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Selectins support the capture and rolling of leukocytes in venules at sites of inflammation and in lymphocyte homing. Gene-targeted mice with null mutations at the L-, E-, or P-selectin locus develop normally and show mild (E/-) to moderate (P/-, L/-) defects in inflammatory cell recruitment. Mice lacking both P- and E-selectin (E/P-/−) have severe neutrophilia and spontaneous skin infections that limit their life span. Other combinations of selectin deficiency have not been investigated. We have generated novel mice lacking L- and P-selectin (L/P-/−), L- and E-selectin (L/E-/−), or all three selectins (E/L/P-/−) by bone marrow transplantation. L/P-/− mice (only E-selectin present) show an absence of leukocyte rolling after trauma and severely reduced rolling (by ~90%) in inflammation induced by TNF-α. Residual rolling in L/P/-− mice was very slow (3.6 ± 0.2 μm/s after TNF-α). L/E-/− mice (only P-selectin present) showed rolling similar to that of L/-− mice at increased velocities (15.1 ± 0.3 μm/s).

The number of adherent leukocytes after 2 or 6 h of TNF-α treatment was not significantly reduced in L/E-/− or L/P/-− mice. L/E/P/-− mice showed very little rolling after TNF-α, all of which was blocked by mAb to α4 integrin. Adherent and emigrated neutrophils were significantly reduced at 6 h after TNF-α. We conclude that any one of the selectins can support some neutrophil recruitment but eliminating all three selectins significantly impairs neutrophil recruitment. The Journal of Immunology, 1999, 162: 6755–6762.

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by transplanting \(P^{−/−}\) bone marrow into lethally irradiated \(P^{−/−}\), \(E^{−/−}\) or \(P/E^{−/−}\) mice. The phenotype of these mice was investigated under conditions of mild trauma caused by exteriorizing the cremaster muscle for intravital microscopy (5) and in two models of TNF-\(\alpha\)-induced inflammation with different selectin requirements (6, 7).

**Materials and Methods**

**Animals**

E-selectin null (\(E^{−/−}\)) (8), P-selectin null (\(P^{−/−}\)) (19), L-selectin null (\(L^{−/−}\)) (1), E- and P-selectin double null (8), and C57BL/6 WT mice were obtained from established colonies maintained at the University of Virginia Health Sciences Center vivarium. All mutant mice used in this study were backcrossed into the C57BL/6 strain for at least six generations.

**Abs and cytokines**

The rat anti-mouse mAb to E-selectin, 9A9 (rat IgG1), has been previously shown to specifically block E-selectin function in vitro (20) and in vivo (9). mAb 9A9 was a generous gift of Dr. B. Wolitzky (Hoffman-La Roche, Nutley, NJ). mAb to P-selectin, RB40.34 (rat IgG1), is a function-blocking mAb against murine P-selectin (13). mAb R1/2 to \(\alpha\)-2 integrin (21) was purified from hybridoma culture supernatant. All Abs were injected i.v. at a dose of 30 \(\mu\)g/mouse. For the two models of TNF-\(\alpha\)-induced inflammation, recombinant murine TNF-\(\alpha\) (Genzyme, Cambridge, MA) was injected intrascrotally at a dose of 500 ng/mouse in a volume of 0.3 ml of sterile saline either 2 or 6 h before the beginning of the intravital microscopic experiments as described previously (7). In the 6-h group, 30 U of heparin saline either 2 or 6 h before the beginning of the intravital microscopic experiments was administered. The cremaster muscle for intravital microscopy (5) and in two pancreatic disease models of TNF-\(\alpha\)-induced inflammation with different selectin requirements (6, 7).

