Sequential Contribution of L- and P-selectin to Leukocyte Rolling In Vivo

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Summary

Leukocyte recruitment into inflammatory sites is initiated by a reversible transient adhesive contact with the endothelium called leukocyte rolling, which is thought to be mediated by the selectin family of adhesion molecules. Selectin-mediated rolling precedes inflammatory cell emigration, which is significantly impaired in both P- and L-selectin gene-deficient mice. We report here that ∼13% of all leukocytes passing venules of the cremaster muscle of wild-type mice roll along the endothelium at <20 min after surgical dissection. Rolling leukocyte flux fraction reaches a maximum of 28% at 40–60 min and returns to 13% at 80–120 min. In P-selectin–deficient mice, rolling is absent initially and reaches 5% at 80–120 min. Rolling flux fraction in L-selectin–deficient mice is similar to wild type initially and declines to 5% at 80–120 min. In both wild-type and L-selectin–deficient mice, initial leukocyte rolling (0–60 min) is completely blocked by the P-selectin monoclonal antibody (mAb) RB40.34, but unaffected by L-selectin mAb MEL-14. Conversely, rolling at later time points (60–120 min) is inhibited by mAb MEL-14 but not by mAb RB40.34. After treatment with tumor necrosis factor (TNF)-α for 2 h, ∼24% of all passing leukocytes roll in cremaster venules of wild-type and P-selectin gene–deficient mice. Rolling in TNF-α–treated mice is unaffected by P-selectin mAb or E-selectin mAb 10E9.6. By contrast, rolling in TNF-α–treated P-selectin–deficient mice is completely blocked by L-selectin mAb. These data show that P-selectin is important during the initial induction of leukocyte rolling after tissue trauma. At later time points and in TNF-α–treated preparations, rolling is largely L-selectin dependent. Under the conditions tested, we are unable to find evidence for involvement of E-selectin in leukocyte rolling in mice.

Leukocyte recruitment into inflammatory sites is known to be a multi-step process, which is characterized by their initial transient contact with the endothelium, or rolling, followed by firm adhesion and transmigration (1–3). Most rolling leukocytes are probably granulocytes (4, 5), but the distinction between granulocytes and mononuclear cells cannot readily be made by intravital microscopy; hence, we use the general term leukocyte rolling. Leukocyte rolling is characterized by rapid formation and subsequent breakage of bonds formed by selectins (6, 7). Selectins are calcium-dependent mammalian lectins that share a high degree of structural homology in their lectin, epidermal growth factor-like, and consensus repeat domains (7). L-selectin is expressed on circulating granulocytes, monocytes, and most lymphocytes (8); E-selectin is induced on cytokine-treated endothelial cells (9); and P-selectin is expressed on the surface of platelets and endothelial

cells shortly after stimulation and is further induced by cytokine treatment of endothelial cells (10, 11).

In mice rendered genetically deficient for either P-selectin (12), or L-selectin (13), granulocyte emigration into experimentally inflamed peritoneum is significantly attenuated. Leukocyte emigration into inflammatory sites is also impaired in animals treated with several blocking selectin antibodies (14–16). These findings indicate that selectin-mediated rolling is an important determinant of the inflammatory response.

Earlier in vitro studies have suggested that granulocyte L-selectin may function as a ligand for endothelial E- and P-selectin (17), and L-selectin has been proposed to be involved in neutrophil adhesion to purified E-selectin under conditions of flow (18). In several studies, a partial inhibition of E- and P-selectin–dependent adhesion was seen by antibodies blocking L-selectin function. Granulocyte adhesion to platelet mono-

layers (19) or histamine-activated human umbilical vein endothelial cells (20) was completely blocked by a P-selectin mAb, and reduced by ∼50% by L-selectin mAb. Also, an L-selectin mAb inhibited ∼65% of granulocyte adhesion to L-cells transfected with E-selectin cDNA (21).

