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Deficiency of P-Selectin or P-Selectin Glycoprotein Ligand-1 Leads to Accelerated Development of Glomerulonephritis and Increased Expression of CC Chemokine Ligand 2 in Lupus-Prone Mice

Xiaodong He,* Trenton R. Schoeb,* Angela Panoskaltsis-Mortari,† Kurt R. Zinn,‡ Robert A. Kesterson,* Junxuan Zhang,* Sharon Samuel,* M. John Hicks,§ Michael J. Hickey,¶ and Daniel C. Bullard²*

The selectins and their ligands mediate leukocyte rolling on endothelial cells, the initial step in the emigration cascade leading to leukocyte infiltration of tissue. These adhesion molecules have been shown to be key promoters of acute leukocyte emigration events; however, their roles in the development of long-term inflammatory responses, including those that occur during chronic inflammatory diseases such as systemic lupus erythematosus, are unclear. To assess participation of P-selectin in such disorders, we studied the progression of systemic lupus erythematosus-like disease in P-selectin-deficient and control MRL/MpJ-Fas<sup>lpr</sup> (Fas<sup>lpr</sup>) mice. Surprisingly, we found that P-selectin deficiency resulted in significantly earlier mortality, characterized by a more rapid development of glomerulonephritis and dermatitis. Expression of CCL2 (MCP-1) was increased in the kidneys of P-selectin mutant mice and in supernatants of LPS-stimulated primary renal endothelial cell cultures from these mice. A closely similar phenotype, including elevated renal expression of CCL2, was also observed in Fas<sup>br</sup> mice deficient in the major P-selectin ligand, P-selectin glycoprotein ligand-1. These results indicate that P-selectin and P-selectin glycoprotein ligand-1 are not required for leukocyte infiltration and the development of autoimmune disease in Fas<sup>br</sup> mice, but rather expression of these adhesion molecules is important for modulating the progression of glomerulonephritis, possibly through down-regulation of endothelial CCL2 expression. The Journal of Immunology, 2006, 177: 8748–8756.
suggesting that these adhesion molecules may be functional in promoting inflammatory disease in this mouse model and SLE (28, 29). In the present study, we analyzed FasLr mice deficient in P-selectin or its major ligand P-selectin glycoprotein ligand-1 (PSGL-1) to determine the roles of these adhesion molecules in IC-mediated SLE-like vascular inflammation. Unexpectedly, we found that loss of expression of P-selectin or PSGL-1 was not protective, but led to accelerated forms of glomerulonephritis and dermatitis. The rapid progression of glomerulonephritis in these adhesion molecule mutant mice was also associated with increased expression of the chemokine CCL2 in both kidney tissue and in purified renal endothelial cells. These findings suggest that P-selectin and PSGL-1 play key roles in regulating the development of glomerulonephritis in this model, possibly through inhibition of CCL2 expression.

Materials and Methods

Mice

P-selectin−/− FasLr−/− double homozygous mice (P-sel/FasLr) were generated as previously described (29). PSGL-1−/− mutant mice were a gift from Drs. B. C. Furie and B. Furie, Harvard Medical School, Boston, MA (30). The PSGL-1 mutation was backcrossed for eight generations onto the MRL/Mpj-FasLr strain (The Jackson Laboratory), followed by intercrossing to generate PSGL-1−/− FasLr−/− double homozygotes (PSGL-1/FasLr). Inbred FasLr−/− and FasLr mice that died between 21 and 37 wk of age. Necropsies were also performed on additional FasLr and P-sel/FasLr mice that died between 16 and 20 wk of age. Necropsies were also performed on additional FasLr and P-sel/FasLr mice that died between 21 and 37 wk of age. Tissues were fixed in 70% ethanol/10% formalin, routinely processed and embedded in paraffin, sectioned at 5 μm, and stained with H&E or periodic acid-Schiff and hematoxylin. For ultrastructural examination, kidney specimens were fixed in glutaraldehyde, embedded in plastic, and analyzed by transmission electron microscopy.

