European Journal of Immunology **Immunomodulation**

α2,3-Sialyltransferase-IV is essential for L-selectin ligand function in inflammation

Markus Sperandio[1], David Frommhold[1], Inna Babushkina[1], Lesley G. Ellies[5], Timothy S. Olson[4], Michael L. Smith[4], Benedikt Fritzsching[3], Eva Pauly[1], David F. Smith[4], Rainer Nobiling[2], Otwin Linderkamp[1], Jamey D. Marth[6] and Klaus Ley[4]

[1] Children's Hospital, University of Heidelberg, Heidelberg, Germany
[2] Dept. of Experimental Surgery, University of Heidelberg, Heidelberg, Germany
[3] German Cancer Research Center, Heidelberg, Germany
[4] Robert M. Berne Cardiovascular Research Center and Dept. of Biomedical Engineering, University of Virginia, Charlottesville, USA
[5] Moores UCSD Cancer Center, University of California-San Diego, La Jolla, USA
[6] Department of Cellular and Molecular Medicine and Howard Hughes Medical Institute, University of California-San Diego, La Jolla, USA

Received 26/4/06
Revised 26/9/06
Accepted 6/10/06

[DOI 10.1002/eji.200636157]

L-selectin belongs to the C-type lectin family of glycoproteins and is constitutively expressed on most leukocytes. L-selectin mediates leukocyte rolling in inflamed microvessels and high endothelial venules (HEV) *via* binding to specific carbohydrate structures on selectin ligands. Previous studies using sialidase treatment suggested a role of sialic acid residues in L-selectin-dependent rolling. To investigate the role of the α2,3-sialyltransferase (ST3Gal)-IV on L-selectin ligand activity *in vivo*, we studied leukocyte rolling in inflamed venules of the cremaster muscle and in Peyer's patch HEV of ST3Gal-IV-deficient mice and littermate control mice. In cremaster muscle venules with or without TNF-α treatment, L-selectin-dependent rolling was almost completely abolished in ST3Gal-IV$^{-/-}$ mice. In both models, L-selectin interacts with P-selectin glycoprotein ligand-1 (PSGL-1) presented by adherent leukocytes and leukocyte fragments, but not with endothelial L-selectin ligands. In contrast, L-selectin-dependent rolling in Peyer's patch HEV, which is mediated by unknown endothelial L-selectin ligands, was not impaired in the absence of ST3Gal-IV. Our *in vivo* data show that PSGL-1, the molecule responsible for L-selectin-mediated leukocyte interactions in inflammation, is dependent on ST3Gal-IV, while α2,3-sialylation by ST3Gal-IV is not necessary for L-selectin ligand activity on high endothelial cells of Peyer's patch HEV.

Key words: Adhesion · Inflammation · Neutrophils

Correspondence: Markus Sperandio, M.D., Division of Neonatal Medicine, Children's Hospital, Universität Heidelberg, INF 150, 69120 Heidelberg, Germany
Fax: +49-6221-56-4208
e-mail: markus.sperandio@med.uni-heidelberg.de

Abbreviations: **core 2 GlcNAcT:** core 2 β1,6-N-acetyl glucosaminyltransferase · **FucT-VII:** fucosyltransferase-VII · **HEV:** high endothelial venules · **PSGL-1:** P-selectin glycoprotein ligand-1 · **RCA-I:** *Ricinus communis* agglutinin I · **RFF:** rolling flux fraction · **sLe^x:** sialyl Lewis x · **ST3Gal:** α2,3-sialyltransferase · **6-Sul-T:** 6-O-sulfotransferase

Introduction

The recruitment of leukocytes follows a well-defined cascade of events beginning with the capture of leukocytes to the vessel wall and subsequent leukocyte rolling along the endothelium [1, 2]. These early steps are mediated by the selectin family of adhesion molecules. Three different selectins have been identified: E-selectin (CD62E), P-selectin (CD62P), and L-selectin (CD62L) [3].

L-selectin is constitutively expressed on most leukocytes including granulocytes, monocytes, naive, and some memory lymphocytes [3]. Studies in L-selectin-deficient mice demonstrated reduced leukocyte rolling in inflamed postcapillary venules of the murine cremaster muscle [4]. In addition, leukocyte recruitment into the peritoneal cavity of L-selectin-deficient mice was significantly impaired after intraperitoneal injection of thioglycollate [5]. Furthermore, small, lymphocyte-depleted peripheral lymph nodes were identified in L-selectin$^{-/-}$ mice with reduced binding of L-selectin$^{-/-}$ lymphocytes to high endothelial venules (HEV) of peripheral lymph node frozen sections (Stamper–Woodruff assay) [5]. These findings clearly demonstrate the importance of L-selectin in inflammation and immune cell trafficking.

L-selectin-dependent rolling requires interactions between L-selectin and glycosylated counter-receptors termed L-selectin ligands, which are found on high endothelial cells, chronically inflamed endothelial cells and leukocytes [6]. There is substantial evidence that L-selectin ligands are differentially regulated in lymphoid and non-lymphoid tissue. This is not only true for the identified protein candidates but also for the specific glycosyltransferases involved in the carbohydrate decoration of L-selectin ligands [6]. Recently, P-selectin glycoprotein ligand-1 (PSGL-1) has been identified to be responsible for L-selectin-dependent leukocyte rolling during inflammation *in vivo* [7]. PSGL-1 is a heavily glycosylated homodimer expressed on most leukocytes [8]. L-selectin-mediated rolling *via* PSGL-1 is mostly dependent on secondary tethering events, *i.e.* binding of free flowing leukocytes to already adherent leukocytes and leukocyte fragments, but independent of endothelial L-selectin ligands [7].

Functional studies on L-selectin ligand activity during inflammation demonstrated that binding is dependent on specific glycosyltransferases such as fucosyltransferase-VII (FucT-VII) and core 2 β1,6-N-acetyl glucosaminyltransferase-I (core 2 GlcNAcT-I) [9, 10], both enzymes being critically involved in the posttranslational glycosylation of L-selectin ligands [6]. Sialylation of neutrophils has also been reported to contribute to L-selectin ligand function during inflammation [11, 12]. In addition, intravital microscopy studies demonstrated that rolling of L-selectin-transfected cell lines was significantly reduced by neuraminidase pretreatment [13]. However, the sialyltransferases required for the posttranslational modification of inflammatory L-selectin ligands are not defined.

L-selectin ligands on HEV of lymphatic tissue carry sialylated and fucosylated sequences on core 2 GlcNAcT modified O-linked glycans [6]. L-selectin ligands on HEV in lymph nodes also require sulfation on C-6 of N-acetylglucosamine and/or C-6 of galactose of sialyl Lewis x (sLe^x) [14, 15]. L-selectin ligand function of HEV can be blocked by the carbohydrate-binding mAb MECA-79, which helped to identify candidate L-selectin ligands on HEV including glycosylation-dependent cell adhesion molecule-1 and CD34 [16]. The scaffold protein(s) presenting L-selectin ligands in Peyer's patch HEV are unknown.

