Absence of Trauma-induced Leukocyte Rolling in Mice Deficient in Both P-Selectin and Intercellular Adhesion Molecule 1

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Summary

Leukocyte recruitment during inflammation is achieved through a multistep paradigm that includes margination, selectin-mediated rolling, β_2 integrin-mediated firm adhesion, emigration, and migration into the site of inflammation. We have used the mouse cremaster muscle as a model of trauma- and cytokine-induced inflammation to study the possible role of intercellular adhesion molecule (ICAM) 1 in leukocyte rolling using gene-targeted mice deficient in ICAM-1, P-selectin, and a combination of P-selectin and ICAM-1. Rolling flux and average leukocyte rolling velocity in ICAM-1–deficient mice was not different from wild-type mice, but P-selectin/ICAM-1–deficient mice showed a total absence of rolling for at least 2 h after surgical trauma. Rolling in both wild-type and ICAM-1–deficient mice 60–120 min after trauma was significantly inhibited by a P-selectin monoclonal antibody (mAb) (RB40.34). In contrast, an mAb (KAT-1) blocking ICAM-1 binding to leukocyte function–associated antigen 1 did not block residual rolling in P-selectin–deficient mice. TNF-α induced leukocyte rolling in P-selectin/ICAM-1–deficient mice, but the rolling flux fraction was significantly lower than in TNF-α–treated ICAM-1–deficient mice. Leukocyte rolling in P-selectin/ICAM-1–deficient mice treated with TNF-α for 3 h was completely blocked by an E-selectin mAb (9A9E3), and partially by an L-selectin mAb (MEL-14). This clearly demonstrates E-selectin–dependent rolling in vivo. Leukocyte rolling velocities were significantly reduced after TNF-α treament and were similar in wild-type and gene-targeted strains. We conclude that the residual trauma-induced leukocyte rolling seen in P-selectin–deficient mice is completely abolished by concomitant ICAM-1 deficiency. This severe defect in leukocyte rolling may explain the absence of leukocyte recruitment into the inflamed peritoneal cavity of P-selectin/ICAM-1–deficient mice at early time points (≤4 h).

The recruitment of leukocytes into a site of inflammation is pivotal to the eventual successful defense and subsequent healing of the host organism. This recruitment is achieved through a multistep paradigm that includes hemodynamic and rheologic margination in the vasculature, transient adhesive interactions with the vascular endothelium, resulting in leukocyte rolling, firm adhesion to the vascular endothelium, emigration through the vascular wall into the extracellular matrix, and the ensuing chemotactic migration to the inflammatory locale (1–3). With the exception of rheological margination, which is thought to be essentially nonlimiting, the aforementioned steps are mediated by specific interactions between constitutive and inducible adhesion molecules found on the leukocyte and the vascular endothelium.

Leukocytes from patients with the clinical syndrome leukocyte adhesion deficiency type II, which is characterized by a defect in fucose metabolism and the inadequate recruitment of granulocytes into sites of inflammation, have a reduced ability to roll along vascular endothelium treated with the cytokine IL-1 to stimulate the expression of inflammatory adhesion molecules (4). Also, mice deficient in selectins, the adhesion molecules that mediate leukocyte rolling, show impaired responses in various models of in-

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flammation (5–7). This evidence identifies rolling as one of the limiting steps in leukocyte extravasation.

Leukocyte rolling has previously been shown to be mediated by transient adhesive interactions between the selectin family of adhesion molecules (L-, P-, and E-selectin) and their respective ligands (8–11), although $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins can also mediate rolling by binding to vascular cell adhesion molecule 1 and mucosal addressin cell adhesion molecule 1, respectively (12–14). The selectins are calcium-dependent mammalian lectins that share a high degree of homology, among each other as well as among species, in their lectin, epidermal growth factor–like, and consensus repeat domains (15). L-selectin is found constitutively on circulating granulocytes, monocytes and most lymphocytes and is shed after activation (16). P-selectin is stored in α granules of platelets and Weibel-Palade bodies of endothelial cells. Its expression is rapidly and transiently induced upon stimulation by mediators such as histamine or thrombin (17). Both P- and E-selectin are expressed by endothelial cells upon stimulation with cytokines such as TNF-α (18, 19).

Prior in vivo work has focused almost solely on the role of the selectins in mediating leukocyte rolling, but the complex interactions occurring during this process leave open the possibility that additional molecules may be involved. Several recent investigations have examined the leukocyte recruitment in models of peritonitis using mice deficient in P-selectin (20), intercellular adhesion molecule (ICAM)[1] (21, 22), and both molecules together (23). ICAM-1 is an immunoglobulin-like molecule with five immunoglobulin domains anchored to the cell by a transmembrane domain and a short cytoplasmic tail. This molecule is expressed constitutively on endothelial cells and is up-regulated upon stimulation with cytokines. ICAM-1 acts as a ligand for the β_2 integrins, lymphocyte function-associated antigen (LFA) 1 and Mac-1, expressed on leukocytes, and is important for the firm adhesion, emigration, and migration of leukocytes (3).

