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Selectin-independent leukocyte rolling and adhesion in mice deficient in E-, P-, and L-selectin and ICAM-1

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Forlow, S. Bradley, and Klaus Ley. Selectin-independent leukocyte rolling and adhesion in mice deficient in E-, P-, and L-selectin and ICAM-1. *Am J Physiol Heart Circ Physiol* 280: H634–H641, 2001.—To study selectin-independent leukocyte recruitment and the role of intercellular adhesion molecule-1 (ICAM-1), we generated mice lacking all three selectins and ICAM-1 (E/P/L/I−/−) by bone marrow transplantation. These mice were viable and appeared healthy under vivarium conditions, although they showed a 97% reduction in leukocyte rolling, a 63% reduction in leukocyte firm adhesion, and a 99% reduction of neutrophil recruitment in a thioglycollate-induced model of peritonitis at 4 and 24 h. Mononuclear cell recruitment was almost unaffected. All residual leukocyte rolling and most leukocyte adhesion in these mice depended on α_4-integrins, but a small number of leukocytes (6% of wild-type control) still became adherent in the absence of all known rolling mechanisms (E-, P-, L-selectin and α_4-integrins). A striking similarity of leukocyte adhesion efficiency in E/P/L−/− and E/P/I−/− mice suggests a pathway in which leukocyte rolling through L-selectin requires ICAM-1 for adhesion and recruitment. Comparison of our data with mice lacking individual or other combinations of adhesion molecules reveal that elimination of more adhesion molecules further reduces leukocyte recruitment but the effect is less than additive.

neutrophil adhesion; thioglycollate-induced peritonitis; intravital microscopy; knockout mice; α_4-integrins

EFFICIENT LEUKOCYTE RECRUITMENT to sites of inflammation requires the selectin family of adhesion molecules. E-, P-, and L-selectin mediate leukocyte capture and rolling on the inflamed vessel wall (4, 12). The generation of mice lacking one, two, or three selectins has provided valuable information in the overlapping and unique functions of each selectin in efficient leukocyte recruitment in inflammation (1, 2, 7, 9, 18, 22, 27). E- and P-selectin double knockout mice (E/P−/−) treated with an L-selectin-blocking antibody or mice made deficient in E-, P-, and L-selectin by transplanting L−/− bone marrow into E/P−/− mice showed drastically reduced leukocyte rolling (9, 11). Residual rolling in selectin-deficient mice was blocked with an α_4-integrin monoclonal antibody (MAb) (9). A similar role of α_4-integrins in leukocyte recruitment was also shown in a model of allergic inflammation (13). Therefore, selectins mediate the majority of leukocyte capture and rolling, and α_4-integrins mediate the capture and rolling of a small fraction of circulating leukocytes.

Selectin-mediated leukocyte capture and rolling has been a generally accepted prerequisite for firm leukocyte adhesion and subsequent transmigration (4, 12). However, despite very few rolling cells in selectin-deficient mice (9, 11), a significant amount of leukocyte adhesion remained. Kubes et al. (15) estimated that rolling must be reduced by >90% to significantly affect firm adhesion.

Firm leukocyte adhesion is mediated primarily through β_2 (CD18)-integrins. β_2-Integrins are a family of four heterodimers, CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), CD11c/CD18 (p150,95), and CD11d/CD18. LFA-1 is the predominant β_2-integrin on lymphocytes and neutrophils (14). Neutrophils also express Mac-1 (30). Both LFA-1 and Mac-1 can interact with intercelular cell adhesion-1 (ICAM-1) expressed on resting (8) and inflamed (5, 6, 8, 21, 29) endothelial cells.

ICAM-1 was recently shown to be important in stabilizing P- and L-selectin-mediated leukocyte rolling. Mice deficient in P-selectin and ICAM-1 did not display leukocyte rolling after trauma-induced inflammation (16), had significantly reduced leukocyte rolling and increased rolling velocities after tumor necrosis factor-α (TNF-α) treatment compared with ICAM-1−/− and wild-type mice (16), and showed almost a complete absence of neutrophil recruitment in a peritonitis model (3). L-selectin/ICAM-1 double-mutant mice also showed increased rolling velocities and decreased neutrophil recruitment in various experimental models of inflammation (31, 32). These data suggest ICAM-1 is synergistic with P- and L-selectin in mediating efficient leukocyte recruitment.

To address the possible role of ICAM-1 in firm leukocyte adhesion and recruitment in selectin-deficient mice and to further investigate selectin-independent mechanisms of leukocyte rolling, firm adhesion, and recruitment, we generated novel mice deficient in four adhesion molecules (E-, P-, and L-selectin and ICAM-

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1). We tested the impact of the absence of these adhesion molecules in two models of inflammation, a TNF-α-induced model of inflammation of the mouse cremaster muscle and thioglycollate-induced peritonitis.

MATERIALS AND METHODS

Animals. L-selectin null (L−/−) (1) and E- and P-selectin and ICAM-1 triple null (E/P/I−/−) (24) mice were obtained from established colonies maintained at the University of Virginia Health Sciences Center vivarium under specific pathogen-free conditions in a barrier facility. All mice used in these studies were back-crossed into the C57BL/6 background for at least six generations. C57BL/6 wild-type mice (Hilltop Lab Animals, Scottdale, PA) were maintained at the University of Virginia Health Sciences Center vivarium until needed for experiments.