Although sialylation was the first described post-translational modification necessary for the synthesis of functional HEV-expressed L-selectin ligands [17], the crucial sialyltransferases involved in L-selectin ligand formation have not been reported so far. In mammals, six sialyltransferases (ST3Gal-I–VI) have been identified that transfer sialic acid residues with an α2,3-linkage to terminal galactose residues [18]. ST3Gal-III, ST3Gal-IV, and ST3Gal-VI can sialylate type II oligosaccharides (Galβ1-4GlcNAc) [19, 20]. Recently, mice deficient in ST3Gal-IV have been generated [21]. *In vivo* studies investigating P- and E-selectin-mediated leukocyte rolling in inflamed cremaster muscle venules of ST3Gal-IV$^{-/-}$ mice revealed no defect in P-selectin-dependent rolling [22]. However, E-selectin-dependent leukocyte rolling velocity was significantly increased with no defect in E-selectin-mediated leukocyte capture, suggesting that ST3Gal-IV regulates E-selectin-dependent rolling velocity while it does not affect the efficiency of E-selectin to attract free flowing leukocytes to the inflamed endothelium [22].

The present study was designed to investigate L-selectin ligand function in the absence of ST3Gal-IV in lymphoid and non-lymphoid tissue. To this end, we investigated L-selectin-dependent leukocyte rolling in unstimulated and TNF-α-stimulated cremaster muscle venules, and in HEV of Peyer's patches, where leukocyte rolling is mediated by L-selectin, P-selectin, and $\alpha_4\beta_7$-integrin binding to the mucosal vascular addressin cell adhesion molecule-1 [23, 24].

Results

L-selectin-IgG chimera binding to Gr1$^+$ leukocytes

As reported earlier, ST3Gal-IV$^{-/-}$ mice appeared healthy and fertile and showed no difference in systemic leukocyte counts compared to control mice [22]. To investigate L-selectin ligand activity on ST3Gal-IV$^{-/-}$ leukocytes and control leukocytes, L-selectin chimera IgG Fc fusion protein was incubated with anti-human IgG Fc conjugated to PE added to peripheral blood leukocytes from ST3Gal-IV-deficient mice and littermate control mice and stained with allophycocyanin-conjugated Gr-1. Flow cytometric analysis showed that binding of L-selectin-IgG chimera to ST3Gal-IV$^{-/-}$ Gr-1$^+$ leukocytes was almost completely absent when com-

pared to L-selectin-IgG chimera binding to littermate control leukocytes (Fig. 1A, B). This suggests that ST3Gal-IV is critical for L-selectin ligand activity on the surface of $Gr1^+$ leukocytes. In addition, pretreatment of $Gr\text{-}1^+$ littermate control leukocytes with sialidase from *Vibrio cholerae* led to a similar reduction in L-selectin binding as seen in ST3Gal-IV-deficient mice, suggesting that L-selectin ligand activity on $Gr\text{-}1^+$ cells is mostly dependent on ST3Gal-IV (data not shown).

Binding of L-selectin chimera to $Gr\text{-}1^+$ cells was dependent on calcium because addition of EDTA completely blocked L-selectin-dependent interactions (Fig. 1A). In ST3Gal-IV-deficient $Gr\text{-}1^+$ cells, addition of EDTA did not lead to any changes in the L-selectin binding signal, suggesting that L-selectin chimera binding to ST3Gal-IV-deficient $Gr\text{-}1^+$ cells is dramatically impaired (Fig. 1B). Binding of L-selectin chimera to wild-type $Gr\text{-}1^+$ cells was specific for L-selectin because replacement of L-selectin chimera by human IgG significantly reduced the intensity signal in $Gr\text{-}1^+$ cells (Fig. 1A). In contrast, no significant changes in fluorescence intensity were observed after replacing L-selectin chimera with human IgG (Fig. 1B). We also performed additional FACS experiments of *Ricinus communis* agglutinin I (RCA-I) binding to ST3Gal-IV-deficient $Gr\text{-}1^+$ leukocytes and compared it to binding of RCA-I to littermate control $Gr\text{-}1^+$ leukocytes. The lectin RCA-I recognizes terminal galactose residuals from desialylated glycoproteins. RCA-I binding was stronger for ST3Gal-IV-deficient $Gr\text{-}1^+$ cells than for $Gr\text{-}1^+$ cells from littermate control mice, suggesting that more terminal galactose residues were exposed in ST3Gal-IV-knockout mice (data not shown).

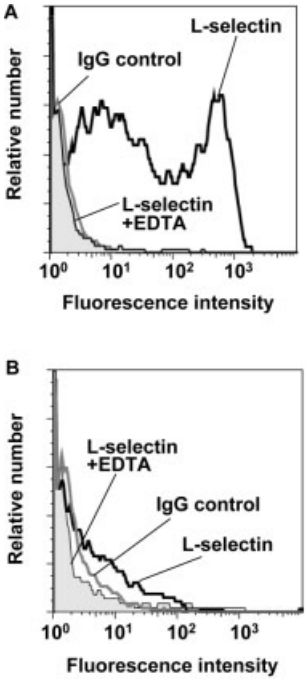

Figure 1. L-selectin ligand activity on ST3Gal-IV$^{-/-}$ mice. Flow cytometric analysis of L-selectin-IgG Fc fusion protein binding to $Gr\text{-}1^+$ cells from wild-type control mice (A) and ST3Gal-IV$^{-/-}$ mice (B). To test that L-selectin binding to its ligand was calcium-dependent, EDTA was added. In addition, human IgG was used instead of L-selectin-IgG chimera to test for non-specific binding. Results are representative of three separate experiments.

Leukocyte rolling in trauma-induced inflammation

Leukocyte rolling was assessed in 73 cremaster muscle venules of eight ST3Gal-IV$^{-/-}$ mice and compared to 56 venules of five littermate controls. Hemodynamic parameters between ST3Gal-IV-deficient mice and control mice showed no significant differences in vascular diameter, blood flow velocity, and wall shear rate (Table 1). In untreated mice, leukocyte rolling in surgically prepared cremaster muscle venules is P-selectin-dependent within the first hour after exteriorization of the cremaster muscle [4]. More than 1 h after surgery, P-selectin- and L-selectin-dependent rolling can be observed [25]. Leukocyte rolling flux fraction (RFF) in untreated animals was significantly higher in ST3Gal-IV$^{-/-}$ mice (46±5%) than in control mice (31±6%). The reason for the difference is unknown at present.