Kidney lesions were evaluated by subjective scoring, without the pathologist’s knowledge of the genotype of the mice, as previously described (12). Specific changes evaluated were glomerular cellularity, necrosis, crescent and synchia formation, neutrophil accumulation, capillary basement membrane thickening and reduplication, mesangial sclerosis, capsular and periglomerular fibrosis, tubular changes, interstitial inflammatory cell infiltrate, tubular epithelial cell degeneration, and interstitial fibrosis. Each was scored 0, 1, 2, or 3 for normal, mild, or severe, respectively. At least 6, and up to 15, glomeruli and adjacent tubules and interstitium were evaluated from both H&E- and periodic acid-Schiff and hematoxylin-stained sections from each mouse, and equal numbers of glomeruli from superficial, middle, and deep cortex were examined. Only glomeruli sectioned through the approximate center of the tuft and including the base of the tuft were included. Overall lesion scores for each mouse were calculated as the mean of summed individual scores for each glomerulus, with scores for necrosis and crescent formation each weighted by a factor of 2. Clinical signs of cutaneous inflammation were assessed weekly by visual inspection of the mice.

Immunohistochemical staining

For the detection of monocytes and macrophages, kidneys were embedded in OCT, snap-frozen in liquid nitrogen, sectioned at 6 μm, fixed in ice-cold acetone, and air dried. After rehydration, the sections were blocked with 10% goat serum, incubated with rat anti-mouse CD68 (Vector Laboratories), and then incubated with HRP-conjugated goat anti-rat IgG (H+L) (Southern Biotechnology Associates) and with peroxidase ABC (Vector Laboratories). Both frozen and paraffin sections were developed with diaminobenzidine (Vector Laboratories) and counterstained with hematoxylin. Immunostained cells were counted in 6–15 glomeruli (equal numbers from superficial, middle, and deep cortex) for each mouse. Only cells within the glomerular tuft were counted and stained cells external to the capsule were excluded.

Kidney function assay

We measured renal function using radioactive 99mTc-mercaptoacetyltriglycine (MAG). Normal functioning kidneys rapidly remove MAG, from the blood via the proximal tubules with immediate excretion in the bladder (31). Dynamic renal studies (10 s/frame for 900 s) were conducted every 2 wk using an Anger 420/550 Mobile Radiosotope Gamma camera (Technicare) equipped with a parallel-hole collimator. Three intact mice were imaged simultaneously by orienting them around a center enflurane anesthesia delivery point on the collimator, and injecting the mice sequentially over ~180 s. The mice were doped with 99mTc-MAG (Birmingham Central Pharmacy) at ~1.0 mCi (37 MBq) per mouse via the tail vein 30 min after 0.5 ml of i.p. saline to insure adequate hydration. Syringes were measured and after injection with a dose calibrator (Atomlab 100; Biodex Medical Systems). The percentage of injected dose (%ID) in each kidney was calculated for each time point after MAG, injection using standard region of interest analyses with background correction. The following parameters were calculated for each mouse at each imaging session: peak kidney uptake as %ID, time to peak, and %ID in each kidney 15 min after initial uptake of MAG. The peak to 15-min ratio was then determined for each kidney. The mean peak to 15-minute-ratio was then calculated for each genotype at each time point.

Cytokine analysis

Kidney extracts were prepared by homogenizing kidney tissue in 2 ml of proteinase inhibitors in PBS (Boehringer Mannheim). Total kidney extract protein was determined by the Lowry method using a total protein determination kit (Sigma-Aldrich). Cytokines from kidney extracts or from cultured endothelial cell supernatants were analyzed by ELISA or multiplex assay using mouse-specific kits and beadsets (R&D Systems). Levels were then interpolated from standard curves of the relevant recombinant mouse proteins (R&D Systems). Endothelial cell supernatant cytokines were presented as picograms per milliliter of supernatant. Kidney cytokines were normalized to total protein and presented as picograms of cytokine per milligram of total protein.

Real-time RT-PCR

Kidney total RNA was prepared using the SV Total RNA Isolation System (Promega). cDNA was synthesized using SuperScript III kit (Invitrogen Life Technologies). Multiplex real-time RT-PCR was conducted using LUX-labeled oligonucleotide primers for CCL2 and β-actin (Invitrogen Life Technologies) analyzed on a Chromo4 Instrument (MJ Research) for each gene of interest. For CCL2 amplification, the primers used were GACCTGATATAAGCATTACACACAGTCCGAG and TTCCACCACACC TCAAGC ACT. The threshold cycle corresponding to exponential growth of PCR product during the log-linear phase for both CCL2 (FAM labeled) and the internal reference gene β-actin (6-carboxy-4',5'-dichloro-2',7'-di-methoxyfluorescein-labeled) were calculated and analyzed for each sample in triplicate using the ΔΔ threshold cycle comparative method to determine relative expression levels. Amplification efficiencies and assay validations were determined from control standard curves for both the gene of interest and the reference RNA generated for each 96-well assay.