**Bone marrow transplantation**

Recipient mice were irradiated in two doses of 550–600 rads each, for a total of 1100–1200 rads, −4 h apart. Donor mice were killed by lethal injection of sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL), and bone marrow cells from both femurs and tibias were harvested under sterile conditions. Approximately 50 million nucleated bone marrow cells were obtained from each donor mouse. Bones were flushed with RPMI (Life Technologies, Grand Island, NY) (without phenol red) with 10% FCS (Atlanta Biologicals, Norcross, GA). Suspended bone marrow cells were obtained from each donor mouse. Bones were flushed with RPMI (Life Technologies, Grand Island, NY) (without phenol red) with 10% FCS (Atlanta Biologicals, Norcross, GA). Suspended bone marrow cells were washed twice and counted using a standard hemocytometer. The bone marrow cells were washed and lysed in 1.5 mM NH4Cl lysis solution. The bone marrow cells were washed twice and counted using a standard hemocytometer. Approximately 1−2 million unfractionated bone marrow cells in 200 \(\mu\)l of media were delivered i.v. through the tail vein of each recipient mouse. For E/P\(^{−/−}\) recipients, 10 million cells were infused per mouse, because some E/P\(^{−/−}\) mice died when reconstituted with 1−2 million unfractionated bone marrow cells. Recipient mice were housed in a barrier facility (individually ventilated cages, high energy particulate arresting filtered air), under pathogen-free conditions before and after bone marrow transplantation. After bone marrow transplantation, the mice were maintained on autoclaved water with antibiotics (0.7 mM neomycin sulfate, 60 \(\mu\)M tetracycline, and 0.37 mM trimethoprim) (Sigma, St. Louis, MO) and fed autoclaved food. These conditions were maintained for 4−5 wk. At this point, peripheral leukocyte counts and differentials had returned to normal, and the mice were ready for intravital microscopic experiments.

**Intravital microscopy**

Mice were anesthetized with an i.p. injection of ketamine hydrochloride (100 mg/kg, Ketalar, Parke-Davis, Morris Plains, NJ) after pretreatment with sodium pentobarbital (30 mg/kg i.p., Nembutal, Abbott Laboratories, North Chicago, IL) and atropine (0.1 mg/kg i.p., Elkins-Sinn). The trachea was intubated, and one jugular vein was cannulated for administration of anesthesia throughout the intravital microscopic experiment. One carotid artery was cannulated for blood pressure monitoring, blood samples, and systemic mAb injections. Mice were kept at a constant temperature of 37°C with a thermo-controlled heating lamp (Physitemp, Clifton, NJ) and received 0.2 mI/diluted pentobarbital in saline i.v. to maintain anesthesia and a neutral fluid balance. The cremaster muscle was prepared for intravital microscopy as described (5). The cremaster muscle was exteriorized. Time 0 was set at the beginning of the cremaster muscle exteriorization. The cremaster muscle was superfused with thermo-controlled (35°C) bicarbonate-buffered saline. Blood samples (10 \(\mu\)l each) were taken throughout the experiment from the carotid catheter at −45-min intervals to analyze systemic leukocyte concentrations. Differential leukocyte counts were obtained by counting. Kimura-stained blood smears in a hemocytometer. Microscopic observations were made on a Zeiss intravital microscope (Axioskop, Carl Zeiss, Thornwood, NY) with a saline immersion objective (SW 40/ 0.75 numerical aperture). Venules with diameters between 20 and 80 \(\mu\)m were observed and recorded via a CCD camera system (model VE-1000CD, Dage-MTI, Michigan City, IN) on a Panasonic S-VHS recorder (Panasonic, Osaka, Japan). Centerline RBC velocity was measured using a dual photodiode and a digital on-line cross-correlation program (22). Centerline velocities were converted to mean blood flow velocities by multiplying with an empirical factor of 0.625 (23). Wall shear rates \(\gamma_w\) were estimated as 2.12 \((8V/bd)\), where \(V_b\) is the mean blood flow velocity, \(b\) is the diameter of the vessel, and 2.12 is a median empirical correction factor obtained from velocity profiles measured in microvessels in vivo (24).

**Rolling and adhesion parameters**

Microvessel diameters, lengths, and rolling leukocyte velocities were measured with a digital image processing system (22). Each rolling leukocyte passing a line perpendicular to the vessel axis was counted, and leukocyte rolling flux was expressed as leukocytes per minute. Rolling flux fraction was calculated as described (5) 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, \(v_b\) is mean blood flow velocity, and \(d\) is the vessel diameter.

Leukocyte rolling velocities were measured over a constant 2-s time window. The rolling velocities of 10 leukocytes were measured in each venule. Adherent cells were defined to be the leukocytes that were not moving for at least 30 s. The total number of adherent cells was measured for each segment of venule (−200 \(\mu\)m long) and expressed per unit area of inside surface area of the venule. The surface area was calculated from diameter and length, assuming cylindrical geometry of the venule.

**Flow cytometry**

Flow cytometry was used to detect L-selectin expression on peripheral blood leukocytes as well as bone marrow cells of the recipient mice. Peripheral blood was obtained as described above using PBS (Life Technologies) with 0.01% azide. For each sample tube, a 200-\(\mu\)l volume of peripheral blood or bone marrow cell suspension was incubated with either MEL-14 conjugated with PE or rat IgG2a conjugated with PE (both Pharmingen, San Diego, CA) for 30 min (0.5 \(\mu\)g/10\(^6\) cells). Cells were centrifuged and aspirated. Bone marrow cells were resuspended in 500 \(\mu\)l of PBS with 0.01% azide. Peripheral blood leukocytes were lysed in 1.5 ml of 1.5 mM NH4Cl solution. Flow cytometry data were analyzed using a four-decade FACScan and Cell Quest software package (Becton Dickinson, San Jose, CA). For each sample, 10,000 cells were analyzed.