To specifically investigate L-selectin-dependent rolling, the P-selectin-blocking mAb RB40.34 was injected into control mice and ST3Gal-IV$^{-/-}$ mice. In control mice, injection of P-selectin-blocking mAb RB40.34 led to a marked reduction in RFF to 5% (Fig. 2). The residual rolling observed after blocking P-selectin was mostly L-selectin-dependent, as the additional injection of $F(ab')_2$ fragments of the L-selectin-blocking antibody MEL-14 almost completely abolished rolling in this setting (Fig. 2). In contrast, injection of P-selectin-blocking mAb RB40.34 completely abolished rolling in ST3Gal-IV$^{-/-}$ mice, suggesting that L-selectin-mediated rolling is absent in ST3Gal-IV$^{-/-}$ mice (Fig. 2).

Previously it had been demonstrated that severe defects in leukocyte adhesion lead to a lower surface expression of L-selectin on the leukocyte surface [26, 27]. Flow cytometric analysis conducted with leukocytes from ST3Gal-IV$^{-/-}$ and littermate controls demonstrated that the expression of L-selectin was similar in ST3Gal-IV$^{-/-}$ and control leukocytes (data not shown), making it unlikely that differences in L-selectin expression could account for the marked decrease in L-selectin-dependent rolling in ST3Gal-IV-deficient mice.

Table 1. Microvascular parameters in cremaster muscle venules and Peyer's patch HEV[a)]

Mouse Genotype	Mice (n)	Venules (n)	Diameter (μm)	Centerline velocity (μm/s)	Wall shear rate (s^{-1})
Untreated cremaster muscles					
ST3Gal-IV$^{-/-}$	8	73	30±1	2800±220	2400±210
Control	5	56	32±1	3200±340	2600±290
			n.s.	n.s.	n.s.
TNF-α-treated cremaster muscles					
ST3Gal-IV$^{-/-}$	8	62	31±1	2400±180	1500±190
Control	8	57	32±2	1800±120	1400±100
			n.s.	n.s.	n.s.
Untreated Peyer's patches					
ST3Gal-IV$^{-/-}$	10	58	19±1	1600±200	1900±200
Control	10	75	20±1	1900±300	2400±400
			n.s.	n.s.	n.s.

[a)] Diameters, centerline velocities, and wall shear rates (mean ± SEM) are presented from experiments conducted in cremaster muscle venules of untreated and TNF-α-treated mice and in HEV of Peyer's Patches; n.s., not significant.

Leukocyte rolling in TNF-α-induced inflammation

Intrascrotal injection of TNF-α leads to L-selectin-, E-selectin-, and P-selectin-dependent rolling with a significant L-selectin-dependent component more than 4 h after injection [28]. We observed leukocyte rolling in 62 venules of eight TNF-α-treated (4–6 h) mice lacking ST3Gal-IV and compared the results to rolling in 57 venules of eight control animals. Microvascular parameters for both groups are presented in Table 1 and show similar vessel diameters, centerline velocities, and wall shear rates. Leukocyte RFF 4 h after TNF-α treatment was similar between ST3Gal-IV$^{-/-}$ mice and control mice (26±8% *vs.* 27±3%, respectively; Fig. 3). Injection of both the P-selectin-blocking mAb RB40.34 and the E-selectin-blocking mAb 9A9 revealed residual L-selectin-mediated rolling in control mice (RFF 3.8%), which was significantly reduced (RFF<1%) by additional injection of F(ab')$_2$ fragments of the L-selectin-blocking mAb MEL-14 (Fig. 3). These results demonstrate that most of the observed residual rolling is L-selectin-dependent.

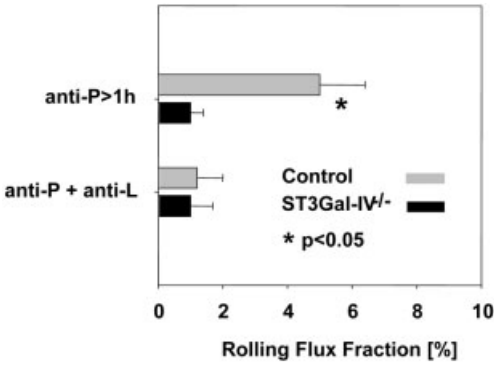

Figure 2. Leukocyte rolling in untreated venules. Leukocyte RFF (mean ± SEM) was assessed in venules of the cremaster muscle of untreated ST3Gal-IV-deficient (black bars) and wild-type mice (gray bars) at >60 min after exteriorization; anti-P, mAb RB40.34; anti-L, mAb F(ab')$_2$ MEL-14. Significant differences ($p<0.05$) in leukocyte RFF between ST3Gal-IV$^{-/-}$ and wild-type group are indicated by asterisks.

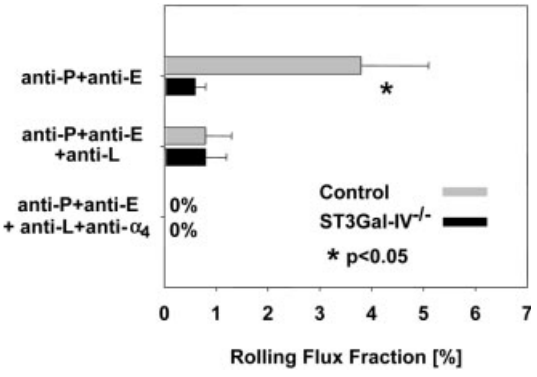

Figure 3. Leukocyte rolling in TNF-α-treated mice. Leukocyte RFF (mean ± SEM) was observed in TNF-α-treated venules at >4 h in ST3Gal-IV-deficient mice (black bars) and littermate control mice (gray bars); anti-P, mAb RB40.34; anti-E, mAb 9A9; anti-L, mAb F(ab')$_2$ MEL-14, anti-α_4, mAb PS/2. Significant differences ($p<0.05$) in leukocyte RFF between ST3Gal-IV$^{-/-}$ and control mice are indicated by asterisks.

In ST3Gal-IV$^{-/-}$ mice treated with P-selectin-blocking mAb RB40.34 and E-selectin-blocking mAb 9A9, leukocyte rolling was dramatically reduced (RFF<1%; Fig. 3) when compared to control mice. Additional injection of F(ab')$_2$ fragments of the L-selectin-blocking mAb MEL-14 did not lead to any further reduction in rolling, suggesting that L-selectin ligand function in ST3Gal-IV-deficient mice is severely defective. The few remaining leukocytes observed in both groups after antibody blockade of all three selectins was dependent on α_4-integrin, as the addition of α_4-integrin-blocking mAb PS/2 completely abolished rolling in both groups (Fig. 3).

