P-Selectin Glycoprotein Ligand 1 Is Not Required for the Development of Experimental Autoimmune Encephalomyelitis in SJL and C57BL/6 Mice

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In multiple sclerosis and in its animal model experimental autoimmune encephalomyelitis (EAE), inflammatory cells migrate across the endothelial blood-brain barrier and gain access to the CNS. The involvement of P-selectin glycoprotein ligand 1 (PSGL-1) and of its major endothelial ligand P-selectin in this process have been controversial. In this study we demonstrate that although encephalitogenic T cells express functional PSGL-1, which can bind to soluble and immobilize P-selectin if presented in high concentrations, PSGL-1 is not involved in cell interaction with P-selectin expressing brain endothelial cells in vitro. Furthermore, neither anti-PSGL-1 Abs nor the lack of PSGL-1 in PSGL-1-deficient mice inhibits the recruitment of inflammatory cells across the blood-brain barrier or the development of clinical EAE. Taken together, our findings demonstrate that PSGL-1 is not required for the pathogenesis of EAE. The Journal of Immunology, 2005, 175: 1267–1275.

The CNS is considered an immuneprivileged site where the endothelial blood-brain barrier (BBB) tightly controls lymphocyte entry into the CNS. Under physiological conditions lymphocyte traffic into the CNS is low whereas during inflammatory diseases of the CNS, such as multiple sclerosis or in the animal model experimental autoimmune encephalomyelitis (EAE), a large number of circulating lymphocytes readily gain access to the CNS. EAE is a CD4+ T cell-mediated autoimmune disease of the CNS, which is initiated by autoaggressive T cells activated outside the CNS. These encephalitogenic T cell blasts enter the CNS parenchyma across the healthy BBB. Upon encounter of Ag, autoaggressive T cells initiate inflammation and the recruitment of inflammatory effector cells across the inflamed BBB into the CNS leading to edema and demyelination. Thus, the interaction of autoaggressive and inflammatory effector cells with the BBB is a critical step in the pathogenesis of EAE.

In general, lymphocyte recruitment across the vascular wall is regulated by the sequential interaction of different adhesion or signaling molecules on lymphocytes and endothelial cells lining the vessel wall (1). An initial transient contact of the circulating leukocyte with the vascular endothelium, generally mediated by adhesion molecules of the selectin family or alternatively by α4 integrins and their respective ligands, slows down the leukocyte in the bloodstream. With greatly reduced velocity the leukocyte rolls along the vascular wall allowing it to recognize chemokines displayed on the endothelial surface. Binding of a chemokine to its G protein-coupled receptor on the leukocyte surface results in a pertussis toxin-sensitive activation of integrins on the leukocyte surface. Activated integrins mediate the firm adhesion of the leukocytes to the vascular endothelium by binding to their endothelial ligands, which belong to the Ig superfamily. This ultimately leads to the extravasation of the leukocyte. Successful recruitment of circulating leukocytes into the tissue depends on the productive leukocyte/endothelial interaction during each of these sequential steps.

The mechanisms mediating leukocyte recruitment into the CNS are not completely understood. mAbs directed against α4 integrin have been shown to successfully interfere with the development of EAE in different animal models (2–5) and moreover to reverse the ongoing disease process (6). As the anti-α4 integrin treatment proved to block adhesion of inflammatory cells to inflamed cerebral venules in vitro (2, 7) and to substantially decrease the recruitment of inflammatory cells across the BBB in vivo, there is general agreement that there is a pivotal role for α4 integrins in leukocyte/BBB interaction during EAE (2, 5).

The predominant involvement of α4 integrins in leukocyte recruitment across the BBB during EAE does not exclude the contribution of other adhesion molecules in this process. The potential involvement of selectins in particular in T lymphocyte interaction with the BBB has been controversial. We have reported a lack of E- and P-selectin expression in CNS microvessels during EAE and demonstrated that anti-E- and anti-P-selectin Abs do not inhibit the development of EAE (8). More recent work of other laboratories using intravital microscopy demonstrated the involvement of E- and P-selectin and their ligand P-selectin protein ligand 1 (PSGL-1) in tethering and rolling of leukocytes or encephalitogenic T cells in superficial brain microvessels in vivo (9, 10). In contrast, when performing intravital microscopy of the spinal cord white matter, we found a lack of rolling and a predominant role of
α4 integrins in the initial capture and the firm adhesion of autoaggressive T cells within the spinal cord white matter microvasculature (11).