**Histology**

To differentiate intravascular and interstitial leukocytes, whole mounts of cremaster muscle were prepared by dropping 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) onto the tissue while tissue was still mounted on the cremaster stage for intravital microscopy. Each cremaster muscle was removed, mounted flat on a poly-l-lysine (Sigma)-treated glass slide, and air dried for 5–10 min, followed by fixation 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 stain (Sigma) at room temperature for 5–10 min, and differentiated in 0.01% acetic acid for contrast. The differentiated slides were sequentially 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 on a Zeiss microscope with a 100×, 1.4 numerical aperture oil immersion objective (Carl Zeiss). Intravascular and interstitial leukocytes were counted and differentiated into neutrophils, eosinophils, and mononuclear cells. The interstitial tissue observed was a circular area (with a diameter of 141 \(\mu\)m) bisected by each venule.

**Statistics**

Average leukocyte rolling flux fractions, leukocyte adhesion, leukocyte rolling velocities, leukocyte counts and differentials between groups were compared with the one-way ANOVA Kruskal-Wallis multiple comparison test. Statistical significance was set at \(p < 0.05\), indicated by ∗.
shown that absence of E- and P-selectin indeed impairs engraftment of bone marrow stem cells (25).

Leukocyte rolling after mild trauma

Mild trauma rapidly induces P-selectin-dependent leukocyte rolling (2, 26), followed by a second phase which is both L-selectin and P-selectin dependent (5, 14, 27). E-selectin is not or very faintly expressed in cremaster muscle vessels under these conditions (28). Here, we assessed leukocyte rolling in 126 venules of 12 mice with velocities, diameters, and shear rates (Table I). Rolling is expressed as leukocyte rolling flux fraction, which represents the number of rolling leukocytes divided by the total number of leukocytes passing through the same venule (29). L/P−/− mice showed a complete absence of leukocyte rolling at >30 min after exteriorization, compared with normal rolling in WT mice (15 ± 1% (5)) and to residual rolling in P−/− mice reported earlier (rolling flux fraction, 4 ± 1% (5)). There were too few rolling leukocytes in L/P−/− mice to measure leukocyte rolling velocity. However, in control mice in which WT bone marrow was injected into lethally irradiated C57BL/6 WT mice, the rolling velocity at >30 min after exteriorization was 48 ± 6 μm/s, similar to the 49 μm/s reported earlier for WT mice under these conditions (14). Rolling velocity in WT mice reconstituted with L−/− bone marrow was slightly reduced to 40 ± 1 μm/s, similar to previous findings in L−/− mice (14, 30). These findings confirm that P-selectin mediates slower leukocyte rolling than L-selectin under these conditions and show that trauma-induced rolling is eliminated when both P- and L-selectin are absent (Table I).

Short term (2 h) TNF-α treatment

Short term treatment with TNF-α is sufficient to induce expression of E-selectin and enhance the expression of P-selectin on the venular endothelium in this model (28). Here, we report leukocyte rolling in 248 venules of 25 TNF-α-treated mice lacking various selectins. Hemodynamic parameters were similar in these mice (Table II). The leukocyte rolling flux fraction after short term TNF-α treatment was slightly reduced in WT mice reconstituted with L−/− bone marrow (Fig. 2), similar to previous findings in L−/− mice (6). E−/− mice reconstituted with WT bone marrow showed increased levels of leukocyte rolling, consistent with previous findings in E−/− mice (6) and in mice pretreated with an E-selectin Ab (6, 31). All rolling in these mice was blocked by a P-selectin Ab (Fig. 2).

L/E−/− mice generated by reconstituting E−/− mice with L−/− bone marrow showed a rolling flux fraction that was not significantly different from that seen in mice lacking only P-selectin (P−/−) or only L-selectin (L−/−) (Fig. 2). Mice lacking endothelial P-selectin were generated by reconstituting P−/− mice with WT bone marrow. Rolling flux fraction in these mice was similar to that previously reported for P−/− mice (5), suggesting that platelet P-selectin does not play an important role in this model of inflammation.