Models of inflammation. Leukocyte recruitment was studied in a TNF-α-induced model of inflammation of the mouse cremaster muscle and in a thioglycollate-induced peritonitis. The long-term (6 h) TNF-α model of inflammation of the mouse cremaster muscle has been described previously (9, 11). TNF-α enhances the expression of P-selectin and ICAM-1 on the endothelial surface. The expression of E-selectin and ligands for L-selectin and α_4-integrins are also induced by treatment with TNF-α (11). This model is accessible to intravital microscopy and allows direct observation of the mechanisms underlying leukocyte recruitment.

An intraperitoneal injection of thioglycollate effectively induces the recruitment of leukocytes into the peritoneal cavity. This assay provides an inflammation endpoint model to investigate the ability of circulating leukocytes to be recruited to a site of inflammation [overall leukocyte recruitment efficiency, (10)] in mice lacking various adhesion molecules.

Antibodies and cytokines. The rat anti-mouse MAb to α_4-integrin PS/2 (rat IgG2b) (23), purified from hybridoma culture supernatant, has been previously shown to specifically block α_4-integrin function in vivo (9). In some experiments, PS/2 (100 μg/mouse) was administered intraperitoneally at the time of TNF-α injection. In the 6-h TNF-α-induced model of inflammation, intravascular coagulation has been described as a consequence of the severe inflammation (9). Therefore, E/P/I−/− and E/P/L/I−/− mice were injected with 10 units of hirudin (Sigma Chemical; St. Louis, MO) at the time of TNF-α administration and 10 units of hirudin 2.5 h before cremaster exteriorization to prevent intravascular coagulation.

Bone marrow transplantation. Mice deficient in E-, P-, and L-selectin and ICAM-1 (E/P/L/I−/−) were generated by transplanting L-selectin-deficient (L−/−) bone marrow into E/P/I−/− mice. Bone marrow transplant recipient mice were 2–3 mo of age.

Bone marrow was harvested from donor mice and transplanted into recipient mice as previously described (9). Briefly, recipient mice were lethally irradiated in two doses of 600 rads each ~4 h apart. Under these conditions, all non-transplanted mice died within 2 wk of irradiation. Donor mice were killed by lethal injection of pentobarbital sodium (Nembutal, Abbott Laboratories; North Chicago, IL), and bone marrow cells from both femurs and tibias were harvested under sterile conditions. Approximately 50 million nucleated cells were obtained from each donor mouse. Bones were flushed with RPMI (GIBCO; Grand Island, NY) (without phenol red) containing 10% fetal calf serum (Atlanta Biologicals; Norcross, GA). Suspended bone marrow cells were washed and erythrocytes were lysed in 0.15 M NH_4Cl lysing solution. Approximately 5 million unfractionated nucleated bone marrow cells in 200 μl of media were delivered intravenously through the tail vein of each recipient mouse. After bone marrow transplantation, mice were maintained on autoclaved water with antibiotics (5 mM sulfamethoxazole, 0.86 mM trimethoprim) (Sigma) and fed autoclaved food. These conditions were maintained for 4–5 wk until intravital microscopy or thioglycollate-induced peritonitis.

Control groups for intravital microscopy were also generated by bone marrow transplantion. E/P−/− mice were transplanted with wild-type bone marrow or L−/− bone marrow to generate the E/P−/− and E/P/L−/− groups (9). E/P/I−/− mice were transplanted with wild-type bone marrow to generate E/P/I−/− control mice.

Intravital microscopy. For the model of TNF-α-induced acute inflammation, mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride (Ketalar, 125 mg/kg, Parke-Davis; Morris Plains, NJ), xylazine (12.5 mg/kg, Vedco; St. Joseph, MO), and atropine (0.25 mg/kg, Elkins-Sinn; Cherry Hill, NJ). The trachea was intubated and anesthetic (diluted pentobarbital sodium in saline) was administered throughout the intravital experiment through one cannulated jugular vein. Blood pressure was monitored, and blood samples were obtained through a cannulated carotid artery. Mice were kept at a constant temperature of 37°C with a thermo-controlled heating pad (Physitemp Instruments; Clifton, NJ) during the intravital microscopic experiment. The cremaster muscle was prepared for intravital microscopy as described (17). Recombinant murine TNF-α (Genzyme; Cambridge, MA) was injected intrascrotally at a dose of 500 ng per mouse in a volume of 0.3 ml of sterile saline 6 h before exteriorization of the cremaster muscle. The cremaster muscle was superfused with thermo-controlled (35°C) bicarbonate-buffered saline. Throughout the experiment, blood samples were taken from the carotid catheter to analyze systemic leukocyte concentrations. Kimura-stained blood samples were analyzed by using a hemocytometer to obtain leukocyte counts. Microscope observations were made with a Zeiss intravital microscope (Axioskop; Thornwood, NY) by using a saline immersion objective (SW 40/0.75 numerical aperture). Venules with diameters between 20 and 80 μm were observed and recorded via a CCD camera system (model VE-1000CD, Dage-MTI; Michigan City, IN) on a Panasonic S-VHS recorder. Centerline red blood cell velocity was measured by using a dual photodiode and a digital on-line cross-correlation program (CircuSoft Instrumentation; Hockessin, DE). Mean blood flow velocities were obtained by multiplying the centerline velocity by an empirical factor of 0.625 (20). Wall shear rates (γ_w) were estimated as 2.12 ($8V_b/d$), where V_b is the mean blood flow velocity, d is the diameter of the vessel, and 2.12 is a median empirical correction factor obtained from velocity profiles measured in microvessels in vivo (26).

