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*J Immunol* 2007; 179:8470-8479; doi: 10.4049/jimmunol.179.12.8470

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E- and P-Selectin Are Not Required for the Development of Experimental Autoimmune Encephalomyelitis in C57BL/6 and SJL Mice

Axinia Döring,* Martin Wild, † Dietmar Vestweber, † Urban Deutsch,* and Britta Engelhardt†

In multiple sclerosis and in its animal model experimental autoimmune encephalomyelitis (EAE), inflammatory cells migrate across the endothelial blood-brain barrier (BBB) and gain access to the CNS. It is well-established that α4 integrins are actively involved in leukocyte recruitment across the BBB during EAE. In contrast, the role of endothelial E- and P-selectin in this process has been a controversial issue. In this study, we demonstrate that P-selectin protein can be detected in meningeal blood vessel endothelial cells in healthy SJL and C57BL/6 mice and on rare parenchymal CNS blood vessels in C57BL/6, but not SJL, mice. During EAE, expression of P-selectin but not E-selectin was found up-regulated on inflamed CNS microvessels surrounded by inflammatory infiltrates irrespective of their meningeal or parenchymal localization with a more prominent immunostaining detected in C57BL/6 as compared with SJL mice. P-selectin immunostaining could be localized to CNS endothelial cells and to CD41-positive platelets adhering to the vessel wall. Despite the presence of P-selectin in wild-type mice, E/P-selectin-deficient SJL and C57BL/6 mice developed clinical EAE indistinguishable from wild-type mice. Absence of E- and P-selectin did neither influence the activation of myelin-specific T cells nor the composition of the cellular infiltrates in the CNS during EAE. Finally, endothelial-specific tetracycline-inducible expression of E-selectin at the BBB in transgenic C57BL/6 mice did not alter the development of EAE. Thus, E- and P-selectin are not required for leukocyte recruitment across the BBB and the development of EAE in C57BL/6 and in SJL mice. The Journal of Immunology, 2007, 179: 8470–8479.
leukocyte recruitment across the BBB has been supported by the observation that therapeutic targeting of leukocyte trafficking across the BBB by blocking \( \alpha_4 \) integrin with the humanized Ab natalizumab has proven beneficial for the treatment of MS (11, 12) and reduces the number of inflammatory cells present in the cerebrospinal fluid of natalizumab-treated MS patients (13).

Despite the predominant involvement of \( \alpha_4 \) integrins in leukocyte interaction with the BBB, a number of studies using intravital microscopy of the brain have convincingly demonstrated that E- and P-selectin are expressed in superficial blood vessels of the brain (10) and by interaction with P-selectin glycoprotein ligand (PSGL)-1 mediate the tethering and rolling of endogenous leukocytes or T cells in these vessels in nicotine (14), TNF-\( \alpha \), or LPS-treated mice (15) or in mice suffering from EAE (8, 9). Other studies have, however, failed to detect the expression of E- and P-selectin in CNS microvessels during EAE (16) or shown that cultured brain microvascular endothelial cells lack storage of P-selectin in Weibel-Palade bodies (17). Also, in apparent contrast to the observation of E- and P-selectin-mediated leukocyte rolling in superficial brain microvessels by intravital microscopy, Ab inhibition studies blocking E- and P-selectin or PSGL-1 failed to inhibit the development of EAE in the SJL mouse model (9, 16, 18).

Furthermore, several studies have now demonstrated that C57BL/6 mice deficient for PSGL-1 or P-selectin develop MOG\( \text{aa35–55} \)-induced EAE indistinguishable from C57BL/6 wild-type mice (9, 18, 19).

The apparent discrepancies on the CNS expression patterns reported for E- and P-selectin and their involvement in leukocyte recruitment across the BBB during EAE prompted us to reinvestigate the expression of endothelial selectins in the CNS of SJL and
Furthermore, we investigated the development of EAE in E/P-selectin-deficient mice of both mouse strains to avoid overlooking strain-specific differences, which have been suggested regarding the function of P-selectin in leukocyte trafficking to the CNS (20). In this study, we demonstrate that in healthy C57BL/6 and SJL mice, P-selectin protein can be detected in endothelial cells of meningeal but not parenchymal CNS blood vessels. During EAE, P-selectin is up-regulated in inflamed microvessels surrounded by inflammatory cuffs during EAE in SJL (C) and C57BL/6 (G) mice. Adjacent control sections were stained for vWF (B, D, F, and H). Hematoxylin counterstain. Representative tissue sections from a SJL mouse sacrificed at day 17 p.i. with a clinical score of 1 and a C57BL/6 mouse sacrificed at day 16 p.i. with a clinical score of 1 are shown. Bar, 20 μm.