Leukocyte rolling in high endothelial venules of Peyer's patches

Leukocyte rolling was analyzed in 58 venules of ten ST3Gal-IV$^{-/-}$ mice and compared to 75 venules in ten control mice. Microvascular parameters are presented in Table 1 and show no significant differences regarding vessel diameter, centerline velocity, and wall shear rate. Leukocyte rolling in Peyer's patch HEV is dependent on L-selectin, P-selectin, and $\alpha_4\beta_7$-integrin [23]. Injection of the L-selectin-blocking F(ab')$_2$ fragments of mAb MEL-14 reduced normalized leukocyte RFF in both ST3Gal-IV$^{-/-}$ and control mice (Fig. 4). This demonstrates that ST3Gal-IV deficiency does not significantly impair L-selectin-mediated rolling in Peyer's patch HEV. Injection of the P-selectin-blocking mAb RB40.34 and the α_4-integrin-blocking mAb PS/2 caused a similar significant reduction in RFF for both groups (Fig. 4). Blocking of α_4-integrin, L-selectin, and P-selectin abolished rolling in both groups. These results demonstrate that L-selectin-mediated rolling in HEV of Peyer's patches is not dependent on ST3Gal-IV.

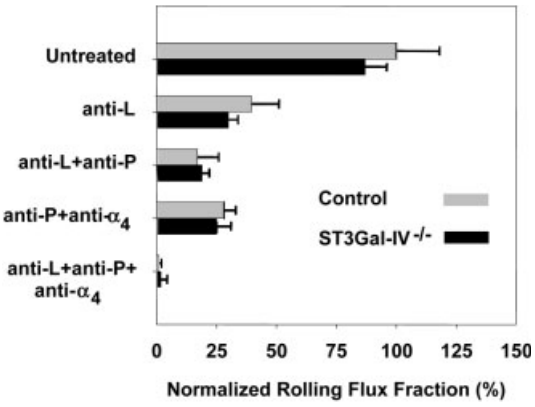

Figure 4. Leukocyte rolling in Peyer's patches. Normalized leukocyte RFF (mean ± SEM) was assessed in HEV of Peyer's patches of ST3Gal-IV-deficient mice (black bars) and littermate control mice (gray bars); anti-P, mAb RB40.34; anti-L, mAb F(ab$_2$) MEL-14; anti-α_4, mAb PS/2. No significant differences (p<0.05) in leukocyte RFF between ST3Gal-IV$^{-/-}$ and control mice.

Discussion

This study demonstrates that L-selectin-mediated leukocyte rolling in inflamed postcapillary venules of the cremaster muscle is abolished in ST3Gal-IV-deficient mice, providing evidence that ST3Gal-IV is necessary for L-selectin ligand function in inflamed venules of non-lymphoid tissue. L-selectin-mediated leukocyte rolling in inflamed postcapillary venules of the cremaster muscle has recently been demonstrated to be dependent on the selectin ligand PSGL-1, which is expressed on most leukocytes [7]. Therefore, L-selectin-dependent interactions do not require L-selectin ligands expressed on inflamed endothelium but are mediated between free flowing leukocytes and already adherent leukocytes or leukocyte-derived fragments deposited on the inflamed endothelium [7]. PSGL-1 binds to all three selectins *in vitro* and *in vivo*. Ramachandran *et al.* [29] showed that a sialylated and fucosylated core 2 decorated O-glycan at a threonine at position 57 of human PSGL-1 is crucial for PSGL-1 binding to P-selectin and L-selectin. Similar results were reported later for murine PSGL-1 [30].

ST3Gal-IV-deficient mice do not demonstrate any defect in P-selectin-mediated rolling in inflamed cremaster muscle venules *in vivo* [22], while L-selectin-mediated rolling, as shown here, is almost completely absent. These findings suggest that lack of α2,3-sialylation of murine PSGL-1 by ST3Gal-IV does not affect P-selectin binding to PSGL-1, but severely curtails the L-selectin ligand function of PSGL-1. *In vitro* studies investigating P-selectin- and L-selectin-IgG chimera binding to a synthetic glycosulfopeptide modeled after the N terminus of human PSGL-1 containing three clustered tyrosine sulfates and a short core 2 decorated and monofucosylated sLe^x demonstrated that desialylation of the glycosulfopeptide was followed by a considerable reduction of glycosulfopeptide binding to P-selectin- and L-selectin-IgG [31, 32]. Since the requirements for P-selectin and L-selectin binding to the N-terminal binding site on PSGL-1 are probably similar in the murine system, it is likely that other ST3Gal such as ST3Gal-VI contribute to the synthesis of the crucial sLe^x containing core 2-dependent O-glycan at the N terminus of PSGL-1 in the absence of ST3Gal-IV. On the other hand, ST3Gal-IV could also modify carbohydrate epitopes on PSGL-1 distinct from the core 2 decorated sLe^x at the N terminus of PSGL-1. Such an epitope, PEN5 (a sulfated polylactosamine), has been reported, but its expression is restricted to NK cells and not found on neutrophils. PEN5 is a unique posttranslational modification of PSGL-1 inducing L-selectin

ligand activity on NK-cells independent of tyrosine sulfation and resistant to mocarhagin treatment [33]. Mocarhagin is a metalloprotease that specifically cleaves a ten-amino acid peptide from the N terminus of PSGL-1 and abolishes the ability of PSGL-1 to bind to P-selectin [34]. Because of the restricted expression of PEN5 on NK cells, it is unlikely that PEN5 contributes to L-selectin-dependent rolling during inflammation *in vivo*.

Evidence for another L-selectin binding site on PSGL-1 distinct from the P-selectin binding site comes from static and dynamic *in vitro* assays where COS mutant cells transfected with FucT-VII, 6-O-sulfotransferase (6-Sul-T), and PSGL-1 were investigated for their ability to bind to selectin-transfected 300.19 cells [35]. In this study, L-selectin binding to COS cells was crucially dependent on 6-Sul-T and FucT-VII transfection, and additional transfection of 6-Sul-T/FucT-VII-positive COS cells with PSGL-1 enhanced L-selectin binding under shear and resistance to shear stress. Interestingly, this did not change after treatment with mocarhagin, suggesting the existence of an L-selectin binding site on PSGL-1 distinct from that on the N terminus [35].

These findings are in conflict with other reports from dynamic *in vitro* assays demonstrating that mocarhagin treatment of PSGL-1 or antibody treatment of the N terminus of PSGL-1 significantly inhibit L-selectin-dependent rolling of leukocytes on other leukocytes [36, 37]. Since other ST3Gal exist, it is likely that those other ST3Gal like ST3Gal-I, ST3Gal-III, and ST3Gal-VI [22] may sialylate core 2 decorated O-glycans in the absence of ST3Gal-IV. Sialylation may be not as efficient as in the presence of ST3Gal-IV but sufficient to support P-selectin- (but not L-selectin-) dependent rolling.