Rolling and adhesion parameters. A digital image processing system was used to measure microvessel diameters, lengths, and leukocyte rolling velocities (25). Leukocyte rolling flux, expressed as leukocytes per minute, was calculated by counting leukocytes rolling past a line perpendicular to the vessel axis (19). Rolling flux fraction was calculated as described (19) by dividing leukocyte rolling flux by total leukocyte flux estimated as $[WBC]V_b\pi(d/2)^2$, where [WBC] is the systemic leukocyte count. Adherent leukocytes were defined as leukocytes that did not move for at least 30 s. The total number of adherent leukocytes was determined for each venule segment (~200 μm) and expressed per unit area of inside surface area of the venule.

Flow cytometry. Expression of L-selectin on mouse neutrophils obtained from both peripheral blood and bone marrow was determined by direct immunofluorescence. Bone marrow cells were harvested as described above using PBS (GIBCO) with 0.01% azide. Whole blood or bone marrow was incubated with fluorescein isothiocyanate (FITC)-labeled MAb Gr-1 (0.5 $\mu g/10^6$ cells, Pharmingen; San Diego, CA) to identify granulocytes, and phycoerythrin-labeled MAb MEL-14 to label L-selectin (0.5 $\mu g/10^6$ cells, Pharmingen) or isotype control (rat IgG2a, 0.5 $\mu g/10^6$ cells, Pharmingen). Samples were incubated for 30 min on ice. Unlabeled antibody was removed by aspiration after centrifugation. Bone marrow cells were resuspended in PBS with 0.01% azide. Peripheral blood was resuspended in 150 mM NH_4Cl, 10 mM $NaHCO_3$, and 1 mM Na_2EDTA in deionized, distilled water to lyse red blood cells. Cells were analyzed by forward scatter, side scatter, FITC fluorescence, and phycoerythrin fluorescence using a laser flow cytometer (FACScan, Becton-Dickinson; San Jose, CA). Neutrophils were identified and gated by expression of Gr-1 antigen measured by incubation with Gr-1-FITC (0.5 $\mu g/10^6$ cells, Pharmingen). Data are presented as fluorescence histograms of MEL-14 expression of Gr-1-positive cells on a four-decade log scale.

Histology. To differentiate intravascular and interstitial leukocytes, cremaster muscle whole mounts were prepared. While the cremaster muscle was still mounted on the stage for intravital microscopy, the tissue was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The cremaster muscle was removed and mounted flat on a gelatanized-treated glass slide, air dried for 5–10 min, and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C. After fixation, the tissue was washed three times in 0.1 M phosphate buffer with 5% ethanol, stained with Giemsa (Sigma) at room temperature for 5 min, and differentiated in 0.01% acetic acid for contrast. The differentiated slides were washed in water, 75% ethanol, 95% ethanol, 100% ethanol, and fresh xylene, followed by mounting in mounting media (Sigma). The Giemsa-stained cremaster muscles were observed using a Zeiss microscope with a ×100, 1.4 numerical aperture oil immersion objective (Zeiss, Germany). Intravascular and interstitial leukocytes were counted and differentiated into neutrophils, eosinophils, and mononuclear cells. The interstitial tissue observed was a circular area (diameter of 183 μm) bisected by each venule.

Thioglycollate-induced peritonitis. Wild-type, E/P/I−/−, and E/P/L/I−/− mice were injected intraperitoneally with 1 ml of 4% thioglycollate (Sigma) in deionized water at *time 0*. At 4 or 24 h mice were killed with a lethal injection of pentobarbital sodium and injected intraperitoneally with 5 ml ice-cold PBS (without Ca^{2+}, Mg^{2+}) containing 2 mM EDTA, their abdomens were massaged, and total lavage fluid was collected through a small slit cut into the peritoneal cavity. Collected cells were pelleted by centrifugation and resuspended in 5 ml of 150 mM NH_4Cl, 10 mM $NaHCO_3$, and 1 mM Na_2EDTA in deionized, distilled water to lyse red blood cells. The number of recruited leukocytes was counted, and leukocyte differentials were obtained from Kimura-stained samples and verified using stained cytospins. Systemic leukocyte concentrations were determined at 0 and 4 or 24 h from Kimura-stained blood samples collected from the tail vein. To obtain a leukocyte recruitment efficiency (10), the leukocyte concentration in the peritoneal lavage was divided by the leukocyte concentration in the circulation. Data obtained from E/P/I−/− mice transplanted with wild-type bone marrow were not significantly different from data obtained in E/P/I−/− mice; therefore, nontransplanted wild-type and E/P/I−/− mice were also used in the thioglycollate-induced peritonitis studies.

Statistics. Average leukocyte rolling velocities, leukocyte adhesion, systemic leukocyte counts, and differentials between groups were compared using one-way ANOVA and Kruskal-Wallis multiple comparison test. Statistical significance was set at $P < 0.05$.