C57BL/6 mice during health and EAE. Furthermore, we investigated the development of EAE in E/P-selectin-deficient mice of both mouse strains to avoid overlooking strain-specific differences, which have been suggested regarding the function of P-selectin in leukocyte trafficking to the CNS (20). In this study, we demonstrate that in healthy C57BL/6 and SJL mice, P-selectin protein can be detected in endothelial cells of meningeal but not parenchymal CNS blood vessels. During EAE, P-selectin is up-regulated in inflamed microvessels surrounded by inflammatory cells, irrespective of their meningeal or parenchymal localization. Stronger immunostaining for P-selectin in inflamed CNS microvessels of C57BL/6 as compared with SJL mice suggested a stronger P-selectin expression in C57BL/6 mice. C57BL/6 and SJL mice deficient for E- and P-selectin developed EAE indistinguishable from the respective wild-type mice. Absence of E- and P-selectin did not influence the activation of myelin-specific T cells nor the composition of the cellular infiltrates in the CNS during EAE. As we did not detect E-selectin protein in CNS blood vessels in wild-type SJL and C57BL/6 mice during EAE, we asked whether endothelial cell-specific tetracycline (TET)-inducible expression of E-selectin in double-transgenic C57BL/6 mice established in our laboratory (Deutsch, U., T. M. Schlaeger, B. Dehouck, A. Döring, S. Tauber, W. Risau, and B. Engelhardt; manuscript in preparation) would influence EAE development. Although E-selectin protein was expressed at the BBB in healthy and diseased double-transgenic mice, its presence did not have any impact on the localization of inflammatory cuffs and the development of EAE. Taken together, our data demonstrate that E- and P-selectin are dispensable for inflammatory cell recruitment across the BBB and the development of EAE.

**Materials and Methods**

**Mice**

SJL mice were obtained from Taconic Farms and Harlan Breeders and C57BL/6 mice were obtained from Harlan Breeders. E/P-selectin-deficient mice were provided by R. O. Hynes (Massachusetts Institute of Technology, Cambridge, MA) and backcrossed into either SJL or C57BL/6 background for at least 10 or 8 generations, respectively, before analysis. Double-transgenic mice capable of TET-inducible expression of E-selectin in double-transgenic C57BL/6 mice established in our laboratory (Deutsch, U., T. M. Schlaeger, B. Dehouck, A. Döring, S. Tauber, W. Risau, and B. Engelhardt; manuscript in preparation) would influence EAE development. Although E-selectin protein was expressed at the BBB in healthy and diseased double-transgenic mice, its presence did not have any impact on the localization of inflammatory cuffs and the development of EAE. Taken together, our data demonstrate that E- and P-selectin are dispensable for inflammatory cell recruitment across the BBB and the development of EAE.

**FIGURE 3.** P-selectin is up-regulated in the vascular endothelium in the CNS during EAE in SJL and C57BL/6 mice. During EAE, increased immunostaining for P-selectin can be detected in endothelial cells surrounded by inflammatory infiltrates in meningeal areas in SJL (A) as well as in C57BL/6 (E) mice. Additionally, positive immunostaining for P-selectin was observed in endothelial cells of parenchymal vessels surrounded by inflammatory cuffs during EAE in SJL (C) and C57BL/6 (G) mice. Adjacent control sections were stained for vWF (B, D, F, and H). Hematoxylin counterstain. Representative tissue sections from a SJL mouse sacrificed at day 17 p.i. with a clinical score of 1 and a C57BL/6 mouse sacrificed at day 16 p.i. with a clinical score of 1 are shown. Bar, 20 μm.

**FIGURE 4.** Dotted P-selectin immunostaining in inflamed CNS vessels in mice afflicted with EAE localizes to CD41⁺ platelets. During EAE P-selectin (red, A and B) immunostaining can be detected on endothelial cells and CD41⁺ platelets (green, C and D) adhering to the vessel wall. In the overlay, double immunofluorescence shows P-selectin-positive platelets in yellow (E and F). Representative tissue sections from a SJL mouse sacrificed at day 16 p.i. with a clinical score of 1 and a C57BL/6 mouse sacrificed at day 22 p.i. with a clinical score of 0.5 are shown. Bar, 20 μm.
expression of both the reporter LacZ and E-selectin could be observed in endothelial cells in several tissues with the highest number of positive vessels in the brain (Deutsch, U., T. M. Schlaeger, B. Dehouck, A. Döring, S. Tauber, W. Risau, and B. Engelhardt; submitted for publication). Mice were housed in our own animal facility under specific pathogen-free conditions in individually ventilated cages. The genotype of mice was confirmed by PCR before and after the experiments. All animal procedures were performed in accordance with the Swiss legislation on the protection of animals and approved by the respective government authorities.

