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Leukocyte-Endothelial Cell Interactions Are Enhanced in Dermal Postcapillary Venules of MRL/fas<sup>lpr</sup> (Lupus-Prone) Mice: Roles of P- and E-Selectin<sup>1</sup>

Michael J. Hickey,* Daniel C. Bullard, † Andrew Issekutz, ‡ and Will G. James*

MRL/fas<sup>lpr</sup> mice are affected by a systemic autoimmune disease that results in widespread leukocytic infiltration of the vasculature, including in the skin. The molecular pathways responsible for this leukocyte recruitment are poorly understood. Therefore, the aim of these experiments was to examine the mechanisms of leukocyte trafficking in the dermal microvasculature of MRL/fas<sup>lpr</sup> mice. Intravital microscopy was used to examine leukocyte rolling and adhesion in dermal postcapillary venules of MRL/fas<sup>lpr</sup> mice at 8, 12, and 16 wk of age. When compared with age-matched BALB/c and MRL/<sup>+/+</sup> (nondiseased) mice, leukocyte rolling and adhesion in MRL/fas<sup>lpr</sup> mice were significantly enhanced at 12 wk of age, and remained elevated at 16 wk of age. At 8 and 12 wk, leukocyte rolling in all three strains was almost entirely inhibited by an anti-P-selectin mAb. In contrast, at 16 wk some (~10%) leukocyte rolling persisted following P-selectin blockade. This residual rolling was predominantly inhabitable with an anti-E-selectin mAb; however, treatment with anti-E-selectin mAb alone had a minimal effect. P-selectin-deficient MRL/fas<sup>lpr</sup> mice also displayed leukocyte rolling that was significantly lower than in wild-type MRL/fas<sup>lpr</sup> mice. However, in these mice, leukocyte adhesion remained at the elevated levels observed in wild-type MRL/fas<sup>lpr</sup> mice. This adhesion was eliminated by chronic treatment with anti-E-selectin mAb. These findings indicate that leukocyte-endothelial cell interactions are enhanced in the dermal microvasculature of MRL/fas<sup>lpr</sup> mice above the age of 12 wk. Furthermore, the data suggest that the endothelial selectins share overlapping roles in mediating this enhanced leukocyte recruitment. The Journal of Immunology, 2002, 168: 4728–4736.

<sup>1</sup>Abbreviations used in this paper: SLE, systemic lupus erythematosus; PSGL-1, P-selectin glycoprotein ligand-1.

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it is clear that additional adhesion molecule pathways are functioning in these animals.

To fully understand the unique roles played by adhesion molecules in the multistep process of leukocyte recruitment in these animals, it is necessary to directly examine the affected microvasculature. Therefore, the aim of these studies was to characterize the adhesion molecule pathways responsible for the vasculitis in the skin of MRL/fas<sup>lpr</sup> mice, via the use of intravital microscopy. We chose to focus on the dermal microvasculature, as the skin is one of the organs affected most commonly in human SLE. Furthermore, previous studies have shown that MRL/fas<sup>lpr</sup> mice develop inflammatory lesions of the skin which have features in common with those in human lupus erythematosus (19). In these experiments, it was observed that infiltration of mononuclear leukocytes into the dermis commences at ∼2–3 mo. Therefore, we examined mice at 8 wk of age, when inflammation was likely to be minimal, and 12 and 16 wk, when further increases in leukocyte trafficking were anticipated. In contrast to earlier studies which had focused on adhesion molecules thought to be responsible for mediating leukocyte adhesion (e.g., ICAM-1 and VCAM-1), the focus of these studies was on the potential role of P- and E-selectin in initiating rolling interactions between leukocytes and endothelial cells. The involvement of these molecules was examined using function-blocking Abs, and MRL/fas<sup>lpr</sup> mice possessing a gene-targeted mutation in the P-selectin gene. These experiments revealed that leukocyte-endothelial cell interactions in dermal postcapillary venules of lupus-prone mice progressively increase as the mice are affected by active disease, and that P- and E-selectin share a common role in mediating these interactions.