RESULTS

E/P/L/I−/− chimeric mice. E/P/L/I−/− mice were generated by transplanting lethally irradiated E/P/I−/− mice with L−/− bone marrow. E/P/I−/− control mice were also generated through bone marrow transplantation (reconstituted with wild-type bone marrow). At 4 wk after reconstitution, peripheral blood (data not shown) and bone marrow of chimeric mice were analyzed for L-selectin expression (Fig. 1). L-selectin expression was undetectable on bone marrow and blood cells in mice transplanted with L−/− bone marrow, confirming that reconstitution was efficient and complete. E/P/I−/− and E/P/L/I−/− mice appeared healthy and did not show ulcerative dermatitis as seen in E/P−/− mice (2).

TNF-α-induced inflammation of the cremaster muscle: systemic leukocyte counts and hemodynamics in mouse cremaster venules. The systemic leukocyte counts in E/P/I−/− and E/P/L/I−/− mice were significantly elevated (approximately a 4-fold increase; Table 1) after TNF-α treatment compared with wild-type mice (4,390 ± 750/μl; see Ref. 11). We investigated leukocyte rolling and adhesion in 81 venules in six E/P/I−/− and seven E/P/L/I−/− mice. The average vessel diameter was 51.8 ± 2.8 μm. The severe inflammatory model using long-term TNF-α treatment (6 h) resulted in reduced blood flow velocities (Table 1) and, therefore, lower wall shear rates than in untreated or short-term TNF-α-treated mice. The average center-

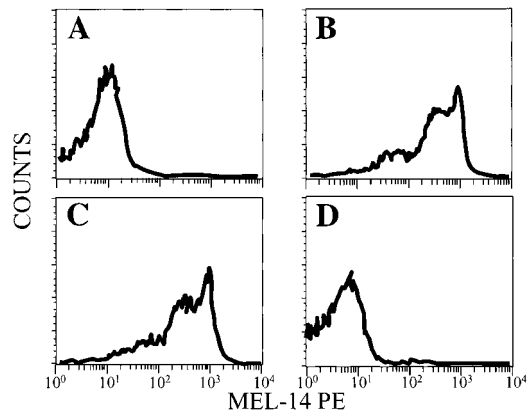

Fig. 1. Expression of L-selectin on Gr-1-positive bone marrow cells (neutrophils and precursors). *A*: isotype control; *B*: monoclonal antibody (MAb) MEL-14 staining in wild-type mice. Lethally irradiated E- and P-selectin and intercellular adhesion molecule-1 (ICAM-1)-deficient (E/P/I−/−) mice transplanted with wild-type bone marrow resulted in L-selectin expression similar to nonirradiated wild-type mice (*C*). Transplantation of L−/− bone marrow into lethally irradiated E/P/I−/− mice resulted in a complete absence of L-selectin expression (*D*).

Table 1. *Hemodynamics and microvascular parameters*

Mouse Type	Systemic Leukocyte Counts, cells/μl	*N*	*n*	Average Diameter, μm	Centerline Blood Velocity, μm/s
E/P/I−/−	18,750 ± 3,290	6	35	55.1 ± 3.2	980 ± 140
E/P/L/I−/−	17,310 ± 4,620	7	46	48.5 ± 2.4	1,240 ± 80

Data includes mice pretreated with an α_4-integrin monoclonal antibody and are expressed as means ± SE; *N*, number of mice; *n*, number of vessels. E/P/I−/−, mice lacking E-, P-, and intercellular adhesion molecule-1 (ICAM-1); E/P/L/I−/−, mice lacking E-, P-, L-selectins and ICAM-1.

line velocity was 1.1 ± 0.1 mm/s, and the calculated wall shear rate was 240 ±20 s^{-1}.

Leukocyte rolling. TNF-α (6 h) induces the expression of E-selectin, enhances the expression of P-selectin, and induces L-selectin- and α_4-integrin-dependent rolling (8, 11). We have previously shown that leukocyte rolling in E/P−/− (2) and E/P/L−/− mice is drastically reduced (9) compared with wild-type mice. Residual rolling in mice lacking all three selectins was shown to be α_4-integrin dependent (9).

The leukocyte rolling flux in E/P/I−/− mice (2.7 ± 0.5 cells/min; Fig. 2*A*) was similar to E/P/L−/− mice (2.0 ± 0.4 cells/min) and lower than in E/P−/− mice (5.2 ± 0.8 cells/min). E/P/L/I−/− mice yielded a leukocyte rolling flux (2.1 ± 0.3 cells/min; Fig. 2*A*) similar to E/P/I−/− and E/P/L−/− mice, which was approximately a 60% reduction from the level of leukocyte rolling in E/P−/− mice and a more than 97% reduction compared with wild-type mice (11).

To determine how efficiently circulating leukocytes were captured and became rolling leukocytes, the rolling flux fraction was calculated to normalize for the differences in systemic leukocyte concentrations between mouse groups. Approximately 10% of the circulating leukocytes in wild-type mice were rolling (Fig. 2*B*). The rolling flux fraction was reduced to 0.9 ± 0.2% in E/P−/− mice (Fig. 2*B*). Removing L-selectin and/or ICAM-1 resulted in a further reduction in the leukocyte rolling flux fraction in E/P/L−/− (0.4 ± 0.6%), E/P/I−/− (0.3 ± 0.9%), and E/P/L/I−/− (0.3 ± 0.6%) mice (Fig. 2*B*).