**Antibodies**

The IgG fraction of a polyclonal rabbit anti-mouse P-selectin Ab, raised against a P-selectin-Ig fusion protein (21), was purified by affinity chromatography and used in a 1/2000 dilution. Specificity of the Ab was confirmed by immunoprecipitation of [35S]methionine/cysteine-labeled P-selectin from inflamed brain endothelioma cells (21) and by negative immunostaining of inflamed brain and spinal cord sections from E/P-selectin-deficient mice and lack of staining of LPS-stimulated E/P-selectin-deficient brain endothelioma cells. Rabbit anti-human von Willebrand factor (vWF) Ab was purchased from Dako Diagnostic and used at 1/350 in immunohistochemistry and 1/800 in immunofluorescence. Rabbit anti-human von Willebrand factor (vWF) Ab was purchased from Dako Diagnostic and used at 1/350 in immunohistochemistry and 1/800 in immunofluorescence. Anti-mouse CD28, Gr-1 (anti-mouse Ly-6G), B220 (anti-mouse CD45R), Lyt.2 (anti-mouse CD8), FITC-conjugated rat anti-mouse CD41 were purchased from BD Pharmingen and used at a final concentration of 10 μg/ml. 7/4 (anti-mouse neutrophil differentiation Ag) and F4/80 were purchased from AbD Serotec. Anti-mouse CD3e and Cy3-conjugated goat anti-rabbit were purchased from Milan Analytica and used at a dilution of 1/200. UZ4 and UZ7 (anti-mouse E-selectin) were provided by R. Hallmann (University of Münster, Münster, Germany). Mec13.3 (anti-mouse PECAM-1) was a gift of Dr. E. Dejana (University of Milan, Milan, Italy). Supernatants of the hybridomas Hermes-1 (9B5, anti-human CD44, used as an isotype-matched control), 10E9.6 (anti-mouse E-selectin), RB40.34 (anti-mouse P-selectin), FD441.8 (anti-mouse LFA-1), M1/9 (anti-mouse CD4), and PS/2 (anti-mouse α4 integrin), and GK1.5 (anti-mouse CD4) were produced in our own laboratory and used undiluted.

**Immunohistology and immunofluorescence**

Mice were anesthetized with isoflurane (Baxter; Arovet) and perfused with 1% formaldehyde (Grogg Chemie) in PBS through the left ventricle of the heart. Brains and spinal cords were removed, embedded in Tissue-Tek (OCT compound; Haslab) and snap-frozen in a dry ice/isopentane bath (Grogg Chemie). Cryostat sections (6 μm) were air-dried overnight, acetone-fixed, and stained for immunohistology using a three-step immunoperoxidase staining kit (Vectastain; Reactolab) according to the manufacturer’s protocol exactly as described before (18). For immunofluorescence staining, sections were blocked for 20 min with skimmed milk, incubated for 1 h each with primary and secondary Ab diluted in skimmed milk, with TBS washing steps in between. After a final TBS wash, sections were mounted in Mowiol (Calbiochem).

**FIGURE 5.** Lack of E- and P-selectin does not affect Ag-induced T cell proliferation in SJL and C57BL/6 mice. A, Line diagrams show PLP<sub>139–151</sub>-induced T cell proliferation in primary cultures of draining lymph node lymphocytes removed from wild-type and E/P-selectin-deficient SJL mice 10 days after immunization with PLP<sub>139–151</sub>/CFA for increasing PLP<sub>139–151</sub> concentrations. B, Line diagrams show MOG<sub>35–55</sub>-induced T cell proliferation in primary cultures of draining lymph node lymphocytes removed from wild-type and E/P-selectin-deficient C57BL/6 mice 10 days after immunization with MOG<sub>35–55</sub>/CFA for increasing MOG<sub>35–55</sub> concentrations. Polyclonal T cell proliferation induced by Con A was indistinguishable in wild-type and E/P-selectin-deficient mice (data not shown). Proliferation normalized against baseline proliferation is shown as incorporation of [3H]thymidine; bars represent mean ± SD (n = 3).

**FIGURE 6.** E/P-selectin-deficient SJL mice develop active EAE indistinguishable from wild-type SJL mice. Average disease scores ± SD (A) and average weight change ± SD (B) assessed daily following immunization with PLP<sub>139–151</sub> are shown. Values represent three mice per group. One representative experiment of four with a total number of 33 E/P-selectin<sup>−/−</sup> SJL mice and 42 SJL wild-type mice examined is shown. Overall disease incidence was 100% for the E/P-selectin<sup>−/−</sup> SJL mice and 90 ± 16% for the wild-type SJL mice.
FACS analysis of inflammatory cells isolated from the brain and spinal cord

Inflammatory cells from the CNS were isolated as described previously (22, 23). Briefly, anesthetized mice were perfused with 15–20 ml of PBS (4°C) to wash out leukocytes present within the blood vessels. Brain and spinal cord tissues were carefully homogenized between glass slides and digested with collagenase VIII (0.2 mg/ml; Sigma-Aldrich) at 37°C for 30 min in the presence of DNase I (1 U/ml). Inflammatory cells were isolated by a Percoll (Amersham Biosciences) gradient (50% and 30% isotonic Percoll; 4°C, 30 min, 1300 × g). Cells were washed twice; immunofluorescence staining with directly labeled Abs and two-color FACS analysis for cell surface Ags was performed exactly as described previously (16).

Induction of EAE in C57BL/6 and SJL mice

Active EAE was induced by immunizing 8- to 12-wk-old female C57BL/6, E/P-selectin-deficient C57BL/6 mice, and TET-inducible E-Sele/LacZ C57BL/6 mice with 200 µg of MOG<sub>aa35–55</sub> in CFA (List; Santa Cruz Biotechnology) supplemented with 4 mg/ml nonviable, desiccated Mycobacterium tuberculosis (H37RA; Difco/BD Biosciences/BD Clontech) s.c. A total of 300 ng of pertussis toxin from Bordetella pertussis (List; LabForce; Santa Cruz Biotechnology) supplemented with 4 mg/ml nonviable, desiccated M. tuberculosis (H37RA; Difco/BD Biosciences/BD Clontech) s.c. A total of 3 × 10<sup>8</sup> organisms of heat-killed B. pertussis (provided by Crucell-Berna Biotech) per mouse was administered i.v. in 0.1 ml of PBS at days 1 and 3 p.i. In agreement with the local government, assessment of clinical disease activity was performed twice daily as described previously (18) using a 4-point-scoring system as follows: 0, healthy; 0.5, limp tail; 1, hind leg paraparesis; 2, hind leg paraplegia; 3, hind leg paraplegia with incontinence. Note, at clinical score 2, the mice are still motile, whereas at clinical score 3, motility of animals begins to be impaired.