In marked contrast, novel mice lacking both P- and L-selectin (L/P−/−) showed very low levels of leukocyte rolling after 2 h of

<table>
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<tr>
<th>Mouse Type</th>
<th>No. of Mice (N)</th>
<th>No. of Vessels (n)</th>
<th>Av. Diameter (μm)</th>
<th>Centerline Blood Velocity (μm/s)</th>
<th>Wall Shear Rate (μm/s)</th>
<th>Rolling Flux Fraction (%)</th>
<th>Av. Rolling Velocity (μm/s)</th>
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<tr>
<td>L−/−</td>
<td>5</td>
<td>26</td>
<td>33.2 ± 1.5</td>
<td>2.1 ± 0.2</td>
<td>710 ± 70</td>
<td>38.7 ± 4.8</td>
<td>39.7 ± 1.2</td>
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<tr>
<td>P−/−</td>
<td>3</td>
<td>54</td>
<td>36.1 ± 1.3</td>
<td>2.9 ± 0.2</td>
<td>930 ± 80</td>
<td>2 ± 1.5*</td>
<td>2 ± 1.5*</td>
</tr>
<tr>
<td>L/P−/−</td>
<td>4</td>
<td>46</td>
<td>40.6 ± 2.1</td>
<td>3.3 ± 0.2</td>
<td>860 ± 50</td>
<td>0.2 ± 0.005*</td>
<td>0.2 ± 0.005*</td>
</tr>
</tbody>
</table>

* Data are for venules in untreated mouse cremaster muscle (trauma-induced rolling). *, p < 0.05 compared with all other groups.
TNF-α treatment (Fig. 2). These findings suggest that L- or P-selectin are required to initiate rolling and that E-selectin cannot serve that particular function. This provides clear evidence of a nonoverlapping function of P- and L-selectin compared with E-selectin. Under these conditions, all residual rolling in L/P/E mice was blocked by injecting a blocking E-selectin Ab, mAb 9A9 (Fig. 2).

Next, we investigated the rolling velocities in these mice. WT mice reconstituted with L/E−/− bone marrow showed rolling at 5.0 ± 0.3 μm/s (Fig. 3), similar to rolling in L−/− mice (6.4 ± 0.5 μm/s (6)). Similarly, rolling velocity in P/E−/− reconstituted with WT bone marrow was 5.9 ± 0.3 μm/s, comparable to 6.4 ± 1.9 μm/s found in P−/− mice (6). However, rolling velocity was dramatically increased in E/E−/− mice reconstituted with WT bone marrow, reaching an average of 31.1 ± 0.9 μm/s. Again, this is similar to rolling velocities found in E−/− mice (6).

In L/E−/− mice reconstituted with L−/− bone marrow (L/E−/−), rolling is mediated by P-selectin (Fig. 2) and has an average rolling velocity of 14.8 ± 0.4 μm/s. Because this is a novel mouse, it can be compared only indirectly with previous data, which showed an average rolling velocity of 14.5 ± 1.4 μm/s in L-selectin-deficient mice treated with an E-selectin Ab (6).

The most interesting result in this model is the finding that residual rolling in L/P−/− mice proceeds at very low velocities, averaging 3.5 ± 0.2 μm/s (Fig. 3). These mice have very low numbers of rolling leukocytes, which use E-selectin for rolling (Fig. 2). This represents the first isolation of purely E-selectin-dependent rolling in vivo.

**Long term (6 h) TNF-α treatment**

Long term TNF-α treatment has previously been shown to induce L-selectin and α4 integrin-dependent rolling in E/P−/− mice (7). Now, we use L−/− bone marrow transplantation into E−/−, P−/−, and E/P−/− mice to investigate how rolling is altered when only P-selectin (L/E−/−), only E-selectin (L/P−/−), and no selectin (E/P−/−) is present (Fig. 4). These investigations were made in 161 hemodynamically comparable venules of 26 mice (Table III).

Similar to previous findings in L−/− mice (7), WT mice reconstituted with L−/− bone marrow showed reduced leukocyte rolling flux fractions after 6 h of TNF-α treatment, 1.2 ± 0.3% (Fig. 4) compared with 10.6 ± 2.7% in WT mice treated in the same way (7). Rolling was also reduced when L−/− bone marrow was transplanted into E−/− mice, but much less so than in WT mice reconstituted with L−/− bone marrow. This probably reflects the increase in leukocyte rolling flux produced by absence of E-selectin under these conditions. The rolling flux fraction seen in these L/P−/− mice, 0.9 ± 0.3% (Fig. 4), is similar to that found in E/P−/− mice reconstituted with WT bone marrow (0.9 ± 0.2%, Fig. 4). This rolling flux fraction was further reduced to 0.4 ± 0.06% in L/P−/− mice lacking all three selectins. These data show that L-selectin plays an important role in leukocyte rolling after long term TNF-α treatment, because E/L/P−/− mice show less rolling than E/P−/− mice (7). In contrast to the findings obtained after TNF-α treatment for 2 h, rolling was not totally abolished even in the absence of all three selectins. Rolling was completely blocked only after a blocking α4 integrin Ab, mAb PS/2, was injected in these mice.