Materials and Methods

Animals

“Lupus-prone” MRL-MpJ/fas<sup>lpr</sup> (MRL/fas<sup>lpr</sup>) mice and MRL-Mp<sup>1+/+</sup> (MRL/+<sup>+</sup>) mice were supplied by The Jackson Laboratory (Bar Harbor, ME). MRL/fas<sup>lpr</sup> mice have recently been renamed MRL/Tnfrsf6<sup>lpr</sup>; however, we will use the more familiar nomenclature in describing these experiments. MRL/+<sup>+</sup> mice are susceptible to autoimmune disease, especially later in life. However, addition of the <i>lpr</i> mutation to this background (MRL/fas<sup>lpr</sup> mice) causes a marked acceleration in development of autoimmune disease and vasculitis. Therefore, both MRL/+<sup>+</sup> and MRL/fas<sup>lpr</sup> mice were examined to determine whether the MRL background alone contributed to any alterations in dermal leukocyte trafficking. A similar approach has been used in previous studies (16). BALB/c mice (purchased from the University of Adelaide, Adelaide, Australia) were used as controls to indicate the level of basal leukocyte trafficking in nondiseased wild-type mice. Mice were housed under quarantine conditions, and used at 8, 12, and 16 wk of age.

Generation of P-selectin<sup>−/−</sup> MRLAs<sup>3H</sup> mice

P-selectin<sup>−/−</sup>-MRL/fas<sup>lpr</sup> mice were generated by backcrossing a gene-targeted P-selectin mutation onto the MRL/fas<sup>lpr</sup> strain background for eight generations (20). Mice were then intercrossed to produce double heterozygotes (P-selectin<sup>−/−</sup>-fas<sup>lpr/+/+</sup>) (21).

Intravital microscopy

Animals were anesthetized by i.p. injection of a mixture of 10 mg/kg xylazine (Bayer Pharmaceuticals, Pymble, Australia) and 200 mg/kg ketamine hydrochloride (Parke-Davis, Carlingbah, Australia). The left jugular vein was cannulated to administer anesthetic, fluorescent dyes, and Abs. The animal was placed on a thermocontrolled heating pad, regulating the core temperature to 37°C. The microcirculation of the ventral abdominal skin was then prepared for microscopy as previously described (22). Briefly, a midline abdominal incision was made extending from the level of the diaphragm to the pelvic region. The skin was carefully separated from the underlying tissue, remaining attached laterally to ensure the blood supply remained intact. The area of skin was then extended over a viewing pedestal and secured along the edges using 2–0 suture. The loose connective tissue on the dermal undersurface was carefully removed by dissection under an operating microscope. The exposed dermal microvasculature was immersed in normal saline and covered with a coverslip held in place with vacuum grease. To visualize leukocytes, animals were injected with 50 μl of 0.05% (i.v.) rhodamine 6G (Sigma-Aldrich, St. Louis, MO) immediately before microscopy. Rhodamine 6G at the dose used labeled leukocytes, and platelets, and has been shown to allow detection of the same number of rolling leukocytes as transmitted light, and has no effect on leukocyte kinetics (23, 24). Therefore, it allows for quantification of leukocyte rolling flux, leukocyte rolling velocity, and leukocyte adhesion via epifluorescence microscopy. Rhodamine 6G-associated fluorescence was visualized by epifluorescence at 510–560 nm, using a 590-nm emission filter (24, 25).

In an additional series of experiments, the dermal microvasculature of the ear was examined using a technique which causes minimal disruption to the tissue (26). Briefly, following anesthesia and insertion of a catheter in the tail vein, the hair on the right ear was removed using depilatory cream. The ear was then gently positioned on a heated pad, immersed in saline, and covered with a coverslip held in place with vacuum grease. To aid in visualization of the vasculature, 10 μl of 5% FITC-7-kDa dextran (Sigma-Aldrich) was administered i.v. as a plasma marker. This fluorescence was visualized by epifluorescence at 450–490 nm, with a 520-nm emission filter. Detection of rhodamine-6G-labeled leukocytes was performed as for the ventral skin preparation.

For preparations of both types, the microvasculature was visualized using an intravital microscope (Axioplan 2 Imaging; Carl Zeiss, Carnegie, Australia) with a ×40 water immersion objective lens (Achromat ×40/0.80 NA, Carl Zeiss) and a ×10 eyepiece. A SIT video camera (Dage-MTI VE-1000; SciTech Pty. Ltd., Preston South, Australia) was used to project the images onto a monitor (Sony PVM-20N5E; Carl Zeiss), and the images were recorded for playback analysis using a videocassette recorder (Panasonic NV-HS950; Panasonic, Secaucus, NJ). One to four dermal venules (25–40 μm in diameter) were selected in each experiment, and to minimize variability, the same section of venule was observed throughout the experiment. Venular diameter and the number of rolling and adherent leukocytes were determined offline during video playback analysis. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. Leukocyte rolling velocity was determined by measuring the time required for a leukocyte to roll along a 100-μm length of venule. Rolling velocity was determined for 20 leukocytes at each time interval. Leukocytes were considered adherent to the venular endothelium whether they remained stationary for 30 s or longer.