T cell proliferation

Wild-type and E/P-selectin-deficient SJL and C57BL/6 mice were immunized exactly as described above except for the omission of injecting B. pertussis toxin or organisms. At day 10 p.i., mice were sacrificed and draining lymph nodes were removed. Single-cell suspensions were produced and cells were seeded in 96-well plates at 2 × 10<sup>5</sup> cells/well in RPMI 1640 supplemented with 10% FCS (Invitrogen Life Technologies/LuBioScience), 10 U/ml penicillin/streptomycin, 2 mM l-glutamine, 1% (v/v) nonessential amino acids, 1 mM sodium pyruvate, and 0.05 mM 2-ME (Grogg Chemie). PLP<sub>aa139–151</sub> was added in the concentrations of 1, 10, 50, or 100 µg/ml to test Ag-specific T cell proliferation in SJL mice, whereas MOG<sub>aa35–55</sub> was added in the concentrations of 1, 10, or 100 µg/ml to test Ag-specific T cell proliferation in C57BL/6 mice. T cell proliferation induced by the mitogen Con A (2.5 µg/ml) was used as positive control for T cell proliferation. All samples

Table I. Semiquantitative analysis of the number of inflammatory infiltrates in brain and spinal cord during EAE

<table>
<thead>
<tr>
<th>Size of Infiltrate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Meningeal localization</th>
<th>Parenchymal localization</th>
<th>Spinal Cord</th>
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<tbody>
<tr>
<td></td>
<td>Small</td>
<td>Medium</td>
<td>Large</td>
</tr>
<tr>
<td>SJL wild-type, n = 2 mice</td>
<td>7.5 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.5 ± 0.7</td>
<td>4 ± 1.4</td>
</tr>
<tr>
<td>SJL E/P-selectin−/−, n = 4 mice</td>
<td>11.5 ± 7.4</td>
<td>13.3 ± 7.3</td>
<td>17.5 ± 14.4</td>
</tr>
<tr>
<td>C57BL/6 wild-type, n = 2 mice</td>
<td>3.5 ± 3.5</td>
<td>3.5 ± 2.1</td>
<td>5 ± 1.4</td>
</tr>
<tr>
<td>C57BL/6 E/P-selectin−/−, n = 4 mice</td>
<td>3.5 ± 1.9</td>
<td>4 ± 1.2</td>
<td>3 ± 1.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Animals with a mean clinical score of 0.7 ± 0.25 between days 13 and 17 p.i. were included into this analysis. CD45<sup>b</sup> inflammatory infiltrates around PECAM-1<sup>b</sup> blood vessels were counted in 6-µm frozen tissue sections immunostained for PECAM-1 or CD45. Three coronal brain sections from the cerebellum, the middle, and the frontal brain were mounted in parallel and counted as one section. The spinal cord was divided in three parts; three longitudinal sections of the lower, middle, and upper spinal cord were mounted in parallel and counted as one section.

<sup>b</sup>Small-sized inflammatory infiltrates <150 µm in diameter; medium-sized infiltrates 150–300 µm in diameter; large size infiltrates >300 µm in diameter. The latter category also includes those areas where several cuffs have fused to one large inflammatory area.
were plated as triplicates. [\(^{3}\text{H}\)]Thymidine (\(^{3}\text{H}\)dT, 1 µCi/ml) was added 16 h before harvesting the cultures on glass-fiber filters using a cell harvester (Inotech) and incorporation of [\(^{3}\text{H}\)]dT was measured by liquid scintillation counting.

**Results**

**P-selectin is expressed in endothelial cells of meningeal blood vessels in the CNS of healthy SJL and C57BL/6 mice**

The expression of P-selectin protein in the brain and spinal cord of healthy SJL and C57BL/6 mice was investigated by immunohistochemistry on frozen tissue sections using a polyclonal rabbit anti-mouse P-selectin Ab. Positive immunostaining for P-selectin was detected in meningeal vessels in brains and spinal cords of C57BL/6 and SJL mice (Figs. 1, A and E). In addition, positive immunostaining for P-selectin could be detected in blood vessels at the base of the choroid plexus in C57BL/6 mice but not in SJL mice (Fig. 1). Occasionally, dotted P-selectin immunostaining was observed in the lumen of CNS parenchymal vessels of healthy SJL and C57BL/6 mice (Figs. 1C, 2C, and 2G). Double-immunofluorescence staining for CD41 showed that in healthy C57BL/6 and SJL mice dotted P-selectin immunostaining detected within parenchymal CNS vessels was localized to CD41-positive platelets adhering to the CNS endothelial cells despite the perfusion of the mice before tissue preparation (Fig. 2). In C57BL/6 but not in SJL mice, P-selectin expression could be detected on rare parenchymal CNS microvessels (Fig. 1G). Thus, in healthy C57BL/6 and SJL mice P-selectin is preferentially expressed in meningeal but not or rarely on parenchymal CNS vessels. In accordance to our previous results, the monoclonal anti-P-selectin Ab RB40.34 and the monoclonal anti-E-selectinAbs UZ4, UZ7, and 10E9.6 did not detect expression of P- and E-selectin protein, respectively, in the brain and spinal cord of healthy mice (data not shown; Ref. 16).

