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Efficient Recruitment of Lymphocytes in Inflamed Brain Venules Requires Expression of Cutaneous Lymphocyte Antigen and Fucosyltransferase-VII[1]

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Lymphocyte migration into the brain represents a critical event in the pathogenesis of multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE). However, the mechanisms controlling the recruitment of lymphocytes to the CNS via inflamed brain venules are poorly understood, and therapeutic approaches to inhibit this process are consequently few. In this study, we demonstrate for the first time that human and murine Th1 lymphocytes preferentially adhere to murine inflamed brain venules in an experimental model that mimics early inflammation during EAE. A virtually complete inhibition of rolling and arrest of Th1 cells in inflamed brain venules was observed with a blocking anti-P-selectin glycoprotein ligand 1 Ab and anti-E- and P-selectin Abs. Th1 lymphocytes produced from fucosyltransferase (FucT)-IV$^{-/-}$ mice efficiently tethered and rolled, whereas in contrast, primary adhesion of Th1 lymphocytes obtained from FucT-VII$^{-/-}$ or Fuc-VII$^{-/-}$FucT-IV$^{-/-}$ mice was drastically reduced, indicating that FucT-VII is critical for the recruitment of Th1 cells in inflamed brain microcirculation. Importantly, we show that Abs directed against cutaneous lymphocyte Ag (CLA), a FucT-VII-dependent carbohydrate modification of P-selectin glycoprotein ligand 1, blocked rolling of Th1 cells. By exploiting a system that allowed us to obtain Th1 and Th2 cells with skin- vs gut-homing (CLA$^+$ vs integrin β_7^+) phenotypes, we observed that induced expression of CLA on Th cells determined a striking increase of rolling efficiency in inflamed brain venules. These observations allow us to conclude that efficient recruitment of activated lymphocytes to the brain in the contexts mimicking EAE is controlled by FucT-VII and its cognate cell surface Ag CLA. *The Journal of Immunology,* **2005, 174: 5805–5813.**

Multiple sclerosis (MS)[4] and its animal model, experimental autoimmune encephalomyelitis (EAE), are inflammatory autoimmune diseases of the CNS mediated by lymphocytes reactive to brain Ags (1–4). Emigration of lymphocytes through the blood-brain barrier (BBB) represents a critical pathogenetic event in the initiation of CNS inflammation. The process leading to leukocyte extravasation involves multiple steps: 1) initial contact (tethering or capture) and rolling along the vessel wall mediated by selectins and integrins, and their ligands; 2) chemoattractant-induced heterotrimeric G_i protein-dependent intracellular biochemical changes leading to integrin activation; 3) integrin-dependent firm arrest; and 4) diapedesis (5–7).

The formidable technical challenges associated with efforts to visualize brain microcirculation in vivo have represented major obstacles to understanding how leukocytes gain access into the brain during inflammation. Our initial efforts in this area, using intravital microscopy, have implicated P-selectin glycoprotein ligand-1 (PSGL-1) as a concomitant of recruitment of phenotypically heterogeneous autoreactive lymphocytes in inflamed brain venules (8). In addition, it has been shown that endothelial E- and P-selectin, both PSGL-1 ligands, might be involved in the recruitment of leukocytes in murine and human brains during inflammatory diseases including EAE and MS (9–15).

Two α-1,3-fucosyltransferases (FucTs), FucT-VII and FucT-IV, are expressed in leukocytes and endothelial cells and catalyze the final reaction in selectin ligand biosynthesis, the addition of fucose to sialylated precursors (16–18). PSGL-1 is fucosylated by both FucT-VII and FucT-IV, but FucT-VII is principally responsible for efficient PSGL-1 interactions with endothelial selectins (19). Expression of cutaneous lymphocyte Ag (CLA), a FucT-VII-dependent carbohydrate modification of PSGL-1, is closely correlated with interactions between PSGL-1 and E-selectin (20–22). It has been demonstrated previously that FucT-VII expression is high in Th1 cells, whereas Th2 lymphocytes express high levels of FucT-IV, but not FucT-VII (23, 24). Moreover, Th1 cells, but not Th2 cells, are able to bind to P-selectin and E-selectin (25). Experiments performed in mice with targeted deletions of the FucT-IV and FucT-VII loci have established that absence of FucT-VII yields a severe attenuation of lymphocyte migration to secondary

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[4] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; BBB, blood-brain barrier; PSGL-1, P-selectin glycoprotein ligand-1; FucT, fucosyltransferase; CLA, cutaneous lymphocyte Ag; WT, wild type; WSR, wall shear rate; WSS, wall shear stress.

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lymphoid organs and to sites of cutaneous inflammation (contact hypersensitivity) (26–28).

The mechanisms controlling lymphocyte capture and rolling in brain microcirculation are not fully understood. There is not a clear consensus in the literature regarding the role of endothelial selectins in lymphocyte recruitment in inflamed brain venules (29). Moreover, no study has been made yet to identify the phenotype of the T cells able to gain access during early vs chronic brain inflammation. In the present paper, we show for the first time that Th1, but not Th2, lymphocytes are preferentially adherent to inflamed brain venules, in an experimental model that mimics early inflammation during EAE. Intravital microscopy studies have not yet been performed to study rolling interactions of lymphocytes that lack FucT activity in brain microcirculation. Using this approach, we observe that expression of FucT-VII is critical for tethering and rolling interactions, whereas FucT-IV has only a minor role. Moreover, our results clearly demonstrate that expression of CLA, a glycosylation epitope of PSGL-1, generates adhesive interactions between Th1 or Th2 lymphocytes and inflamed brain endothelium.

Materials and Methods

Mice

C57BL/6 young females were obtained from Charles River. FucT-IV$^{-/-}$, FucT-VII $^{-/-}$, and FucT-IV$^{-/-}$VII$^{-/-}$ mice were obtained as previously described (27, 28). FucT-deficient mice were on a C57BL/6 genetic background (backcrossed for nine generations). Mice were housed and used according to current European Community rules.

Antibodies

mAbs anti-mouse L-selectin (Mel-14), anti-α4 (PS/2), anti-LFA-1 α-chain (TIB213), anti-CLA (HECA-452), and anti-human L-selectin (DREG56) were from American Type Culture Collection. A control rat IgM was obtained from Caltag. Abs anti-E- and P-selectin (RME-1 and RMP-1) were kindly provided by Dr. A. Issekutz from Dalhousie University (Halifax, Nova Scotia, Canada), whereas anti-PSGL-1 (4RA10) was provided by Dr. D. Vestweber (Max Plank Institute, Muenster, Germany). PL1, anti-human PSGL-1 was kindly provided by Dr. R. McEver from Oklahoma University (Oklahoma City, OK).