On the other hand, several data sets suggest that this interaction, although formally possible, does not appear to contribute to selectin-mediated adhesion under most conditions. Specifically, HL-60 cells, which express L-selectin at very low or undetectable levels, bind very well to P-selectin (22, 23), and can roll in mesenteric venules in vivo (24). Also, specific glycoprotein ligands for E-and P-selectin distinct from L-selectin have been discovered on myeloid cells (25–27). Furthermore, the ability of human neutrophils to bind to P-selectin is not reduced by stimulation with phorbol ester, which leads to shedding and loss of L-selectin from the cell surface (22), and L-selectin from a myeloid cell line does not bind to E- or P-selectin columns (28). In addition, several endothelial ligands for L-selectin have been described which are unrelated to P-selectin (29–31), and L- and E-selectin function independently in granulocyte adhesion to cytokine-treated endothelial cells in vitro (32). Taken together, it is not clear whether the L-selectin- and P-selectin–dependent pathways of leukocyte adhesion are distinct, overlapping, or synergistic.

Leukocyte rolling was reported to be reduced in mesenteric venules of L-selectin gene–deficient mice (13), and to be almost completely absent in venules of P-selectin gene–deficient mice (12). In this latter study, no hemodynamic data such as microvascular blood flow velocity, flow rate, or wall shear rate were measured. However, hemodynamic forces are known to be an important determinant of rolling leukocyte flux (4, 33, 34). Here, we address this issue by measuring leukocyte rolling in a large number of venules present in the mouse cremaster muscle, a thin striated muscle with a well-developed microcirculation (35). Specifically, we show that hemodynamic parameters account for ∼40% of the total variation seen in leukocyte rolling flux in venules in vivo.

The present study is designed to resolve the apparent overlap of phenotypes of selectin-deficient mice with respect to leukocyte rolling. Here, we investigate the time-course of leukocyte rolling after acute trauma in L-selectin and P-selectin-gene-deficient mice, because leukocyte rolling in internal organs is not constitutively present, but is rapidly induced by tissue exteriorization (5, 36). We find that leukocyte rolling in mice is P-selectin–dependent shortly after surgical trauma, shows a prominent L-selectin–dependent component at later time points, and appears to be largely mediated by L-selectin in cytokine-treated venules.

Materials and Methods

mAbs and Cytokines. We used a mAb against murine P-selectin, RB40.34 (rat IgG1, 60 μg per mouse), which in other experiments completely blocked adhesion of HL-60 promyelocytes to immobilized P-selectin, and a mAb against murine E-selectin, 10E9.6 (rat IgG2a, 60 μg per mouse), which completely inhibited E-selectin–dependent binding of HL-60 cells to TNF-activated murine endothelial cells (36a). mAb MEL-14 (IgG1, 80 μg per mouse) recognizing

murine L-selectin was a gift of Dr. E. C. Butcher (Stanford University, Palo Alto, CA). This antibody is known to effectively block L-selectin–mediated adhesion to high endothelial venules in peripheral lymph nodes (37). An antibody to murine E-cadherin, DECMA-1 (38), was used as a binding isotype-matched control antibody. Human recombinant TNF-α was a gift of Dr. J. A. Baker (Genentech, Inc., San Francisco, CA).

Animals. Experiments were performed on a total of 34 male mice between 8 and 15 wk of age and weighing 28–38 g. Mice genetically deficient for expression of L-selectin or P-selectin, respectively, were generated as described earlier by targeted gene disruption (13, and Bullard, D. C., L. Qin, I. Lorenzo, W. M. Quinlin, N. A. Doyle, D. Vestweber, C. M. Doerschuk, and A. L. Beaudet, manuscript submitted for publication). In both mouse strains, the presence of the mutation was confirmed by Southern blot analysis and PCR, and mAbs failed to detect expression of L- and P-selectin, respectively. All selectin-deficient and control mice used in this study were of a mixed 129/Sv × C57BL/6 background.

Intravital Microscopy. For intravital microscopic experiments, mice were anesthetized with ketamine (Ketavet®, 80 mg/kg i.p.) after premedication with pentobarbital (Nembutal®, 20 mg/kg i.p.) and atropine (0.1 mg/kg i.p.). The mice were thermocontrolled and received an i.v. infusion as described (34) to maintain anesthesia and a neutral fluid balance. Some animals were pretreated with an intrascrotal injection of 0.5 μg TNF-α in 0.3 ml saline.

The cremaster muscle was prepared for intravital microscopy as described (35), with the exception that the vein connecting the cremaster muscle with the epididymis was left intact. The testis was gently pushed back into the peritoneal cavity, and the epididymis was deflected to a lateral position. The surgical procedure took ∼10 min. Throughout the experiment, the muscle was superfused with thermocontrolled (37°C) bicarbonate-buffered saline as described (34).