In contrast to inflammatory L-selectin ligands expressed on leukocytes, L-selectin ligand activity on Peyer's patch HEV was not impaired in ST3Gal-IV-deficient mice. This is in agreement with the normal structure and size of secondary lymphoid organs observed in ST3Gal-IV-deficient mice [21]. The reason why L-selectin-mediated rolling is completely absent in inflamed venules of the cremaster muscle while L-selectin-dependent rolling is normal in HEV of Peyer's patches is unknown. However, several lines of evidence indicate that posttranslational glycosylation of L-selectin ligands is differentially regulated in various tissues [6]. In Peyer's patch HEV of ST3Gal-IV-deficient mice, as shown here, L-selectin ligand function was preserved. Because α2,3-sialylation is crucial for L-selectin ligand function on HEV, our results suggest that other ST3Gal such as ST3Gal-VI may contribute to L-selectin ligand function in lymphoid tissue [20]. ST3Gal-VI is ubiquitously distributed throughout the body and may significantly contribute to L-selectin ligand activity in Peyer's patch HEV. Engineering mice to be deficient in ST3Gal-VI may therefore help to identify the role of α2,3-sialylation on L-selectin ligand function in lymphatic tissue. In addition, it may provide further insights into the regulation of the inflammatory P-selectin ligand PSGL-1.

In conclusion, the present study shows that ST3Gal-IV is critical for L-selectin ligand activity during inflammation. In contrast, L-selectin ligand function in Peyer's patch HEV is not impaired in ST3Gal-IV$^{-/-}$ mice. These findings support the concept that L-selectin ligand activity is differentially regulated in lymphoid and non-lymphoid tissues *in vivo*.

Material and methods

Animals

Mice lacking a functional gene encoding the leukocyte ST3Gal-IV (E.C.2.4.99.4) were generated as described previously [22] and backcrossed for at least seven generations into the C57BL/6 strain. Mice were maintained as a heterozygous breeding colony at the University of Virginia, Charlottesville, VA, and the University of Heidelberg, Germany. The wild-type ST3Gal-IV allele was detected using PCR primers located adjacent to the deleted region (LE-94: 5′-GACGCCATCCACC-TATGAG-3′ and LE-130: 5′-GGCTGCTCCCATTCCACT-3′), resulting in a 260-bp fragment, while the mutant allele was detected using LE-94 and a primer at the 3′ end of the Kpn 4500-bp fragment, downstream of the sialyl motif encompassing exons 12 and 13 (LE-99: 5′-GGCTCTTTGTGGGAC-CATCAG-3′), yielding a 450-bp fragment. Heterozygous (+/–) and homozygous (+/+) littermates, which did not show any significant differences in leukocyte rolling, served for control experiments.

All ST3Gal-IV$^{-/-}$ mice and control mice used in this study were healthy and at least 8 wk of age. Mice were housed in a barrier facility under SPF conditions. All animal experiments were approved by the University of Virginia Animal Care and Use Committee, and by the Regierungspräsidium Karlsruhe, Baden-Württemberg, Germany, AZ 35-9185.81/G-67/03.

Antibodies and cytokines

The P-selectin-blocking mAb RB40.34 (rat IgG1, 30 μg/mouse) was a gift from Dr. Dietmar Vestweber (University of Münster, Germany). RB40.34 blocks P-selectin-dependent adhesion and recruitment [38] and P-selectin-dependent leukocyte rolling *in vivo* [4]. E-selectin mAb 9A9 (rat IgG1, 30 μg/mouse), which blocks E–selectin-mediated rolling *in vivo* [39], was a gift from Dr. B. Wolitzky (Hoffmann-La Roche, Inc., Nutley, NJ). The L-selectin mAb MEL-14 (rat IgG2a) was purified from hybridoma supernatant (American Type Culture Collection, Manassas, VA). This antibody blocks L-selectin-dependent leukocyte rolling *in vivo* [4, 23]. We noted an inconsistent drop in the leukocyte count after injection of MEL-14. Therefore $F(ab')_2$ fragments were used for experiments generated using the Pierce Immunoglobulin $F(ab')_2$ Preparation Kit (Pierce, Rockford, IL). The mAb PS/2 (rat IgG2b, 30 μg/mouse) was purified from hybridoma supernatant

(American Type Culture Collection) and blocks α_4-dependent binding of lymphocytes to recombinant mucosal vascular addressin cell adhesion molecule-1 *in vitro* [40] and lymphocyte homing to Peyer's patches *in vivo* [41]. In certain experiments, recombinant murine TNF-α (R&D, Minneapolis, MN) was injected intrascrotally at a dose of 500 ng per mouse in a volume of 0.3 mL of sterile saline 4 h before the beginning of the intravital microscopic experiment.

Flow cytometry

Flow cytometry was conducted to detect L-selectin and L-selectin ligand expression on peripheral blood leukocytes of control and ST3Gal-IV-deficient mice. To investigate L-selectin expression, whole blood was centrifuged and red blood cell lysis was conducted with PharM-Lyse-10× solution (PharMingen, San Diego, CA). After centrifugation, cells were suspended in PBS/1% BSA (Sigma, St. Louis, MO) solution and incubated in the dark with either MEL-14 conjugated with PE or rat IgG2a conjugated with PE (both PharMingen, San Diego, CA) for 30 min on ice (0.5 μg/10^5 cells). Thereafter, cells were washed twice in PBS/1% BSA solution and incubated with allophycocyanin-conjugated mAb Ly-6G directed against the neutrophilic surface antigen Gr-1 (PharMingen) for 30 min on ice (0.5 μg/10^5 cells). L-selectin expression on cells was determined on 10 000 leukocytes per mouse using a four-decade FACSCalibur with Cell Quest software package (Becton Dickinson, San Jose, CA).

To investigate binding of L-selectin to Gr-1^+ leukocytes, recombinant mouse L-selectin fused to human IgG Fc (R&D) was incubated with PE-labeled anti-human IgG Fc for 30 min on ice (1 μg/10^5 cells) [23]. Subsequently, isolated peripheral blood leukocytes from ST3Gal-IV$^{-/-}$ mice and littermate controls were incubated with L-selectin-IgG Fc complexed with PE-labeled anti-human IgG Fc with or without EDTA (5 mM; Sigma) for 30 min on ice. As a negative control, PE-labeled anti human IgG Fc was incubated with human IgG (Sigma) for 30 min on ice and then added to isolated peripheral blood leukocytes for 30 min on ice. After washing twice, cells were incubated with allophycocyanin-conjugated mAb Ly-6G. L-selectin ligand activity on Gr-1^+ leukocytes was assessed by flow cytometry. Additional flow cytometric experiments on L-selectin ligand activity were conducted on littermate control leukocytes and ST3Gal-IV-deficient leukocytes pretreated with V. cholerae sialidase (Calbiochem, Darmstadt, Germany).