Production of murine Th1 and Th2 cells

Naive CD4$^+$ T cells were positively selected from spleens and lymph nodes of C57BL/6 mice by anti-CD4-coated magnetic microbeads (Miltenyi Biotec) and by anti-CD62-coated magnetic microbeads (Miltenyi Biotec). Naive CD4$^+$ T cells (2×10^5 cells/well) were stimulated in culture with Con A (2 μg/ml) and mitomycin C-treated splenocytes (5×10^6 cells/well) as APC, in the presence of either 0.1 ng/ml recombinant mouse IL-12 (R&D Systems) and 10 μg/ml anti-mouse IL-4 (11B11; American Type Culture Collection), or 20 ng/ml mouse rIL-4 (Roche Diagnostics) and 10 μg/ml anti-mouse IL-12 mAb (10F6; Hoffmann-La Roche), to obtain Th1 or Th2 cell lines, respectively (30). Cells were cultured in RPMI 1640 supplemented with 50 μM 2-ME, 2 mM L-glutamine, 50 μg/ml gentamicin, and 10% FCS (Sigma-Aldrich). After 3 days in vitro, T cells were expanded and grown in complete medium containing 100 U/ml recombinant human IL-2 (Hoffmann-La Roche). The phenotype of Th1 and Th2 cell lines was determined by intracytoplasmic staining for IFN-γ and IL-4 (see *Cell surface and intracellular staining*).

Human cell isolation and culture

Human PBMCs were isolated from healthy blood donors by Ficoll-Paque (Pharmacia Biotech) density gradient centrifugation. Briefly, CD4$^+$ T cells were first isolated with CD4$^+$ T cell isolation kit (Miltenyi Biotec), and memory/effector T cells were depleted by incubation with anti-CD45RO-conjugated magnetic beads (Miltenyi Biotec). Purity of cells was typically >95%. Mature monocyte-derived human dendritic cells were mixed with allogenic naive CD4$^+$ T cells (ratio, 1:25) in XVIVO-15 medium (BioWhittaker) or XVIVO-15 supplemented with 5% FCS. Th1 and Th2 cells were differentiated by addition of 2 μg/ml PHA (Sigma-Aldrich) and cytokine and anti-cytokine Ab mixtures as previously described (31–33).

Cell surface and intracellular staining

Following culture in the presence of polarizing cytokines, mouse T cells were restimulated with 50 ng/ml PMA and 1 μg/ml ionomycin for 4 h, with 10 μg/ml brefeldin A (Novartis) added for the last 2 h. After fixing, the cells were stained for intracytoplasmic IFN-γ and IL-4 by using FITC-conjugated rat anti-mouse IFN-γ (XMG1.2; BD Pharmingen) and PE-conjugated rat anti-mouse IL-4 (11B11; BD Pharmingen) or with FITC- and PE-labeled rat IgG1 isotype controls (R3-34; BD Pharmingen) (30).

The following Abs and reagents were used for human cell surface staining: anti-CLA-FITC, anti-CLA-biotin, anti-integrin β_7-PE, anti-β_7 integrin-allophycocyanin (BD Biosciences). Stimulation and intracellular staining were performed as described previously using anti-IFN-γ-FITC and anti-IL-4-PE Abs (BD Biosciences) (33). Flow cytometry analysis was performed on a BD LSR using CellQuest software.

Intravital microscopy

Lymphocytes were labeled with either green 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes) or orange 5-(and-6)-(((chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR; Molecular Probes). Cells were kept for 6–8 h in cytokine-free medium before intravital microscopy experiments to reduce background adhesiveness and to allow inside-out signaling generated by local proadhesive agonists (8). Flow cytometry experiments showed a constantly high expression of α_4 integrins, LFA-1 and PSGL-1 during the starvation period (data not shown).

Wild-type (WT) C57BL/6 mice were injected i.p. with 12 μg of LPS (*Escherichia coli* 026:B6; Sigma-Aldrich) 5–6 h before starting the intravital experiment (8). Animals were anesthetized and a heparinized PE-10 catheter was inserted into the right common carotid artery toward the brain. To exclude noncerebral vessels from the analysis, the right external carotid artery and pterygopalatine artery, a branch from the internal carotid, were ligated (8).

The preparation was placed on an Olympus BX50WI microscope and a water immersion objective with long focal distance (Olympus Achroplan; focal distance, 3.3 mm; numerical aperture, 0.5 ∞) was used. Blood vessels were visualized through the bone by using fluorescent dextrans. A total of 2×10^6 fluorescent labeled cells/condition was slowly injected into the carotid artery by a digital pump. The images were visualized by using a silicon-intensified target video camera (VE-1000 SIT; Dage MTI) and a Sony SSM-125CE monitor and recorded using a digital VCR (Panasonic NV-DV10000).

Image analysis

Video analysis was performed by playback of digital videotapes. Vessel diameter (D), hemodynamic parameters, and the velocities of rolling were determined by using a PC-based system (8). The velocities of ≥20 consecutive freely flowing cells/venule were calculated, and from the velocity of the fastest cell in each venule (V_{fast}), we calculated the mean blood flow velocities (V_m): $V_m = V_{fast}/(2 - \epsilon^2)$. The wall shear rate (WSR) was calculated from WSR $= 8 \times V_m/D$ (s^{-1}), and the wall shear stress (WSS) acting on rolling cells was approximated by WSR $\times$ 0.025 (dyn/cm^2), assuming a blood viscosity of 0.025 Poise. Lymphocytes that remained stationary on venular wall for ≥30 s were considered adherent. At least 140 consecutive cells/venule were examined. Rolling and firm-arrest fractions were determined as the percentage of cells that rolled or firmly arrested within a given venule in the total number of cells that entered that venule during the same period.

Statistics

A two-tailed Student's t test was used for statistical comparison of two samples. Multiple comparisons were performed using Kruskal-Wallis test with the Bonferroni correction of p. Velocity histograms were compared using Mann-Whitney U test and Kolmogorov-Smirnov test. Differences were regarded as significant with a value of $p < 0.05$.

Results

Murine Th1 cells preferentially tether, roll, and arrest in inflamed brain venules

The phenotype of T cells able to gain access during early brain inflammation in autoimmune diseases is not fully understood. We have previously shown that in mice treatment with TNF-α or LPS induces expression of P- and E-selectin, ICAM-1, and VCAM-1 on brain endothelium (8). This experimental model resembles early inflammation during EAE, because we and others brought evidence that brain endothelium expresses E- and P-selectin,