Microscopic observations were made on a Leitz intravital microscope (water immersion objective SW 25/0.60 numerical aperture) modified for telescopic imaging (39). Venules with diameters between 17 and 69 μm were observed for ∼90 s each, and video recordings were made through a multidiode camera (RCA) on a Panasonic S-VHS recorder. Microvascular centerline red blood cell velocity was measured using a dual phototransistor and an automatic tracking correlator (model 102B; Instrumentation for Physiology and Medicine, San Diego, CA). Centerline velocities were converted to mean blood flow velocities by multiplication with an empirical factor of 0.625 as described earlier (40). Throughout the experiment, small 20-μl blood samples were withdrawn from the carotid catheter at ∼45-min intervals and analyzed for leukocyte concentration after detergent lysis of erythrocytes using a Coulter counter (Coulter, Herts, UK). Additional samples were taken after administration of mAbs and at the end of each experiment. Differential leukocyte counts were obtained by microscopy of Giemsa-stained blood smears.

Microvessel diameter was measured interactively using a digital image processing system (41), and rolling leukocyte flux was determined by counting the number of leukocytes passing each venule as described (13, 36). Total leukocyte flux was estimated as the product of measured systemic leukocyte concentration and blood volume flow. Microvessel blood flow was calculated using the circular cross-sectional area multiplied with mean blood flow velocity. Leukocyte rolling flux fraction is defined as the flux of rolling leukocytes in percent of total leukocyte flux. By way of its definition, leukocyte rolling flux fraction is independent of variations of systemic leukocyte counts.

Statistical Analysis. The dependence of leukocyte rolling flux frac-

tion on hemodynamic parameters was analyzed using multiple linear regression after appropriate transformation, followed by analysis of covariance (ANCOVA) (42). Leukocyte rolling in different experimental groups was compared using Student's t-test and Bonferroni correction for multiple comparisons where appropriate. Results are expressed as mean ± SEM.

Results and Discussion

L-selectin and P-selectin gene–deficient as well as wild-type mice appeared healthy under vivarium conditions and did not show obvious abnormalities. Systemic leukocyte counts and differentials were similar in the three groups, averaging 7.4 ± 1.4 × 10^6/ml with 34% PMN in wild type, 6.7 ± 1.3 × 10^6/ml with 36% PMN in L-selectin–deficient and 6.9 ± 0.8 × 10^6/ml with 35% PMN in P-selectin–deficient mice. This is in agreement with an earlier study on L-selectin-deficient mice (13). The moderate elevation of neutrophil counts reported in P-selectin–deficient mice (12) was not obvious in the present study. Leukocyte counts did not change significantly during the course of the experiments or after pretreatment with TNF-α (data not shown). Mean arterial blood pressure ranged between 60 and 120 mm Hg, which is in the normal range for anesthetized mice.

Hemodynamic Modulation of Leukocyte Rolling. Leukocyte rolling was investigated in a total of 234 venules in 18 wild-type mice, 134 venules in 11 P-selectin–deficient mice, and 65 venules in 8 L-selectin–deficient mice. Venular diameters ranged between 17 and 69 μm with an overall mean of 42 ± 1 μm. Centerline erythrocyte velocities in these venules averaged 2.1 ± 0.1 mm/s, ranging from 0.3 to 4.9 mm/s. This corresponds to Newtonian wall shear rates between 32 and 990 s^{-1} and estimated wall shear stress values from 1 to 25 dyn/cm^2. Microhemodynamic parameters were similar in all investigated groups.