Leukocyte rolling in E/P/L−/−, E/P/I−/−, and E/P/L/I−/− mice was completely blocked by a MAb to α_4-integrin, showing that α_4-integrins are capable of capturing a very small fraction of circulating leukocytes (Fig. 2, *A* and *B*). These data also show that the L-selectin-mediated rolling present in E/P−/− mice (11) does not occur in the absence of ICAM-1 because the leukocyte rolling flux and flux fraction are the same for E/P/I−/− and E/P/L−/− mice.

Leukocyte rolling velocities after 6 h of TNF-α treatment in wild-type, E/P−/−, and E/P/L−/− mice were previously determined to be 13.3 ± 1.0, 15.7 ± 1.2, and 13.6 ± 1.2 μm/s, respectively (9). E/P/I−/− and E/P/L/I−/− mice showed average leukocyte rolling velocities of 19.9 ± 1.8 and 19.7 ± 2.5 μm/s, respectively (Fig. 3). All leukocyte rolling in E/P/L−/−, E/P/I−/−, and E/P/L/I−/− mice was α_4-integrin dependent (Fig. 2*B*). The marginally elevated leukocyte rolling velocities seen in E/P/I−/− mice and the significantly elevated leukocyte rolling velocities seen in E/P/L/I−/− mice, therefore, suggest a role of ICAM-1 in leukocyte rolling for the subset of leukocytes captured by α_4-integrins.

Leukocyte adhesion and transmigration. Although there were dramatic reductions of leukocyte rolling in E/P−/−, E/P/L−/−, E/P/I−/−, and E/P/L/I−/− mice (Fig. 2, *A* and *B*), TNF-α treatment for 6 h still resulted in a substantial amount of leukocyte adhesion in all the mutants (400–600 adherent leukocytes/mm^2) (Fig. 2*C*).

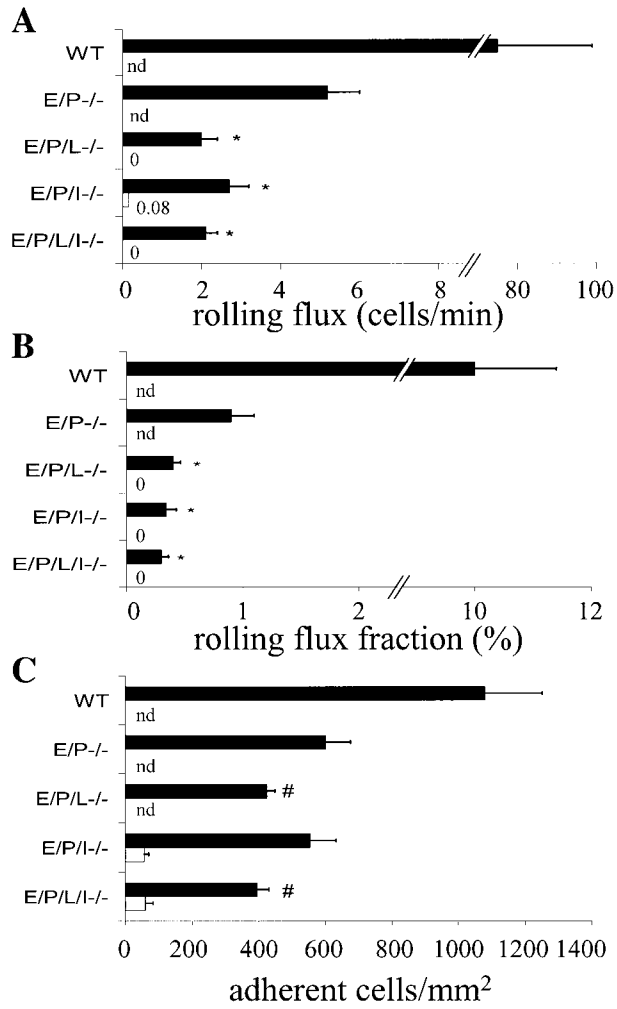

Fig. 2. Leukocye rolling and adhesion in venules of the cremaster muscle treated with tumor necrosis factor-α (TNF-α) for 6 h. Leukocyte rolling flux (cells/min) (*A*), leukocyte rolling flux fraction (rolling cells as percentage of all circulating cells in the same vessel) (*B*), and leukocyte adhesion (cells/mm^2) (*C*) were investigated in wild-type (WT), E- and P-selectin deficient (E/P−/−), E-, P-, and L-selectin deficient (E/P/L−/−), E- and P-selectin and ICAM-1 deficient (E/P/I−/−), and E-, P-, and L-selectin and ICAM-1 deficient (E/P/L/I−/−) mice (solid bars). Pretreatment with an α_4-integrin MAb (PS/2) at the time of TNF-α administration (open bars) completely blocked rolling and severely reduced adhesion. Data from wild-type, E/P−/−, and E/P/L−/− mice were reported previously (9, 11). *Significantly different from E/P−/− mice. #Significantly different from E/P−/− and E/P/I−/− mice. Wild-type mice were significantly different from all other groups ($P < 0.05$).