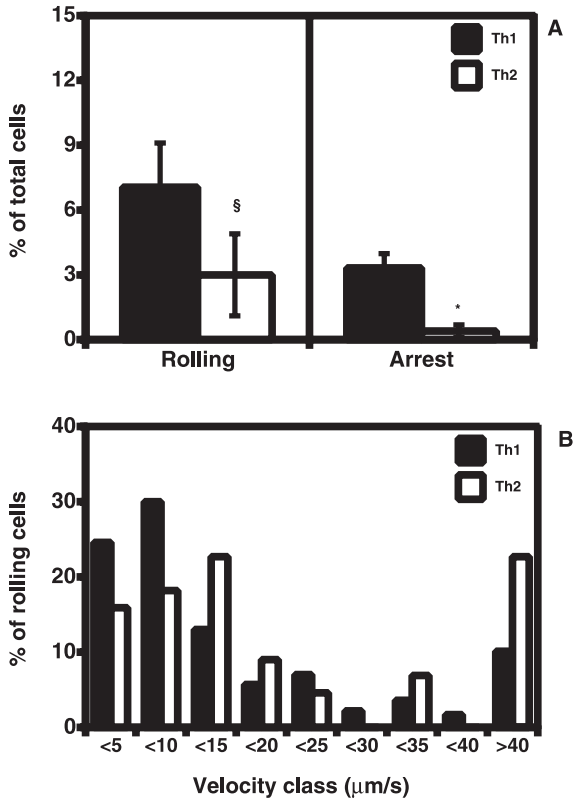

FIGURE 1. Mouse Th1, but not Th2, cells efficiently interact with inflamed brain endothelium in vivo. Mice were treated with 12 μg of LPS 5–6 h before starting the intravital experiments. *A*, Rolling and arrest fractions were calculated. The number of venules/animals for cell type is described in Table I. Groups were compared using Student's *t* test. §, $p < 0.01$ for the rolling fraction, and ∗, $p < 0.001$ for the arrest fraction of Th1 cells when compared with Th2. Data shown are mean ± SEM. *B*, Velocity histograms were generated by measuring rolling velocities. Frequency distributions were calculated after cells were assigned to velocity classes from >0 to 5, 5–10, 10–15 μm/s, and so on. A total of 244 rolling cells was examined for Th1, and 54 rolling cells were considered for Th2.

ICAM-1, and VCAM-1 during early inflammation in preclinical phase of autoimmune disease (8, 11, 12, 34). Moreover, we have previously shown that encephalitogenic CD4 T cells, but not naive lymphocytes, are able to roll and arrest in inflamed brain microcirculation (8). For details regarding our transcranial imaging method and the resolution of images during intravital microscopy performed in murine cerebral superficial vessels, see Reference 8. As autoreactive T cell lines contain cells with different phenotypes, we now asked whether T cells with Th1 or Th2 phenotype, each of which comprise variable fractions of the phenotypically heterogeneous encephalitogenic or autoreactive T cells, have different adhesive capacities in inflamed brain venules.

By using intravital microscopy, we first compared rolling and sticking of WT murine Th1 cells vs Th2 cells (Fig. 1). The comparison between the two populations was performed in the same venules, thus in similar hemodynamic conditions and in the presence of equivalent expression of endothelial ligands. Notably, microvascular hemodynamics were similar during the injection of Th1 cells or after administration of Th2 cells, supporting the accuracy of our in vivo results (Table I). The rolling fraction of Th1 cells was two times higher than for Th2 cells (mean ± SEM, 7 ± 2 vs 3 ± 1.9%; $p < 0.02$) (Fig. 1*B*, Table I). We next analyzed the quality and strength of rolling by measuring the rolling velocities (V_{roll}) of Th1 and Th2 lymphocytes. The median rolling velocity of Th1 cells was 8.7 μm/s, whereas the median rolling velocity of Th2 cells was 17.1 μm/s, suggesting an increase of the fraction of slow-rolling cells in Th1 cells. V_{roll} from different experiments were pooled in velocity classes to better analyze potential rolling differences between Th1 and Th2 cells. Considering the pool of cells that displays a V_{roll} <10 μm/s, there was a clear difference between Th1 and Th2 lymphocytes, because the percentage of Th1 cells with low rolling velocities was 54%, whereas the percentage of slow-rolling Th2 was 34% ($p < 0.03$). Moreover, the percentage of Th2 cells rolling at velocities >40 μm/s was 2-fold higher than the percentage of Th1 cells rolling at this highest velocity, suggesting that different molecular mechanisms account for rolling interactions in the two lymphocyte populations (Fig. 1*C*).

In addition, we also analyzed the arrest of lymphocytes in brain vessels. The percentage of Th1 cells that firmly adhered was 3.3 ± 0.6 (mean ± SEM). By contrast, the mean percentage (±SEM) of adherent cells was 0.41 ± 0.2 for Th2 cells (Fig. 1*B*, Table I). These data clearly show that Th1, but not Th2, cells preferentially tether, roll, and arrest in inflamed brain venules, and imply a corresponding relative reference for recruitment.

Human Th1 cells efficiently interact with inflamed brain endothelium

To define the nature of human T lymphocytes that are relevant to lymphocyte recruitment in the context of inflammatory diseases of the CNS, we took advantage of the fact that human adhesion molecules expressed by leukocytes are able to efficiently interact with their endothelial ligands expressed by mouse endothelium in an intravital microscopy setting (13, 35–37). This allowed us to compare the rolling and arrest behaviors of human Th1 cells vs Th2 cells in inflamed brain venules. Similar to the results obtained with

Table I. *Diameter, hemodynamics, behavior, and rolling velocities in cerebral venules*[a]

Cell Type	Murine Th1	Murine Th2	Human Th1	Human Th2
Number of venules/animals	11/4	11/4	14/5	14/5
Diameter (μm)	58 ± 24	58 ± 24	55 ± 24	55 ± 24
V_{fast} (μm/s)	5577 ± 756	5689 ± 1426	4448 ± 1218	3954 ± 1119
V_m (μm/s)	2824 ± 388	2878 ± 717	2302 ± 435	2010 ± 538
WSS (dyn/cm^2)	10.8 ± 4.8	10.6 ± 4.1	7.9 ± 1.9	7.1 ± 2.3
% Rolling	7 ± 2	3 ± 1.9	6.2 ± 2	2.4 ± 1.2
% Arrest	3.3 ± 0.6	0.41 ± 0.2	3.2 ± 0.9	0.5 ± 0.1
V_{roll} (μm/s)	8.7	17.1	11.1	20.4

[a] Mice were treated with LPS (12 μg) in order to induce a subacute inflammation of brain microcirculation. Venules were analyzed by individual velocity measurement of at least 20 consecutive noninteracting T cells in each venule. The velocity of the fastest cell in the sample (V_{fast}) was used to determine the mean blood flow velocity (V_m). Venular WSR and WSS and the percentage of rolling and arrested cells were calculated as described in *Materials and Methods.* The velocity of rolling cells was measured by digital frame-by-frame analysis of videotapes. V_{roll} are presented as median. Data are arithmetic mean ± SD for hemodynamic parameters and mean ± SEM for the percentages of rolling and arrest.

murine T cells, the rolling fraction of human Th1 cells was increased 2.6-fold when compared with Th2 cells (mean ± SEM, 6.2 ± 2 vs 2.4 ± 1.2%; $p < 0.01$) (Fig. 2*A*, Table I). The median rolling velocity for Th1 cells was 11.1 μm/s, whereas the median rolling velocity of Th2 cells was 20.4 μm/s, implying an increase in the fraction of slow-rolling cells in Th1 cells. When we pooled the V_{roll} in velocity classes, we observed a similar pattern of distribution as for murine Th1 and Th2 cells (Fig. 2*B*). Considering the pool of cells that display a V_{roll} <10 μm/s, the percentage of Th1 cells with low rolling velocities was 45%, whereas the percentage of slow-rolling Th2 cells was 26% ($p < 0.02$). Moreover, similar to the results obtained with murine cells, the percentage of Th2 cells rolling at velocities >40 μm/s was higher than the percentage of Th1 cells rolling in this rapid velocity class (Fig. 2*B*).