The wide variety of velocities and diameters of venules in vivo necessitated the analysis of the impact of these parameters on leukocyte rolling flux fraction. Two separate control groups (five animals each) were used for P- and L-selectin–deficient animals, consisting of age-matched control animals with a similar genetic background. Since the two control groups did not differ significantly in any of the measured parameters, they were combined for subsequent analysis. Multiple regression analysis and ANCOVA showed that 37% of the total variation of leukocyte rolling flux fraction could be attributed to variations of venular diameter and flow velocity. Least square correlations yielded r = 0.49 (p <0.001) for the dependence of leukocyte rolling flux on 1/diameter, and r = 0.46 (p <0.001) for that on 1/velocity, with an overall r for multiple regression of 0.61. The microhemodynamic variability seen in the present study is similar to that reported in other microvascular beds. The hyperbolic negative correlation between microvessel diameter and rolling flux fraction (Fig. 1 *a*) can be explained by the decreasing surface-to-volume ratio with increasing microvessel size. The negative dependence of leukocyte rolling flux fraction on microvessel blood flow velocity (Fig. 1 *b*) reflects the shear sensitivity of the

rolling interaction, which has been documented in earlier studies in other tissues (33, 34). The systematic influence of microvessel diameter and flow velocity on rolling flux fraction variability was adjusted using the results of multiple correlation. The present analysis underlines the necessity to account for hemodynamic variation among the microvessels in which leukocyte rolling is studied.

Leukocyte Rolling in Wild-type Mice. In cremaster venules of wild-type animals, leukocyte rolling flux fraction was found to average 13.3 ± 1.8% <20 min after the onset of surgical preparation of the cremaster muscle (Fig. 2). Due to the nature of the surgery necessary to expose the cremaster muscle, leukocyte rolling could not be observed at very early time points (<10 min) in this preparation. Leukocyte rolling flux fraction increased to reach a maximum of 27.7 ± 3.4% at 40–60 min after surgery, whereafter it declined and remained at 13.0 ± 1.4% at 80–120 min. This is in agreement with earlier findings in the hamster cheek pouch, the mouse mesentery, and the rat mesentery (4, 36).

Leukocyte Rolling in Selectin Gene-deficient Mice. In stark contrast with these findings, leukocyte rolling was initially absent in mice deficient for expression of P-selectin, consistent with an earlier report on leukocyte rolling in P-selectin deficient mice (12). However, in contrast to that report, leukocyte flux fraction increased significantly with time after surgery, reaching detectable levels at 40–60 min and maximal levels of 4.9 ± 1.9% at 80–120 min (Fig. 2). This discrepancy cannot be attributed to tissue-specific differences between the mesenteric microcirculation used in the earlier study (12) and the cremaster muscle used here, because we observed a similar pattern of induction of leukocyte rolling in venules of the exteriorized mesentery of P-selectin–deficient mice. In mesenteric venules of P-selectin–deficient mice, no rolling leukocytes were seen between 0 and 40 min after exteriorization, and leukocyte rolling flux fraction reached 3.9 ± 1.2% at >60 min.

In L-selectin gene–deficient mice, leukocyte rolling flux fraction was indistinguishable from that observed in wild-type animals initially, but, in marked contrast to wild-type mice, leukocyte rolling flux fraction dropped with time to reach 5.2 ± 1.2% at 80–120 min (Fig. 2). This finding underscores the important role of L-selectin for leukocyte rolling in vivo, which has also been documented by various studies using blocking antibodies (43, 44) and transfected cell lines (24, 45). The present data suggest that P-selectin dominates early leukocyte rolling and continues to contribute to leukocyte rolling at later time points, at which L-selectin significantly contributes to leukocyte rolling.