The average rolling velocity in WT mice reconstituted with L−/− bone marrow and treated with TNF-α for 6 h before surgery was 10.7 ± 1.2 μm/s (Fig. 5), similar to values seen in L−/− mice under the same conditions (8 ± 0.8 μm/s (7)). L/E−/− mice showed elevated rolling velocities of 15.2 ± 0.9 μm/s, suggesting that this may be the characteristic velocity of P-selectin-mediated rolling under long term TNF-α treatment. Isolated P-selectin-dependent rolling has not previously been investigated in this model. The L/P−/− mouse is also novel, showing a rolling velocity for E-selectin of 4.5 ± 0.3 μm/s (Fig. 5), which is not quite as low as the 3.6 μm/s seen in short term TNF-α model (see Fig. 3), but significantly lower than rolling in WT mice after 6 h of TNF-α treatment (Fig. 5). Transplanting E/P−/− mice with WT bone marrow yields rolling at an average velocity of 15.7 ± 1.2 μm/s, not different from the velocity seen in E/P−/− mice (19 ± 2 μm/s (7)). This finding suggests that the presence of P-selectin on platelets in the bone marrow-transplanted mice shown here has no major impact on leukocyte rolling velocity (Fig. 5) or flux (see Fig. 4) in this model.

The most interesting rolling velocity was observed in mice lacking all three selectins. In E/L/P−/− mice, we find an average rolling velocity of 13.6 ± 1.2 μm/s, similar to the 15 ± 1 μm/s found

![FIGURE 2](http://www.jimmunol.org/) Roll these certain parameters for bone marrow-transplanted mice treated with TNF-α for 2 h before surgery. The dotted lines indicate the rolling flux fraction (mean ± SEM) in WT mice as reported previously (5, 6, 9). P-selectin (P-sel.) mAb is RB40.34, and E-selectin (E-sel.) mAb is 9A9. Number of mice and vessels as shown in Table II. *, significantly different from all other groups, p < 0.05; #, significantly different from L/E−/− and L/P−/− without mAb, respectively (p < 0.05).

**Table II. Hemodynamics and microvascular parameters for bone marrow-transplanted mice**

<table>
<thead>
<tr>
<th>Mouse Type</th>
<th>No. of Mice (n)</th>
<th>No. of Vessels (n)</th>
<th>Av. Diameter (μm)</th>
<th>Centerline Blood Velocity (mm/s)</th>
<th>Wall Shear Rate (s−1)</th>
<th>Rolling Flux Fraction (%)</th>
<th>Av. Rolling Velocity (μm/s)</th>
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<tbody>
<tr>
<td>L−/−</td>
<td>5</td>
<td>45</td>
<td>37.3 ± 1.4</td>
<td>2.4 ± 0.1</td>
<td>680 ± 30</td>
<td>10.1 ± 1.7</td>
<td>5.0 ± 0.3*</td>
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<tr>
<td>E−/−</td>
<td>5</td>
<td>54</td>
<td>40.3 ± 1.4</td>
<td>2.7 ± 0.1</td>
<td>710 ± 30</td>
<td>48.1 ± 5.5*</td>
<td>31.1 ± 0.9*</td>
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<tr>
<td>L/E−/−</td>
<td>7</td>
<td>67</td>
<td>41.6 ± 1.6</td>
<td>2.7 ± 0.1</td>
<td>720 ± 30</td>
<td>11.3 ± 1.3</td>
<td>14.8 ± 0.4*</td>
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<tr>
<td>P−/−</td>
<td>3</td>
<td>38</td>
<td>40.5 ± 1.7</td>
<td>2.3 ± 0.2</td>
<td>610 ± 30</td>
<td>11.5 ± 2.2</td>
<td>5.9 ± 0.3*</td>
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<tr>
<td>L/P−/−</td>
<td>5</td>
<td>44</td>
<td>49.1 ± 2.1</td>
<td>3.2 ± 0.1</td>
<td>730 ± 30</td>
<td>1.3 ± 0.2*</td>
<td>3.5 ± 0.2*</td>
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</table>

* Data are for venules in cremaster muscles pretreated with TNF-α for 2 h. *, p < 0.05 compared with all other groups.
in E/P<sup>−/−</sup> treated with a blocking Ab to L-selectin, MEL-14 (7). The value of 13.6 ± 1.2 μm/s represents the first in vivo estimate of α<sub>4</sub> integrin-mediated rolling, because all rolling in E/L/P<sup>−/−</sup> mice was blocked by a mAb to α<sub>4</sub> (Fig. 4).