We also analyzed the arrest of human Th1 and Th2 cells in brain vessels. The percentage of human Th1 cells that arrest was 3.2 ± 0.9 (mean ± SEM). In contrast, the mean percentage (± SEM) of firmly adhered Th2 cells was 0.5 ± 0.1 (Fig. 1*A*, Table I). These data, together with the results obtained with murine cells, clearly demonstrate that Th1, but not Th2, preferentially roll and firmly adhere in inflamed brain microcirculation.

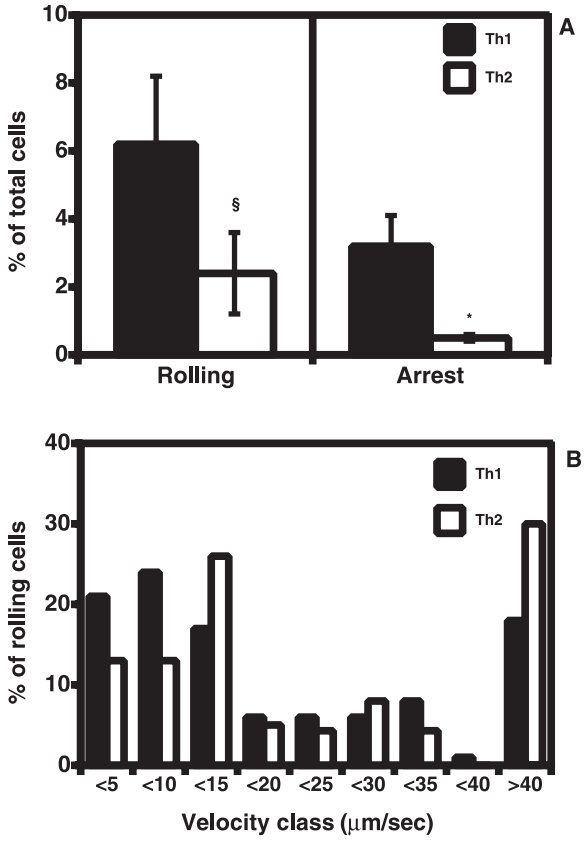

FIGURE 2. Efficiency of rolling and arrest of human Th1 cells on inflamed brain venules exceeds rolling and arrest of Th2 cells. The experiments were performed in five mice. Fourteen venules were examined. Animals were prepared as described at Fig. 1. *A*, Rolling and arrest fractions were compared between human Th1 and Th2 cells. Mean values ± SEM are presented. §, $p < 0.02$ for the rolling fraction, and *, $p < 0.001$ for the arrest fraction. *B*, Velocity histograms were generated by measuring rolling velocities, whereas frequency distributions were calculated as described at Fig. 1. A total of 100 rolling cells was examined for Th1, and 43 rolling cells were considered for Th2.

PSGL-1/endothelial selectins are critical for tethering and rolling of Th1 cells

We next set out to identify the molecular mechanisms controlling adhesion of murine and human Th1 cells to the inflamed brain microvasculature. We first performed intravital microscopy experiments using murine Th1 cells. We observed that rolling interactions were blocked by 93% using an anti-PSGL-1 Ab. By contrast, treatment of cells with a mAb to L-selectin had no statistically significant effect on rolling of Th1 cells, in keeping with others' observations that L-selectin is poorly expressed on Th1 cells and is not believed to contribute meaningfully to Th1-endothelial cell interactions (24, 38, 39). (Fig. 3*A*). Blocking effects similar to those observed with anti-PSGL-1 Abs were obtained with anti-P- and E-selectin mAbs used together (inhibition of 88%) (Fig. 3*A*). Individual blockade of E- and P-selectin drastically blocked tethering and rolling, confirming that both selectins are required for efficient adhesive interactions in inflamed brain venules (data not shown) (8, 10). These results correlated with the fact that our Th1, but not Th2, cells are able to bind E- and P-selectin-IgM chimeras in vitro (data not shown) (26). The diameters of the microvessels

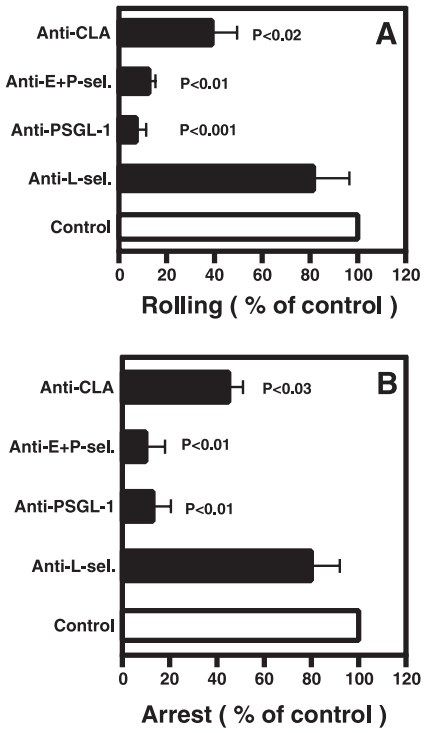

FIGURE 3. Murine Th1 cell rolling and arrest in inflamed cerebral venules is primarily mediated by PSGL-1. Rolling (*A*) and arrested (*B*) cell fractions were analyzed after treatment of cells and animals with anti-adhesion molecules blocking mAbs. In some experiments, cells were pretreated with 100 μg/ml mAb for 15 min at 25°C in a total volume of 300 μl and then injected through the right carotid catheter. Then, a supplement up to 100 μg of mAb was administered together with Ab-treated cells. Control cells received no Ab treatment. To block adhesion molecules expressed by the endothelium, mice received 100 μg of mAb before the injection of the cells. Control cells were injected before the mAb administration i.v. Bars depict rolling fractions as percentage of control cells that rolled/arrested in the same venule. Data are expressed as mean values ± SEM. The hemodynamic parameters did not differ significantly between the experimental conditions (data not shown). The following number of venules/animals was examined: 11/4 for anti-PSGL-1, 5/2 for anti-L-selectin, 6/3 for anti-E- and P-selectin Abs. Groups were compared with control using Kruskal-Wallis test followed by Bonferroni correction of *p*.