This finding reconciles seemingly discrepant results obtained in earlier studies on the relative importance of P- and L-selectin for leukocyte rolling in vivo (43–47). In these studies, the time course of induction of leukocyte rolling after surgery was not examined. Comparison of the time course of leukocyte rolling in wild-type and L-selectin–deficient mice suggests that functional ligand(s) for L-selectin are induced within ~30–60 min after surgical trauma, which is in agreement with earlier findings for the time course of rolling of L-selectin–transfected cells in rat mesenteric venules (24). The complete ab-
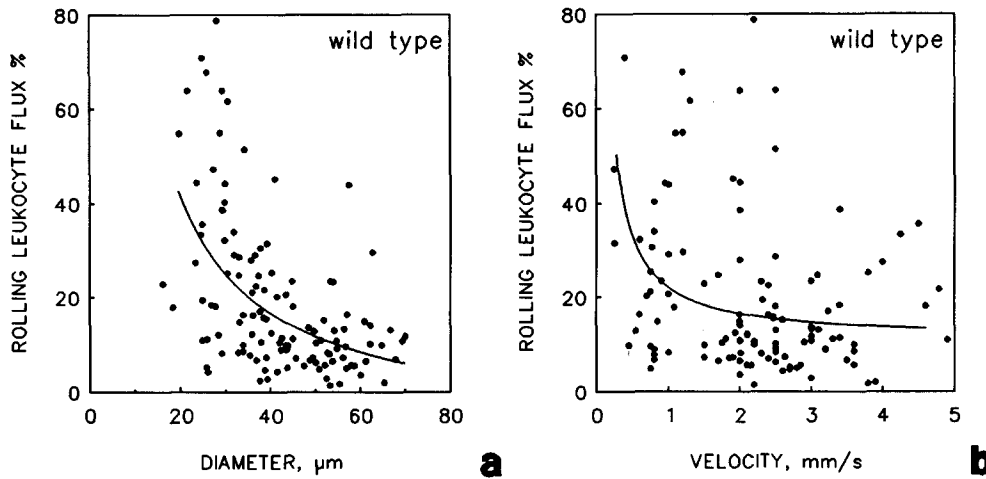
sence and partial reduction of leukocyte rolling seen at 30–60 min in P- and L-selectin–deficient mice, respectively, suggests that P- and L-selectin can promote rolling in a synergistic fashion. The average leukocyte rolling flux fraction in wild-type mice exceeds the sum of the rolling flux fractions present in L- and P-selectin–deficient mice at all but the earliest and latest time points, suggesting that the L- and P-selectin–dependent pathways show not only temporal overlap but operate synergistically during this time interval. The observed pattern of leukocyte rolling may also be caused by different leukocyte classes participating at different times as suggested by an earlier histological study in the rabbit mesentery (5).
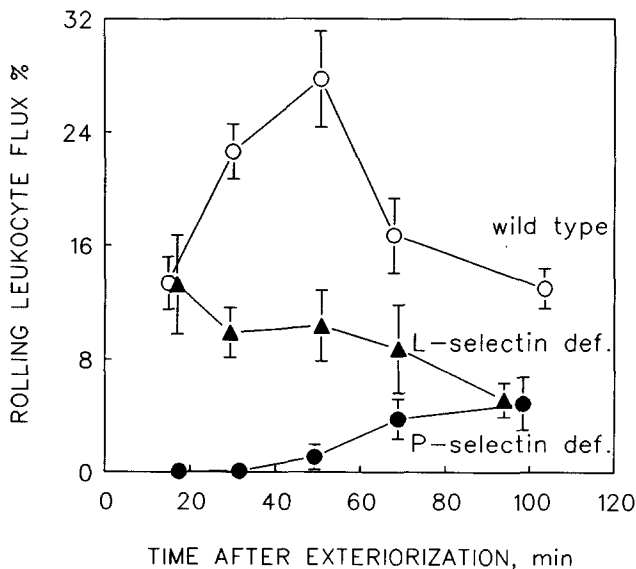

Figure 2. Time course of induction of leukocyte rolling. Beginning of surgery at $t = 0$ min. Wild-type mice (*open symbols*); L-selectin gene–deficient mice (*closed triangles*); P-selectin–deficient mice (*closed circles*); five animals each. Mean ± SEM for 20-min classes (last class 40 min), containing 7–48 venules per class. P-selectin gene–deficient mice significantly different from wild-type mice at all time points ($p < 0.01$), L-selectin gene–deficient mice different from wild type at all times >20 min and from P-selectin gene–deficient mice at times <60 min ($p < 0.01$).
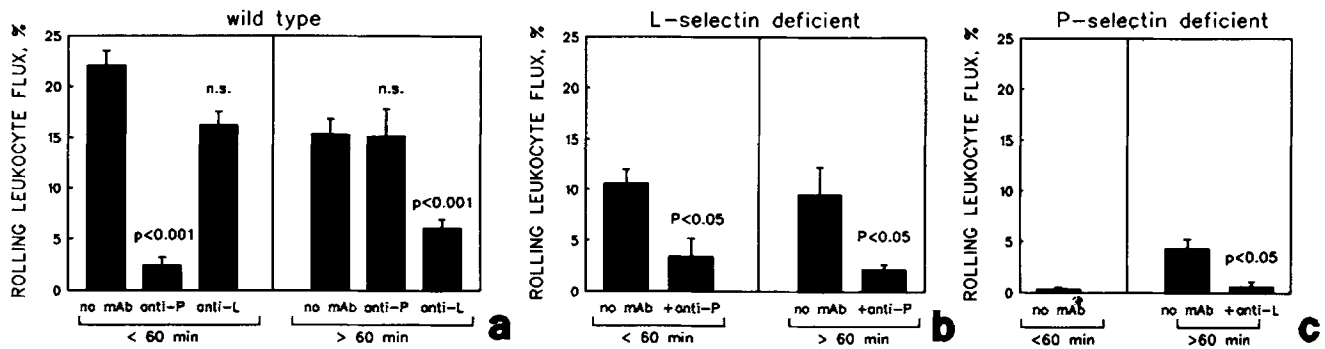
The intravital microscopic techniques used in the present study cannot distinguish between different classes of rolling leukocytes.