These apparent discrepancies prompted us now to investigate the functional expression of the homodimeric sialomucin PSGL-1 on encephalitogenic T cells and its potential involvement in inflammatory cell recruitment across the BBB during EAE. In this study we demonstrate that although encephalitogenic T cells express functional PSGL-1 on their surface as exemplified by the ability to bind purified P-selectin, PSGL-1 is not involved in interactions of these T cells with brain endothelium in vitro. Furthermore, PSGL-1 is not required for the development of EAE in SJL or C57BL/6 mice.

Materials and Methods

Mice

SJL mice were obtained from M&B and C57BL/6 mice were obtained from Harlan Winkelmann. PSGL-1-deficient C57BL/6 mice were described (12) and were continually crossed into C57BL/6 mice from Harlan Winkelmann. They were used for EAE experiments after seven additional generations of breeding into C57BL/6 in our mouse facility. The genotype of mice was confirmed by PCR.

Monoclonal Abs and P-selectin Ig chimera

The monoclonal rat anti-mouse PSGL-1 Abs 4RA10 (rat IgG1, blocking), 4R12 (rat IgG2a, nonblocking) (13), 2PH1 (rat IgG1, blocking) (14) as well as 5C7.8 (rat IgG1 anti-mouse endomucin) (15) and RB40.34 (rat IgG1, anti-mouse P-selectin) (16) and 6C7.1 (rat IgG1, anti-mouse VCAM-1) (17) were raised in the D. Vestweber laboratory. MK2.7 (rat IgG1, anti-mouse VCAM-1) (18) and 9B5 (rat IgG2a, anti-human CD44, used as a control) were a kind gift of E. C. Butcher (Stanford University School of Medicine, Stanford, CA). FD4/1 (rat LFA-1) was purchased from American Type Culture Collection, Mcl13.3 (anti-mouse PECAM-1/CD31) was a gift of Dr. E. Dejana (Istituto di Ricerche Farmacologiche, Milano, Italy) (19). Abs directed against B220, Gr-1, CD3e, Mac-1, Mac-1 FITC, CD8 and CD4, PE-labeled rat anti-mouse γ-IFN, as well as control rat IgG1, PE- rat IgG1, and rat IgG2a were purchased from BD Biosciences.

The P-selectin IgG fusion protein has been described before (20). Tig2 IgG fusion protein was kindly provided by Urban Deutsch and was used as a negative control (our unpublished observations).

Cells

The proteolipid protein (PLP)-specific Tp.1 effector/memory T cell lines SJL.PLP2 to SJL.PLP9 raised against the PLP peptide aa139 –153 were previously described in detail (5, 17).

The E-selectin/P-selectin−/− brain endothelioma cell line bEndEP.5 was established by infection of primary brain endothelial cells (derived from E-selectin double-deficient mice kindly provided by R. Hynes (Massachusetts Institute of Technology, Cambridge, MA) with a recombinant retrovirus coding for the polonya middle T oncogene as described (21, 22).

With the exception of the expression of E- and P-selectin, surface expression levels of vascular endothelial-cadherin, PECAM-1, ICAM-2, endoglin, and the mouse endothelial-specific Ag Meca-32 as well as inducibility of ICAM-1 and VCAM-1 on bEndEP.5 were found to be indistinguishable from that on wild-type endothelium cell lines such as bEnd5 (22).

Bone marrow-derived neutrophils and Chinese hamster ovary cells triple-transfected with mPC7 (murine PSGL-1, core 2 β1–6-G-glucosaminyltransferase VII) and fucosyltransferase (FUC-TVII) were used as positive control for optimal P-selectin binding. mPC7 cells have been previously described in detail (23, 24).

Induction of EAE and PLP-specific T cell proliferation

All animal experiments were performed in accordance with the requirements of the local government in Münster, Germany (permission number G15/2002) and Bern, Switzerland (permission number 55/04).

C57BL/6 mice and PSGL-1-deficient C57BL/6 mice were immunized sc. with 200 μg of myelin oligodendrocytic glycoprotein (MOG) aa35−55 in CFA supplemented with 4 mg/ml inactivated Mycobacterium tuberculosis (H37RA; Difco). At 1 and 3 days after immunization, 300 ng of pertussis toxin were applied i.p. Passively transferred EAE (tEAE) was induced in SJL mice by i.v. injection of 3 × 106 freshly activated syngeneic encephalitogenic CD4+ PLP-specific T line cells (5, 8). Clinical disease was checked daily and scored as follows: 0.5 (limp tail), 1 (hind leg weakness), 2 (hind leg paraparesis), 3 (hind leg paraparesis and incontinence). PLP-specific T cell proliferation was measured by the incorporation of [3H]thymidine as described (5).