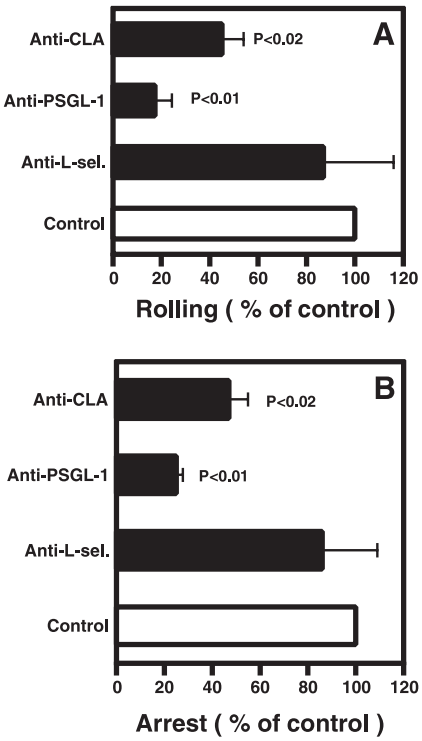

FIGURE 4. Expression of PSGL-1 and CLA are required for human Th1 cell rolling and arrest. Rolling (*A*) and arrested (*B*) cell fractions were analyzed after treatment of cells and animals with anti-adhesion molecules blocking mAbs as described for Fig. 3. Bars depict rolling/arrest fractions as percentage of control cells that rolled/arrested in the same venule. Data are expressed as mean values ± SEM. The following number of venules/animals were examined: 7/3 for anti-PSGL-1, 6/3 for anti-L-selectin, 6/3 for anti-CLA. The hemodynamic parameters did not differ significantly between the experimental conditions (data not shown). Groups were compared with control using Kruskal-Wallis test followed by Bonferroni correction of *p*.

studied did not differ significantly between the experimental conditions, nor did the shear rate or shear stress within these vessels (data not shown). These data show that PSGL-1 expression on Th1 lymphocytes is critical for capture and rolling in the inflamed microvasculature, and that expression of E- and P-selectin on brain endothelium is required for efficient primary adhesion. Arrest was not blocked after treatment with anti-L-selectin Ab, whereas anti-PSGL-1 and anti-E- and P-selectin Abs significantly blocked sticking, suggesting that rolling interactions mediated by PSGL-1-selectins are mandatory for the arrest of Th1 cells (Fig. 3*B*). CLA has been shown to be a glycosylation epitope of PSGL-1 able to mediate interactions with E- and P-selectin (20–22). We further investigated the involvement of PSGL-1 in rolling interactions by using HECA-452, an Ab that recognizes the CLA carbohydrate epitope in human and mouse leukocytes (21, 22, 40). As shown in Fig. 3, HECA-452 mAb significantly blocked 61% of rolling, whereas a negative control IgM mAb (see *Materials and Methods*) had no effect on the adhesive interactions (data not shown). The fact that HECA-452 Ab (IgM) blocked rolling to a slightly lesser extent than anti-PSGL-1 Ab (IgG), suggest that other glycosylation epitopes of PSGL-1 might be involved or that the mAbs used might have different blocking efficiency.

We also sought to define the mechanisms mediating rolling interactions of human Th1 cells. We observed that anti-PSGL-1 mAb (PL-1) blocks 83% of rolling interactions, whereas in contrast, L-selectin blockade has no significant effect (Fig. 4*A*). Anti-E- and P-selectin mAbs inhibited to an extent that was similar to that observed with anti-PSGL-1 mAb (data not shown). The arrest of human Th1 cells was also markedly blocked by anti-PSGL-1, whereas anti-L-selectin Ab had no significant effect (Fig. 4*B*). Furthermore, HECA-452 mAb significantly blocked rolling and arrest, whereas a negative control IgM mAb had no effect on the adhesive interactions (data not shown). These observations imply that this sialylated, fucosylated sLex surrogate is required for the recruitment of human and murine Th1 cells in inflamed brain venules.

Expression of FucT-VII is crucial for lymphocyte recruitment

It has been shown previously that FucT-VII expression is high in Th1 cells, whereas Th2 lymphocytes express high levels of FucT-IV, but generally not FucT-VII (19, 23). Moreover, the blocking effect obtained with HECA-452 mAb recognizing CLA, a PSGL-1 epitope that has been shown to correlate with FucT-VII expression, suggested to us that FucT-VII may have a role in lymphocyte recruitment in inflamed brain venules.

We therefore used intravital microscopy to determine whether either FucT contributes to PSGL-1 ligand activities relevant to lymphocyte tethering, rolling, and arrest in inflamed brain venules. Th1 cells were used exclusively for these experiments, because Th2 cells interact poorly with inflamed brain endothelium. We produced Th1 cells from mice deficient in FucT-IV, or deficient in FucT-VII, or deficient in both enzymes. As previously shown, in vitro polarization toward the Th1 phenotype was retained in the absence of FucT-IV, FucT-VII, and FucT-IV and -VII (data not shown) (26). Rolling and arrest fractions of Th1 from Fuc-T-IV-deficient mice were not significantly different from the behavior of cells obtained from WT mice (Table II and Fig. 5*A*). By contrast, rolling and arrest of Th1 cells from FucT-VII$^{-/-}$ mice were markedly reduced (inhibition of 75 and 87%, respectively), suggesting a critical role for FucT-VII in the recruitment of lymphocytes in subacutely inflamed brain venules (Fig. 5*A*). Rolling interactions in cells from double-deficient mice were further decreased when

Table II. *Venular microhemodynamics, effect of FucT deficiencies, and rolling velocities*[a]

Mouse Type	No. Venules/Animals	Diameter (μm)	V_m (μm/s)	WSS (dyn/cm^2)	% Rolling	% Arrest	Mean V_{roll}	Median V_{roll}
WT	8/3	51.1 ± 15	1897 ± 586	8 ± 3.7	12.6 ± 2.9	4.2 ± 2.6	22.6	11.9
FucT-IV$^{-/-}$	8/3	51.1 ± 15	1716 ± 704	7.1 ± 2.9	11.5 ± 3.6	3.4 ± 2.3	17.5	9.7
WT	6/3	38.5 ± 11.9	2506 ± 316	13.6 ± 2.7	11.3 ± 2.1	3.4 ± 0.4	19	11.4
FucT-VII$^{-/-}$	6/3	38.5 ± 11.9	2040 ± 797	10.9 ± 4.2	2.7 ± 0.8	0.4 ± 0.01	40	13.1
WT	8/3	42.5 ± 10.5	1902 ± 561	9.5 ± 2.9	12.2 ± 3.1	3 ± 1.9	ND	ND
FucT-IV&VII$^{-/-}$	8/3	42.5 ± 10.5	1896 ± 385	9.2 ± 2.6	1.1 ± 0.3	0.8 ± 0.6	ND	ND

[a] Mice were treated with LPS (12 μg) in order to induce a subacute inflammation of brain microcirculation. The velocity of the fastest cell in the sample (V_{fast}), mean blood flow velocity (V_m), WSS, and the percentage of rolling and arrested cells were calculated as described in *Materials and Methods.* V_{roll} was measures in micrometers per second. The small number of residual rolling of cells from double-deficient mice was insufficient for statistical analysis (ND). Data are arithmetic mean ± SD for hemodynamic parameters and mean ± SEM for the percentages of rolling and arrest.