RBC velocity was determined via analysis of the velocity of 1-μm diameter fluorescent polystyrene microspheres (FluoSpheres-yellow/green, Molecular Probes, OR) injected i.v. in small boluses (27). Beads were visualized via epifluorescence as for FITC-dextran. Video sequences showing microspheres moving through postcapillary venules were digitized using an image analysis computer with an IC-PCI video capture card (Imaging Technology, Bedford, MA) controlled by the Sequence Snap video acquisition software (Adept Electronic Solutions, Perth, Australia). Following calibration appropriate to the magnification under examination, RBC velocity was measured using Scion Image (Scion, Frederick, MD). Calibration was a procedure developed by Dr. K.K. (University of Sheffield, Sheffield, U.K.) (28). The mean velocity (V<sub>MEAN</sub>) of 10 randomly selected microspheres was determined, and venular wall shear rate (γ) was calculated based on the Newtonian definition: γ = 8 (V<sub>MEAN</sub>/D<sub>l</sub>) (29).

Circulating leukocyte counts

At the end of each experiment, whole blood was drawn via cardiac puncture. Total leukocyte counts were performed, using a Neubauer hemocytometer (U-Lab, Eltham, Australia).

Antibodies

The Abs used in vivo in this study were RB40.34, an mAb against murine P-selectin (BD Biosciences, San Diego, CA; 20 μg/mouse); R1–2, an mAb against the murine α4 integrin (BD Biosciences; 75 μg/mouse); and RME-1, an mAb against rat and mouse E-selectin (Issekutz Laboratory, Halifax, Nova Scotia, Canada). The doses of RB40.34 and R1–2 used have been shown previously to be effective in specifically blocking their respective target molecules in vivo (11, 30). RME-1 inhibits binding of HL-60 promyelocytic cells to the ventral abdominal skin of the nude mouse (31). Therefore, it block E-selectin function in vivo in a murine model of endotoxin-induced leukocyte rolling (31, 32). For acute blockade of E-selectin, 100 μg of RME-1 was administered i.v., as previously described (32). For studies assessing effects of chronic E-selectin blockade, 200 μg RME-1 was administered i.v. via the tail vein, 18 h before microscopic observation. This dose has been observed to provide effective E-selectin blockade for at least 22 h (A. Issekutz, unpublished observations). Abs used for flow cytometry and immunohistochemistry were: PE-conjugated anti-murine P-selectin glycoprotein ligand-1 (PSGL-1) (2PH1), FITC-conjugated anti-murine...
CD3 (17A2), and anti-murine E-selectin (10E9.6) (all from BD Biosciences); Cy5-conjugated RB6-8C5 (Gr-1) purified from hybridoma supernatant; and affinity-purified rabbit polyclonal Ab raised against human P-selectin, generated as previously described (generously provided by Dr. M. Berndt, Baker Medical Research Institute, Melbourne, Australia) (33).

Histopathology
Areas of ventral skin were fixed in formalin, and 3-μm sections were prepared and stained with H&E according to standard techniques. Profiles of postcapillary venules located immediately subjacent to the dermis, corresponding to those viewed in vivo, were identified and the presence of leukocytes in close apposition to the endothelium determined. All venular profiles with at least one leukocyte closely apposed to the endothelium were defined as containing leukocytes interacting with the endothelium. These leukocytes were then classified as either granulocytic or mononuclear according to their morphology.

Flow cytometry and immunohistochemistry
Expression of PSGL-1 on circulating leukocytes was examined via flow cytometry. Heparinized blood samples were collected via cardiac puncture and 100-μl blood samples were treated with PE-conjugated 2PH1 at 1:100 for 20 min. To determine PSGL-1 expression by lymphocytic and granulocytic populations, samples were cocultivated with FITC-conjugated anti-murine CD3 and Cy5-conjugated anti-Gr-1 (RB6-8C5, granulocyte marker). Leukocyte fixation and erythrocyte lysis were performed using a Q-Prep Workstation (Beckman Coulter, Miami, FL) and samples were analyzed using a MoFlo flow cytometer (Cytomation, Fort Collins, CO). In addition, mononuclear and granulocytic populations were differentiated on the basis of forward and side scatter.

