CD8+ T cells from acute multiple sclerosis patients display selective increase of adhesiveness in brain venules: a critical role for P-selectin glycoprotein ligand-1

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Abstract

Multiple Sclerosis (MS) is considered an autoimmune inflammatory disease of the central nervous system. We compared for the first time the adhesiveness of CD4+ and CD8+ lymphocytes from acute, non-treated patients with the relapsing remitting form of the disease (RRMS) and from healthy donors under physiological conditions. We show that in patients with RRMS CD8+, but not CD4+, T cells display increased rolling and arrest in inflamed murine brain venules. Moreover, CD8+, but not CD4+, lymphocytes from MS patients show increased rolling on P-selectin in vitro. Anti-P-selectin glycoprotein ligand-1 (PSGL-1) antibodies dramatically block the recruitment of CD8+ cells from MS patients in brain vessels, suggesting that PSGL-1 represents a novel pharmaceutical target which may be exploited to block the selective entrance of CD8+ cells during early inflammation. VCAM-1, but not PSGL-1, is critical for the adhesion of CD4+ cells from MS patients, highlighting a fundamental dichotomy in the mechanisms governing the recruitment of lymphocyte subsets in RRMS. Importantly, seven-colour FACS analysis together with functional data indicates that a large fraction of CD8+ cells from MS patients display the characteristics of memory effector phenotype. In conclusion, our results show that CD8+, but not CD4+, T cells from patients with RRMS in the acute phase of the disease display increased ability to be recruited in inflamed brain venules.

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Introduction

Multiple sclerosis (MS) is a common often-disabling disease of the central nervous system that has initially a relapsing-remitting course (RRMS) in most patients.\textsuperscript{1} MS is believed to be of autoimmune origin, and until now research has focused nearly exclusively on the role of CD$^+$ T cells in the induction of the disease.\textsuperscript{2} However, inflammatory infiltrates from brain and spinal cord from MS patients and from animals with experimental autoimmune encephalomyelitis (EAE), the animal model of MS, include both CD$^+$ and CD$^+$ T cells.\textsuperscript{3-5} Moreover, CD$^+$ T cells may also play a major role in the induction of autoimmune demyelination in EAE.\textsuperscript{6,7} Recent data has shown that in actively demyelinating lesions CD$^+$ T cells are far more numerous than CD$^+$ T cells.\textsuperscript{8} Moreover, analysis of TCR-β gene rearrangements by PCR of individual cells from frozen sections of lesional tissue revealed that CD$^+$ T cell population expansion was oligoclonal, while, in contrast, CD$^+$ T cells were more heterogeneous.\textsuperscript{8}

Lymphocyte migration into the brain represents a crucial moment in the initiation of the inflammatory process in the central nervous system (CNS). The process leading to lymphocyte extravasation is a finely regulated sequence of steps controlled by both adhesion molecules and activating factors. It involves: 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\textsubscript{i} protein-dependent intracellular biochemical changes leading to integrin activation; 3) integrin-dependent firm arrest; and 4) diapedesis.\textsuperscript{9-11} We recently developed a novel intravital microscopy model to directly analyze through the skull the interactions between lymphocytes and the endothelium in cerebral venules of mice.\textsuperscript{12} In this new model we have previously shown that P-selectin glycoprotein ligand-1 (PSGL-1)/endothelial selectins are critical in the recruitment of mouse autoreactive lymphocytes in inflamed brain microvessels.
We examined for the first time the adhesive capabilities of CD4+ and CD8+ T cells derived from acute MS patients in a physiological experimental setting. 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.13-15 Our results reveal an increased adhesive capacity of CD8+, but not CD4+, T cells from acute MS patients in brain vessels, supporting a role for CD8+ T cells in early inflammation in RRMS. Importantly, we show that PSGL-1 is responsible for the preferential recruitment of CD8+ cells in brain vessels, whereas VCAM-1 has a critical role in the recruitment of CD4+ T cells. The dichotomy in the adhesion mechanisms of CD8+ versus CD4+ lymphocytes provides important information for the potential selective blocking of the recruitment of lymphocyte subpopulations in MS.
Materials and Methods

Patients

Peripheral blood samples from 20 patients, (16 women and 4 men; mean age 34.4, range 19-51) with clinically definite relapsing-remitting MS\textsuperscript{16,17} were obtained from the Centre for Multiple Sclerosis of the Neurological Unit “Lancisi” of the San Camillo Hospital in Rome, Italy, following informed consent procedures approved by the scientific ethics committee of the Hospital. Patients were in the initial phase of the disease (mean duration of disease, 2.9 years; range 0-9) and scored low on the expanded disability status scale (EDSS) (mean 0.66, range 0-3). None of the patients had previously undergone immuno-modulating therapy. The diagnosis of relapse was defined according to the diagnostic criteria currently recommended for multiple sclerosis.\textsuperscript{16,17} Peripheral blood samples was also obtained from 12 apparently healthy subjects age- and sex-matched with the patients.