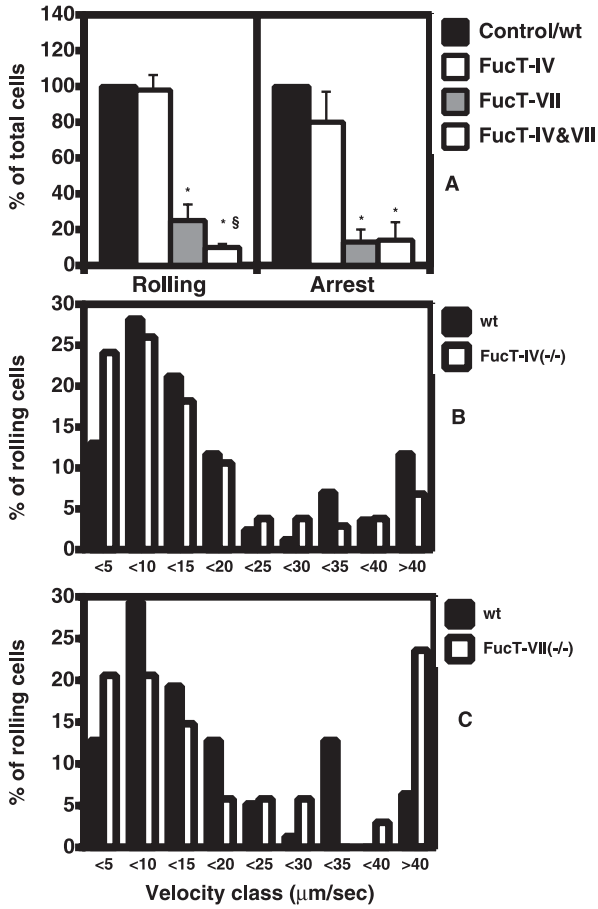

FIGURE 5. FucT-VII controls lymphocyte tethering, rolling, and arrest in inflamed brain venules. Animals were prepared as described at Fig. 1. *A*, Rolling and arrested cell fractions were compared between Th1 cells from WT mice and Th1 cells from FucT-deficient mice. Mean values ± SEM are presented. Groups were compared with control using Kruskal-Wallis test followed by Bonferroni correction of *p*. ∗, $p < 0.001$, and §, $p < 0.02$ (FucT-VII$^{-/-}$ vs FucT-IV$^{-/-}$VII$^{-/-}$). *B* and *C*, Velocity histograms were generated by measuring rolling velocities, whereas frequency distributions were calculated as described at Fig. 1. For *B*, 85 rolling cells were examined for WT and 104 rolling cells were considered for FucT-IV-deficient cells. For *C*, 80 rolling cells were examined for WT, and 44 rolling cells were considered for FucT-VII-deficient cells.

compared with cells obtained from FucT-VII$^{-/-}$ mice (Fig. 5*A*). These results suggest that FucT-IV contributes to rolling interactions but has a minor role, relative to the contribution made by FucT-VII.

Although we observed only a slight increase of median V_{roll}, when analyzing FucT-VII$^{-/-}$ Th1 cells, the mean (±SEM) of rolling velocities of Th1 cells from FucT-VII-null mice was high (40 ± 14 μm/s), relative to that of cells from WT mice (19 ± 6 μm/s). The fraction of rolling cells <5 μm/s was slightly increased, but overall, the percentage of cells that rolled <10 μm/s was similar to that observed in cells from WT mice. However, we observed a clear increase of the fraction of rolling cells at higher velocities (Fig. 5*C*). Unexpectedly, Th1 cells from FucT-IV$^{-/-}$ mice had a statistically significant increase of the percentage of cells at <5 μm/s, whereas the fraction of rolling cells at >40 μm/sec was proportionally decreased ($p < 0.05$) (Fig. 5*B*).

Expression of CLA enhances recruitment efficiency

To further explore the contribution of FucT-VII and CLA to lymphocyte recruitment in inflamed brain venules, we produced human Th1 and Th2 cells with distinct subsets of skin-homing (CLA$^+$integrin β_7^-) and gut-homing (integrin β_7^+CLA$^-$) phenotypes (Fig. 6*A*) (41). Th1 and Th2 polarization of the cells was confirmed by flow cytometry experiments assessing the expression of intracellular IFN-γ vs IL-4 production (data not shown) (41). Although Th2 CLA$^-$ cells were completely devoid of CLA expression, residual CLA was observed on Th1 CLA$^-$ cells (5–10% of the total cells), in agreement with previous studies showing that Th1 phenotype induces expression of CLA (23). We first compared rolling fractions of Th1 cells with the CLA$^+$ phenotype vs Th1 cells of the CLA$^-$ phenotype (Fig. 6*B*). Microvascular hemodynamic parameters were similar during injection of CLA$^+$ Th1 cells or CLA$^-$ Th1 cells (Table III). The percentage of Th1 CLA$^+$ rolling cells increased 2.5-fold when compared with Th1 CLA$^-$ (mean ± SEM, 12.1 ± 2.3 vs 4.7 ± 1%; $p < 0.01$) (Fig. 6*B*). Similarly, the efficiency of arrest was increased in CLA$^+$ Th1 cells. The median rolling velocity of Th1 CLA$^+$ cells was 9.3 μm/s (mean, 14.9 μm/s), whereas median rolling velocity of Th1 CLA$^-$ cells was 19.4 μm/s (mean, 48.9 μm/s), showing that expression of CLA markedly decreases V_{roll}, presumably by enhancing the interactions between CLA and endothelial selectins. Induced augmentation of CLA expression determined a clear decrease in the percentage of rolling cells >25 μm/s and an increase of cells in the velocity classes <25 μm/s.

When we analyzed the results obtained with human Th2 cells in vivo, the differences between CLA$^+$ and CLA$^-$ cells were remarkable. The percentage of rolling cells was increased 6-fold in Th2 cells in which CLA expression was induced (mean ± SEM, 6.3 ± 2.2 vs 0.4 ± 0.1; $p < 0.001$) (Fig. 6*D*). Median V_{roll} of CLA-Th2 cells was 21.6 μm/s, whereas the expression of CLA in CLA$^+$ Th2 cells was accompanied by a clear drop of V_{roll} to 5.9 μm/s. Moreover, velocity histograms showed a marked shift toward slower rolling velocities and a drastic increase of rolling cells <5 μm/s in CLA$^+$ Th2 cell population (Fig. 6*E*). In addition, we observed a striking increase (increment of 15-fold) of the capacity of CLA$^+$ Th2 cells to arrest in inflamed brain venules (mean ± SEM, 0.4 ± 0.1), relative to CLA$^-$ Th2 cells (6.3 ± 2.2). These results demonstrate that CLA expression is a clear concomitant to the establishment of adhesive interactions at the contact with inflamed brain endothelium.