Leukocyte adhesion and transmigration

In spite of the massive changes in leukocyte rolling parameters, the number of adherent neutrophils inside microvessels was similar in all mutants after 2 h of TNF-α treatment (data not shown). This finding suggests that sufficient overlap exists among the functions of the selectins to ensure neutrophil delivery in acute inflammation even when one or two of three selectins are absent. After 6 h of TNF-α treatment, neutrophil adhesion was slightly but not significantly reduced in each of the single and double mutants (Fig. 6). A significant reduction was seen only in E/L/P<sup>−/−</sup> mice lacking all three selectins. Remarkably, the composition of adherent leukocytes was drastically altered in E/L/P<sup>−/−</sup> mice. In E/L/P<sup>−/−</sup> mice, neutrophils accounted for only 63% of all intravascular leukocytes, with a balance of 25% mononuclear cells and 8% eosinophils. This shows that absence of selectins specifically impairs neutrophil recruitment but leaves mononuclear cell and eosinophil recruitment relatively intact. These findings were also reflected in the number of neutrophils found in the tissue surrounding the venules (data not shown).

Discussion

Our data show that the three selectins have both overlapping and unique functions. The defects seen in leukocyte rolling under inflammatory conditions are mild in single mutants and substantial in two of the three double mutants. Rolling in L/P<sup>−/−</sup> is drastically reduced, to an extent similar to that found in E/P<sup>−/−</sup> mice. These data suggest that E-selectin alone can mediate slow leukocyte rolling but is inefficient at capturing leukocytes to the venule wall in the absence of P- and L-selectin. This idea is supported by in vitro studies showing that L-selectin is particularly efficient at initiating leukocyte contact with the substrate (15–17). Under more severe inflammatory conditions, modeled here by treatment with TNF-α for >6 h, the rolling defects seen in both single- and double-mutant mice are insufficient to significantly block leukocyte recruitment to cremaster muscle venules or into

FIGURE 3. Histogram of the velocity of rolling leukocytes in mice treated with TNF-α for 2 h. Data for WT mice (A), L<sup>−/−</sup> mice generated by transplanting L<sup>−/−</sup> bone marrow into WT recipients (B), E<sup>−/−</sup> mice generated by transplanting WT bone marrow into E<sup>−/−</sup> recipients (C), L/E<sup>−/−</sup> mice generated by transplanting L<sup>−/−</sup> bone marrow into E<sup>−/−</sup> recipients (D), P<sup>−/−</sup> mice generated by transplanting WT bone marrow into P<sup>−/−</sup> recipients (E), and L/P<sup>−/−</sup> mice generated by transplanting L<sup>−/−</sup> bone marrow into P<sup>−/−</sup> recipients (F). D, rolling mediated by P-selectin only; F, rolling mediated by E-selectin only. Under these conditions, there is no rolling mediated by L-selectin only as reported previously in E/P<sup>−/−</sup> mice (8). Arrows indicate arithmetic mean velocity, SEM, and number of cells indicated; all velocity histograms significantly different (p < 0.05).

FIGURE 4. Rolling flux fraction (mean ± SEM, rolling leukocytes as percent of all leukocytes passing through the same vessel) in venules of the cremaster muscle of bone marrow-transplanted mice treated with TNF-α for 6 h before surgery. The dotted lines indicate the rolling flux fraction (mean ± SEM) in venules of WT mice as reported previously in the same model (7). α<sub>4</sub> integrin mAb is PS/2. Number of mice and vessels as shown in Table III. * significant differences from all other groups (p < 0.05); #, significantly different from E/P<sup>−/−</sup> (p < 0.05).
the tissue. This is remarkable and surprising in view of the significant rolling defects seen in these mice. Apparently, under inflammatory conditions, even a small number of rolling leukocytes can suffice to deliver neutrophils to sites of inflammation. This is reminiscent of findings obtained in a study exploring the therapeutic potential of inhibiting P- and L-selectin (32). In that study, Kubes et al. reported that rolling must be inhibited by at least 90% to see therapeutic effects in terms of reduced neutrophil recruitment to inflammatory lesions. With TNF-α treatment for 6 h, leukocyte rolling is reduced by ~95% in mice lacking all three selectins (E/L/P−/−). In E/L/P−/− mice, neutrophil recruitment is significantly impaired (Fig. 6).