To confirm successful desialylation of leukocytes by sialidase, FITC-labeled RCA-I (0.2 μg/10^5 cells; Vector Laboratories, Burlingame, CA) was used to detect terminal galactose residues of desialylated glycoproteins. L-selectin ligand expression on cells was assessed by flow cytometry on 10 000 leukocytes per mouse using the LSR II flow cytometer with FACSDiva (both Becton Dickinson) and FlowJo (www.flowjo.com) software package.

Intravital microscopy

After anesthesia with intraperitoneal injection of ketamine (125 mg/kg body weight; Ketalar; Parke-Davis, Morris Plains, NJ), xylazine (12.5 mg/kg body weight; Phoenix Scientific, Inc., St. Joseph, MO), and atropin sulfate (0.25 mg/kg body weight; Elkins-Sinn, Inc., Cherry Hill, NJ), mice were placed on a heating pad to maintain body temperature. Intravital microscopy was conducted on an upright microscope (Axioskop; Zeiss, Thornwood, NY) with a saline immersion objective (SW 40/0.75 numerical aperture). Mice were intubated and the left carotid artery was cannulated for blood sampling and systemic mAb administration. During the experiment mice received 0.2 mL/h normal saline i.v. to maintain neutral fluid balance.

Cremaster muscle preparation

The surgical preparation of the cremaster muscle for intravital microscopy was conducted as described [39]. Briefly, after opening the scrotum the cremaster muscle was exteriorized and spread over a cover glass. The epididymis and testis were gently pinned to the side, giving full microscopic access to the cremaster muscle microcirculation. Experiments were recorded *via* a CCD camera system (model VE-1000; Dage-MTI, Inc., Michigan City, IN) on a Panasonic S-VHS recorder. The cremaster muscle was superfused with thermocontrolled (35°C) bicarbonate-buffered saline. Postcapillary venules under observation ranged from 20 to 50 μm in diameter. Systemic blood samples (10 μL) were taken after each mAb injection and assessed for white blood cell count. Blood samples were diluted 1:10 with Kimura (11 mL of 5% w/w toluidine blue, 0.8 mL of 0.03% light green SF yellowish, 0.5 mL of saturated saponin, and 5 mL of 0.07 M phosphate buffer, pH 6.4; all Sigma), and leukocyte concentration was expressed as number of leukocytes per μL of whole blood.

Peyer's patch preparation

The surgical preparation to study leukocyte rolling in HEV of the Peyer's patch was conducted as described previously [42, 43]. Briefly, after opening the peritoneal cavity, the caecum and small intestine were exposed and superfused with thermocontrolled (35°C) bicarbonate-buffered saline throughout the experiment. The intestines were pushed out of the peritoneal cavity by gently pressing on the back. After locating a Peyer's patch on the small intestine, it was placed between two parallel strips of clear silicone, high vacuum grease and covered by a plastic coverslip (Baxter, Deerfield, IL).

After systemic injection of 0.15 mL of 2 mg/mL rhodamine 6G (Molecular Probes, Eugene, OR) intravital epifluorescence microscopy was used to investigate leukocyte rolling in HEV of the Peyer's patch. The intravital microscope (Axioskop; Zeiss) was equipped with a saline immersion objective (SW 63/0.9 numerical aperture). For observation of Peyer's Patch HEV, epifluorescence illumination (60/s; Strobex 236; Chadwick Helmuth, Mountain View, CA, and filter block Zeiss 9) was used. Each venule was observed for 60 s. Venules with diameters between 12 and 35 μm were observed and recorded *via* a signal-intensified CCD camera (model SIT-66; Dage-MTI, Inc.) on a Panasonic S-VHS recorder.

Data analysis of intravital experiments

Vessel diameter and vessel segment length of postcapillary venules or HEV were measured using a digital image processing system [44]. 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, Hockessin, DE). An empirical factor of 0.625 was used to convert centerline velocities to mean blood flow velocities [45]. In Peyer's patch HEV, centerline blood flow velocity was determined using 2-μm diameter fluorescent YG microspheres (Polysciences, Warrington, PA) and measuring frame-to-frame displacement of the bead (three microspheres per venule). Wall shear rates (γ_w) were estimated as $4.9 \times (8v_b/d)$, where v_b is the mean blood flow velocity and d the diameter of the vessel. The constant 4.9 is a mean empirical correction factor obtained from velocity profiles measured in microvessels *in vivo* [46, 47].

Leukocyte RFF was defined as the percentage of rolling leukocytes to all leukocytes passing through the same venule per unit time. RFF in cremaster muscle venules was calculated as described [4] by dividing leukocyte rolling flux by total leukocyte flux estimated as [WBC] v_b π $(d/2)^2$. [WBC] is the actual systemic leukocyte count, v_b is the blood flow velocity and d is the venular diameter.

Due to the preferential delivery of leukocytes to the terminal capillaries in microvascular networks [48, 49], leukocyte rolling flux is consistently higher than the product of the flow rate and the systemic leukocyte concentration. Therefore, we set the RFF in venules of Peyer's patch from untreated control mice as 100% and expressed flux fractions of mAb-treated controls and untreated and mAb-treated ST3Gal-IV-deficient mice relative to control.

Statistics

Sigma Stat 2.0 software package (SPSS Science, Chicago, IL) was used for statistical analysis. Vessel diameters, leukocyte RFF, leukocyte rolling velocities, and shear rates between groups and treatments were compared with the one-way ANOVA on ranks (Kruskal–Wallis) with a multiple pairwise comparison test (Dunn's test). Leukocyte counts and differentials were compared with Student's *t*-test or by the Wilcoxon rank-sum test as appropriate. Statistical significance was set at $p<0.05$, indicated by an asterisk.

Acknowledgements: We thank Dr. Dietmar Vestweber, University Münster (Münster, Germany), for providing mAb RB40.34, and Dr. Barry Wolitzky, MitoKor (San Diego, CA), for providing the E-selectin-blocking antibody 9A9. This work was supported by NIH grants HL-64381 and HL-54136 (K.L.), by NIH grant P01-HL57345 (J.D.M.), and by German Research Foundation (DFG) grant SP621/3-1 (M.S.). L.G.E is supported by NCI Awards F32CA79130 and K08CA88035, B.F. is supported by a Young Investigator Award from the University of Heidelberg (Heidelberg, Germany).

References

1 **Butcher, E. C.,** Leukocyte-endothelial cell recognition – three (or more) steps to specificity and diversity. *Cell* 1991. **67:** 1033–1036.