Endothelial cell culture and purification

Kidney endothelial cells were isolated from newborn mice. Three-day-old mice were sacrificed and the kidneys were removed aseptically and placed in 10% FBS HBSS. Under a dissection microscope, vessels and connective tissue surrounding the kidneys were teased off and the kidneys were minced into small pieces. Pieces of tissue were then moved to a gelatin-coated 6-well-cell culture plate containing DMEM with 10% FBS, 5 mM HEPES buffer, 1 mM sodium pyruvate, nonessential amino acids, 100 μg/ml endothelial mitogen (Biomedical Technologies), 10 μg/ml heparin, and 1 μg/ml hydrocortisone (Sigma-Aldrich). Each well contained 10–20 pieces of tissue. The plate was then placed in a 5% CO2, 37°C incubator and cultured for 2–3 days. Initially, the fibroblasts grew out of the tissues in single layers. Proliferating endothelial cells were then observed to grow in masses on the top of fibroblasts or on the coating gelatin. Endothelial cells were then separated from the fibroblasts by physical dislocation using repeated pipetting. The cell supernatants were then collected, centrifuged down, and the cells were then resuspended in fresh medium and seeded on gelatin-coated plates.

Endothelial cell purity was determined by uptake of 1.1'-diocadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI)-conjugated acetylated low-density lipoprotein (DiI-Ac-LDL) and positive staining with Von
Willebrand factor (vWF) Ab (32, 33). For the assessment of Dil-Ac-LDL uptake, endothelial cells were cultured on gelatin-coated glass slides coated overnight in fresh medium. The cells were then incubated for 4 h with 10 μg/ml Dil-Ac-LDL (Biomedical Technologies) at 37°C, washed with PBS, and mounted with fluorescence mounting medium (Vector Laboratories). For vWF staining, endothelial cells were also cultured on slides overnight, air dried, fixed with 5% acetic acid in ethyl alcohol, and incubated with primary rabbit anti-human vWF Ab, a biotinylated-goat anti-rabbit IgG (Vector Laboratories), and streptavidin HRP (Southern Biotechnology Associates), respectively. Only endothelial cell cultures that showed >95% purity were used for additional experiments. The slides were then developed with diaminobenzidine (Vector Laboratories) and counterstained with hematoxylin.

**Endothelial cell stimulation with LPS and CCL2-releasing assay**

Purified endothelial cells were suspended at 0.5 × 10⁶ cells/ml in medium and added to 24-well plates precoated with gelatin at 1 ml/well. After overnight incubation, and with the cells attached to the bottom of the plate, the supernatant was removed and 1 ml of fresh medium containing 10 μg/ml LPS (Sigma-Aldrich) was added per well. The plate was then incubated at 37°C and 22 μl/well supernatant was collected hourly for CCL2 analysis.

**Statistics**

Survival data were determined by Kaplan-Meier curves and analyzed by the log-rank test. Overall lesion scores were compared by ANOVA with Tukey’s test for mean comparisons. Scores for individual lesion components were analyzed with the Kruskal-Wallis test with supplemental comparisons by ANOVA of ranks and the Z test. The Student t test was used for all other comparisons.