Mice genetically engineered to be deficient in ICAM-1 have been shown to have elevated blood neutrophil counts, decreased neutrophil emigration into thioglycolate-induced peritonitis, and a severely attenuated delayed-type hypersensitivity response (21). These data suggest that ICAM-1–deficient mice have severely impaired leukocyte extravasation. No studies were done to identify which step in the recruitment process is affected by ICAM-1 deficiency, however. Hence, the specific role of ICAM-1 deficiency in this extravasation dysfunction remains unknown. Previous work has demonstrated that P-selectin–deficient mice have a threefold increase in systemic neutrophil counts and an absence of rolling immediately after tissue trauma (20). Later work has found that rolling in these mice begins to increase after ~1 h, although it remains below the levels seen in wild-type mice (24).

Mice deficient in P-selectin and ICAM-1 have been produced by mating P-selectin– and ICAM-1–deficient mice (23). These mice show a complete lack of neutrophil emigration into an inflamed peritoneal cavity for at least 4 h. This lack of emigration is in contrast to both wild-type mice and mice deficient in only P-selectin or only ICAM-1, which do show neutrophil emigration, although less than wild-type mice (23). Again, this work does not address whether combined ICAM-1 and P-selectin deficiency affects leukocyte rolling or a later step such as emigration from the vasculature or migration into the inflammatory locus.

To assess the role of ICAM-1 in leukocyte recruitment, and, more specifically, the rolling step, we have examined the time course of the rolling leukocyte flux in venules of the cremaster muscle of mice deficient in ICAM-1, P-selectin, and both P-selectin and ICAM-1. Additionally, we have used function-blocking mAbs in these mice to assess which molecules mediate any residual rolling. As in previous work (24), hemodynamic variations in the data are effectively controlled through measurements of blood flow velocity and venular diameter. Within this framework, we have found that a deficiency in both ICAM-1 and P-selectin causes a total absence of leukocyte rolling for at least 2 h after tissue insult due to surgery.

Materials and Methods

mAbs and Cytokines. Several different mAbs were used in this study, including an mAb against murine P-selectin, RB40.34 (rat IgG1, 30 μg per mouse), which has been shown in other experiments to completely block adhesion of HL-60 promyelocytes to immobilized P-selectin (25); an mAb against murine E-selectin, 10E9.6 (rat IgG2a, 30 μg per mouse), which completely inhibited E-selectin–dependent binding of HL-60 cells to TNF-α–activated murine endothelial cells (25); an mAb against murine E-selectin, 9A9E3.F10 (rat IgG1, 30 μg per mouse), which blocks HL-60 promyelocyte adhesion to transfected COS monolayers (26); mAb MEL-14 (rat IgG1, 100 μg per mouse), recognizing murine L-selectin and effectively blocking L-selectin–mediated lymphocyte adhesion to high endothelial venules in peripheral lymph nodes (27); and an LFA-1 function-blocking mAb against murine ICAM-1, KAT-1 (IgG2a, 30 μg per mouse) (CALTAG Laboratories, South San Francisco, CA) (28). Murine recombinant TNF-α (0.5 μg per mouse) was obtained from Genzyme Corp. (Cambridge, MA).

Animals. A total of 36 male mice >8 wk in age and weighing between 28 and 47 *g* were used in these experiments. Mice genetically engineered to be deficient in expression of P-selectin (23) or ICAM-1 (21) were prepared as described earlier by targeted gene disruption. Mice deficient in both P-selectin and ICAM-1 were produced by breeding the single-mutant mice as described (23). All mice used in this study were of a mixed 129/Sv × C57BL/6 background or from a fifth-generation back-cross onto C57BL/6. Leukocyte rolling in C57BL/6 × 129/Sv wild-type mice was found to be indistinguishable from that in C57BL/6 mice; therefore, the results of the control experiments in these strains were combined.

Before the experiment, mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine hydrochloride (Ketalar; Parke-Davis, Detroit, MI) after premedication (given intraperitoneally) with 30 mg/kg sodium pentobarbital (Nembutal;

[1] *Abbreviations used in this paper:* ICAM, intercellular adhesion molecule; LFA, leukocyte function–associated antigen.

Abbott Laboratories) and 0.1 mg/kg atropine (Elkins-Sinn, Inc., Cherry Hill, NJ). Some mice were pretreated (2–2.5 h before surgery) with an intrascrotal injection of 0.5 μg TNF-α in 0.40 ml isotonic saline. Mice were prepared for the ensuing surgery and data procurement by tracheal cannulation to facilitate normal respiration and use of a respirator (if required), jugular cannulation for injection of anesthetic, antibodies, and saline, and carotid cannulation for blood pressure monitoring and blood sampling. During the experiment, the mice were thermocontrolled using a small animal heating pad (model 50-7503; Harvard Apparatus, South Natick, MA) and received an intravenous infusion of isotonic saline to maintain anesthesia and a neutral fluid balance. Blood pressure was monitored (model BPMT-2; Stemtech, Inc., Menomonee Falls, WI) and maintained in the range of 60–100 mmHg.