2 **Springer, T. A.,** Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annu. Rev. Physiol.* 1995. **57:** 827–872.

3 **Vestweber, D. and Blanks, J. E.,** Mechanisms that regulate the function of the selectins and their ligands. *Physiol. Rev.* 1999. **79:** 181–213.

4 **Ley, K., Bullard, D. C., Arbones, M. L., Bosse, R., Vestweber, D., Tedder, T. F. and Beaudet, A. L.,** Sequential contribution of L- and P-selectin to leukocyte rolling *in vivo*. *J. Exp. Med.* 1995. **181:** 669–675.

5 **Arbones, M. L., Ord, D. C., Ley, K., Ratech, H., Maynard-Curry, C., Otten, G., Capon, D. J. and Tedder, T. F.,** Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity* 1994. **1:** 247–260.

6 **Rosen, S. D.,** Ligands for L-selectin: Homing, inflammation, and beyond. *Annu. Rev. Immunol.* 2004. **22:** 129–156.

7 **Sperandio, M., Smith, M. L., Forlow, S. B., Olson, T. S., Xia, L., McEver, R. P. and Ley, K.,** P-selectin glycoprotein ligand-1 mediates L-selectin-dependent leukocyte rolling in venules. *J. Exp. Med.* 2003. **197:** 1355–1363.

8 **McEver, R. P. and Cummings, R. D.,** Perspectives series: Cell adhesion in vascular biology. Role of PSGL-1 binding to selectins in leukocyte recruitment. *J. Clin. Invest.* 1997. **100:** 485–491.

9 **Maly, P., Thall, A. D., Petryniak, B., Rogers, C. E., Smith, P. L., Marks, R. M., Kelly, R. J. *et al.*,** The α(1,3)fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin ligand biosynthesis. *Cell* 1996. **86:** 643–653.

10 **Sperandio, M., Forlow, S. B., Thatte, J, Ellies, L. G., Marth, J. D. and Ley, K.,** Differential requirements for core2 glucosaminyltransferase for endothelial L-selectin ligand function *in vivo*. *J. Immunol.* 2001. **167:** 2268–2274.

11 **Bargatze, R. F., Kurk, S., Butcher, E. C. and Jutila, M. A.,** Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells *via* L-selectin on the rolling cells. *J. Exp. Med.* 1994. **180:** 1785–1792.

12 **Jones, D. A., Smith, C. W., Picker, L. J. and McIntire, L. V.,** Neutrophil adhesion to 24-hour IL-1-stimulated endothelial cells under flow conditions. *J. Immunol.* 1996. **157:** 858–863.

13 **von Andrian, U. H., Chambers, J. D., Berg, E. L., Michie, S. A., Brown, D. A., Karolak, D., Ramezani, L. *et al.*,** L-selectin mediates neutrophil rolling in inflamed venules through sialyl Lewis^x-dependent and -independent recognition pathways. *Blood* 1993. **82:** 182–191.

14 **Imai, Y., Lasky, L. A. and Rosen, S. D.,** Sulphation requirement for GlyCAM-1, an endothelial ligand for L-selectin. *Nature* 1993. **361:** 555–557.

15 **Hemmerich, S. and Rosen, S. D.,** Carbohydrate sulfotransferases in lymphocyte homing. *Glycobiology* 2000. **10:** 849–856.

16 **Hemmerich, S., Butcher, E. C. and Rosen, S. D.,** Sulfation-dependent recognition of high endothelial venules (HEV)-ligands by L-selectin and MECA 79, an adhesion-blocking monoclonal antibody. *J. Exp. Med.* 1994. **180:** 2219–2226.

17 **Rosen, S. D., Singer, M. S., Yednock, T. A. and Stoolman, L. M.,** Involvement of sialic acid on endothelial cells in organ-specific lymphocyte recirculation. *Science* 1985. **228:** 1005–1007.

18 **Lowe, J. B. and Marth, J. D.,** A genetic approach to Mammalian glycan function. *Annu. Rev. Biochem.* 2003. **72:** 643–691.

19 **Sasaki, K., Watanabe, E., Kawashima, K., Sekine, S., Dohi, T., Oshima, M., Hanai, N. *et al.*,** Expression cloning of a novel Gal beta (1-3/1-4) GlcNAc alpha 2,3-sialyltransferase using lectin resistance selection. *J. Biol. Chem.* 1993. **268:** 22782–22787.

20 **Okajima, T., Fukumoto, S., Miyazaki, H., Ishida, H., Kiso, M., Furukawa, K., Urano, T. and Furukawa, K.,** Molecular cloning of a novel alpha2,3-sialyltransferase (ST3Gal VI) that sialylates type II lactosamine structures on glycoproteins and glycolipids. *J. Biol. Chem.* 1999. **274:** 11479–11486.

21 **Ellies, L. G., Ditto, D., Levy, G. G., Wahrenbrock, M., Ginsburg, D., Varki, A., Le, D. T. and Marth, J. D.,** Sialyltransferase ST3Gal-IV operates as a dominant modifier of hemostasis by concealing asialoglycoprotein receptor ligands. *Proc. Natl. Acad. Sci. USA* 2002. **99:** 10042–10047.

22 **Ellies, L. G., Sperandio, M., Underhill, G. H., Yousef, J., Smith, M., Priatel, J. J., Kansas, G. S. *et al.***, Sialyltransferase specifity in selectin ligand formation. *Blood* 2002. **100:** 3618–3625.

23 **Kunkel, E. J., Ramos, C. L., Steeber, D. A., Muller, W., Wagner, N., Tedder, T. F. and Ley, K.**, The roles of L-selectin, β_7 integrins, and P-selectin in leukocyte rolling and adhesion in high endothelial venules of Peyer's patches. *J. Immunol.* 1998. **161:** 2449–2456.

24 **Berlin, C., Berg, E. L., Briskin, M. J., Andrew, D. P., Kilshaw, P. J., Holzmann, B., Weissman, I. L. *et al.***, $\alpha_4\beta_7$ integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* 1993. **74:** 185–195.

25 **Jung, U., Bullard, D. C., Tedder, T. F. and Ley, K.**, Velocity difference between L-selectin and P-selectin dependent neutrophil rolling in venules of the mouse cremaster muscle *in vivo*. *Am. J. Physiol.* 1996. **271:** H2740–H2747.

26 **Bullard, D. C., Kunkel, E. J., Kubo, H., Hicks, M. J., Lorenzo, I., Doyle, N. A., Doerschuk, C. M. *et al.***, Infectious susceptibility and severe deficiency of leukocyte rolling and recruitment in E-selectin and P-selectin double mutant mice. *J. Exp. Med.* 1996. **183:** 2329–2336.