**Results**

**Survival analyses and comparative histopathology**

MRL/MpJ-Fasbr mice show accelerated mortality compared with MRL/MpJ and other inbred mouse strains due to complications from glomerulonephritis and vasculitis (34–36). To determine the long-term effects of P-selectin deficiency on the development of these inflammatory processes, we performed survival analyses on a large cohort of P-sel/Fasbr and control Fasbr mice. Surprisingly, we found that P-selectin mutant mice showed an overall decreased survival compared with controls (median ± SEM for P-sel/Fasbr = 20.50 ± 0.47 vs Fasbr = 26.14 ± 0.79, p < 0.0001; Fig. 1A). To determine whether this phenotype was associated with the accelerated development of inflammatory disease, we evaluated and compared both dermatitis and glomerulonephritis in both strains of mice. We found that P-selectin-deficient mice also showed a more rapid onset of cutaneous inflammation compared with controls (Fig. 1B). Comparative histopathology of kidney sections taken during the initial phases of glomerulonephritis (16 wk) did not reveal any significant differences between P-selectin-deficient and control mice (Fig. 1C). However, P-sel/Fasbr mice did show significantly higher lesion scores than controls at 20 wk of age. At this time point, lesions in P-selectin mutant mice were characterized by intense neutrophil infiltration of the glomerular tuft, proliferation of mesangial and endothelial cells, increased amounts of mesangial proteinic material, and mild multifocal tubular atrophy and interstitial mixed inflammatory cell accumulation (Fig. 2). There also were more severely affected glomeruli with necrosis of large portions of the tuft, fibrin deposition, proliferation of the lining epithelium of Bowman’s capsule with fusion with the tuft (synechia), capsular fibrosis, and pericapsular lymphocyte accumulation. In contrast, most glomeruli of Fasbr mice had only mild neutrophil infiltration and hypercellularity. The remaining glomeruli that were more severely affected had moderate to intense neutrophil infiltration and hypercellularity similar to the less severely affected glomeruli of P-sel/Fasbr mice, but tuft necrosis, capsular proliferation, and fibrosis were less common and less severe.

Comparison of glomerular ultrastructure between P-sel/Fasbr and Fasbr mice at 20 wk of age revealed considerable differences. Glomeruli from Fasbr mice had a mild increase in mesangial cells and matrix (Fig. 3, A and B), and there were occasional small parameginal deposits and small well-defined intramembranous deposits. The foot processes were only focally effaced with the majority being thin and delicate. In contrast, P-sel/Fasbr glomeruli had markedly expanded mesangium, primarily due to mesangial matrix increase and large electron dense deposits (Fig. 3, C and D). The degree of cellularity in the mesangium was moderately increased. The foot processes were diffusely effaced and fused, and both subepithelial and subendothelial electron dense deposits were readily identified. Renal lesions at the time of death were severe in both P-sel/Fasbr and Fasbr mice, and the mean scores were not significantly different.
These findings suggest that loss of P-selectin expression does not alter the initiation of glomerulonephritis, but results in a more rapid progression to end-stage renal disease via an unknown mechanism. Examination of other organs in P-sel/Fas$^{lpr}$ mice at these various time points did not reveal any other types of inflammatory lesions, other than vasculitis, which is typical of Fas$^{lpr}$ mice. Furthermore, P-selectin-deficient mice did not have evidence of infection or complications of vasculitis such as hemorrhage or thrombosis, suggesting that these factors did not significantly contribute to the accelerated mortality.

To determine whether the accelerated renal disease in P-selectin deficient mice correlated with altered leukocyte recruitment, we assessed the overall numbers of intraglomerular T cells, monocyte/macrophages, and neutrophils in kidney sections. At 20 wk of age, when P-selectin mutant mice showed higher renal lesion scores than control mice, mean numbers of CD3$^+$ and CD68$^+$ cells were not significantly different between genotypes as determined by immunohistochemistry (data not shown). In contrast, neutrophil accumulation was severe in P-sel/Fas$^{lpr}$ mice at 20 wk, whereas it was mild to moderate in Fas$^{br}$ mice at this time point (data not shown).

**Real-time assessment of renal function**

To further examine the development of glomerulonephritis in P-selectin deficient mice, we conducted renal function studies using $^{99m}$Tc-mercaptoacetyltriglycine (MAG3), an established radioactive tracer for this purpose (31). Starting at 18 wk of age, P-selectin mutant and control mice were dosed every 2 wk with MAG3 and simultaneously imaged with a dynamic gamma camera. At 18 wk, we did not observe any significant differences between the different genotypes in the uptake and clearance of MAG3 from the kidneys, as determined by comparing the mean peak to 15-min ratios (see Materials and Methods and Table I). Fig. 4A shows a representative imaging profile for a P-sel/Fas$^{lpr}$ mouse at 18 wk of age. At subsequent time points, both P-selectin deficient and control mice showed progressive declines in renal function (data not shown). However, by 26 wk, all of the P-sel/Fas$^{lpr}$ mice had died compared with only two Fas$^{br}$ controls. A significant defect in