For immunohistochemical analysis of endothelial selectin expression, skin samples were fixed in peridate-lysine-parafomaldehyde for 4 h at 4°C and cryoprotected via washing in 7% sucrose/PBS for 48 h. Samples were then embedded in OCT compound, frozen over liquid nitrogen, and 7-μm cryostat sections prepared. To determine P-selectin expression, sections were stained using a three-layer peroxidase-anti-peroxidase technique, as previously described (34). Briefly, rabbit anti-P-selectin Ab (10 μg/ml, overnight at 4°C) was used as primary Ab. The secondary Ab was peroxidase-conjugated swine anti-rabbit IgG (1:50, 60 min), followed by rabbit peroxidase anti-peroxidase (1:100, 60 min). To determine E-selectin expression, rat anti-mouse E-selectin (10E9.6) (1:25, overnight at 4°C) was used as primary Ab, rabbit anti-rat IgG (1:100) as secondary Ab, and HRP-conjugated swine anti-rabbit IgG as tertiary Ab. All secondary and tertiary Abs were supplied by DAKO (Carpinteria, CA). All sections were developed by incubation in diaminobenzidine, and counterstained with hematoxylin. Selectin expression was analyzed by quantitating the number of vascular profiles displaying positive staining per ×10 field.

Statistics
For parameters such as leukocyte rolling flux, rolling velocity, and adhesion, comparison between the three mouse strains were performed using one-way analysis of variance, or Student’s t tests using the Bonferroni correction for multiple comparisons. Velocity analyses before and after administration of mAbs were performed using paired t tests. A value of p < 0.05 was deemed significant.

Results
Alterations in leukocyte trafficking in MRL/fas<sup>lpr</sup> mice
In initial experiments, leukocyte trafficking was characterized in dermal postcapillary venules of BALB/c, MRL<sup>+/−</sup>, and MRL/fas<sup>lpr</sup> mice at 8, 12, and 16 wk of age. Data are shown as mean ± SEM of 6-12 animals per group. *p < 0.05 vs BALB/c. **p < 0.05 vs BALB/c and MRL<sup>+/−</sup>.

0.7, and 9.1 ± 0.8 × 10<sup>6</sup>/ml, respectively. In addition, venular shear rate was significantly reduced in 16-wk MRL/fas<sup>lpr</sup> mice relative to BALB/c mice, but not relative to MRL<sup>+/−</sup> mice (Table I). Given that the rolling observed in 16-wk MRL<sup>+/−</sup> mice was <50% than in age-matched MRL/fas<sup>lpr</sup> mice despite comparable shear rates, this indicates that reduced shear rate alone was insufficient to explain the elevation in rolling in the lupus-prone mice.

Similarly, the numbers of adherent leukocytes in BALB/c and MRL<sup>+/−</sup> mice did not significantly differ between 8, 12, and 16 wk of age (Fig. 2). However, in MRL/fas<sup>lpr</sup> mice, leukocyte adhesion was significantly enhanced in 12- and 16-wk-old mice, indicating that the alteration in leukocyte rolling observed in these mice was associated with an increase in the number of cells ultimately progressing to adhesion (Fig. 3). Although the increased number of adherent cells in 12- and 16-wk-old MRL/fas<sup>lpr</sup> mice (−4/100-μm venule) is quite low relative to other tissues, this is comparable to the amount of adhesion we have seen in skin with optimal levels of Ag challenge (22). Leukocyte-endothelial cell interactions in arteries were also assessed in these mice. However, leukocytes were rarely observed to interact with the endothelium in arterial vessels, apart from an occasional cell in severely diseased MRL/fas<sup>lpr</sup> mice at 16 wk of age (data not shown).