**P-selectin is up-regulated in CNS blood vessels of SJL and C57BL/6 mice during EAE**

To investigate whether the expression of E- and P-selectin on CNS blood vessels changes during EAE, we performed immunohistochemistry on frozen brain and spinal cord sections from SJL and C57BL/6 mice afflicted with EAE. The polyclonal anti-P-selectin Ab detected up-regulated expression of P-selectin in inflamed meningeal microvessels surrounded by inflammatory cells in SJL (Fig. 3A) and C57BL/6 (Fig. 3E) mice. Additionally, during EAE expression, P-selectin was induced in parenchymal CNS vessels surrounded by inflammatory cuffs in both mouse strains (Fig. 3). Interestingly, immunostaining for P-selectin in inflamed CNS parenchymal vessels always appeared stronger in C57BL/6 mice than in SJL mice. To define the source of P-selectin, i.e., endothelial cells vs platelets, double immunofluorescence stainings for CD41 were performed and demonstrated that during EAE, P-selectin immunostaining was due to endothelial and platelet P-selectin (Fig. 4). In accordance with our previous results, the monoclonal anti-P-selectin Ab RB40.34 and the monoclonal anti-E-selectin Abs UZ4, UZ7, and 10E9.6 did not detect expression of P- and E-selectin protein, respectively, in the brain and spinal cord of C57BL/6 and SJL mice during EAE (data not shown; Ref. 16).

**Myelin-specific T cell activation is not impaired in E/P-selectin-deficient SJL and C57BL/6 mice**

As P-selectin is up-regulated on inflamed CNS endothelium during EAE and we may have failed to detect expression of E-selectin due to the lack of a polyclonal anti-E-selectin Ab, we next asked whether the development of EAE is impaired in C57BL/6 or SJL mice in the absence of both E- and P-selectin. To this end, E/P-selectin-deficient mice were backcrossed into the EAE-susceptible SJL and C57BL/6 strains and the development of EAE was investigated. As expected, E/P-selectin-deficient mice developed EAE with a clinical score similar to that of the wild-type SJL mice. This suggests that the lack of P-selectin does not interfere with the recruitment of inflammatory cells across the BBB during EAE in SJL mice.
Lack of E- and P-selectin does not affect the composition of CD45\(^+\) cellular infiltrates in the brains and spinal cords of SJL mice during EAE. Inflammatory cells were isolated from the brains (A) and spinal cords (B) of wild-type and E/P-selectin-deficient SJL mice (average disease score in both groups = 0.7 ± 0.25) at day 17 p.i. during active clinical EAE. Cellular composition of the CD45\(^+\) inflammatory cell infiltrates was investigated by immunofluorescence staining and FACS analysis. Inflammatory cells are scatter gated and density blots of double immunofluorescence stainings for CD45 and the respective subpopulation markers (CD4\(^+\) T cells, CD8\(^+\) T cells, Mac-1\(^+\) macrophages, B220\(^+\) B cells, Gr-1\(^+\) granulocytes) are shown. Microglial cells, which are coisolated by this protocol, can easily be distinguished from the CD45\(^{high}\) inflammatory cell population by their characteristically lower staining for CD45 and their positive staining for Mac-1. The percentages given on the top right of the density blots refer to the percentage of cells staining positive for the respective marker among the CD45\(^{high}\) inflammatory cell population.