Intravital Microscopy. The cremaster muscle was prepared for intravital microscopy as described previously (29). If the artery–vein pair connecting the epididymis to the cremaster was a major feed-drainage pair, then the epididymis and testes were pinned to the side; otherwise, these vessels were pinched off and severed, and the testis was gently pushed back into the peritoneal cavity. The surgical procedure was completed in ~10 min. During and after this procedure, the muscle was superfused with thermocontrolled (36°C) bicarbonate-buffered saline with the following composition: 131.9 mM NaCl, 18 mM $NaHCO_3$, 4.7 mM KCl, 2.0 mM $CaCl_2 \cdot 2H_2O$, and 1.2 mM $MgCl_2$.

Microscopic observations were made using an intravital microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY) with a saline immersion objective (SW 40, 0.75 numerical aperture). Venules with diameters between 15 and 80 μm were observed and recorded through a CCD camera system (model VE-1000CD; Dage-MTI, Inc., Michigan City, IN) for ~60 s (S-VHS recorder; Panasonic, Osaka, Japan). Centerline red blood cell velocity in the recorded microvessels was measured using a dual photodiode and a digital on-line cross-correlation program (30) running on a 486DX2-66 (Winlabs, Manassas, VA) personal computer. Centerline velocities were converted to mean blood flow velocities by dividing the centerline velocity by an empirical factor of 1.6 (31). Throughout the experiment, 10-μl blood samples were withdrawn at ~45-min intervals from the carotid catheter and analyzed for leukocyte concentration (expressed as number of leukocytes per microliter of whole blood). Blood smears were stained with a three-step stain (LeukoStat; Fisher Scientific Co., Pittsburgh, PA) from which a differential leukocyte count was obtained. Additional blood samples were taken, using the same procedure outlined above, before and after mAb administration and at the termination of the experiment.

Microvessel diameter was measured from video recordings using a digital image processing system (30), and the rolling leukocyte flux was determined by counting the number of leukocytes passing through a stationary plane perpendicular to the axis of each venule (24) over a given time period. The total leukocyte flux through a given venule was estimated by the product of the systemic leukocyte count and the volumetric flow rate of blood through the venule, which was calculated using the microvessel diameter and the mean blood flow velocity, assuming cylindrical geometry. The leukocyte rolling flux fraction is defined as the flux of rolling leukocytes as a percentage of the total leukocyte flux, and, because of this definition, it is independent of variations in systemic leukocyte counts.

Individual leukocyte rolling velocities were measured from video recordings by analyzing approximately five leukocytes per venule and measuring the distance each traveled in a given time using a digital image-processing system (30). In TNF-α–treated mice with large numbers of rolling and adherent leukocytes, those leukocytes that appeared to have rapid, transient interactions with rolling or adherent leukocytes were not chosen for rolling velocity measurements.

Histology. Whole cremaster muscles obtained from mice injected with or without primary antibody (30 μg per mouse) were laid flat on gelatinized glass slides (32) and fixed in an acetone bath overnight at −18°C. After fixation, the tissues were washed in 0.05 M Tris buffer with 0.03% saponin to permeabilize the tissues. All antibody and reagent incubations were performed for 45 min at room temperature in a humid chamber, and 0.05 M Tris buffer was used to wash the tissues between each antibody or reagent incubation. The tissues were incubated with a 1:500 dilution of a biotin-conjugated rabbit antibody directed against rat IgG (DAKO, Carpinteria, CA) in 0.05 M Tris buffer followed by peroxidase-conjugated streptavidin (DAKO). The tissues were then incubated with 0.3% H_2O_2 in methanol for 1 h. The staining reaction was done using 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) tablets at a concentration of 1 mg/ml in 0.05 M Tris buffer with 0.01% H_2O_2. The tissues were then washed with 0.04% osmium tetraoxide in 0.05 M Tris buffer to enhance contrast. The samples were counterstained with Giemsa stain (Sigma Chemical Co.) and mounted using Permount (Fisher Scientific Co.).

Statistical Analysis. The dependence of the leukocyte rolling flux fraction on hemodynamic parameters was analyzed using a multiple linear regression after appropriate transformation, followed by an analysis of the covariance (33). Leukocyte rolling flux and average leukocyte rolling velocities in different experimental groups were compared using Student's t test with a Bonferroni correction for multiple comparisons when appropriate. All results are expressed as mean ± SEM, and statistical significance was set at $P < 0.05$.

Results

All mice used in these experiments appeared healthy and active under the conditions in the vivarium, and no obvious abnormalities were observed. Systemic leukocyte counts averaged 5,660 ± 350/μl with 53 ± 3% neutrophils and 46 ± 4% lymphocytes in wild-type mice, 7,670 ± 740/μl with 62 ± 3% neutrophils and 36 ± 3% lymphocytes in ICAM-1–deficient mice, 9,800 ± 1,530/μl with 61 ± 5% neutrophils and 37 ± 5% lymphocytes in P-selectin–deficient mice, and 17,130 ± 3,380/μl with 79 ± 4% neutrophils and 20 ± 4% lymphocytes in P-selectin/ICAM-1–deficient mice. These values are comparable with previously published data (20, 21, 23, 24). The percentage of neutrophils in all mice strains are somewhat higher than reported previously, which may be attributable to partial recruitment of the sequestered granulocyte pool in these mice due to anesthesia and surgery. Systemic leukocyte counts were relatively constant during the course of the experiment, throughout mAb treatments, and after the addition of TNF-α, with the exception of a decrease in leukocyte counts after the addition of MEL-14 mAb.