To determine whether the alterations in rolling resulted from a change in the cell type undergoing interactions with the endothelial surface, histology was performed (Table II). Histopathological analysis of skin in 16-wk-old MRL<sup>+/−</sup> and MRL/fas<sup>lpr</sup> mice revealed that in MRL<sup>+/−</sup> mice, 14% of postcapillary venular profiles displayed leukocytes in close apposition to the endothelial surface,

<p>| Table I. Shear rates and diameters in dermal postcapillary venules examined in BALB/c, MRL&lt;sup&gt;+/−&lt;/sup&gt;, and MRL/fas&lt;sup&gt;lpr&lt;/sup&gt; mice at 8, 12, and 16 wk |
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<th></th>
<th>Age</th>
<th>BALB/c</th>
<th>MRL&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>MRL/fas&lt;sup&gt;lpr&lt;/sup&gt;</th>
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<td>8 wks</td>
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<td>Venular shear rate (s&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>180 ± 47</td>
<td>134 ± 18</td>
<td>143 ± 26</td>
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<td>Venular diameter (μm)</td>
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<td>12 wks</td>
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<td>Venular shear rate (s&lt;sup&gt;−1&lt;/sup&gt;)</td>
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<td>146 ± 23</td>
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<td>Venular diameter (μm)</td>
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<td>31 ± 1</td>
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<td>16 wks</td>
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<td>Venular shear rate (s&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>178 ± 31</td>
<td>116 ± 17</td>
<td>93 ± 14</td>
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<tr>
<td>Venular diameter (μm)</td>
<td>33 ± 2</td>
<td>34 ± 1</td>
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* Data are shown as mean ± SEM.
* p < 0.05 vs age-matched BALB/c.
whereas in MRL/fas<sup>−/−</sup> mice, 46% of venules displayed leukocytes adjacent to the endothelium. However, in both strains of mice, 60–75% of these cells were mononuclear, suggesting that while the number of interacting cells was increased in MRL/fas<sup>−/−</sup> mice, this increase was not due to a relative increase in the proportion of one particular subgroup of leukocytes interacting with the endothelium.

**Rolling is also increased in MRL/fas<sup>−/−</sup> mice in the absence of surgically induced inflammation**

To determine whether the elevation in leukocyte rolling and adhesion in MRL/fas<sup>−/−</sup> mice was related to trauma induced by surgical preparation of the ventral skin, leukocyte rolling was also analyzed in the microvasculature of the ear in the absence of surgical manipulation (26). Similar to findings using the ventral skin preparation, leukocyte rolling in dermal postcapillary venules in the noninflamed ears of 16-wk-old MRL/fas<sup>−/−</sup> mice was greater than double that in age-matched MRL<sup>+/+</sup> mice (Fig. 4).

**Molecular mechanisms of leukocyte rolling in dermal postcapillary venules**

To determine a role for the endothelial selectins in leukocyte rolling in these animals, mice were treated with function-blocking Abs to P- and E-selectin. Fig. 5 illustrates the effect of P-selectin inhibition on dermal leukocyte rolling in the three mouse strains. At both 8 and 12 wk, leukocyte rolling in all three strains was almost entirely eliminated by P-selectin blockade. In contrast, at 16 wk, residual rolling of the order of 3–5 cells/min persisted following P-selectin blockade. This finding was the same in all three strains examined. Finally, in BALB/c, MRL<sup>+/+</sup> (data not shown), and MRL/fas<sup>−/−</sup> mice at 16 wk (Fig. 6), the residual P-selectin-independent rolling was eliminated by subsequent E-selectin blockade.

We performed additional experiments with E-selectin blockade alone to determine a role for this molecule in lupus-prone mice. In all three strains of mice at all time points examined, E-selectin blockade had no effect on leukocyte rolling flux (Table III). However, in accord with previous studies (35), E-selectin blockade displayed some effects on rolling velocity (Table III). At 8 wk, E-selectin blockade increased rolling velocity significantly in both BALB/c and MRL<sup>+/+</sup> mice. The same treatment was without effect on rolling velocity in MRL/fas<sup>−/−</sup> mice. At 12 wk of age, small but significant increases were observed in BALB/c and MRL/fas<sup>−/−</sup> mice following E-selectin blockade, but not in MRL<sup>+/+</sup> mice. At 16 wk, E-selectin blockade was without effect on any of the strains examined.

In additional experiments, the role of the α<sub>4</sub> integrin in mediating the increased rolling in venules in dermal venules of MRL/
mice was examined. Treatment of 12- and 16-wk-old MRL/fas\textsuperscript{pr} mice with a mAb against the \( \mu \)-selectin had no effect on either leukocyte rolling flux or rolling velocity (Table III).