![FIGURE 3](image-url)
Cytokine and chemokine analyses

Cytokines and chemokines play important roles in the development of renal inflammatory diseases including glomerulonephritis in Fas\textsuperscript{br} mice (9). To investigate whether the accelerated renal disease in P-selectin mutant mice correlated with altered expression of different inflammatory mediators, we analyzed the overall levels of IFN-γ, CCL2, CCL3, CCL4, MIP-2, KC, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-13 in kidney tissue collected at 20 wk of age by ELISA. In these analyses, we found significantly higher levels of CCL2 in the kidneys of P-sel/Fas\textsuperscript{br} mice compared with controls (Table II). No significant differences in the levels of the other cytokines/chemokines were observed. We next examined CCL2 levels in kidneys collected at earlier ages to determine whether P-selectin mutant mice showed elevated expression of this chemokine in renal tissue during the initiation phases of glomerulonephritis. However, significant differences in CCL2 expression were only observed between groups at 20 wk of age, and not at 12 and 16 wk (Fig. 5A). Finally, we used quantitative real-time RT-PCR analysis to determine whether CCL2 transcription was also increased in the kidneys of P-sel/Fas\textsuperscript{br} mice. We found that P-selectin-deficient mice showed significantly higher levels of CCL2 mRNA transcripts compared with controls at 20 wk of age (Fig. 5B).

Previous studies have strongly implicated CCL2 as a key proinflammatory molecule in the development of many different forms of glomerulonephritis (37–42). In addition, loss or inhibition of this chemokine in Fas\textsuperscript{br} mice significantly inhibits the progression of renal disease and protects from early lethality (43, 44). Based on these previous observations, one possible interpretation of our findings is that loss of P-selectin expression in renal endothelial cells leads to increased production of CCL2 over time, which ultimately results in an accelerated progression of glomerulonephritis. Alternatively, the differences in CCL2 expression may simply be indicative of a more advanced form of renal disease in the kidneys collected from P-selectin mutant mice at 20-wk of age, and similar levels of this chemokine may be observed in older Fas\textsuperscript{br} mice at similar stages of glomerulonephritis. To determine whether CCL2 expression is somehow regulated by P-selectin during glomerulonephritis, we measured production of this chemokine in LPS-stimulated primary renal endothelial cell cultures purified from P-selectin mutant and control mice by ELISA. Endothelial cell cultures were derived from newborn mice using the procedures outlined in Materials and Methods and Fig. 6 shows representative photomicrographs of these cultures, including DiI-Ac-LDL and anti-vWF Ab staining patterns. Following stimulation with LPS, we observed a significant induction of CCL2 expression in control cultures (Fig. 5C). However, even greater levels of CCL2 were detected in the supernatants of P-selectin-deficient endothelial cells treated with LPS (Fig. 5D), with significant increases observed at 4–8 h poststimulation compared with control cultures. These data strongly suggest that P-selectin expression down-regulates CCL2 production in activated endothelial cells, independent of engagement of this adhesion molecule by one of its ligands.

Analysis of PSGL-1/Fas\textsuperscript{br} mice

Leukocyte-expressed PSGL-1 serves as the major rolling receptor for P-selectin (45). To test whether loss of PSGL-1 expression

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Table I. Renal imaging studies

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Peak to 15-Min Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 wk of age</td>
<td></td>
</tr>
<tr>
<td>P-sel/Fas\textsuperscript{br} (n = 5)</td>
<td>5.67 ± 1.56\textsuperscript{ab}</td>
</tr>
<tr>
<td>Fas\textsuperscript{br} (n = 5)</td>
<td>7.36 ± 1.70</td>
</tr>
<tr>
<td>Time point before death</td>
<td></td>
</tr>
<tr>
<td>P-sel/Fas\textsuperscript{br} (n = 4)</td>
<td>1.20 ± 0.09</td>
</tr>
<tr>
<td>Fas\textsuperscript{br} (n = 2)</td>
<td>1.07 ± 0.04</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Values listed represent the mean ± SEM values for each genotype.

\textsuperscript{b} One of the mice in this group died at 19 wk of age and was only imaged once.