Effect of Selectin mAbs on Leukocyte Rolling. When mAb RB40.34, a blocking P-selectin antibody, was applied to wild-type mice soon (<60 min) after surgical trauma, it significantly reduced rolling leukocyte flux by 89%. In contrast, the adhesion-blocking L-selectin mAb MEL-14 had no significant effect when applied at <60 min (Fig. 3 *a*). This pattern was reversed at later time points (>60 min after surgery), at which anti–P-selectin had no effect, while anti–L-selectin blocked leukocyte rolling flux fraction by 60%. A control antibody binding to E-cadherin, mAb DECMA-1, had no effect on leukocyte rolling (data not shown). In L-selectin–deficient mice, leukocyte rolling was almost completely abolished by anti–P-selectin at <60 min. Beyond 60 min, residual leukocyte rolling in L-selectin–deficient mice was also reduced by addition of anti–P-selectin (Fig. 3 *b*). P-selectin deficient mice did not show any leukocyte rolling <60 min after surgery. Residual leukocyte rolling seen in P-selectin gene–deficient mice at later time points was completely abolished by anti–L-selectin (Fig. 3 *c*). These findings support the results obtained in wild-type animals and show that L- and P-selectin together account for almost all of the "spontaneous" leukocyte rolling induced by surgical trauma.

Leukocyte Rolling in TNF-α–treated Venules. In venules of wild-type mice pretreated with TNF-α (2 h, intrascrotal), massive leukocyte adhesion was observed along the microvessel walls. Intrascrotal injection of vehicle (saline) did not affect leukocyte rolling or adhesion. In TNF-α–treated wild-type mice, leukocyte rolling flux fraction averaged 24.4 ± 2.9% (Fig. 4 *a*). This value is similar to the maximum of leukocyte rolling observed without TNF-α at 40–60 min after tissue exteriorization (27.7 ± 3.4%) and corresponds to about twice the sustained level of leukocyte rolling measured in untreated cremaster at 80–120 min (13.0 ± 1.4%). TNF-α treatment may also recruit other classes of leukocytes which may not be rolling subsequent to surgical trauma alone.

TNF-α–induced leukocyte rolling was not reduced by injection of anti–P-selectin (mAb RB40.34) or anti–E-selectin

Figure 3. Effect of function-blocking selectin mAbs on trauma-induced leukocyte rolling. In wild-type mice, rolling is inhibited by the P-selectin mAb RB40.34 (anti-P), but not by L-selectin mAb MEL-14 (anti-L) at <60 min. At later time points, L-selectin mAb reduces rolling significantly, and P-selectin mAb has no effect (a). In L-selectin–deficient mice, residual rolling is largely abolished by P-selectin mAb (b). In P-selectin mice, residual rolling at >60 min is completely blocked by L-selectin mAb (c).

(mAb 10E9.6, Fig. 4 a). Both antibodies are known to be function blocking in promyelocyte adhesion assays in vitro. The absence of an inhibitory effect of anti-E- and P-selectin suggests that leukocyte rolling in TNF-α–stimulated venules does not require P- and E-selectin function. This does not exclude the possibility that minor differences in leukocyte rolling patterns, e.g., in the distribution of rolling velocities, may occur when P- and/or E-selectin function are blocked. In agreement with the absence of an effect of anti-P-selectin on leukocyte rolling in TNF-α–treated mice, leukocyte rolling flux fraction in TNF-α–treated P-selectin gene–deficient mice was very similar to that in TNF-α–treated wild-type mice (Fig. 4 b). The L-selectin mAb MEL-14 blocked TNF-α–induced rolling in P-selectin gene deficient mice by more than 90%. This finding indicates that L-selectin is responsible for the majority of leukocyte rolling in TNF-α–treated venules in the present model.