Hemodynamic Variation in Leukocyte Rolling. Leukocyte rolling was investigated in 149 venules in 10 wild-type mice, 101 venules in eight ICAM-1–deficient mice, 82 venules in four P-selectin/ICAM-1–deficient mice, and 79

venules in four P-selectin–deficient mice. Venular diameters ranged from 13 to 86 μm, with an overall mean ± SEM of 36 ± 1 μm. Centerline erythrocyte velocities in these venules ranged from 0.35 to 8.3 mm/s and averaged 1.6 ± 0.1 mm/s. This corresponds to wall shear stresses ranging from 0.9 to 20 dynes/cm^2. Hemodynamic parameters were similar in all investigated mouse genotypes. The effect of these hemodynamic parameters on leukocyte rolling were controlled as described previously (24). The rolling leukocyte flux fraction (number of rolling leukocytes as a fraction of the total leukocytes flowing through the observed venule) was examined in age-matched control mice and evaluated for variance due to hemodynamic parameters. Rolling flux fraction is inherently sensitive to the surface-to-volume ratio in these venules, which is reflected by a significant correlation between rolling flux fraction and 1/diameter ($r = 0.54$; $P < 0.001$). There was also a small dependence of rolling flux fraction on 1/velocity ($r = 0.04$; NS). Multiple linear regression analysis showed that ~30% of the observed variation in the rolling leukocyte flux fraction in control mice was due to hemodynamic variations in venule diameter and blood flow velocity. All the data were then adjusted for this variation using the correlation found in the control group. Measurements obtained at very low blood flow velocities (<0.3 mm/s) or in venules with very small diameters (<15 μm) were eliminated because these measurements were deemed inaccurate (amounting to <2% of all measurements).

Leukocyte Rolling in Wild-type Mice. The rolling flux fraction in wild type mice remained relatively constant at ~45% for the entire 2-h observation period (Fig. 1 *A*). Because the surgery to exteriorize the cremaster muscle required ~10 min, no leukocyte rolling time course measurements are available at very early time points. Leukocyte rolling velocity averaged ~35 μm/s and was similar to values reported in other preparations (Table 1).

Leukocyte Rolling in ICAM-1, P-Selectin, and P-Selectin/ICAM-1–deficient Mice. The rolling leukocyte flux fraction seen in ICAM-1–deficient mice (Fig. 1 *A*) was similar to that of wild-type mice for the entire 2-h observation period. The flux fraction in these mice averaged ~52% for the first 60 min after surgical insult and reached 42 ± 5% by the end of the observation period.

Mice deficient in both P-selectin and ICAM-1 (Fig. 1 *A*) showed essentially no leukocyte rolling for the entire 2-h observation period ($P < 0.001$ compared with wild-type mice at all time points). The complete lack of rolling leukocytes in P-selectin/ICAM-1–deficient mice is in stark contrast to observations made in mice deficient in P-selectin only. These mice have no rolling for the first h after tissue exteriorization but regain a significant amount of rolling by the end of 2 h (24). This previous finding was confirmed in the present set of experiments (data included in Fig. 1 *B*; $P < 0.01$ vs P-selectin/ICAM-1–deficient mice at 80–120 min). Leukocytes rolling in P-selectin–deficient mice show intermittent interactions with the endothelium, causing a significant increase in average rolling velocity to ~129 μm/s (Table 1).

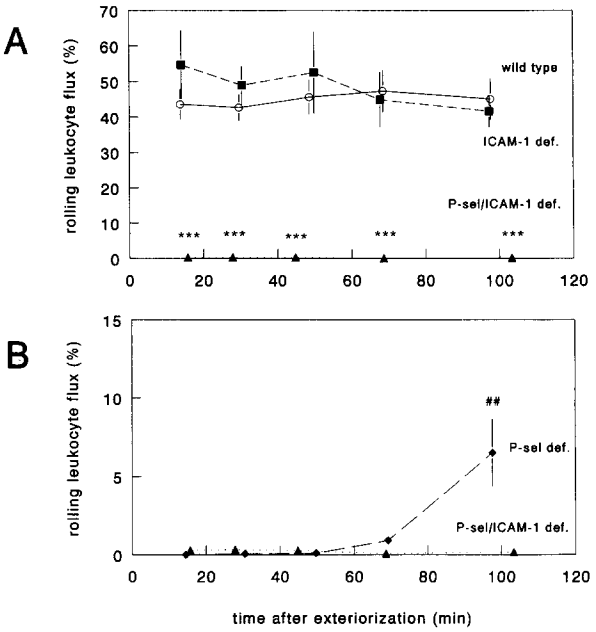

Figure 1. Time course of leukocyte rolling in wild-type, ICAM-1–deficient, P-selectin–deficient, and P-selectin/ICAM-1–deficient mice. Surgery at $t = 0$. Wild-type mice (10 animals): open circles; ICAM-1–deficient mice (8 animals): filled squares; P-selectin–deficient mice (4 animals): filled diamonds; P-selectin/ICAM-1–deficient mice (4 animals): filled triangles. Mean ± SEM for 20-min classes plotted at mean time for each class, with 11–39 venules per class. (*A*) Leukocyte rolling flux fraction in P-selectin/ICAM-1–deficient mice significantly differed from that in wild-type mice at all times from 0 to 120 min (*** $P < 0.001$). (*B*) Leukocyte rolling flux fraction in P-selectin/ICAM-1–deficient mice significantly different from P-selectin deficient mice (## $P < 0.01$) at 80–120 min. Notice ordinate scale change.