Immunohistology, immunofluorescence, and quantitative analysis of inflammation

Immunohistology was performed exactly as described (7, 8). Severity of inflammation was assessed by counting the number of inflammatory cuffs having diameters ranging from 25 to 150 μm in CD45-immunostained brain and spinal cord sections under the microscope by a blinded observer. The area of the evaluated tissue sections was measured to calculate the number of inflammatory cuffs per mm2 of CNS tissue. For double immunofluorescence stainings, frozen tissue sections were incubated sequentially with rat anti-mouse PSGL-1, Cy3-labeled secondary goat anti-rat IgG, and anti-Mac-1 FITC, each at 10 μg/ml for 30 min each step in a humidified chamber, with PBS washes in between the single steps. Sections were mounted in Moviol (Calbiochem) and immediately analyzed.

Ab inhibition studies in EAE

For Ab inhibition studies mAbs were injected i.v. Endotoxin levels of mAb preparations were routinely determined by Fresenius or in our laboratory using the Endosafe IPT in vitro pyrogen test detection kit from Charles River Breeding Laboratories. Mice were fed Abs specifically with rat anti-mouse PSGL-1, Cy3-labeled secondary goat anti-rat IgG, and anti-Mac-1 FITC, each at 10 μg/ml for 30 min each step in a humidified chamber, with PBS washes in between the single steps. Sections were mounted in Moviol (Calbiochem) and immediately analyzed.

Flow cytometry

FACS analysis for cell surface Ags was performed exactly as previously described (8). For flow cytometric analysis of γ-IFN production by PLP-specific T cells brefeldin A (10 μg/ml) was added to the culture 5 h before harvesting the T cells. T cells were fixed (4% paraformaldehyde/PBS for 20 min at 20°C), washed in PBS, permeabilized (0.1% saponin/azide in PBS), and stained with PE-rat anti-mouse γ-IFN or the respective control Ab following the protocol by Austrup et al. (25). Flow cytometric analysis was performed on a FACSCalibur using CellQuest software (BD Biosciences).

Adhesion and transmigration assays with brain endothelioma cells

Adhesion and transmigration assays were performed in the presence of divalent cations in triplicate as described (17, 21, 22). Endotheliomas were stimulated with 5 nM TNF-α or 1 μg/ml LPS for 3–4 h to induce optimal P-selectin cell surface expression (22). Assays were repeated at least five times.

Adhesion assay to immobilized P-selectin IgG

Selectin Ig chimeras were coated (25–100 μg/ml) in 20 μl of PBS onto 18-field (diameter = 4 mm) glass slides at 4°C in a humidified chamber overnight. Fields were washed twice with PBS and blocked with 100% bovine calf serum for 30 min at 20°C. T cells were cultured in RPMI 1640/25 mM HEPES and 5% bovine calf serum (5 × 104 cells/ml). A total of 20 μl of cell suspension (1 × 105 cells/field) was incubated for 30 min at 20°C under shear (50 rpm on an IKA Laboratories rotating platform, model KS250 basic). Nonadherent cells were washed off by dipping the slides twice into PBS; adherent cells were fixed in 2.5% glutaraldehyde in PBS. For Ab inhibition assays, cells or slides were reincubated with mAb at 20 μg/ml for 20 min at 20°C, washed, and adhesion assays were performed as described. Samples were analyzed in triplicate. Assays were analyzed by video-associated light microscopy (NIH Image software, National Institutes of Health, Bethesda, MD) and bound cells per predefined field were determined by counting 5 fields per well. Assays were repeated at least five times.

Statistical analysis

Within each assay, parameters were tested at least in triplicate. Quantitative data are given as mean values ± SD. For analysis of differences between the assays, one-way ANOVA followed by unpaired Student’s t test and Tukey-Kramer correction for repeated measurements was performed using...
the Macintosh software Instat, with results considered as follows: significant, \( p < 0.05 \); very significant, \( p < 0.01 \); extremely significant, \( p < 0.001 \).

**Results**

*PSGL-1 is expressed in the CNS of mice afflicted with EAE*

To find out whether PSGL-1 expression can be detected in the brains and spinal cords of mice afflicted with EAE, we performed immunohistochemistry using three different anti-PSGL-1 Abs. During EAE immunostaining for PSGL-1 was observed on the majority of inflammatory cells localized within perivascular cuffs (Fig. 1A). Additionally immunostaining for PSGL-1 was observed in the choroid plexus and the CNS parenchyme resembling immunostaining for CD45 (Fig. 1B). PSGL-1-positive parenchymal cells were characterized as Mac-1-positive microglial cells by double-immunofluorescence staining (Fig. 1B). Thus, PSGL-1 might be involved in inflammatory cell recruitment into the CNS, whereas its up-regulated expression on microglial cells during EAE. Immunoperoxidase staining, hematoxylin counterstaining, and immunofluorescence staining are represented.