The L/P−/− and L/E−/− mice isolate the function of E-selectin and P-selectin in vivo, respectively. Clearly, E-selectin alone is not nearly as good at mediating capture, rolling, and recruitment as P-selectin is. The rolling defect described here for L/P−/− mice appears to be of similar severity to that seen in E/P−/− mice described elsewhere (7, 8). By contrast, L/E−/− mice show only a moderate reduction of leukocyte rolling. The present data indicate that P-selectin is the most versatile of the selectins, because it alone can mediate reasonable levels of neutrophil rolling in all three models. This is achieved neither by L-selectin alone (as seen in E/P−/− mice) nor by E-selectin alone (as seen in L/P−/− mice), which show significant restrictions in the number of rolling leukocytes.

In E/L/P−/− mice, neutrophil recruitment is significantly impaired (Fig. 6). This suggests that the multiple inflammatory defects in the human disease, leukocyte adhesion deficiency II (33), must likely be attributed to defects in all selectins contributing to neutrophil recruitment. A previous report has shown that neutrophils obtained from these patients have impaired ligand function for E- and P-selectin and show impaired rolling in a rabbit model of inflammation (34). The present data suggest that in addition to having defective E- and P-selectin ligands, L-selectin ligand function may also be impaired in these patients.

The generation of E/L/P−/− mice for the first time isolates α4-dependent leukocyte rolling in vivo. This rolling occurs at a reduced efficiency, as seen by a low leukocyte rolling flux, and at an intermediate velocity of ~13–14 μm/s. Previous studies have shown that α4 integrins can mediate capture and rolling on activated endothelial cells (35) and on isolated VCAM-1 (36) in flow chamber-based in vitro assays. In vivo, a role for α4 integrins in leukocyte rolling has previously been shown in a model of Mycobacterium butyricum-induced vasculitis (37) and in lymphocyte

Table III. Hemodynamics and microvascular parameters for bone marrow-transplanted mice

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<td>5</td>
<td>43</td>
<td>36.8 ± 1.1</td>
<td>1.9 ± 0.1</td>
<td>560 ± 20</td>
<td>1.2 ± 0.3</td>
<td>10.7 ± 1.2</td>
</tr>
<tr>
<td>L/E−/−</td>
<td>5</td>
<td>34</td>
<td>39.5 ± 1.7</td>
<td>2.5 ± 0.2</td>
<td>660 ± 30</td>
<td>5.0 ± 1.0*</td>
<td>15.2 ± 0.9**</td>
</tr>
<tr>
<td>L/P−/−</td>
<td>6</td>
<td>30</td>
<td>42.6 ± 2.2</td>
<td>2.4 ± 0.2</td>
<td>590 ± 30</td>
<td>0.9 ± 0.3</td>
<td>4.8 ± 0.4**</td>
</tr>
<tr>
<td>E/P−/−</td>
<td>5</td>
<td>19</td>
<td>43.4 ± 2.0</td>
<td>2.0 ± 0.2</td>
<td>480 ± 40</td>
<td>0.9 ± 0.2</td>
<td>15.7 ± 1.2**</td>
</tr>
<tr>
<td>E/L/P−/−</td>
<td>5</td>
<td>35</td>
<td>45.1 ± 2.1</td>
<td>2.3 ± 0.1</td>
<td>540 ± 30</td>
<td>0.4 ± 0.06†</td>
<td>13.6 ± 1.2</td>
</tr>
</tbody>
</table>

* Data are for venules in cremaster muscle pretreated with TNF-α for 6 h. †, p < 0.05 compared with all other groups; **, p < 0.05 compared with L−/−; ††, p < 0.05 compared with E/P−/−.