**Role of E-selectin in mediating leukocyte rolling and adhesion in P-selectin\textsuperscript{−/−}-MRL/fas\textsuperscript{pr} mice**

Finally, to further examine the relative roles of P- and E-selectin in mediating leukocyte rolling and subsequent adhesion in MRL/fas\textsuperscript{pr} mice, we examined the dermal microvasculature of P-selectin\textsuperscript{−/−}-MRL/fas\textsuperscript{pr} mice (Fig. 7). In agreement with the results of P-selectin blockade in wild-type MRL/fas\textsuperscript{pr} mice, leukocyte rolling was dramatically reduced, although not entirely absent in P-selectin\textsuperscript{−/−}-MRL/fas\textsuperscript{pr} mice. At all time points examined, some residual rolling was observed. However, despite the marked reduction in leukocyte rolling in the P-selectin-deficient mice, leukocyte adhesion remained at the elevated levels observed in wild-type MRL/fas\textsuperscript{pr} mice, indicating that leukocyte adhesion could occur in these animals via a P-selectin-independent pathway (Fig. 7B).

Acute studies with RME-1 indicated that the residual rolling in P-selectin\textsuperscript{−/−}-MRL/fas\textsuperscript{pr} mice was E-selectin-dependent (Fig. 7C). We next wanted to test whether E-selectin-dependent rolling resulted in leukocyte adhesion in this model. The relatively short time course of the acute experiments with RME-1 was insufficient to observe the impact of E-selectin blockade on leukocyte adhesion, as adherent cells which may have required E-selectin for rolling no longer use E-selectin to remain firmly attached to the endothelium, and consequently would not be affected by E-selectin blockade. Therefore, we chronically treated both wild-type MRL/fas\textsuperscript{pr} mice and P-selectin\textsuperscript{−/−}-MRL/fas\textsuperscript{pr} mice with RME-1 to prevent E-selectin-dependent rolling for 18 h, thus preventing cells from progressing to adhesion via this mechanism (Fig. 8). In wild-type MRL/fas\textsuperscript{pr} mice, extended E-selectin blockade had no effect on rolling flux, rolling velocity (data not shown), and leukocyte adhesion (Fig. 8A). In sharp contrast, in P-selectin\textsuperscript{−/−}-MRL/fas\textsuperscript{pr} mice treated chronically with RME-1, leukocyte rolling and adhesion were eliminated (Fig. 8B).

**Endothelial selectin expression**

One potential mechanism to explain the increase in selectin-dependent leukocyte rolling was an alteration in endothelial expression of P- and E-selectin. This possibility was assessed by immunohistochemical analysis of selectin expression in MRL/+/+ and MRL/fas\textsuperscript{pr} mice. Fig. 9 shows the level of P-selectin expression observed in the skin of the two strains of mice at various ages. At all ages examined, the number of vascular profiles which stained positively for P-selectin was consistently higher in MRL/fas\textsuperscript{pr} mice than in MRL/+/+ mice. In contrast, E-selectin expression was detected only sporadically in both strains of mice, with the number of labeled vessels not differing between the two strains. However, after systemic treatment with LPS, E-selectin expression was observed in dermal vessels in both strains of mice (data not shown), indicating that these tissues had the capacity to express this molecule given appropriate stimulation.

**PSGL-1 expression by circulating leukocytes**

Alterations in leukocyte expression of PSGL-1, the major ligand of P-selectin, could conceivably also be responsible for alterations in
and T cells (CD3-MRL/fas−/− leukocytes were positive for PSGL-1, whereas in similarly aged granulocyte and mononuclear populations in the two strains indicated that this vasculitis is initiated by an influx of mononuclear leukocytes followed by neutrophil infiltration at later stages of disease (39). The critical function of these leukocytes to these sites of inflammation remains poorly understood. In many years, the mechanisms responsible for recruitment of leukocytes to the microvasculature of the skin of these animals. These experiments provided the first indication that one of the effects of this systemic autoimmune disease is to enhance leukocyte-endothelial cell interactions in the peripheral microvasculature.

This study enabled us to determine the role of the endothelial selectins in mediating leukocyte recruitment to inflamed venules undergoing a chronic inflammatory stimulus. Several observations have been made that P-selectin was highly important in mediating leukocyte rolling in these vessels. Firstly, P-selectin blockade was highly effective at preventing rolling in dermal vessels in MRL/fas−/− mice. Even when rolling was significantly elevated above basal levels at 12 and 16 wk, P-selectin blockade inhibited rolling by <90%. Furthermore, the number of rolling leukocytes was reduced to a comparable degree in P-selectin−/−MRL/fas−/− mice. Thirdly, our data indicated that P-selectin expression was increased in the dermal microvasculature of MRL/fas−/− mice. Using an immunohistochemical approach, we found that although P-selectin was rarely expressed at detectable levels in MRL−/− mice, in MRL/fas−/− mice vascular expression of P-selectin was consistently higher. This is in contrast to previous experiments which indicated that expression of P- and E-selectin, determined using an in vivo radiolabeled immunoassay, did not differ between BALB/c, MRL−/−, and MRL/fas−/− mice (36). The reasons for this discrepancy are unclear, but could include differences in the sensitivity of the detection technique, or differences in housing conditions of the mice. Nevertheless, together the current data support the hypothesis that P-selectin has a key role in mediating leukocyte rolling in dermal postcapillary venules in these chronically inflamed mice.