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**FIGURE 1.** A subpopulation of inflammatory cells and activated microglial cells express PSGL-1. Immunohistology of the spinal cord (A) or brain (B) taken from SJL mice suffering from EAE. A, Control staining as well as staining for PECAM-1, CD45, and PSGL-1 is shown. PSGL-1 is expressed on a majority but not on all CD45-positive inflammatory cells localized within the perivascular cuffs. Immunoperoxidase staining and hematoxylin counterstaining are represented. B, Outside of inflammatory cuffs up-regulated staining for CD45 was observed in the choroid plexus and the CNS parenchyme, with a similar staining pattern observed for PSGL-1, suggesting PSGL-1 expression on activated microglial cells. Double immunofluorescence staining confirmed PSGL-1 expression on Mac-1-positive activated microglial cells during EAE. Immunoperoxidase staining, hematoxylin counterstaining, and immunofluorescence staining are represented.

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**FIGURE 2.** Encephalitogenic T cells express PSGL-1 and can bind P-selectin. Expression of PSGL-1 on freshly activated PLP-specific T cell blasts (line SJL.PLP7) (A), mPC7 cells (B), and PMNs (C) as determined by FACS analysis using the mAb 4RA10 is shown. A, Overlays show negative staining using control IgG (thin line) and positive staining for PSGL-1 or P-selectin binding (thick line). After incubation with P-selectin IgG at a concentration of 50 \( \mu \text{g/ml} \) 15% of SJL.PLP7 demonstrated high binding of P-selectin, whereas 85% of SJL.PLP7 showed low binding of P-selectin IgG. In contrast, 100% of mPC7 or PMNs bound soluble P-selectin IgG at 10 \( \mu \text{g/ml} \) (B and C). Binding of P-selectin IgG to SJL.PLP7, mPC7, or PMNs was dependent on divalent cations as demonstrated by the loss of P-selectin binding in the presence of 2 mM EDTA, when at the same time surface expression of PSGL-1 remained unaffected. Cells are scatter gated on live cells.
Encephalitogenic T cells express functional PSGL-1

To define whether encephalitogenic T cells express PSGL-1 on their surface, we performed immunofluorescence stainings using three different mAbs directed against murine PSGL-1 (4RA10, 4RB12, 2PH1). Encephalitogenic PLP-specific T cells were found to display surface expression levels of PSGL-1 comparable to those observed on mPC7 cells or on bone marrow-derived murine neutrophils (Fig. 2). Surface expression levels of PSGL-1 did not change depending on the activation status of the T cells (data not shown). Selectin ligands including PSGL-1 require posttranslational modifications for selectin binding. To determine whether encephalitogenic T cells bind P-selectin we studied the binding of P-selectin IgG to encephalitogenic T cells as compared with mPC7 cells or bone marrow-derived neutrophils by FACS analysis. Binding of P-selectin IgG to encephalitogenic T cells could only be observed if P-selectin IgG was offered in high concentrations (30–50 μg/ml). Under these experimental conditions only a minor population of encephalitogenic T cell blasts (15%) were found to bind P-selectin IgG in high amounts whereas binding of P-selectin IgG to the majority of T cells was found to be very low (Fig. 2A). In contrast, P-selectin binding to mPC7 cells or bone marrow-derived neutrophils was already found to be optimal at lower concentrations of P-selectin IgG fusion protein (10–25 μg/ml) with 100% of the cells binding P-selectin homogeneously (Fig. 2, B and C). Dependence of P-selectin binding on the presence of divalent cations was confirmed by the loss of P-selectin binding to all three investigated cell populations in the presence of 2 mM EDTA, whereas detection of PSGL-1 protein surface expression remained unaffected (Fig. 2). Thus, although encephalitogenic T cells can bind soluble P-selectin, it is less efficient than P-selectin binding to mPC7 cells or polymorphonuclear granulocytes (PMNs).