Table II. Kidney cytokine analysis

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>P-sel/Fas\textsuperscript{br}</th>
<th>Fas\textsuperscript{br}</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>KC</td>
<td>10.82 ± 1.92</td>
<td>7.13 ± 1.03</td>
<td>NS</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.11 ± 0.03</td>
<td>0.13 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td>CCL3</td>
<td>2.40 ± 0.98</td>
<td>1.36 ± 0.26</td>
<td>NS</td>
</tr>
<tr>
<td>CCL4</td>
<td>9.67 ± 3.20</td>
<td>7.17 ± 1.30</td>
<td>NS</td>
</tr>
<tr>
<td>MIP-2</td>
<td>1.09 ± 0.22</td>
<td>0.60 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>IL-1β</td>
<td>3.05 ± 1.03</td>
<td>1.78 ± 0.90</td>
<td>NS</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.55 ± 0.77</td>
<td>0.42 ± 0.20</td>
<td>NS</td>
</tr>
<tr>
<td>IL-4</td>
<td>3.15 ± 0.76</td>
<td>4.26 ± 1.87</td>
<td>NS</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.11 ± 0.03</td>
<td>0.08 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>IL-6</td>
<td>6.87 ± 1.14</td>
<td>5.77 ± 1.97</td>
<td>NS</td>
</tr>
<tr>
<td>IL-10</td>
<td>8.45 ± 1.71</td>
<td>6.67 ± 1.46</td>
<td>NS</td>
</tr>
<tr>
<td>IL-13</td>
<td>1.45 ± 0.39</td>
<td>1.10 ± 0.36</td>
<td>NS</td>
</tr>
<tr>
<td>CCL2</td>
<td>14.07 ± 2.35</td>
<td>5.71 ± 0.64</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Kidney cytokine levels were normalized to total protein. The values shown are the mean picograms of cytokine per milligram of total kidney protein ± SEM; n = 6–7/genotype. NS = p > 0.05.
would result in a similar phenotype to that observed in the P-selectin mutants, we generated and analyzed PSGL-1/Fas<sup>lpr</sup> mice. We found that PSGL-1-deficient mice also showed early lethality (median &plusmn; SEM for PSGL-1/Fas<sup>lpr</sup> mice = 21.40 &plusmn; 0.86 vs Fas<sup>lpr</sup> mice = 26.40 &plusmn; 0.86, p < 0.0001; Fig. 7A), accelerated onset of cutaneous skin inflammation (Fig. 7B), and significantly increased

**FIGURE 5.** Increased CCL2 expression in kidneys and primary renal endothelial cells from P-sel/Fas<sup>lpr</sup> mice. A, Kidney extracts were isolated from P-sel/Fas<sup>lpr</sup> and sex- and age-matched Fas<sup>lpr</sup> mice at the 12, 16, and 20 wk of age (n = 5–7 for 12 and 16 wk, n = 15 for 20 wk). CCL2 levels were determined as described in Materials and Methods (*, p < 0.03). B, Normalized levels of CCL2 mRNA transcripts. Total kidney RNA was isolated from P-sel/Fas<sup>lpr</sup> and Fas<sup>lpr</sup> mice at 20 wk of age and CCL2 transcript levels were measured by reverse transcriptase real-time PCR and normalized to β-actin expression in each sample (n = 8/group; *, p = 0.007). C, Significant induction of CCL2 from Fas<sup>lpr</sup> renal endothelial cells following stimulation with 10 μg/ml LPS. D, P-sel/Fas<sup>lpr</sup> renal endothelial cells show significantly increased production of CCL2 compared with Fas<sup>lpr</sup> controls following LPS stimulation (*, p < 0.05).