To evaluate whether lack of E- and P-selectin has any influence on the number or localization of cellular infiltrates present in the CNS of wild-type and E/P-selectin-deficient SJL and C57BL/6 mice during EAE, we performed a semiquantitative analysis counting the number of small, medium-sized, and large CD45\(^+\) inflammatory foci present in meningeal and parenchymal areas of the brain and in the spinal cord during EAE. Although we observed significant differences in the number of inflammatory foci present during EAE in the C57BL/6 vs SJL mouse model, within each mouse strain the absence of E- and P-selectin had neither an effect on the localization nor on the size of CD45\(^+\) inflammatory infiltrates present in the CNS during EAE (Table I). To address whether lack of E- and P-selectin may have any influence on the cellular composition of the inflammatory infiltrates, we first performed immunostainings of frozen brain and spinal cord sections of mice suffering from EAE. CD45\(^+\) cellular infiltrates were composed of comparable numbers of Mac-1\(^+\) macrophages, CD4\(^+\) T cells, scattered Gr-1\(^+\) granulocytes, and PSG1L\(^+\) inflammatory cells in the brains and spinal cords of both, wild-type and E/P-selectin-deficient SJL (Fig. 8) and C57BL/6 mice (data not shown) during the first clinical episode of EAE but also at later stages during the disease, i.e., at day 44 p.i. in the SJL model and at day 58 p.i. in compared with E/P-selectin-deficient SJL mice. Similarly, induction of EAE by immunization with MOG\(_{aa\,35-55}\) in CFA induced clinical EAE in both wild-type and E/P-selectin-deficient C57BL/6 mice. In six individual experiments, investigating a total of 25 wild-type and 29 E/P-selectin-deficient C57BL/6 mice, we did not detect any significant difference in the day of onset of clinical EAE, the disease course, or the severity of EAE in C57BL/6 wild-type mice compared with E/P-selectin-deficient C57BL/6 mice (Fig. 7). Even when following the EAE course until day 44 p.i. in the SJL mouse model and until day 58 p.i. in the C57BL/6 mouse model, we failed to detect any difference comparing wild-type and E/P-selectin-deficient mice. Taken together, these data demonstrate that E- and P-selectin are not required for inflammatory cell recruitment across the BBB and the development of clinical EAE in both SJL and C57BL/6 mice.
The C57BL/6 model. The number of Gr-1$^+$ granulocytes detected in CD45$^+$ cellular infiltrates in the CNS tissue sections of C57BL/6 mice seemed higher when compared with that observed in inflammatory infiltrates in the CNS of SJL mice. This observation was in fact confirmed when CD45$^+$ inflammatory cells were isolated from the brains and spinal cords of wild-type and E/P-selectin-deficient C57BL/6 and SJL mice and their subcellular composition was quantified by two-color-FACS analysis (Fig. 9 and Table II). Compared with SJL mice, C57BL/6-mice harbor about twice as many Gr-1$^+$ CD45$^{high}$ granulocytes within their CD45$^{high}$ inflammatory cell infiltrates in their brains and spinal cords during EAE. Neither in C57BL/6 (Table II) nor in SJL mice (Fig. 9) did the presence or absence of E- and P-selectin demonstrate any influence in the percentage of CD3$^+$ T cells, CD4$^+$ T cells, CD8$^+$ T cells, Mac-1$^+$ macrophages, B220$^+$ B cells, and Gr-1$^+$ granulocytes infiltrating the brains and spinal cords during EAE.

Taken together, the absence of E- and P-selectin did neither exert any influence on the number and localization of inflammatory cuffs within the CNS nor on the recruitment of different inflammatory cell subsets across the BBB during EAE.

Endothelial cell-specific TET-induced expression of E-selectin at the BBB does not influence the development of EAE

Based on our previous detection of E-selectin mRNA in inflamed CNS microvessels isolated from C57BL/6, but not from SJL, mice suffering from EAE by gene array analysis (24), we considered the possibility that we failed to detect E-selectin protein in CNS microvessels during EAE in C57BL/6 mice due to a failure to detect low levels of E-selectin protein with the

<table>
<thead>
<tr>
<th>Brain, wild-type</th>
<th>CD3 % ±</th>
<th>CD4 % ±</th>
<th>CD8 % ±</th>
<th>Mac-1 % ±</th>
<th>B220 % ±</th>
<th>Gr1 % ±</th>
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<tbody>
<tr>
<td></td>
<td>60 ± 3</td>
<td>38.8 ± 1.6</td>
<td>11.7 ± 5</td>
<td>32 ± 3.5</td>
<td>18 ± 0.8</td>
<td>31.8 ± 9.3</td>
</tr>
<tr>
<td>Brain, E/P-selectin$^{-/-}$</td>
<td>60.1 ± 1.9</td>
<td>41.8 ± 2.1</td>
<td>7.8 ± 2.4</td>
<td>27.6 ± 3.8</td>
<td>19.7 ± 0.9</td>
<td>27.3 ± 7.1</td>
</tr>
<tr>
<td>Spinal cord, wild-type</td>
<td>58.3 ± 7.5</td>
<td>42.6 ± 2.1</td>
<td>10.9 ± 3.9</td>
<td>24.8 ± 5.8</td>
<td>14.3 ± 3.3</td>
<td>25.6 ± 7.4</td>
</tr>
<tr>
<td>Spinal cord, E/P-selectin$^{-/-}$</td>
<td>66.3 ± 14.4</td>
<td>46.2 ± 18.6</td>
<td>9.3 ± 2.5</td>
<td>22.4 ± 10.4</td>
<td>17.9 ± 3.9</td>
<td>21.2 ± 9.5</td>
</tr>
</tbody>
</table>

*a* A total number of 17 C57BL/6 wild-type and 17 E/P-selectin$^{-/-}$C57BL/6 were included into this study performed in three independent EAE experiments. Inflammatory cells were isolated on day 15 or 16 p.i. The average disease score was 0.74 ± 0.07 for the C57BL/6 wild-type and 0.95 ± 0.4 for the E/P-selectin$^{-/-}$C57BL/6 mice and is considered not significant.

*b* The percentage of CD45$^{high}$ inflammatory cells excluding CD45$^{low}$ microglial cells as determined by FACS analysis is given.