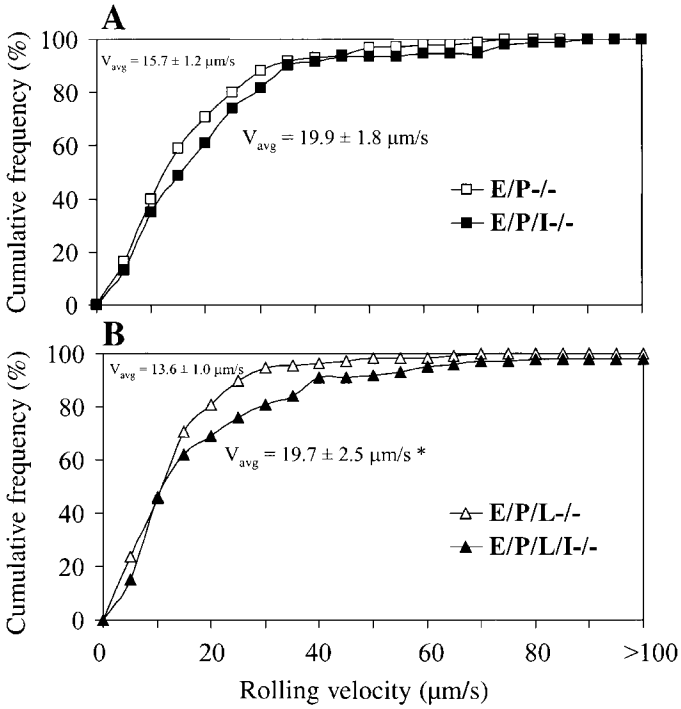

Fig. 3. Cumulative histograms of leukocyte rolling velocities in cremaster muscle venules treated with TNF-α for 6 h before exteriorization. Data for E/P/I−/− (n = 92 cells; *A*) and E/P/L/I−/− (n = 100 cells; *B*) mice are compared with E/P−/− and E/P/L−/− mice reported previously (9). Average leukocyte rolling velocities (V_{avg}) (means ± SE) are indicated next to curves. *Significantly different from E/P/L−/− mice ($P < 0.05$).

Leukocyte adhesion was reduced by ~40–50% in mutant mice compared with wild-type mice (1,080 ± 170 adherent leukocytes/mm^2) (Fig. 2*C*). Blocking all leukocyte rolling in E/P/I−/− and E/P/L/I−/− mice by pretreatment with a MAb to α_4-integrin at the time of induction of inflammation almost completely abolished leukocyte adhesion (Fig. 2*C*).

Giemsa-stained cremaster whole mounts were used to differentiate intravascular and transmigrated leukocytes. E/P/L−/− mice showed a significant reduction in neutrophil recruitment (63%) (9) compared with that observed in wild-type mice (81%) (9). Neutrophil recruitment was further reduced in the absence of ICAM-1 (43% in E/P/I−/− and 36% in E/P/L/I−/− mice; Table 2). To appreciate the impact of these adhesion molecules on neutrophil and mononuclear cell recruitment, the number of adherent neutrophils and mononuclear cells per squared millimeter of vessel surface area was estimated by multiplying the intravascular differential counts obtained by histology (Table 2) with the leukocyte adhesion density obtained by intravital microscopy (Fig. 2*C*). These data show that despite similar leukocyte adhesion levels (cells/mm^2) in E/P−/− compared with E/P/I−/− and in E/P/L−/− compared with E/P/L/I−/− mice, the elimination of ICAM-1 significantly impairs neutrophil recruitment (Fig. 4).

Thioglycollate-induced peritonitis. A thioglycollate-induced peritonitis model of inflammation was used to further investigate selectin-independent leukocyte recruitment. Peritoneal lavage fluid was collected at 4 or 24 h following thioglycollate injection, and the number of neutrophils and mononuclear cells recruited was counted (Fig. 5*A*). Neutrophil recruitment was severely impaired in E/P/I−/− and E/P/L/I−/− mice compared with that in wild-type mice at 4 and 24 h (Fig. 5*A*). Mononuclear cell recruitment in E/P/I−/− and E/P/L/I−/− mice was unaffected (Fig. 5*A*).

Mutant mice showed severe leukocytosis, primarily because of elevated neutrophil concentrations (Fig. 5*B*). In all mice, circulating mononuclear cell numbers were reduced during thioglycollate-induced peritonitis at 4 h, from 7,500 to 4,000 cells/μl in wild-type mice, from 10,200 to 7,600 cells/μl in E/P/I−/− mice, and from 17,200 to 8,600 cells/μl in E/P/L/I−/− mice. At 24 h, circulating mononuclear cells counts had largely recovered to baseline levels.