27 **Scharffetter-Kochanek, K., Lu, H. F., Norman, K., Vannood, N., Munoz, F., Grabbe, S., McArthur, M. *et al.***, Spontaneous skin ulceration and defective T cell function in CD18 null mice. *J. Exp. Med.* 1998. **188:** 119–131.

28 **Jung, U., Ramos, C. L., Bullard, D. C. and Ley, K.**, Gene-targeted mice reveal importance of L-selectin-dependent rolling for neutrophil adhesion. *Am. J. Physiol.* 1998. **274:** H1785–H1791.

29 **Ramachandran, V., Nollert, M. U., Qiu, H, Liu, W. J., Cummings, R. D., Zhu, C. and McEver, R. P.**, Tyrosine replacement in P-selectin glycoprotein ligand-1 affects distinct kinetic and mechanical properties of bonds with P- and L-selectin. *Proc. Natl. Acad. Sci. USA* 1999. **96:** 13771–13776.

30 **Xia, L., Ramachandran, V., McDaniel, J. M., Nguyen, K. N., Cummings, R. D. and McEver, R. P.**, N-terminal residues in murine P-selectin glycoprotein ligand-1 required for binding to murine P-selectin. *Blood* 2003. **101:** 552–559.

31 **Leppanen, A., Penttila, L., Renkonen, O., McEver, R. P. and Cummings, R. D.**, Glycosulfopeptides with O-glycans containing sialylated and polyfucosylated polylactosamine bind with low affinity to P-selectin. *J. Biol. Chem.* 2002. **277:** 39749–39759.

32 **Leppanen, A., Yago, T., Otto, V. I., McEver, R. P. and Cummings, R. D.**, Model glycosulfopeptides from P-selectin glycoprotein ligand-1 require tyrosine sulfation and a core 2-branched O-glycan to bind to L-selectin. *J. Biol. Chem.* 2003. **278:** 26391–26400.

33 **Andre, P., Spertini, O., Guia, S., Rihet, P., Dignat-George, F., Brailly, H., Sampol, J. *et al.***, Modification of P-selectin glycoprotein ligand-1 with a natural killer cell-restricted sulfated lactosamine creates an alternate ligand for L-selectin. *Proc. Natl. Acad. Sci.* 2000. **97:** 3400–3405.

34 **De Luca, M., Dunlop, L. C., Andrews, R. K., Flannery, J. V., Jr., Ettling, R., Cumming, D. A., Veldman, M. and Berndt, M. C.**, A novel cobra venom metalloproteinase, mocarhagin, cleaves a 10-amino acid peptide from the mature N terminus of P-selectin glycoprotein ligand receptor, PSGL-1, and abolishes P-selectin binding. *J. Biol. Chem.* 1995. **270:** 26734–26737.

35 **Kanamori, A., Kojima, N., Uchimura, K., Muramatsu, T., Tamatani, T., Berndt, M. C., Kansas, G. S. and Kannagi, R.**, Distinct sulfation requirements of selectins disclosed using cells that support rolling mediated by all three selectins under shear flow. L-selectin prefers carbohydrate 6-sulfation to tyrosine sulfation, whereas P-selectin does not. *J. Biol. Chem.* 2002. **277:** 32578–32586.

36 **Spertini, O., Cordey, A. S., Monai, N., Giuffre, L. and Schapira, M.**, P-selectin glycoprotein ligand 1 is a ligand for L-selectin on neutrophils, monocytes, and $CD34^+$ hematopoietic progenitor cells. *J. Cell Biol.* 1996. **135:** 523–531.

37 **Walcheck, B., Moore, K. L., McEver, R. P. and Kishimoto, T. K.**, Neutrophil-neutrophil interactions under hydrodynamic shear stress involve L-selectin and PSGL-1 – a mechanism that amplifies initial leukocyte accumulation on P-selectin *in vitro*. *J. Clin. Invest.* 1996. **98:** 1081–1087.

38 **Bosse, R. and Vestweber, D.**, Only simultaneous blocking of the L- and P-selectin completely inhibits neutrophil migration into mouse peritoneum. *Eur. J. Immunol.* 1994. **24:** 3019–3024.

39 **Kunkel, E. J. and Ley, K.**, Distinct phenotype of E-selectin deficient mice: E-selectin is required for slow leukocyte rolling *in vivo*. *Circ. Res.* 1996. **79:** 1196–1204.

40 **Berlin, C., Bargatze, R. F., Campbell, J. J., von Andrian, U. H., Szabo, M. C., Hasslen, S. R., Nelson, R. D. *et al.***, α_4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell* 1995. **80:** 413–422.

41 **Hamann, A., Andrew, D. P., Jablonski-Westrich, D., Holzmann, B. and Butcher, E. C.**, Role of alpha 4-integrins in lymphocyte homing to mucosal tissues *in vivo*. *J. Immunol.* 1994. **152:** 3282–3293.

42 **Bargatze, R. F., Jutila, M. A. and Butcher, E. C.**, Distinct roles of L-selectin and integrins $\alpha_4\beta_7$ and LFA-1 in lymphocyte homing to Peyer's patch-HEV *in situ*: The multistep model confirmed and refined. *Immunity* 1995. **3:** 99–108.

43 **Wagner, N., Löhler, J., Kunkel, E. J., Ley, K., Leung, E., Krissansen, G., Rajewsky, K. and Müller, W.**, Critical role for β_7 integrins in formation of the gut-associated lymphoid tissue. *Nature* 1996. **382:** 366–370.

44 **Pries, A. R.**, A versatile video image analysis system for microcirculatory research. *Int. J. Microcirc. Clin. Exp.* 1988. **7:** 327–345.

45 **Lipowsky, H. H. and Zweifach, B. W.**, Application of the "two-slit" photometric technique to the measurement of microvascular volumetric flow rates. *Microvasc. Res.* 1978. **15:** 93–101.

46 **Long, D. S., Smith, M. L., Pries, A. R., Ley, K. and Damiano, E. R.**, Microviscometry reveals reduced blood viscosity and altered shear rate and shear stress profiles in microvessels after hemodilution. *Proc. Natl. Acad. Sci. USA* 2004. **101:** 10060–10065.

47 **Smith, M. L., Long, D. S., Damiano, E. R. and Ley, K.**, Near-wall micro-PIV reveals a hydrodynamically relevant endothelial surface layer in venules *in vivo*. *Biophys. J.* 2003. **85:** 637–645.

48 **Ley, K., Pries, A. R. and Gaehtgens, P.**, Preferential distribution of leukocytes in rat mesentery microvessel networks. *Pfluegers Arch.* 1988. **412:** 93–100.

49 **Ley, K., Meyer, J. U., Intaglietta, M. and Arfors, K.-E.**, Shunting of leukocytes in rabbit tenuissimus muscle. *Am. J. Physiol.* 1989. **256:** H85–H93.