Effect of mAbs on Leukocyte Rolling. We investigated which adhesion molecules are involved in mediating leukocyte rolling in wild-type, ICAM-1–deficient, and P-selectin–deficient mice after surgical trauma by using function-blocking mAbs. In wild-type mice (Fig. 2), the addition of a P-selectin antibody (RB40.34) decreased the basal rolling

Table 1. *Average Trauma- and TNF-α–induced Leukocyte Rolling Velocities by Phenotype*

Mouse genotype	Trauma-induced	TNF-α pretreated
	μm/s	
Wild type	34.5 ± 1.4	5.1 ± 1.1‡
ICAM-1 deficient	29.4 ± 3.3	5.1 ± 0.8‡
P-selectin deficient	129 ± 9*	3.3 ± 0.4‡
P-selectin/ICAM-1 deficient	No rolling	6.7 ± 0.8

Data are expressed as mean ± SEM.
*Significantly faster than trauma-induced rolling in wild-type and ICAM-1–deficient mice ($P < 0.001$).
‡Significantly slower than trauma-induced rolling in same genotype ($P < 0.001$). Average rolling velocities after TNF-α treatment are not significantly different between mouse strains.

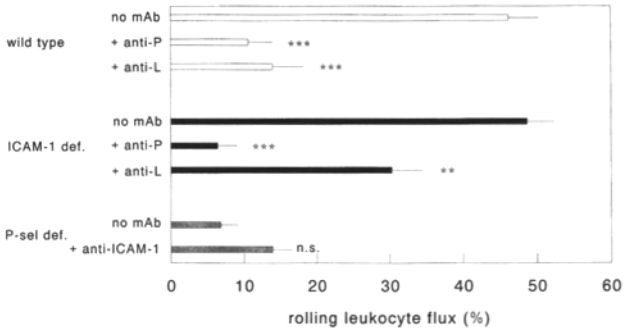

Figure 2. Effect of function-blocking mAb on rolling in wild type, ICAM-1–deficient, and P-selectin–deficient mice at times >60 min after surgical trauma. Rolling in wild-type mice (55 venules) was reduced significantly (12 venules; *** $P < 0.001$) by a P-selectin mAb (RB40.34) and significantly (25 venules; $P < 0.001$) by an L-selectin mAb (MEL-14). In ICAM-1–deficient mice (61 venules), L-selectin mAb (MEL-14) had a partial effect (19 venules; ** $P < 0.01$), whereas P-selectin mAb (RB40.34) blocked rolling by ~80% (10 venules; $P < 0.001$). Addition of ICAM-1 mAb (KAT-1) to P-selectin deficient mice (8 venules) caused a nonsignificant (*n.s.*) increase in rolling, presumably due to the release of previously firmly adherent cells. Data are shown as mean ± SEM.

flux fraction significantly, from 46 ± 4% to 11 ± 3% ($P < 0.001$), but did not completely abolish rolling. The addition of an L-selectin antibody (MEL-14) decreased rolling leukocyte flux fraction to a similar extent (to 14 ± 4%; $P < 0.01$) but did not block rolling completely either. These blocking data, taken together with previous data (24), suggest that P-selectin does mediate rolling at times <1 h after tissue exteriorization with a possible smaller contribution of L-selectin, whereas rolling at late time points (60–120 min) requires both P-selectin and L-selectin function.

In ICAM-1–deficient mice (Fig. 2), blocking L-selectin had a much smaller effect, lowering the rolling flux fraction to 30 ± 4% from 49 ± 4%, whereas blocking P-selectin reduced the rolling leukocyte flux fraction to a much greater extent (to 6 ± 3%; $P < 0.001$). The reduction in the rolling flux fraction in ICAM-1–deficient mice after the addition of an L-selectin mAb was significantly smaller ($P < 0.01$) than the reduction seen in wild-type mice. These data show that rolling in ICAM-1–deficient mice is more sensitive to a P-selectin mAb and less dependent on L-selectin function than rolling in wild-type mice.