To obtain an overall leukocyte recruitment efficiency, the concentration of leukocytes recruited to the peritoneum was divided by the concentration of available circulating leukocytes and normalized to a wild-type efficiency of 1.0 (equalling 100%) (Fig. 5*C*). E/P/I−/− and E/P/L/I−/− mutants had drastically reduced neutrophil recruitment efficiency at 4 h (2.2% and 1.1%

Table 2. *Intravascular and transmigrated leukocytes*

	Wild-Type	E/P−/−	E/P/L−/−	E/P/I−/−	E/P/L/I−/−
Systemic counts					
% Neutrophils	61 ± 7	57 ± 4	69 ± 4	78 ± 2	73 ± 2
% Mononuclear	ND	43 ± 4	31 ± 4	21 ± 1	28 ± 2
Intravascular					
% Neutrophils	81 ± 2	88 ± 2*	63 ± 4*	43 ± 2†	36 ± 3†
% Mononuclear	15 ± 1	10 ± 2*	28 ± 4*	56 ± 2†	62 ± 3†
Transmigrated					
% Neutrophils	ND	94 ± 1‡	70 ± 3‡	27 ± 3†	30 ± 4†
% Mononuclear	ND	5 ± 1‡	19 ± 2‡	72 ± 3†	68 ± 4†

Data are expressed as means ± SE. Wild-type, E/P−/−, and E/P/L−/− data were previously reported (9, 11). ND, not determined. *Significantly different from all other knockout groups; †Significantly different from E/P−/− and E/P/L−/− groups; ‡Significantly different from E/P/I−/− and E/P/L/I−/− groups ($P < 0.05$).

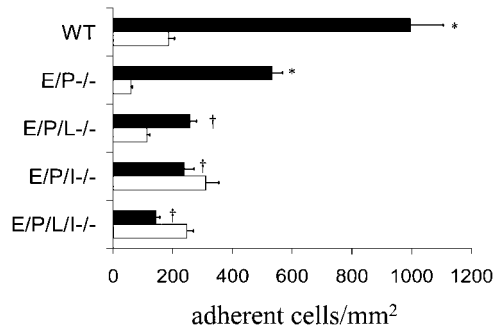

Fig. 4. Leukocyte differentials of adherent leukocytes in cremaster muscle venules treated with TNF-α for 6 h before exteriorization. Levels of neutrophil (solid bars) and mononuclear cell (open bars) recruitment were calculated by multiplying Giemsa-stained, whole-mount differentials (Table 2) by the leukocyte adhesion density (cells/mm^2 of vessel surface area) obtained by intravital microscopy (Fig. 2*C*). *Significantly different from all other groups. †Significantly different from wild-type and E/P−/− groups ($P < 0.05$).

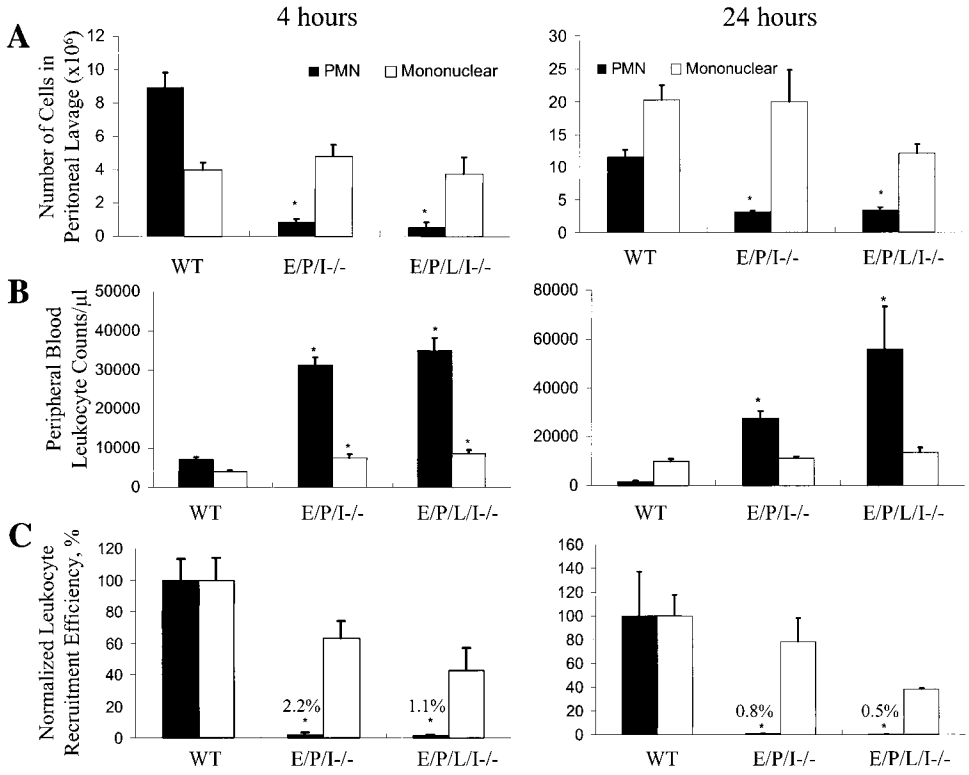

Fig. 5. Leukocyte accumulation following thioglycollate-induced peritonitis in wild-type, E/P/I−/−, and E/P/L/I−/− mice. *A*: number of neutrophil (PMN) and mononuclear cells was determined 4 (*left*) and 24 h (*right*) after thioglycollate injection by Kimura-stained lavage samples. *B*: systemic leukocyte counts were determined at 4 and 24 h from Kimura-stained blood samples obtained from the tail vein. To obtain an overall leukocyte recruitment efficiency, the concentration of cells in the lavage was divided by the concentration of cells in circulation and normalized to a wild-type value of 1.0 (equalling 100%) (*C*). The percentage of neutrophil accumulation in E/P/I−/− and E/P/L/I−/− mice relative to wild-type mice is shown. *Significantly different from wild-type mice ($P < 0.05$).

of wild-type, respectively). The reduction was even more severe at 24 h (0.8% and 0.5% of wild-type, respectively) (Fig. 5*C*). The progressive reduction in the percentage of neutrophil recruitment relative to the wild-type controls for E/P/I−/− and E/P/L/I−/− mice shows a cumulative effect of the absence of adhesion molecules on effective neutrophil recruitment.