**FIGURE 6.** Kidney endothelial cell cultures from Fas<sup>lpr</sup> mice. A, Phase contrast microscopy showing a kidney explant culture containing proliferating endothelial cells from a Fas<sup>lpr</sup> mouse growing on top of fibroblasts (×20). B, Purified kidney endothelial cells grown on gelatin-coated plates (phase contrast, ×40). C, Fluorescent micrograph of purified endothelial cells stained with DiI-Ac-LDL (×100). D, Endothelial cells stained with anti-vWF Ab (×100).
glomerular lesion scores at 20 wk of age compared with controls (Fig. 7C). In addition, examination of renal CCL2 expression revealed significant increases in PSGL-1-deficient kidneys collected from mice at 20 wk of age compared with control mice (Fig. 7D). However, in contrast to our analyses of P-selectin mutant mice, significant increases in the levels of this chemokine were also detected at 16 wk of age in kidneys of PSGL-1/Fas<sup>br</sup> mice. Finally, PSGL-1 mutants, like P-sel/Fas<sup>br</sup> mice, showed a significant increase in CCL2 mRNA levels in the kidneys compared with Fas<sup>br</sup> mice at 20 wk of age, as assessed by real-time RT-PCR (data not shown).

**Discussion**

These studies demonstrate that P-selectin and PSGL-1 play key roles in regulating the progression of inflammatory disease in Fas<sup>br</sup> mice. Our analyses of P-selectin and PSGL-1 mutant mice showed that loss of expression of these adhesion molecules was not protective in this model, but resulted in the accelerated development of both dermatitis and renal disease leading to earlier lethality. Similar phenotypes have been reported in P-selectin mutant mice. Our analyses of P-selectin and PSGL-1 mutant mice showed a significant increase in CCL2 mRNA levels in the kidneys compared with Fas<sup>br</sup> mice at 20 wk of age, as assessed by real-time RT-PCR (data not shown).

The current investigations strongly suggest that one of the functions of these adhesion molecules is to limit the intensity or severity of the inflammatory response, especially in the kidney following IC deposition. Our data indicate that one of the major mechanisms by which P-selectin and PSGL-1 control the progression of chronic renal inflammation is through reducing expression of CCL2. This chemokine has been shown to be an important chemoattractant for monocytes and T cells, is involved in the regulation of Th1/Th2 lymphocyte differentiation, and can restrict Th1 cell responses (48–52). An expanding body of evidence strongly suggests that CCL2 promotes the development of glomerulonephritis in many different human diseases, including SLE, and is a key mediator of renal inflammation in different models of glomerulonephritis, including Fas<sup>br</sup> mice (37–42, 53). In this model, loss or inhibition of CCL2 significantly inhibited the progression to end-stage renal disease and resulted in longer life spans compared with controls (43, 44).

CCL2 is produced by multiple cell types in the kidney, but is coexpressed with P-selectin in renal endothelial cells and PSGL-1 in infiltrating leukocytes such as macrophages and lymphocytes (41, 54). To test for a possible relationship between P-selectin and CCL2 expression in endothelial cells, we first established primary endothelial cultures from P-sel/Fas<sup>br</sup> and Fas<sup>br</sup> mice. We then examined and compared CCL2 induction in response to LPS stimulation, without the presence of leukocytes or anti-P-selectin Abs, and found that P-selectin-deficient endothelial cells consistently produced significantly higher levels of secreted CCL2 compared with controls. This novel observation suggests that one of the P-selectin-dependent mechanisms for down-regulating CCL2 in vivo occurs independently of the engagement of this receptor, and simply requires the expression of this adhesion molecule in endothelial cells. Although we do not yet understand the cellular pathways responsible for suppression of CCL2 in this system, it may occur…
through signaling events following translocation of P-selectin to the endothelial cell membrane (55–57).

PSGL-1/Fas<sup>lpr</sup> mice also showed a similar accelerated glomerulonephritis phenotype characterized by higher CCL2 levels, despite the presence of a normal P-selectin gene. One possible explanation for this observation is that engagement of P-selectin by PSGL-1 during leukocyte rolling events also leads to reduced endothelial CCL2 expression through activating P-selectin-dependent intracellular signaling pathways (57). It is interesting to note that we observed significantly higher levels of CCL2 in kidney tissue collected from 16- and 20-wk-old PSGL-1/Fas<sup>lpr</sup> mice compared with controls, while CCL2 expression was only increased in 20-wk-old samples taken from P-sel/Fas<sup>lpr</sup> mice. PSGL-1 also interacts with E-selectin on endothelial cells, and it is possible that the combined loss of PSGL-1 interactions with both of these endothelial selectins is responsible for the increase in CCL2 expression (58, 59). Support for this model comes from preliminary studies in our laboratory where we have observed that E-selectin mutant Fas<sup>lpr</sup> mice also show accelerated lethality associated with the rapid development of glomerulonephritis, and increased CCL2 expression in LPS-stimulated E-selectin-deficient primary endothelial cells (X. He and D. C. Bullard, manuscript in preparation).