Encephalitogenic T cells can bind to high concentrations of immobilized P-selectin in vitro

To determine whether P-selectin binding to encephalitogenic T cells is mediated via PSGL-1, we analyzed their binding to P-selectin IgG immobilized on glass slides. Optimal binding of mPC7 cells to immobilized P-selectin IgG could already be observed when 20 μl of 25 μg/ml fusion protein was used for coating (data not shown). T cell binding to immobilized P-selectin IgG was only observed at concentrations of 50 μg/ml and reached an optimum at 100 μg/ml. Under these experimental conditions encephalitogenic T cells were found to bind to P-selectin, which could be inhibited by the anti-P-selectin Ab RB40 (Fig. 3). PSGL-1 was found to be the major P-selectin ligand on encephalitogenic T cells as the PSGL-1 blocking mAbs 4RA10 and 2PH1 almost completely inhibited T cell binding. At the same time, the nonblocking mAb 4RB12 or the anti-LFA-1 Ab FD441.8 had no effect on T cell binding to immobilized P-selectin. Thus, provided P-selectin is available in high density, encephalitogenic T cells can bind via PSGL-1 to immobilized P-selectin.

FIGURE 3. Adhesion of PLP-specific T cell lines to P-selectin is mediated by PSGL-1. One representative experiment demonstrating the adhesion of encephalitogenic T cells (TC) to P-selectin IgG is shown. Adhesion to immobilized P-selectin (100 μg/ml added in 20 μl per 4 mm field) was completely inhibited in presence of mAbs directed against P-selectin (RB40.34; slide + Ab = preincubation of the P-selectin IgG-coated fields) or against PSGL-1 (4RA10, 2PH1; TC + Ab = preincubation of T cells), demonstrating that PSGL-1 is the major ligand for P-selectin on encephalitogenic T cells. The nonblocking anti-PSGL-1 mAb 4RB12 and the anti-LFA-1 mAb FD441.8 did not interfere with T cell adhesion to P-selectin. Error bars represent mean ± SD (n = 3). **, p < 0.01, Very significant compared with the respective controls. The depicted experiment is representative of ten similar experiments.

FIGURE 4. PSGL-1 is not involved in the adhesion of T cells to brain endothelium in vitro. One representative experiment of eight comparing the adhesion of encephalitogenic T cells to wild-type bEnd5 and E- and P-selectin-deficient bEndEP.5 stimulated with either TNF-α or LPS for 3 h is shown. As shown, the presence of P-selectin on LPS-stimulated bEnd5 does not increase the number of bound T cells when compared with LPS-stimulated E/P-selectin-deficient bEndEP.5. On both endothelial cells, adhesion of T cell lines can be dramatically inhibited by the mAb FD441.8 directed against LFA-1 but not with mAbs directed against PSGL-1. Error bars represent mean ± SD (n = 3).
PSGL-1 is not involved in adhesive interactions of T cells with brain endothelium in vitro

We next asked whether encephalitogenic T cells can use PSGL-1 to adhere to P-selectin on activated brain endothelial cells in vitro. Therefore we performed adhesion assays with freshly activated PLP-specific T cell blasts and the brain endothelium cell line bEnd5, which upon 3 h of stimulation with LPS or TNF-α expresses P-selectin on its surface (22). PLP-specific T cell blasts readily bound to stimulated bEnd5. Pretreatment of T cell blasts with a mAb directed against PSGL-1 had no effect on T cell adhesion to stimulated bEnd5 (Fig. 4). Supporting a lack of involvement of PSGL-1/P-selectin interaction in T cell adhesion to brain endothelium after 3 h of stimulation, T cell binding to E/P-selectin double-deficient bEndEP.5 was found to be indistinguishable from T cell adhesion to wild-type bEnd5 when compared within the same assay (Fig. 4). At the same time blocking LFA-1 significantly inhibited T cell adhesion to both, stimulated bEnd5 and bEndEP.5 (Fig. 4).

Comparing T cell migration across stimulated bEnd5 in the presence or absence of PSGL-1 blocking Abs confirmed a lack of involvement of PSGL-1 in this process (data not shown). Finally, the pretreatment of bEnd5 with the P-selectin blocking Ab RB40.34 had no influence on T cell adhesion to and migration across stimulated brain endothelium confirming the lack of involvement of PSGL-1/P-selectin binding during these processes (data not shown).

Anti-PSGL-1 Abs do not inhibit PLP-induced T cell proliferation or IFN-γ production

Adhesion molecules on autoaggressive T cells could be involved in the pathogenetic processes during EAE other than T cell migration into the CNS. Therefore we asked whether PSGL-1 is involved in the Ag-dependent activation of encephalitogenic T cells. PLP-specific T cells were restimulated with their specific PLP peptide using irradiated syngeneic splenocytes as APCs in the presence or absence of anti-PSGL-1 Abs. Blocking PSGL-1 did neither influence Ag-induced T cell proliferation (Fig. 5A) nor IFN-γ production (Fig. 5B).