![FIGURE 10](http://www.jimmunol.org/)

**FIGURE 10.** TET-induced expression of E-selectin at the BBB does not influence the development of active EAE in C57BL/6 mice. A, TET-induced E-selectin can be detected in CNS vessels in the presence (arrows) or absence (arrowhead) of perivascular CD45$^+$ inflammatory cells in the inflamed brain of double-transgenic (A) but not of single-transgenic or wild-type C57BL/6 mice (data not shown) during EAE. Immunoperoxidase staining, hematoxylin counterstain. Representative tissue sections from a double-transgenic C67BL/6 mouse sacrificed at day 22 p.i. with a clinical score of 0.5 are shown. Bar, 20 μm. B and C, TET-induced E-selectin at the BBB does not influence the development of EAE. Average disease scores ± SD (B) and average weight change ± SD (C) assessed daily following immunization with MOG$_{35-55}$ are shown. Values represent three mice per group. One representative experiment of five with a total number of 32 double-transgenic mice, 51 single-transgenic mice, and 36 wild-type mice examined is shown. Overall disease incidence was 98.4 ± 3.6% for double-transgenic, 97.4 ± 5.6% for single-transgenic, and 95.6 ± 9.8% for wild-type C57BL/6 mice in these experiments.
Abs available to us. Therefore, we asked whether transgenic expression of E-selectin at the BBB would influence inflammatory cell recruitment across the BBB and thus EAE development. To this end, we established double-transgenic C57BL/6 mice with TET-inducible endothelial cell-specific expression of E-selectin using the TET-OFF system (Deutsch, U., T. M. Schlaeger, B. Dehouck, A. Döring, S. Tauber, W. Risau, and B. Engelhardt; manuscript in preparation). In healthy double transgenic but not single transgenic C57BL/6 mice, E-selectin can readily be detected on CNS blood vessels by immunohistology with the Abs available to us (Deutsch et al., submitted for publication). Immunization of double-transgenic, single-transgenic, and nontransgenic C57BL/6 littermates with MOGaa35–55 in CFA induced clinical EAE in all three groups in an indistinguishable manner. In five individual experiments, comparing a total of 32 double-transgenic, 51 single-transgenic, and 36 nontransgenic/wild-type C57BL/6 mice, we did not detect any significant difference in the day of onset of clinical EAE, the disease course, or the severity of EAE in C57BL/6 control mice compared with double-transgenic C57BL/6 mice with TET-induced E-selectin expression at the BBB (Fig. 10). Immunohistological analysis of the inflamed brains and spinal cords of double-transgenic mice suffering from EAE did not show a correlation with E-selectin expression and localization of the inflammatory cuffs (Fig. 10). Thus, TET-induced expression of E-selectin at the BBB did neither influence inflammatory cell recruitment across the BBB nor the development of clinical EAE (Fig. 10).

Discussion

The involvement of endothelial E- and P-selectin and their common ligand PSGL-1 in leukocyte recruitment across the BBB during EAE has been a controversial issue during the past decade. Discrepant observations were already made regarding the presence or absence of E- and P-selectin in CNS microvessels during EAE. Using a polyclonal anti-P-selectin Ab (21), our present study confirms previous observations showing constitutive expression of P-selectin in meningeal but not parenchymal blood vessels (17) in the brains of healthy mice. Also, in accordance to previous reports (8), we detected up-regulated expression of P-selectin in venules surrounded by inflammatory infiltrates—irrespective of their localization in the meninges or the parenchyma—in the CNS of both SJL and C57BL/6 mice during EAE. It should be noted that part of the P-selectin immunostaining observed in CNS blood vessels in our study was due to the presence of CD41+ platelets adhering to the vessel walls despite the perfusion of the mice before tissue preparation. The present detection of P-selectin in inflamed CNS vessels during EAE using a polyclonal anti-P-selectin Ab allows us to assign our previous failure to detect P-selectin immunostaining in CNS microvessels during EAE (16) to the usage of the function blocking monoclonal rat anti-mouse P-selectin Ab RB40.34. As i.v. injection of a radiolabeled version of this Ab detects P-selectin in brain vessels of C57BL/6 mice suffering from EAE (8), RB40.34 seems to detect native P-selectin in vivo, whereas in frozen tissue sections of the inflamed brain and the spinal cord its epitope on P-selectin may be masked. In contrast to P-selectin, we did not detect any positive immunostaining for E-selectin, neither in endothelial cells in the healthy CNS nor on inflamed CNS vessels during EAE, although the anti-E-selectin Abs used in this study detected TET-induced E-selectin on CNS blood vessels of double transgenic TET-E-selectin-C57BL/6 mice. The observation that E-selectin expression can be induced in meningeal brain vessels of mice upon cytokine injection (25) (10) suggests that E-selectin is induced at least in meningeal blood vessels under certain inflammatory conditions, but obviously not during EAE.

E- and P-selectin-mediated leukocyte-endothelial interaction has been directly observed in superficial brain venules by several intravital microscopy studies performed in mice using a cranial window preparation or through the intact skull of young mice. In these experimental setups, Ab inhibition studies demonstrate that CD41+ Th1 cells roll via PSGL-1 on endothelial E- and P-selectin in superficial brain vessels of SJL mice pretreated with TNF-α or LPS (10). In accordance with our present findings detecting P-selectin but not E-selectin in inflamed CNS microvessels during EAE, P-selectin-, but not E-selectin-, mediated rolling of endogenous leukocytes was observed in superficial brain vessels of C57BL/6 mice suffering from EAE (8). The important role of P-selectin in mediating leukocyte rolling in this vascular compartment during EAE has been further supported by the recent observation that leukocyte rolling is completely abrogated in superficial brain vessels of P-selectin-deficient mice (9).