To investigate whether ICAM-1 binding to LFA-1 was involved in mediating leukocyte rolling, we administered an anti-ICAM-1 antibody (KAT-1) in P-selectin–deficient mice 90–120 min after surgical trauma, when rolling is clearly present (a rolling leukocyte flux of ~5–10%) and is mostly L-selectin dependent (24). Although ICAM-1 is clearly expressed on the microvessel endothelium of the cremaster under these conditions (Fig. 3), KAT-1 had no effect on the rolling flux fraction in these mice (Fig. 2) except for a possible slight increase due to the release of previously firmly adherent or adhering leukocytes. Hence, addition of an antibody blocking LFA-1 binding to ICAM-1 in P-selectin–deficient mice did not reproduce the phenotype of P-selectin/ICAM-1–deficient mice, suggesting that

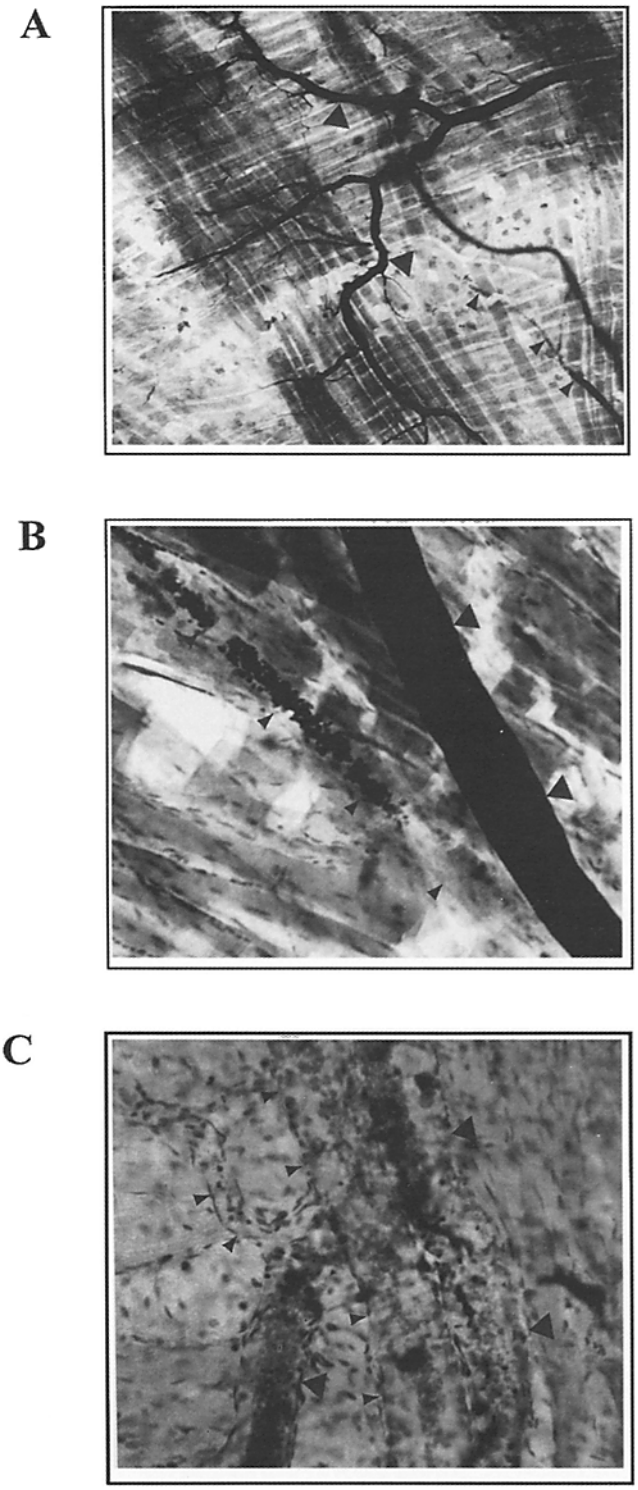

Figure 3. Expression of ICAM-1 in exteriorized mouse cremaster muscle microvessels. P-selectin–deficient mice were injected with an ICAM-1 mAb KAT-1 (*A* and *B*) or an irrelevant IgG2a (*C*). After intravital microscopic investigation, cremaster muscles were removed, mounted on slides, and fixed in cold acetone. After incubation with secondary antibody, 3,3′-diaminobenzidine, and OsO_4, strong expression of ICAM-1 (*black stain*) was detected in all venules (*A, large arrowheads*), but only partially in arterioles (*A, small arrowheads*). Most arterioles were negative for ICAM-1 (*B, small arrowheads*), although adjacent venules were strongly stained (*B, large arrowheads*). Labeling was specific because irrelevant IgG stained neither arterioles (*C, small arrowheads*) nor venules (*C, large arrowheads*).

the role of ICAM-1 in these mice involves some function of the molecule other than the ability to bind LFA-1.

Leukocyte Rolling in TNF-α–Treated Venules. The proinflammatory cytokine TNF-α causes a marked up-regulation of adhesion molecules on vascular endothelium, including E-selectin, P-selectin, and ICAM-1 (3). We have examined rolling in wild-type, ICAM-1–deficient, and P-selectin/ICAM-1–deficient mice after treatment with TNF-α for 2–2.5 h (Fig. 4). TNF-α treatment did not significantly affect the systemic leukocyte counts in any of the mouse lines (data not shown). The rolling flux fraction in TNF-α–treated wild-type mice was 32 ± 5%; which is in agreement with previous data (24). In treated ICAM-1–deficient mice, the rolling flux fraction of 36 ± 7% was not significantly different from wild-type mice. In all mice investigated, TNF-α treatment significantly reduced leukocyte rolling velocity to ~5 μm/s (Table 1).