Discussion

In this study, we demonstrate that expression of CLA and FucT-VII are critical for the preferential recruitment of Th1 lymphocytes in inflamed brain in an experimental model that resembles early inflammation during EAE (8). The identification of mechanisms that control the passage of leukocytes through the BBB is of enormous interest because the blockade of leukocyte recruitment during EAE and MS leads to inhibition of inflammation and brain damage (42–45). However, the mechanisms controlling the recruitment of leukocytes in inflamed brain venules are not well understood. Previous studies performed by Engelhardt et al. (29) indicated that brain endothelium does not express E- and P-selectin during EAE and subacute inflammation induced with LPS and TNF. In contrast with this study, others and we have shown that endothelial selectins are expressed in various models of acute and subacute inflammation of brain vessels, as well as during EAE and MS (8–12, 14). Th1 and Th2 cells have different cytokine production phenotypes and have distinct roles during EAE: Th1 cells have been implicated in the induction of EAE, whereas Th2 cells have a blocking effect on the disease (43, 46, 47). Our data obtained with murine and human Th1 and Th2 cells clearly demonstrate that Th1, but not Th2, cells are preferentially recruited in subacutely inflamed brain venules, suggesting that, during early

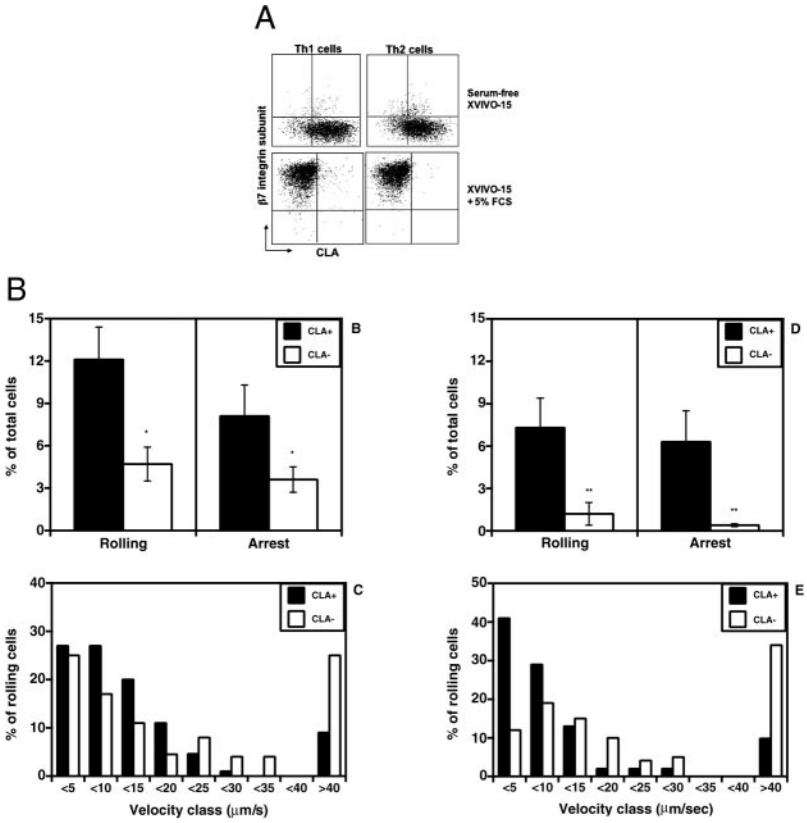

FIGURE 6. Expression of CLA enhances efficiency or rolling and arrest in inflamed brain venules. Animals were prepared as described at Fig. 1. The comparison between CLA^+ and CLA^- cells was performed in the same venules, thus in similar hemodynamic conditions and in the presence of equivalent expression of endothelial ligands (Table III). *A*, Shown are cell surface phenotype of Th1 and Th2 cells. After 10–15 days of culture, T cells were stained for cell surface expression of CLA and integrin β_7. Two-parameter flow cytometry histograms obtained in a representative experiment are shown. *B–E*, Rolling and arrest fractions were compared between Th1 CLA^+ cells and Th1 CLA^- cells, and between Th_2 CLA^+ cells and Th_2 CLA^- cells. Mean values ± SEM are presented. Groups were compared with control using two-tailed *t* test (paired). *, $p < 0.01$; **, $p < 0.001$. Velocity histograms were generated by measuring rolling velocities, whereas frequency distributions were calculated as described at Fig. 1. For *B*, 81 rolling cells were examined for Th1 CLA^+, and 64 rolling cells were considered for CLA^-. For *C*, 90 rolling cells were examined for Th2 CLA^+, and 45 rolling cells were considered for Th2 CLA^-.

phases of brain inflammation during autoimmune diseases, Th1 cells, but not Th2 cells, might gain access to the brain parenchyma and initiate the inflammatory process.

Anti-PSGL-1 and anti-endothelial selectin Abs drastically blocked rolling interactions in both human and murine Th1 cells. These results demonstrate that, as shown in other experimental models, brain endothelium expressing E- and P-selectins recruits T cells able to present functional ligand for endothelial selectins (25). Results obtained by Carrithers et al. (11) suggest that P-selectin, a PSGL-1 ligand, may be involved in the early recruitment of encephalitogenic lymphocytes into the brain. Moreover, Kerfoot and Kubes (12) have recently shown that P-selectin mediates leukocyte-endothelial cell interactions controlling leukocyte trafficking in the brains of EAE mice. PSGL-1 binds both E- and P-selectin in vivo, and we recently documented positivity for both P- and E-selectin before the onset of clinical EAE (8). Abs to PSGL-1 inhibit interactions of leukocytes to areas of inflammation in other animal models (48, 49). Thus, we speculate that blockade of PSGL-1, the molecule responsible for the preferential recruitment of Th1 cells and encephalitogenic lymphocytes in inflamed brain venules, might interfere with the pathogenesis of autoimmune diseases of the brain.