Isolation and Immunostaining of blood-derived cells

PBMC were isolated from blood samples according to standard procedures. 0.5 x 10\textsuperscript{6} cells were used for each staining. Monoclonal antibodies (conjugated with the appropriate fluorochrome) were added to predetermined optimal concentrations, and cells were incubated for 20 minutes at 4 °C. Cells were then washed and analyzed at the flow cytometer.

Antibodies:

The following antibodies were used: anti-CD3 PE-TxRed\textsuperscript{®}, anti-CD3 APC, anti-CD3 TC (Pe-Cy5) anti-CD4 PE-Cy7, anti-CD4 PE, anti-CD8 APC-Cy7, anti-CD11a FITC were from Caltag (Burlingame, CA, USA); anti-CD49d FITC, anti-CD49d APC, anti-CD162/PSGL-1 (KPL1) PE, anti-CD11a APC, anti-CD8 PE were from Pharmingen (La Jolla, CA, USA), and anti-CD62L ECD\textsuperscript{TM} (PE-TxRed\textsuperscript{®}) was from Beckman Coulter (USA). Isotype controls were as follows: IgG2a PE-Tx Red\textsuperscript{®}, IgG2a APC, IgG2a TC, IgG2a PE-Cy7, IgG2a PE, IgG2a APC-Cy7, IgG1 FITC from Caltag; IgG1 FITC, IgG1 APC and IgG1
PE were from Becton Dickinson-Pharmingen, and IgG1 ECD™ was from Beckman Coulter. For intravital microscopy studies we used the following mAbs: PL1, anti-human PSGL-1\textsuperscript{18}, and MK2.7, anti-mouse VCAM-1 from ATCC.

*Flow cytometry*

7-color FACS analysis was performed on a Cytomation MoFlo Cytofluorimeter (Cytomation, Fort Collins, CO, USA). Data was compensated and analyzed using FlowJo software (TreeStar, San Carlos, CA, USA).

*Ex vivo purification of CD+4 and CD+8 T cells*

PBLs were divided in two aliquots and CD4\textsuperscript{+} or CD8\textsuperscript{+} subsets were isolated using MACS MicroBeads (Miltenyi Biotech, GmbH, Germany). A purity of >98% was achieved for both subsets.

*Intravital microscopy*

Lymphocytes were labeled with either green CMFDA (5-chloromethylfluorescein diacetate) (Molecular probes) or orange CMTMR (5-(and-6)-(((chloromethyl)benzoyl)amino)tetramethylrhodamine) (Molecular probes).

SJL young females (Harlan-Nossan, Udine, Italy) were housed and used according to current European Community rules. Mice were injected intraperitoneally with 12µg LPS (E Coli 026:B6, Sigma) 5-6 hours before starting the intravital experiment. Animals were anesthetized and a heparinized PE-10 catheter was inserted into the right common carotid artery toward the brain. In order to exclude non-cerebral vessels from the analysis, the right external carotid artery and pterygopalatine artery, a branch from the internal carotid, were ligated.\textsuperscript{12}

The preparation was placed on an Olympus BX50WI microscope and a water immersion objective with long focal distance (Olympus Achromplan, focal distance 3.3 mm, NA 0.5 ∞) was used. Blood vessels
were visualized through the bone by using fluorescent dextrans.\textsuperscript{12} $2 \times 10^6$ fluorescent labeled cells/condition were slowly injected into the carotid artery by a digital pump. The images were visualized by using a silicon-intensified target videocamera (VE-1000 SIT, Dage MTI, Michigan, IL) and a Sony SSM-125CE monitor and recorded employing a digital VCR (Panasonic NV-DV10000).