Interestingly, TNF-α treatment induced leukocyte rolling in P-selectin/ICAM-1–deficient mice even though spontaneous rolling in these mice was completely absent. The rolling flux fraction was only 22 ± 4% in treated P-selectin/ICAM-1–deficient mice, which differs significantly from the value found in ICAM-1–deficient mice ($P < 0.01$). Again, leukocyte rolling was relatively slow, averaging ~7 μm/s (Table 1). Wild-type and P-selectin–deficient mice had at least threefold higher numbers of stationary, adherent leukocytes after TNF-α treatment than ICAM-1–deficient and P-selectin/ICAM-1 double mutant mice, which had little firm adhesion. An intrascrotal injection of vehicle (isotonic saline) 2–2.5 h before surgery had no effect on leukocyte rolling in wild-type or double mutant mice (data not shown).

Effect of mAbs on TNF-α–induced Leukocyte Rolling. In P-selectin/ICAM-1–deficient mice (Fig. 5), the addition of an L-selectin antibody (MEL-14) blocked a significant fraction of TNF-α–induced rolling, reducing the flux fraction to 5 ± 3% ($P < 0.01$). The addition of 9A9E3, an E-selectin antibody (26), totally ablated rolling ($P < 0.001$). The addition of 9A9E3 to TNF-α–treated wild-type mice yielded

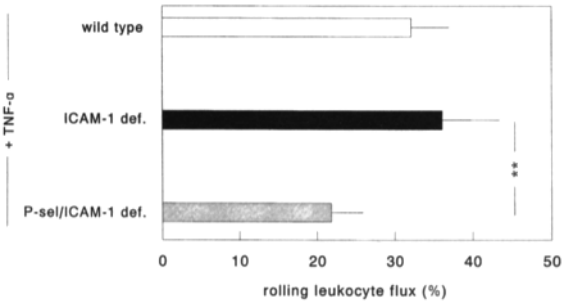

Figure 4. Leukocyte rolling flux fraction in TNF-α–treated venules in wild-type, ICAM-1–deficient, and P-selectin/ICAM-1–deficient mice. Mice were pretreated with an intrascrotal injection of TNF-α 2–2.5 h before the initiation of surgery. Rolling in ICAM-1–deficient mice (29 venules) was not significantly different from that in wild type mice (17 venules). Rolling in P-selectin/ICAM-1–deficient mice (49 venules) was significantly lower (**$P < 0.01$) than rolling in ICAM-1–deficient mice. Data are shown as mean ± SEM.

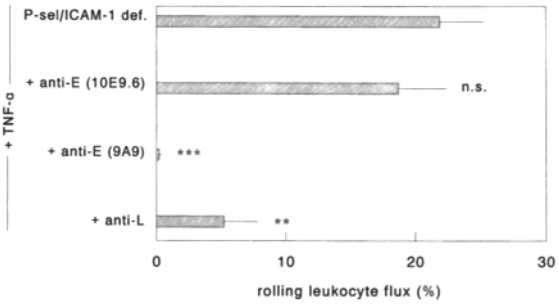

Figure 5. Effect of function-blocking mAbs on rolling in TNF-α–treated P-selectin/ICAM-1–deficient mice. Mice were pretreated with an intrascrotal injection of TNF-α 2–2.5 h before the initiation of surgery. 9A9E3 (blocking E-selectin mAb) totally ablated rolling (9 venules; ***$P < 0.001$) compared with no mAb (49 venules). The addition of 10E9.6 had no effect on leukocyte rolling (13 venules), although this antibody blocks adhesion of myeloid cells to TNF-α–activated endothelial cells in vitro. Adding an L-selectin mAb (MEL-14) significantly reduced rolling (6 venules; **$P < 0.01$). Data are shown as mean ± SEM. *n.s.*, not significant.

no significant difference in the rolling flux fraction (data not shown), which is consistent with previous findings indicating redundancy between E- and P-selectin function (5). Another E-selectin mAb, 10E9.6, known to block E-selectin–dependent murine myeloid cell adhesion to activated endothelial cells (25), had no effect on leukocyte rolling in this assay. This is in agreement with previous findings in wild-type and P-selectin–deficient mice (24). The mAb 9A9E3 binds to the lectin domain of E-selectin, whereas 10E9.6 binds to a different epitope (unpublished data).

Discussion

In this study we have examined the role of ICAM-1 alone and in conjunction with P-selectin in trauma- and TNF-α–induced leukocyte rolling in the cremaster muscle of mice. We observed that the rolling flux and average leukocyte rolling velocity in ICAM-1–deficient mice is not different from that in wild-type mice. In contrast, rolling is completely absent in P-selectin/ICAM-1–deficient mice for at least 2 h. Rolling in ICAM-1–deficient mice also appears to be less L-selectin dependent than in wild type mice. TNF-α treatment induced leukocyte rolling in P-selectin/ICAM-1–deficient mice to a lesser degree than in ICAM-1–deficient or wild-type mice, and TNF-α–induced rolling in P-selectin/ICAM-1–deficient mice is completely E-selectin dependent and partially L-selectin dependent.