Analysis of PSGL-1 expression by circulating leukocytes provided an additional potential mechanism to explain the increase in selectin-dependent interactions observed. In the present study, the proportion of circulating cells expressing PSGL-1 was significantly higher in MRL/fas−/− mice compared with MRL−/− mice. This alteration was due to an increase in the proportion of CD3-negative mononuclear cells in the lupus-prone strain expressing the molecule. The increased PSGL-1 expression in the lupus-prone strain correlated well with the increase in selectin-dependent interactions observed in vivo in the dermal microvasculature of these mice. PSGL-1 is the major leukocyte ligand for P-selectin, and is also capable of interacting with E-selectin (37–39). The critical role of PSGL-1 in mediating P-selectin-dependent leukocyte rolling is demonstrated by the observation that PSGL-1-deficient mice show markedly reduced leukocyte rolling in the acutely inflamed microvasculature and delayed neutrophil recruitment in the thiglycollate model of peritonitis, comparable to that seen in...
Therefore, it is conceivable that alterations in the distribution or expression of this molecule may lead to comparable changes in leukocyte rolling in peripheral vascular beds. Further work is required to determine the functional role of PSGL-1 in the altered leukocyte trafficking in this model.

However, despite the suggestions of a key role for P-selectin in mediating leukocyte recruitment in this response, in P-selectin−/− MRL/fasΔ mice the number of leukocytes which progressed onto adhesion was not different from that observed in wild-type MRL/fasΔ mice. This indicated that P-selectin was not required for leukocyte adhesion to reach the elevated levels observed in these animals. Therefore, to determine the role of the other endothelial selectin (E-selectin) in this response, we analyzed adhesion after chronic anti-E-selectin treatment. Chronic blockade of E-selectin completely eliminated both rolling and adhesion in P-selectin−/− MRL/fasΔ mice, clearly illustrating that in the absence of P-selectin, E-selectin-dependent rolling was required to enable leukocytes to become adherent. Interestingly, when wild-type MRL/fasΔ mice underwent the same anti-E-selectin treatment, leukocyte adhesion was not reduced. Presumably, in wild-type MRL/fasΔ mice in which E-selectin function was inhibited, rolling mediated by P-selectin was sufficient to enable leukocyte adhesion to reach elevated levels. This indicates that in the dermal microvasculature in this model of chronic inflammation, the functions of P- and E-selectin are interchangeable. These findings provide a plausible explanation for the observation that P-selectin−/− MRL/fasΔ mice are not protected from the pathology and premature death which affects wild-type MRL/fasΔ mice (D. Bullard, manuscript in preparation). In these animals, the absence of P-selectin does not affect the ability of pathogenic leukocytes to be recruited to vasculitic sites. Our data indicate that in the absence of

FIGURE 7. Leukocyte rolling flux (A) and adhesion (B) in wild-type MRL/fasΔ mice (n = 6–13) and P-selectin−/−MRL/fasΔ mice (n = 4 at each age) at 8, 12, and 16 wk; and C, Effect of acute treatment with anti-E-selectin mAb on leukocyte rolling in 16-wk-old P-selectin−/−MRL/fasΔ mice (n = 3). Data are shown as mean ± SEM. *, p < 0.05 vs pre-E-selectin Ab data.

FIGURE 8. Effects of chronic (overnight) treatment with anti-E-selectin mAb on leukocyte rolling flux and adhesion in wild-type MRL/fasΔ mice (A), and P-selectin−/−MRL/fasΔ mice (B). Mice were examined at 16 wk of age. On the day before microscopic evaluation, mice were treated with 200 μg RME-1 i.v. Data are shown as mean ± SEM. n = 4 (14 venules) for wild-type MRL/fasΔ mice, and n = 2 (8 venules) for P-selectin−/− wild-type MRL/fasΔ mice.