Earlier in vivo studies have argued in favor of a role for endothelial E-selectin in mediating leukocyte rolling in cytokine-treated rabbit mesenteric venules (44, 48). E-selectin–dependent rolling has been shown in a number of in vitro studies (18, 49), and one study suggesting involvement of E-selectin in leukocyte rolling in vivo has been published (49a).

Our inability to demonstrate a role of E-selectin for rolling in inflamed venules is in agreement with the absence of apparent defects of inflammatory cell recruitment reported recently in E-selectin gene–deficient mice (50). On the other hand, a mAb blocking E-selectin function has been shown to block ~70% of granulocyte accumulation subsequent to glycogen-induced peritonitis and to attenuate plasma leakage and hemorrhage in an IgG immune complex-induced model of pulmonary inflammation in the rat (51).

The distinct time course of P-selectin and L-selectin–mediated leukocyte rolling and the additive effects of L-selectin mAb in P-selectin gene–deficient mice and of anti-P-selectin in L-selectin gene–deficient mice indicate that these two adhesion systems function in a separate but temporally overlapping fashion in vivo. Together, L- and P-selectin account for at least 90% of leukocyte rolling induced in mouse venules either by surgery or by TNF-α treatment. "Early" leukocyte rolling is entirely dependent on P-selectin function, L- and P-selectin synergize to produce rolling in an intermediate time period, and L-selectin dominates rolling in cytokine-stimulated venules. The role of E-selectin in leukocyte rolling in mice remains to be established. Our findings establish clearly distinct phenotypes of P- and L-selectin gene–deficient mice with
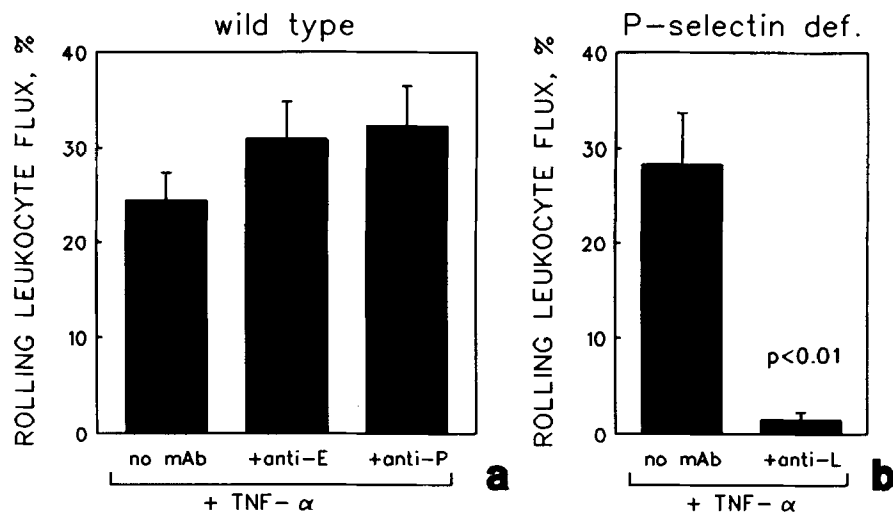

Figure 4. Leukocyte rolling in TNF-α–treated cremaster venules. Mice were pretreated with an intrascrotal injection of TNF-α 2–2.5 h before the experiment. In wild-type mice, mAbs 10E9.6 (anti-E-selectin) and RB40.34 (anti-P-selectin) slightly increase leukocyte rolling (a). In TNF-α–treated P-selectin gene–deficient mice (P-deficient), leukocyte rolling flux fraction is similar to that in wild-type mice, and mAb MEL-14 (anti-L) against murine L-selectin almost completely abolishes leukocyte rolling (b).

respect to the kinetics and pattern of leukocyte rolling. Taken together, the present data help clarify the molecular mecha- nisms underlying leukocyte rolling during the early inflammatory response.

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