Although ICAM-1 has been shown to be inadequate to support rolling in vitro (34), its in vivo role in leukocyte rolling has recently been addressed in an intravital microscopic study of the microcirculation in the rat liver. In this study, an mAb against ICAM-1 was found to reduce leukocyte rolling in the liver sinusoids after ischemia/reperfusion by ~50% (35). We find that mice deficient in only ICAM-1, with and without the addition of TNF-α, show a rolling leukocyte flux time course that is not different

from wild-type mice. Recently, alternatively spliced forms of ICAM-1 have been detected that are expressed in ICAM-1–deficient mice after cytokine treatment (36). We cannot exclude that these forms of ICAM-1 may contribute to normal rolling. However, the complete absence of rolling in P-selectin/ICAM-1 mutant mice shows that the loss of ICAM-1 in addition to P-selectin effectively alters leukocyte recruitment. This finding is in agreement with recent findings regarding the inflammatory defect seen in a peritonitis model in P-selectin/ICAM-1–deficient mice (23).

Although in vitro reconstitution assays (34) have directly implicated P-selectin in leukocyte rolling, the role of ICAM-1 is less clear. Since residual rolling in P-selectin–deficient mice cannot be blocked by a function-blocking antibody against ICAM-1, and since rolling under baseline and TNF-α–activated conditions is normal in ICAM-1–deficient mice, the LFA-1–binding function of ICAM-1 is apparently not directly involved in mediating rolling. However, mice deficient only in P-selectin regain a significant amount of rolling after ~1 h after surgical trauma, whereas P-selectin/ICAM-1–deficient mice do not. Therefore, in the absence of P-selectin, ICAM-1 appears to contribute to rolling in ways that cannot be carried out by other molecules and that are related to L-selectin function. The absence of rolling in P-selectin/ICAM-1–deficient mice, along with the reduced L-selectin–dependent rolling in ICAM-1–deficient mice, suggests that the absence of ICAM-1 appears to incapacitate L-selectin–dependent rolling under baseline conditions, but an L-selectin–dependent rolling component reappears after TNF-α treatment.

The differential L-selectin dependence of leukocyte rolling in ICAM-1–deficient mice could be caused by the shift of leukocyte populations seen in these mice (23). Although the majority of rolling leukocytes are known to be granulocytes (37), mononuclear cells clearly can roll in vivo (32). The higher percentage of neutrophils found in ICAM-1–deficient mice may explain the lesser L-selectin dependence of leukocyte rolling in these mice, if indeed a larger fraction of rolling leukocytes are neutrophils. This question cannot be resolved by intravital microscopy and requires further study.

After TNF-α treatment, rolling in the P-selectin/ICAM-1–deficient mice is significantly lower than that seen in TNF-α–treated ICAM-1–deficient mice. In the absence of P-selectin, which is known to be up-regulated after cytokine treatment (19, 38), rolling seen in these mice is only mediated by E-selectin, L-selectin, and possible selectin-independent mechanisms. Antibody-blocking experiments done in P-selectin/ICAM-1–deficient mice after TNF-α treatment show a complete cessation of rolling after the addition of an antibody against E-selectin. This indicates an absolute requirement for E-selectin function in mediating rolling in P-selectin/ICAM-1–deficient mice. Interestingly, leukoctyes rolled at significantly reduced velocities in mice treated with TNF-α, irrespective of their genotype. Taken together with the sensitivity of TNF-α–induced rolling to E-selectin antibody treatment, these findings suggest that E-selectin may support rolling at lower velocities than L- or P-selectin. This is consistent with previous in vitro findings (39) showing that neutrophils roll on recombinant E-selectin at lower velocities than on P-selectin at comparable site densities.

In addition, leukocyte rolling in the P-selectin/ICAM-1–deficient mice is partially blocked by an L-selectin mAb, which is consistent with TNF-α–induced up-regulation of putative L-selectin ligand(s). In vitro studies (39) have shown that E-selectin can be a very good substrate for mediating leukocyte rolling, but the role of E-selectin in vivo has been less clear. An ~40% decrease in rolling leukocytes has been observed when an E-selectin mAb was introduced into IL-1–treated rabbit mesenteric venules (10), suggesting that other adhesion molecules also contribute to leukocyte rolling on cytokine-stimulated endothelium. In the current study, the E-selectin component was clearly uncovered in mice lacking both P-selectin and ICAM-1. This is consistent with earlier findings, which show a defect in inflammatory cell recruitment to a site of peritonitis in E-selectin–deficient mice only when P-selectin function is also blocked (5). Hence, P-selectin and E-selectin function are at least partially redundant in TNF-α–treated mice.

This work suggests that a complete absence of spontaneous and trauma-induced leukocyte rolling underlies the inflammatory defect seen in P-selectin/ICAM-1–deficient mice. This study also provides the first clear in vivo evidence of purely E-selectin–dependent rolling, seen in TNF-α–treated mice lacking both P-selectin and ICAM-1.

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