DISCUSSION

Quadruple adhesion molecule-deficient E/P/L/I−/− mice are viable and, without challenge, apparently healthy. However, they display a remarkable impairment in leukocyte capture, rolling, adhesion, and recruitment. Mononuclear cell recruitment appeared largely unaffected in the absence of selectins and ICAM-1, indicating a greater role of selectin- and ICAM-1-mediated recruitment for neutrophils than for mononuclear cells.

Our previous work (9) and that of others (27) have shown the role of selectins in mediating efficient neutrophil recruitment to sites of inflammation. Leukocyte capture and rolling, largely mediated by selectins, is the generally accepted prerequisite for firm leukocyte adhesion. Surprisingly, a substantial amount of leukocyte adhesion occurred in E/P/L−/− or E/P/L/I−/− mice (~40% of wild-type) despite very few rolling leukocytes (~3% of wild-type). This is consistent with previous studies using antibody blockade of selectin function. Kubes et al. (15) showed that rolling must be blocked by at least 90% to significantly affect recruitment. Mice lacking all three selectins by repeated gene targeting (27) or by bone marrow chimerism (9) also show very low rolling but still significant recruitment. The E/P/I−/− and E/P/L/I−/− mice studied here, however, reveal much more severely compromised adhesion and recruitment of neutrophils in the cremaster muscle. This represents a very severe impairment of neutrophil recruitment.

Interestingly, some leukocyte adhesion remained in E/P/L/I−/− mice when leukocyte rolling was completely removed with a MAb to α_4-integrins. These data suggest the existence of both neutrophil and mononuclear cell recruitment mechanisms that completely bypass all known rolling mechanisms through selectins and α_4-integrins.

The number of adherent leukocytes found in cremaster venules reflects the steady-state balance between new leukocyte adhesion from the rolling pool and leukocyte transmigration. We have investigated the mechanisms leading to leukocyte adhesion and found a reduction of leukocyte rolling that was more severe than the reduction in the number of adherent leukocytes. The substantial amount of selectin- and ICAM-1-independent leukocyte adhesion could therefore result from a defect in cell transmigration in these mice. Such an additional effect on transmigration is suggested by the data obtained in the peritonitis model. Recruitment of neutrophils in E/P/L/I−/− mice in the peritonitis model was reduced by more than 95% compared with wild-type controls despite only a 83% reduction in the number of firmly adhered cells in the TNF-α model of inflammation. The mechanisms underlying these differential effects remain to be explored by detailed studies of the transmigration process.

Leukocyte adhesion in E/P/L/I−/− mice was similar to the level of leukocyte adhesion in selectin-deficient

mice (E/P/L−/−). This suggests that ICAM-1 is not important in mediating firm adhesion of leukocytes recruited through selectin-independent mechanisms. However, the leukocyte differentials reveal a preferential reduction in neutrophil recruitment in the absence of ICAM-1.

The leukocyte rolling data reveal a unique cooperative function between ICAM-1 and L-selectin in mediating efficient leukocyte recruitment. The majority of leukocyte rolling in E/P−/− mice was previously shown to be L-selectin dependent (11). Here, E/P/I−/− mice showed leukocyte rolling similar to E/P/L−/− mice, and this level was not further reduced in E/P/L/I−/− mice. These data suggest that the L-selectin-mediated rolling seen in E/P−/− mice requires the presence of ICAM-1. ICAM-1 appears necessary for stabilizing rolling of leukocytes captured through L-selectin. This is consistent with earlier findings in a different model. Mice lacking both P-selectin and ICAM-1 show almost no trauma-induced rolling (16) and severely reduced neutrophil recruitment in a peritonitis model (3). The disruption of leukocyte rolling in the absence of ICAM-1 is further substantiated in the present study by the elevated leukocyte rolling velocities in E/P/I−/− and E/P/L/I−/− mice compared with E/P−/− and E/P/L−/− mice. Taken together, these data suggest a model of neutrophil recruitment in which preferential pathways may exist. Leukocytes initially captured by and rolling on L-selectin appear to require ICAM-1 for adhesion and ultimately recruitment. This requirement for ICAM-1 is much less stringent when P- and/or E-selectin are also present, as can be seen for the mild inflammatory defect in ICAM-1−/− mice when all selectins are present (28, 33).

In conclusion, novel E/P/L/I−/− mice were used in two models of inflammation to investigate selectin- and rolling-independent mechanisms of leukocyte recruitment. The data show that selectins and ICAM-1 are all involved in efficient neutrophil recruitment. Eliminating more adhesion molecules further reduces neutrophil recruitment efficiency, but the effect is less than additive. These mice clearly show and highlight leukocyte adhesion and recruitment through α_4-integrin, which may allow E/P/L/I−/− mice to be alive and viable. The similar phenotype seen in E/P/L−/− mice and E/P/I−/− mice without a further defect in E/P/L/I−/− mice suggests that ICAM-1 is necessary for recruitment of leukocytes that use L-selectin to roll.

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