Anti-PSGL-1 Abs do not inhibit the development of tEAE in the SJL mouse

To investigate whether, regardless of the lack of PSGL-1 contribution to T cell activation or T cell interaction with brain endothelium in vitro, PSGL-1 could still be involved in the pathogenesis of EAE, we investigated the effect of the PSGL-1 blocking mAb 4RA10 on the development of EAE in the SJL mouse. Only 30 μg of 4RA10 injected into a mouse were previously shown to be sufficient to completely block PSGL-1-mediated rolling of leukocytes in vivo (26). EAE was transferred by the injection of 3 × 10^6 syngeneic PLP-specific T cell blasts and animals were monitored daily for the development of clinical disease. In seven different experiments, repeated infusions of 4RA10 injected at dosages up to 400 μg/injection failed to reproducibly interfere with the onset of clinical EAE or the recruitment of inflammatory cells into the CNS when compared with animals treated with control Abs (Fig. 6). Quantitative assessment of the number of perivascular inflammatory cuffs present in CD45-immunostained frozen brain and spinal cord sections of mice treated with anti-PSGL-1 Abs in comparison to control EAE animals did not reveal any difference between the different treatment groups (Table I). Also, no difference was found in the composition of the CD45-positive inflammatory infiltrates, which consisted mainly of CD4^+ T cells, Mac-1^+ macrophages, some B220^+ B cells and rare CD8^+ T cells (data not shown). Gr-1^+ positive granulocytes were not observed. Thus, inhibition of the interaction of PSGL-1 with its respective selectin ligands in vivo did not significantly alter the development of iEAE in the SJL mouse. Taken together, these observations demonstrate that PSGL-1 is not required for the development of EAE in SJL mice.

PSGL-1-deficient C57BL/6 mice develop EAE

To confirm the lack of requirement of PSGL-1 in EAE pathogenesis, PSGL-1-deficient mice were backcrossed into the C57BL/6 background. Active EAE was induced by immunization with the MOG peptide (aa 35–55) in CFA and mice were monitored daily

### FIGURE 5. Anti-PSGL-1 mAbs do not interfere with Ag-induced proliferation or IFN-γ production of PLP-specific T cell lines. A. One representative experiment of three showing the proliferative response of PLP-specific T cells (TC) is shown. In the presence of irradiated syngeneic spleen cells as APCs the T cells proliferated in response to the specific Ag PLP, however, there was no proliferation in the absence of Ag. Abs directed against PSGL-1 did not inhibit Ag-dependent proliferation of PLP-specific T cells. Proliferation is shown as incorporation of [3H]thymidine. Error bars represent mean ± SD (n = 3). B. Abs directed against PSGL-1 also did not interfere with IFN-γ production by PLP-specific T cells upon Ag-specific activation. Intracellular cytokine staining as determined by FACS analysis from one representative experiment of two is shown. Overlays show negative staining using a control IgG1 (thin line) and positive staining for intracellular IFN-γ (thick line). 4RA10 (rat IgG1, PSGL-1 blocking), 4RB12 (rat IgG2a, PSGL-1 nonblocking).
for clinical symptoms of EAE. In five different experiments PSGL-1-deficient C57BL/6 mice did not show any significant difference in the onset of actively induced EAE when compared with C57BL/6 wild-type mice (Fig. 7). Both groups of mice exhibited chronic disease, which is typical of MOG-induced EAE in the C57BL/6 model. Quantitative assessment of the number of perivascular cellular infiltrates in CD45-immunostained frozen brain and spinal cord sections of wild-type and PSGL-1-deficient mice did not reveal any significant difference between both groups (Table I). Immunohistological analysis of the cellular composition of the inflammatory infiltrates confirmed the presence of mainly Mac-1+ macrophages, CD4+ T cells, and scattered Gr-1-positive granulocytes in the brain and spinal cord of both wild-type and PSGL-1-deficient mice (Fig. 8). Thus, as absence of PSGL-1 neither influences the recruitment of different inflammatory cell subsets across the BBB during EAE nor inhibits the development of clinical EAE, PSGL-1 is not required for the development of clinical EAE in the C57BL/6 mouse.

Discussion

In this study we show that although encephalitogenic T lymphocytes display surface expression of PSGL-1, their PSGL-1-mediated binding to P-selectin is apparently poor as it was only observed when P-selectin was available at high concentration or density. Furthermore, PSGL-1 was not involved in the interaction of encephalitogenic T cells with P-selectin on stimulated brain endothelial cells in vitro, which was dominated by LFA-1-mediated adhesion. In accordance with our in vitro findings we demonstrate that PSGL-1 is not required for the development of EAE in two different mouse models. Neither blocking PSGL-1 with anti-PSGL-1 mAbs in the transfer EAE model in the SJL mouse nor deficiency of PSGL-1 in the C57BL/6 actively induced EAE model resulted in an obvious impact on leukocyte infiltration of the CNS or the development of the clinical disease.

