectin-dependent rolling. Most likely, L-selectin-dependent rolling can occur only in areas where E-selectin expression has resulted in sufficient accumulation of rolling and adherent leukocytes and leukocyte fragments to provide PSGL-1 as a substrate for rolling.

A previous study found that both mouse and human platelets express PSGL-1 (23). In that study, a polyclonal antibody to mouse PSGL-1 bound to 20% of platelets, while preimmune serum bound to only 5%. PSGL-1 was also demonstrated by Western blot in platelet preparations, but platelet preparations can contain contaminating leukocytes and leukocyte fragments. Indeed, some CD14 staining (a monocyte marker) and some CD11b staining (a neutrophil and monocyte marker) was detected in the previous study (23). In platelet preparations free of leukocyte fragments, we were unable to confirm any significant PSGL-1 expression on mouse platelets.

The finding that all L-selectin-dependent leukocyte rolling in cremaster venules requires PSGL-1 also explains the recent finding that L-selectin-mediated rolling in inflamed cremaster venules, but not in secondary lymphatic organs like Peyer's patches, requires posttranslational modification by core2 N-acetylglucosaminyl transferase (core2 GlcNAcT; reference 20). In light of the present findings, this core2 GlcNAcT requirement is most likely related to PSGL-1 requiring the core2 modification in order to function as an L-selectin ligand. Conversely, L-selectin mediates rolling in secondary lymphatic organs by binding to different, core2 GlcNAcT- and PSGL-1-independent ligands including those defined by reactivity with MECA-79 mAb (5).

L-selectin-dependent capture and rolling events on PSGL-1-expressing leukocytes and leukocyte fragments not only amplify the recruitment of neutrophils to sites of inflammation, but may also be able to initiate binding of other L-selectin-expressing cells that do not express ligands for endothelial P- and E-selectin. This mechanism would lead to representation of a broader range of white blood cells recruited during the course of inflammation. A recent study (39) reported a significant defect of CD8⁺ T

cell recruitment to the inflamed skin in a model of delayed-type hypersensitivity. In this model, injection of the L-selectin blocking antibody Mel-14 (intact IgG2a) reduced recruitment of CD8⁺ T cells to the inflamed skin in E- and P-selectin double-deficient mice. In these mice, CD8⁺ T cell recruitment was independent of PSGL-1 expression on CD8⁺ T cells. However, in this model, neutrophils or other inflammatory cells derived from the recipient mice may be present in microvessels of inflamed skin and may provide PSGL-1 that could support L-selectin-dependent recruitment of CD8⁺ T cells through the mechanism described here.

Our study demonstrates that L-selectin-mediated rolling in inflamed venules in vivo is entirely dependent on PSGL-1 expressed on bone marrow-derived cells. Leukocyte-endothelial interactions following these secondary tethering events require E-selectin. Our findings reconcile previous observations of a pronounced defect in inflammatory cell recruitment in L-selectin^{-/-} mice (7–9) or in mice in which L-selectin function was blocked (6, 9, 39) with the failure to find expression of functionally relevant L-selectin ligands on inflamed endothelial cells.

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