FIGURE 5. Histogram of the velocity of rolling leukocytes in mice treated with TNF-α for 6 h before surgery. Data for WT mice (A), L−/− mice generated by transplanting L−/− bone marrow into WT recipients (B), L/E−/− mice generated by transplanting L−/− bone marrow into E−/− recipients (C), E/P−/− mice generated by transplanting WT bone marrow into E/P−/− recipients (D), L/P−/− mice generated by transplanting L−/− bone marrow into P−/− recipients (E), and E/L/P−/− mice generated by transplanting L−/− bone marrow into E/P−/− recipients (F). C, rolling mediated by P-selectin and α4 integrin; D, rolling mediated by L-selectin and α4 integrin; E, rolling mediated by E-selectin and α4 integrin; F, rolling mediated by α4 integrin only. Arrows indicate arithmetic mean velocity, SEM, and number of cells indicated. Rolling velocity in L/P−/− significantly lower than in all other groups (p < 0.05).
attachment to high endothelial venules in Peyer’s patches (38, 39). In these studies, the role of α4 integrins in leukocyte rolling was not clearly separated from the contribution of the selectins to rolling. Here, we show that α4 integrins mediate leukocyte rolling in E/L/P−/− mice, although at a drastically reduced efficiency compared with selectin-competent mice.

Although our experiments were not specifically designed to address the role of P-selectin expressed on platelets for leukocyte rolling and recruitment, the present data suggest that platelet P-selectin may not be very important under the conditions tested. In the trauma-induced and the short term TNF-α-induced models, P−/− mice reconstituted with WT bone marrow, which express P-selectin on platelets, show rolling and adhesion similar to that seen in P−/− mice (5), which lack P-selectin on both platelets and endothelial cells. In the long term (6 h) TNF-α model, P−/− mice have not been tested; therefore the current data cannot be compared. Our findings are consistent with a recent study (40) in which reconstitution of E/P−/− mice with WT bone marrow failed to correct the elevated neutrophil counts, development of spontaneous skin lesions, and reduced neutrophil recruitment into peritonitis. However, negative findings with respect to platelet P-selectin in Ref. 40 and in the present study do not rule out the possibility that platelet P-selectin may be involved in leukocyte recruitment in other models, as has recently been shown for lymphocyte homing to peripheral lymph nodes (41).

Based on our new findings, best estimates can be given of the quantitative parameters describing rolling in vivo (Table IV). A previous version of this table (6) contained estimates based on data from gene-targeted mice combined with Ab blockade. Table IV now lists the best estimates of rolling fluxes and rolling velocities as mediated by individual selectins entirely based on gene-targeted mice. The data listed are valid for the indicated adhesion molecules at the expression levels seen under three conditions, mild trauma, short term acute inflammation, and long term acute inflammation. This table shows strong synergistic effects for the selectins, because, possibly with the exception of trauma-induced rolling, the sum of the leukocyte rolling fluxes produced by each selectin is significantly less than the rolling flux observed in intact mice. The new estimates are entirely consistent with the previous ones (6), but the data are more complete and now for the first time include estimates for α4 integrin-mediated leukocyte rolling.

In conclusion, the generation of chimeric mice has resulted in three novel and unique mice, E/L−/−, L/P−/−, and E/L/P−/−. These mice have informative phenotypes as evaluated by intravital microscopy. Since the molecular details of initiation and progression of inflammation and the requirement for adhesion molecules are different for different tissues, organs, and inflammatory stimuli, these mice are valuable and defined reagents to investigate the isolated function of each individual selectin in various models of inflammation.

Acknowledgments

We thank Dr. Daniel C. Bullard, University of Alabama Birmingham, for providing breeding pairs of E-selectin, P-selectin, and E-selectin/P-selectin double-deficient mice, and Dr. Thomas F. Tedder, Duke University, for providing L-selectin-deficient mice. Dr. Barry Wolitzky, Hoffmann-La Roche, provided the E-selectin blocking mAb 9A9. We thank Nick Douris and Jennifer Bryant for mouse husbandry.

Table IV. Best estimates of leukocyte rolling flux, expressed as percent of rolling flux seen in WT mice and rolling velocity mediated by each selectin individually or by α4 integrin for three models of acute inflammation

<table>
<thead>
<tr>
<th>Mouse Type</th>
<th>Contribution to Rolling Flux (%)</th>
<th>Rolling Velocity (μm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trauma 2 h TNF-α 6 h TNF-α</td>
<td>Trauma 2 h TNF-α 6 h TNF-α</td>
</tr>
<tr>
<td>L-selectin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-selectin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α4 integrin</td>
<td>E/L/P−/− 0 0 4</td>
<td>No rolling</td>
</tr>
<tr>
<td>L,P,E,α4</td>
<td>WT 100 100 100</td>
<td>43 (14) 5 (6) 12 (7)</td>
</tr>
</tbody>
</table>

* Rolling in WT mice set as 100% in each model. Data from present study shown in bold, other data from references as indicated.