In light of the above-described findings which demonstrate the functional expression of E- and P-selectin in superficial brain vessels, our present results demonstrating that EAE develops in E/P-selectin-deficient mice indistinguishably from wild-type mice, and that TET-induced expression of E-selectin in CNS microvessels fails to have any influence on CNS inflammation during EAE are quite surprising. Our observations are, however, in accordance with previous findings made by us and others, demonstrating that targeting of either E- or P-selectin (alone or both together) or their common ligand PSGL-1 by blocking Abs does not influence inflammatory cell recruitment into the CNS and the development of EAE in the SJL or the C57BL/6 mouse model (9, 16, 18). Additionally, three independent studies demonstrated that PSGL-1-deficient C57BL/6 mice develop MOGaa35–55-induced EAE indistinguishable from wild-type C57BL/6 mice (9, 18, 19). Similarly, P-selectin-deficient C57BL/6 mice develop MOGaa35–55-induced EAE just like wild-type mice (9). Taken together, these data convincingly demonstrate that targeting PSGL-1 or its major endothelial ligands E- and P-selectin fails to block inflammatory cell trafficking into the CNS and consequently EAE pathogenesis in both the SJL and C57BL/6 mouse models.

In contrast, it has been shown that targeting leukocyte trafficking into the CNS by blocking α4 integrins blocks CNS inflammation and clinical EAE in a number of EAE models in SJL mice (3, 6), rats (2), and guinea pigs (5). Intravital microscopy studies have confirmed that α4 integrins contribute to rolling (8) and firm adhesion (9) of endogenous leukocytes in superficial mouse brain vessels during EAE in the C57BL/6 mouse. Additionally, in vivo homing studies tracing fluorescently labeled lymphocytes into the CNS parenchyma of mice suffering from EAE demonstrated that Abs blocking α4 integrins but not Abs blocking P-selectin inhibit inflammatory cell recruitment across the BBB into the brain parenchyma (9). These findings underline the predominant role of α4 integrin in mediating leukocyte trafficking across the BBB into the CNS parenchyma during EAE.

Considering the apparently discrepant observations when comparing the adhesion molecule contribution to leukocyte trafficking in superficial brain vessels, on one hand to the lack of success in therapeutic targeting of E/P-selectin and PSGL-1 in EAE on the other hand, one may ask whether the therapeutic efficacy of targeting α4 integrins in EAE is truly dependent on the inhibition of leukocyte trafficking across the BBB. In fact, as we and others have previously demonstrated that α4 integrins are involved in T cell activation (6, 26), some other α4 integrin-dependent pathomechanisms entirely independent of leukocyte trafficking may contribute to the therapeutic success of targeting α4 integrins in
EAE. Alternatively, one may question the relevance of leukocyte trafficking across superficial brain vessels for EAE pathogenesis. When performing intravital microscopy of the brain, researchers have usually observed superficial brain microvessels localized in the pia mater. Although pial microvessels form a functional BBB with diffusion characteristics similar to the BBB in the CNS parenchyma, they differ from parenchymal microvessels by the structure of their tight junctions and the lack of an astrocytic ensheathment (27). Interestingly, the differences between meningeal and parenchymal CNS blood vessel endothelial cells extend to differences in their expression patterns for P- and E-selectin. In the healthy CNS, meningeal blood vessel endothelial cells can be distinguished from those in the CNS parenchyma by their constitutive expression of P-selectin, which is absent from endothelial cells of parenchymal blood vessels (17, 28). Furthermore, injection of proinflammatory cytokines into mice induced expression of E-selectin in meningeal but not in parenchymal CNS blood vessel endothelial cells (25). It is therefore tempting to speculate that during CNS inflammation, selectin-mediated leukocyte recruitment plays a predominant role in the meningeal but less so in the parenchymal compartment, where α₄ integrin-mediated trafficking mechanisms may predominate. Nevertheless, during EAE, selectin mediated leukocyte recruitment into the meninges will readily be replaced by other, most likely α₄ integrin-dependent mechanisms, as we did not observe reduced meningeal infiltrates in the CNS of E/P-selectin-deficient mice during EAE compared with wild-type mice. In accordance to its expression in both meningeal- and parenchymal endothelium of mice, E-selectin is not observed reduced meningeal infiltrates in the CNS of E/P-selectin-deficient mice to us. Therese Périmat, Matthias Meier, and Sorin Ciocan are acknowledged for expert technical assistance and Carsten Minten and Dr. Friederike Pfeiffer for their tireless help in daily scoring of the EAE experiments.

Acknowledgments

We are very grateful to Dr. Richard Hynes for providing the E/P-selectin-deficient mice to us. Therese Périmat, Matthias Meier, and Sorin Ciocan are acknowledged for expert technical assistance and Carsten Minten and Dr. Friederike Pfeiffer for their tireless help in daily scoring of the EAE experiments.

Disclosures

The authors have no financial conflict of interest.

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