Our observations are in apparent contradiction to the observations made by Piccio et al. (10). By performing intravital microscopy of the brain surface through the intact skull of young mice they demonstrated that autoreactive T lymphocytes rolled and arrested on LPS- or TNF-α-stimulated endothelium within the observed CNS microvessels. Abs blocking PSGL-1 or its endothelial ligands P- and E-selectin blocked tethering and rolling of the observed T lymphocytes, suggesting that PSGL-1/selectin interactions are critical for the recruitment of encephalitogenic T cells in inflamed brain venules. Using the same experimental approach the research group also demonstrated that CD8+ but not CD4+ T cells from patients with acute multiple sclerosis tether and roll in inflamed brain venules of mice via PSGL-1 (27).

PSGL-1-mediated rolling requires the presence of P-selectin or E-selectin on the observed brain endothelium. Performing immunohistoology and in situ hybridizations, we failed to detect expression of E- and P-selectin in parenchymal vessels of the brain and spinal cord during preclinical or clinical EAE in the SJL mouse (8). In contrast, i.v. injection of fluorescently labeled anti-E- and anti-P-selectin Abs documented the presence of both selectins within superficial brain microvessels during the preclinical phase

Table I. Quantitative assessment of the number of inflammatory cuffs in the brain and spinal cord

|                | Brain                          | Spinal Cord                |
|----------------|-------------------------------|----------------------------|
| aEAEb IC/mm²  | C57BL/6                       | C57BL/6 - PSGL-1/c-/-      | C57BL/6                       | C57BL/6 - PSGL-1/c-/- |
| 0.22 ± 0.16   | 0.43 ± 0.33                   | 4.1 ± 3.6                  | 4.0 ± 2.3                     |
| tEAEc IC/mm²  | SJL.PLP7 + control Ab         | SJL.PLP7 + anti-PSGL-1     | SJL.PLP7 + control Ab         | SJL.PLP7 + anti-PSGL-1 |
| 0.9 ± 0.6     | 1.1 ± 0.4                     | 7.4 ± 2.9                  | 8.8 ± 3.6                     |

a Quantitative assessment of the number of inflammatory cuffs in the brain and spinal cord during active EAE (aEAE) in presence or absence of PSGL-1 and during tEAE ± blocking PSGL-1.

b C57BL/6 (n = 6) and C57BL/6-PSGL-1/c-/- (n = 4) mice with a clinical score of 0.5 were analyzed at day 16 postinduction of active EAE.
c Number of microvessels surrounded by inflammatory cuffs (IC, diameter 25-150 µm) per mm² of tissue section.
d Control (n = 4) and four anti-PSGL-1 (4RA10)-treated mice with a clinical score of 0.5 and 1 were analyzed at days 10 and 15 posttransfer of SJL.PLP7, respectively.
should be noted though that leukocyte infiltration of the meninges into the CNS remains to be shown. It so far was only observed in superficial brain microvessels (9, 10), forming immunohistochemistry with a polyclonal P-selectin Ab. The choroid plexus was in fact reported by Carrithers et al. (29) per- formed immunohistochemistry with a polyclonal P-selectin Ab. The overall levels of P-selectin expression detected in the brains and spinal cords of mice before the onset of EAE, and E-selectin but not P-selectin during EAE (10). Quantifying P-selectin expression in brain and spinal cord homogenates from mice during preclinical or clinical EAE after injection of radiolabeled P-selectin Abs revealed increased P-selectin expression in the brains and spinal cords of mice before the onset of EAE with elevated levels remaining until at least 2 wk post EAE symptoms (9). The overall levels of P-selectin expression detected in the CNS of mice after induction of EAE were, however, extremely low. Kerfoot and Kubes (9) argue that comparable levels of P-selectin have been shown in other organs such as muscle and skin to suffice for basal leukocyte rolling. As the latter technique does not allow localization of the P-selectin expression within the CNS the apparent discrepancies of the individual studies might be explained such that in EAE low levels of E- and P-selectin can be induced within the CNS and expression might be restricted to sur- face CNS vessels within the meninges or the superficial brain cortex where the PSGL-1/P-selectin-mediated rolling of lymphocytes was observed by intravital microscopy (9, 10). Expression of P-selectin in murine brains restricted to the meningeal sites and the choroid plexus was in fact reported by Carrithers et al. (29) performing immunohistochemistry with a polyclonal P-selectin Ab.