Genetic evidence derived from analysis of mice with targeted mutations indicated that two FucTs, FucT-VII and FucT-IV, have relevance to selectin counterreceptor activity (19). Intravital microscopy studies in brain microcirculation have not yet been performed to analyze rolling interactions of lymphocytes that lack FucT activity. In the present paper, we show that lymphocytes from mice deficient of FucT-VII have a marked decrease of rolling interactions in inflamed brain venules. Our data is in agreement with previous studies showing that accumulation of lymphocytes in sites of cutaneous inflammation in a model of contact sensitivity

Table III. *Diameter, hemodynamics, behavior, and rolling velocities of CLA^+ and CLA^- T cells*[a]

	Cell Phenotype			
	Th1 cells		Th2 cells	
	CLA^+	CLA^-	CLA^+	CLA^-
Number of venules/animals	8/3	8/3	9/4	9/4
Diameter (μm)	33.4 ± 8.8	33.4 ± 8.8	51.3 ± 19.4	51.3 ± 19.4
V_{fast} (μm/s)	3448 ± 982	3115 ± 448	3820 ± 662	3977 ± 992
V_m (μm/s)	1824 ± 546	1647 ± 265	2020 ± 384	2080 ± 583
WSS (dyn/cm^2)	11.7 ± 5.8	10.5 ± 3.9	9.1 ± 4.6	9.2 ± 4.9
% Rolling	12.1 ± 2.3	4.7 ± 1	7.3 ± 2.1	1.2 ± 0.8
% Arrest	8.1 ± 2.2	3.6 ± 0.9	6.3 ± 2.2	0.4 ± 0.1
V_{roll} (μm/s)	9.3	19.4	5.9	21.6

[a] Cerebral microcirculation was subacutely inflamed. The mean blood flow velocity (V_m), WSS, and the percentage of rolling and arrested cells were calculated as described in *Materials and Methods.* The velocity of rolling cells was measured by digital frame-by-frame analysis of videotapes. V_{roll} are presented as median. Data are arithmetic mean ± SD for hemodynamic parameters and mean ± SEM for the percentages of rolling and arrest.

is mainly dependent on FucT-VII (26). In our experimental conditions, residual rolling is present in the absence of FucT-VII, and this is dependent in part on the contribution of FucT-IV, which becomes evident in double-deficient cells. Our data suggest that, for most lymphocytes, FucT-VII-dependent carbohydrates determine tethering efficiency, whereas FucT-IV products are more relevant during established rolling (50). Moreover, we observed that, although in previous studies by Maly et al. (27), neutrophils from FucT-VII mice are devoid of rolling at <10 μm/s, the percentage of lymphocytes that roll at slow velocities (<10 μm/s) is unchanged, suggesting that, in lymphocytes, FucT-IV synthesizes selectin ligands that contribute to slow rolling. Thus, FucT-IV generates selectin ligands that support rolling of some lymphocytes in inflamed brain venules, similar to results obtained with neutrophils in other models of inflammation (28). It has been shown previously that neutrophils from FucT-IV$^{-/-}$ mice have an increase of V_{roll}, whereas the rolling fraction is not altered (28, 50). In contrast with data obtained with neutrophils, we find that rolling velocity is not decreased in Th1 cells lacking FucT-IV activity. Instead, we observed a significant increase in the percentage of slow-rolling cells. We speculate that a compensatory increase in FucT-VII expression/activity might account for changes in the chemistry and/or abundance of carbohydrates leading to an increase of the percentage of slow-rolling cells (50). As recently discussed by M'Rini et al. (51), FucT-IV might attenuate rolling interactions by diverting some terminal lactosamine acceptor moieties toward the Lewis^X and thus potentially away from the FucT-VII route, thus decreasing FucT-VII production of selectin ligands. Taken together, these observations implicate that neutrophils and lymphocytes have different compensation mechanisms that account for the clear differences observed in the absence of FucT activity. Moreover, whereas neutrophils from mice deficient of FucT-IV and -VII are devoid of any rolling activity, ~10% of double-deficient lymphocytes still have rolling capacity, suggesting that other rolling receptors such as VLA-4 might be involved (8, 37).

It has been shown previously that CLA represents a uniquely glycosylated isoform of PSGL-1 (20). CLA was originally defined by its immunoreactivity with the HECA-452 mAb, which recognizes an incompletely defined carbohydrate modification within the distal tip of PSGL-1 (20, 52, 53). Previous studies using human and mouse T cells show a correlation of the CLA epitope with the capacity to bind E- and P-selectin. Our data represent the first demonstration that CLA expression is required for the rolling and arrest of T lymphocytes in inflamed brain microcirculation, where both endothelial selectins are required for efficient tethering and rolling (8, 10). These results are supported by previous studies showing that the human Th1 phenotype correlates with an increased expression of FucT-VII and in parallel with an increased HECA-452 reactivity (23). To better explore the role of CLA and to demonstrate the concept that CLA is required for lymphocyte recruitment in brain venules, we produced and characterized human Th1 and Th2 with distinct subsets of skin-homing (CLA$^+$integrin β_7^-) and gut-homing (integrin β_7^+CLA$^-$) (41). We observed that the percentage of Th1 CLA$^+$ cells that rolled and arrested is significantly increased when compared with Th1 CLA$^-$ cells. However, Th1 cells defined as CLA$^-$ have residual expression of CLA that might account for the lack of marked differences between Th1 CLA$^-$ and Th1 CLA$^+$ cells. To more precisely define the involvement of CLA in lymphocyte adhesive interactions in inflamed brain venules, we also used Th2 cells that, in the form of gut-homing phenotype, have no HECA-452 immunoreactivity. The percentage of rolling cells was dramatically increased in Th2 CLA$^+$ cells. These results clearly demonstrate that CLA glycosylation epitope promotes adhesive interactions in inflamed brain venules. Although CLA expression was associated with Th1 phenotype, under some circumstances, human Th2 cells have been found to express CLA and FucT-VII. Our data show that, when expressed by either Th1 or Th2 cells, CLA might determine recruitment efficiency at the BBB level (24). The velocity histograms showed a clear increase of rolling cells <5 μm/s in the Th2 CLA$^+$ population. These data suggest that induced CLA expression on Th2 cells leads to an increase in slow-rolling interactions (<5 μm/s) mediated by E-selectin. It has been shown previously that slow rolling of leukocytes on E-selectin is mandatory for the subsequent arrest in vivo (54). In fact, we observed a striking increase (increment of 15-fold) of the capacity of Th2 CLA$^+$ cells to arrest in inflamed brain venules. Slow rolling mediated by CLA may permit longer interactions between the lymphocytes and the endothelial surface and consequently better contact with activating factors triggering the arrest (8). In addition, our results also unveil that the capability to be recruited into the CNS is independent of the acquisition of Th1 vs Th2 cytokine synthesis profiles, but is dictated by quantitative and qualitative differences in the expression of adhesion molecules on lymphocyte surface (41). Our data, together with evidence showing that E-selectin is expressed in vessels from acute plaques of MS patients, suggest that the CLA epitope might represent a key element in breaching the inflamed BBB (14).

In conclusion, our observations provide novel insight into the mechanisms controlling the recruitment of lymphocytes in brain venules. We demonstrate that FucT-VII expression is crucial for the preferential recruitment of Th1 cells in a model that has been shown previously to mimic early inflammation during EAE. Moreover, generation of efficient adhesive interactions at the contact with inflamed brain endothelium appears to be dominated by the expression of CLA epitope of PSGL-1. A more profound understanding of the specific role of CLA and FucTs in autoimmune diseases of the CNS awaits further experimentation.

Disclosures

The authors have no financial conflict of interest.

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