Alternatively, other PSGL-1-dependent mechanisms may also be responsible for regulating CCL2 expression in PSGL-1/Fas<sup>lpr</sup> mice. For example, engagement of PSGL-1 with the selectins may elicit signaling pathways in infiltrating leukocytes themselves, leading to reduced CCL2 production in these cells, which in turn could inhibit or slow the progression of glomerulonephritis in Fas<sup>lpr</sup> mice. Loss of expression of PSGL-1 would thus lead to increased expression of leukocyte-derived CCL2 and promote the rapid development of end-stage renal disease.

We did not observe significant differences in the overall numbers of infiltrating T cells and monocytes in the kidneys between P-selectin mutant and control mice at 20 wk of age, despite our findings that P-sel/Fas<sup>lpr</sup> mice displayed higher levels of CCL2 in kidneys during the progression phases of glomerulonephritis. This may indicate that the concentration of this chemokine has reached saturation and that additional numbers of leukocytes cannot be recruited through CCL2-dependent pathways. Alternatively, increased numbers of infiltrating T cell and monocytes may occur in mice lacking P-selectin or PSGL-1 compared with controls, but at an earlier stage of glomerulonephritis. Due to the chronic nature of this model, it is difficult to accurately assess and compare specific numbers of infiltrating leukocytes between and within groups, since individual mice are not all synchronized for the same stage of glomerulonephritis. Finally, it is possible that the increased levels of CCL2 observed in P-selectin and PSGL-1 mutant mice act primarily to alter Th1/Th2 differentiation and activity, which may in turn promote the more rapid development of glomerulonephritis (48, 60–62).

CCL2-independent mechanisms may also be altered in P-selectin and PSGL-1 mutant Fas<sup>lpr</sup> mice and contribute to the accelerated disease phenotype. These adhesion molecules may modulate the expression or activity of other proinflammatory mediators not analyzed in our studies that contribute to the pathogenesis of renal and vascular disease in this model. In addition, it is possible that loss of P-selectin and PSGL-1 expression may inhibit the recruitment of regulatory T cells or monocyte/macrophage populations to the kidney, thus allowing a more rapid development of glomerulonephritis (63).

Our findings further suggest that P-selectin/PSGL-1-mediated rolling is not absolutely required for leukocyte infiltration and tissue damage in response to IC deposition in this model. Our previous intravital microscopy analyses of P-sel/Fas<sup>lpr</sup> mice support this hypothesis (28, 29). We found that the numbers of rolling leukocytes were reduced, but not completely inhibited, in the skin and brain microvasculature in these mutant mice compared with controls, and significant differences in the numbers of firmly adherent leukocytes were not observed. Thus, other adhesion molecules, such as VCAM-1, L-selectin, or CD44 may compensate for the loss of P-selectin or PSGL-1 and mediate rolling in postcapillary venules. Alternatively, leukocyte rolling may not be required for leukocyte firm adhesion and transendothelial migration in all tissues affected in this model, especially in the glomeruli during the development of renal disease. Evidence from studies in which leukocyte-endothelial cell interactions have been assessed in glomeruli of hydropnephrotic kidneys have shown that selectin antagonists did not reduce inflammatory leukocyte recruitment to glomeruli (64), suggesting that rolling interactions may not be important in glomerular capillaries. Furthermore, we have recently observed using intravital microscopy that leukocytes undergo immediate adhesion in inflamed glomerular capillaries without undergoing a prior rolling step (65).

In summary, our analyses of P-selectin and PSGL-1 mutant Fas<sup>lpr</sup> mice suggest that these adhesion molecules negatively regulate the development of IC-mediated disease in this model, possibly through modulating expression of inflammatory mediators such as CCL2. These findings also implicate these proteins as potentially important inflammatory modulators during the progression of lupus nephritis in SLE. Further investigations of P-selectin and PSGL-1 are necessary to define the inflammatory pathways regulated by these adhesion molecules and to determine their specific roles in the pathogenesis of SLE.

Disclosures

The authors have no financial conflict of interest.

References