As PSGL-1/P-selectin-mediated rolling of inflammatory T cells so far was only observed in superficial brain microvessels (9, 10), its occurrence elsewhere in the CNS remains to be shown. It should be noted though that leukocyte infiltration of the meninges can always be observed in tissue sections of brains and spinal cords taken from mice afflicted with EAE. Infiltration of leukocytes across meningeal vessels might be regulated by mechanisms distinct of those directing inflammatory cells across microvessels in the CNS parenchyme. Also, intravital microscopy of the brain only allows investigation of leukocyte interaction with microves- sels of the CNS gray matter. In the mouse, EAE starts in the lower spinal cord ascending to the brain with inflammatory cuffs localized within the CNS white matter. Performing intravital microscopy of the spinal cord white matter we have failed to detect rolling of encephalitogenic T cells in the white matter microvessels and rather observed their $\alpha_4$ integrin-mediated capture to endothe- lial VCAM-1 (11). Complementing these findings, blocking $\alpha_4$ integrin has been shown to successfully inhibit the development of EAE (2, 5).

Although the cell surface expression levels of PSGL-1 on the encephalitogenic T cells were found to be similar to that on PMNs or mPC7 cells, only PSGL-1 on PMNs and mPC7 cells mediated efficient binding to P-selectin. Binding of soluble P-selectin or immob- ilized P-selectin to encephalitogenic T cells could only be observed at concentrations that were 2- to 4-fold above those allowing maximal P-selectin binding to PMNs or mPC7 cells. Therefore a lack of a detectable contribution of PSGL-1 in T cell adhesion to stimulated brain endothelium is not too surprising given the pre- dominant involvement of LFA-1 and $\alpha_4$ integrin in T cell adhesion to brain endothelium (17, 22).

PSGL-1 requires certain posttranslational modifications for binding to P-selectin, such as fucosylation, tyrosine-sulfation, and branched carbohydrate side chains generated by the core2 $\beta$-1,6-N-acetylgalactosaminyltransferase (core-2 enzyme) (reviewed in Ref. 30). Naïve CD4+ T cells do not express functional PSGL-1, which is only induced by the up-regulated expression of fucosyl- transferase (FucT) VII and core-2 enzyme upon activation and differentiation in effector/memory T cells (31). Skin homing effec- tor/memory T cells have been demonstrated to use PSGL-1 for their immigration into inflamed skin (32). Thus, based on the ob- servation that encephalitogenic T cells fail to efficiently bind to P-selectin it is tempting to speculate that they fail to up-regulate the functional expression of the enzymes required to decorate PSGL-1 with the relevant sugars necessary for P-selectin binding. In agreement with our in vitro observations we found that the anti-PSGL-1 Ab 4RA10, which has been used successfully by us and others to inhibit the interactions of leukocytes in areas of inflamma- tion in other animal models (14, 26, 32), fails to inhibit adoptively transferred EAE in the SJL mouse model. Supporting our Ab inhi- bition studies development of actively induced EAE in PSGL-1-deficient C57BL/6 mice was indistinguishable from the disease course induced in wild-type C57BL/6.

Taken together, this investigation demonstrates that PSGL-1 is not required for the development of cellular infiltrates within the CNS and the development of clinical EAE. PSGL-1/P-selectin-mediated rolling of inflammatory T lymphocytes in superficial brain microvessels might therefore have little clinical relevance. Accordingly, therapeutic targeting of PSGL-1 to inhibit leukocyte infiltration of the CNS during multiple sclerosis does not seem promising.

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**Disclosures**

The authors have no financial conflict of interest.
FIGURE 8. Lack of PSGL-1 does not interfere with the recruitment of leukocyte subpopulations across the BBB during EAE. Immunohistology of inflammatory cuffs at the same periventricular location in the brain of a wild-type (WT) and a PSGL-1-deficient (−/−) C57BL/6 mouse suffering from EAE (day 16 postinfection; clinical score, 0.5) is shown for comparison. Control staining as well as staining for PECAM-1, CD45, Mac-1, CD4, CD3ε, Gr-1, and PSGL-1 is shown. Lack of PSGL-1 does not interfere with the recruitment of Mac-1-positive macrophages, CD3-positive T cells, CD4-positive T cells or macrophages, or Gr-1-positive neutrophils into the CNS during EAE. Note: the number of CD45-positive inflammatory cells stained in the brain section derived from the PSGL-1-deficient mouse is solely lower due to the smaller size of the inflammatory cuff, when compared with the size of the cuff shown for the wild-type mouse. Immunoperoxidase staining and hematoxylin counterstaining are represented.
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