FIGURE 9. P-selectin expression in skin sections from MRL+/+ (E) and MRL/fasΔ mice (f) at 8, 12, and 16 wk of age. Each data point represents an individual animal. Data represent the number of positively labeled vascular profiles observed per 10× field.
P-selectin, at least in the dermal microvasculature, E-selectin provides an alternative molecular pathway which is capable of mediating leukocyte recruitment. However, this function is not apparent in wild-type MRL/fas<sup>br</sup> mice, under conditions of normal P-selectin expression.

It was clear from the examination of the P-selectin<sup>−/−</sup>-MRL/fas<sup>br</sup> mice, that a dramatic reduction in the number of rolling leukocytes did not affect the number of cells able to undergo adhesion in dermal postcapillary venules. This indicates that in the prolonged inflammatory response occurring in these animals, the number of leukocytes which undergo adhesion is not affected by marked reductions in leukocyte rolling flux. This finding is supported by studies of leukocyte recruitment induced by IL-4 over a 24-h time course, in which reduction in leukocyte rolling to as low as 2 cells/min (>95% reduction) did not reduce the number of leukocytes recruited to the cremaster muscle (30). Together with the present findings, these data indicate that in contrast to acute inflammatory responses, during more prolonged or chronic inflammatory responses, leukocyte adhesion and subsequent entry of leukocytes to the inflamed area can occur in an efficient manner even when leukocyte rolling has been almost entirely abolished. This has important implications if leukocyte rolling is to be the target of pharmacological therapy for chronic inflammatory disease.

In peripheral vascular beds under basal conditions, and indeed during most inflammatory responses, leukocytes rarely interact with the endothelium on the arterial side, but predominantly undergo rolling and adhesion on the venular side of the microcirculation (40, 41). However, given that arterial vessels are one of the primary targets of the vasculitis in these mice, it is conceivable that under these inflammatory conditions, leukocytes will undergo rolling and adhesive interactions on the arterial endothelial surface. In the present study, direct observation of arterial vessels revealed that leukocytes rarely interacted with the endothelial surface of dermal arterioles in these animals. On the rare occasions that these interactions were detected, it was only in the most severely diseased MRL/fas<sup>br</sup> mice. This might be considered a surprising observation; however, similar findings were observed in a study of adjuvant-induced vasculitis in rats in which leukocytes did not undergo interactions in arterioles, despite a 10-fold increase in leukocyte rolling and adhesion in adjacent postcapillary venules (9). There are several possible explanations for our observations in the MRL/fas<sup>br</sup> model. Arterial interactions contributing to vasculitis may occur rarely and over extended periods, making detection of these events beyond the scope of conventional intravital microscopy experiments. Alternatively, there is evidence to suggest that leukocytes infiltrating the wall of arteries affected by vasculitis do not enter by interacting with the endothelial lining of the affected vessel, but instead emigrate from alternative vascular sites such as the adventitial microvasculature or adjacent postcapillary venules (4). Histopathological studies of MRL/fas<sup>br</sup> mice show that the initial mononuclear vasculitis predominantly affects the perivascular/adventitial zones of arteries, i.e., the area of the vessel most distant from the endothelial surface (4). Finally, the lack of luminal interactions in arteries in these vasculitic mice could be unique to the tissue under examination. The skin of MRL/fas<sup>br</sup> mice has been shown to have a low, or at least delayed, incidence of vasculitis compared with more severely affected organs such as the lungs, kidneys, and salivary glands (3). It is possible that the dermal arteries examined in the present study were only minimally affected by vasculitis, and consequently, the rate of leukocyte entry into the vascular wall was exceedingly low. These issues may be resolved in the future by direct examination of arteries in more severely affected tissues of MRL/fas<sup>br</sup> mice.

In conclusion, we have shown that one of the consequences of the systemic inflammatory disease which affects MRL/fas<sup>br</sup> mice is an enhancement in leukocyte-endothelial cell interactions in the dermal microvasculature. This increase in leukocyte trafficking is potentially an important contributor to the development of the systemic inflammation which affects these mice. Currently it remains unknown whether comparable alterations in leukocyte trafficking occur in other organs. However, it is clear that in the MRL/fas<sup>br</sup> model of systemic autoimmune disease, and indeed in clinical SLE, a wide range of organs can be affected by inflammatory vascular disease. The aim of future studies will be to determine the existence and mechanisms of aberrant leukocyte trafficking in other critical organs in these mice.

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References
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