\textit{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.\textsuperscript{12} The velocities of $\geq 20$ consecutive freely flowing cells/venule were calculated, and from the velocity of the fastest cell in each venule ($V_{\text{fast}}$), we calculated the mean blood flow velocities ($V_m$): $V_m = V_{\text{fast}} / (2 - \varepsilon^2)$. The wall shear rate (WSR) was calculated from WSR=$8xV_m/D$ (s\textsuperscript{-1}), and the wall shear stress (WSS) acting on rolling cells was approximated by WSR x 0.025 (dyn/cm\textsuperscript{2}), assuming a blood viscosity of 0.025 Poise.

Lymphocytes that remained stationary on venular wall for $\geq 30$ 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.\textsuperscript{12}

\textit{In vitro rolling assay under flow}

100 $\mu$l microcap glass capillary tubes were coated with human P-selectin or VCAM-1 kindly provided by Dr. Daniele D'Ambrosio from Bioxell S.p.A., Milan, Italy. Lymphocytes were suspended in PBS, CaCl\textsubscript{2} 1 mM, MgCl\textsubscript{2} 1 mM, 10\% FCS, pH 7.2, and passed through the coated capillary tubes at a flow rate of 1250 $\mu$l/min (WSS of 2 dynes/cm\textsuperscript{2}). Images were recorded on S-VHS videotape and analyzed.\textsuperscript{19}

\textit{Statistics:}
A two-tailed Student's \( t \) test was employed for statistical comparison of two samples. Multiple comparisons were performed employing Kruskall-Wallis test with the Bonferroni correction of P. Velocity histograms were compared using Man-Whitney U-test and Kolmogorov-Smirnov test. Differences were regarded significant with a value of P<0.05.
Results

Patients

The present international guidelines for MS therapy indicate early treatment in patients with RRMS.\textsuperscript{20,21} The patients with RRMS used in this study had never previously undergone immuno-modulating therapy. The group of 20 patients with relapsing-remitting MS was extremely homogenous as they all were in the initial phase of the disease and scored low on the expanded disability status scale (EDSS). A group of age- and sex-matched healthy subjects was used as controls. All MS patients donated blood within 24 hours after the onset of the first clinical symptom of neurological dysfunction. Consequently, CD4\textsuperscript{+} and CD8\textsuperscript{+} cells obtained from these patients represent a precious human material and give high relevance to the results obtained in functional assays. All new symptoms and/or aggravation of preexisting symptoms of neurological dysfunction lasted more than 24h in agreement with the recommended diagnostic criteria for relapse in RRMS.\textsuperscript{16, 17}

Seven-color FACS analysis of CD4\textsuperscript{+} and CD8\textsuperscript{+} cells

We obtained a purity of $>98\%$ for CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells isolated from acute MS patients and from control donors (Fig. 1A).

We first compared the expression of rolling and sticking receptors between CD4\textsuperscript{+} T cells obtained from healthy donors and acute MS patients (Fig. 1 B, Table 1). We observed no statistically significant differences in L-selectin, LFA-1 and $\alpha_4$ integrins expression. Although the percentage of CD4\textsuperscript{+} cells expressing PSGL-1 was similar between the two populations, the mean fluorescence intensity (MFI) was significantly increased in MS patients (P$<0.05$).

We next compared the expression of rolling and sticking receptors between CD8\textsuperscript{+} T cells obtained from healthy donors and acute MS patients (Fig. 1 B, Table 1). In contrast with data obtained
with CD4+ cells, we observed a significant decrease in the percentage of L-selectin positive cells in acute MS patients (P<0.05). Moreover, a significant increase of the MFI for PSGL-1 was revealed in acute MS patients (P<0.02). We observed no statistically significant difference in LFA-1 and α4 integrin expression.

Finally, we compared the expression of various adhesion receptors between the CD4+ and CD8+ T cells from acute MS patients. Importantly, we observed that the percentage of CD8+ T cells expressing L-selectin is statistically lower when compared to that of CD4+ cells (P<0.005). MFI for L-selectin was also decreased in CD8+ population (P<0.03). Moreover, CD8+ T cells expressed statistically higher MFI for PSGL-1 (P<0.0002), LFA-1 (P<0.03) and α4 integrins (P<0.05). Interestingly, we observed that CD8+ cells that were negative for both CCR7 and L-selectin were responsible for the increased MIF of PSGL-1 (data not shown). Furthermore, CD27CD8+, but not CD27CD8+, T cells isolated from RRMS patients showed an increased expression of PSGL-1 (data not shown). Taken together, these results show that at the initiation of a clinical relapse in RRMS CD8+ T cells are more activated than CD4+ T cells and display an increase of the characteristics of memory effector phenotype.

**CD4+ T cells from acute MS patients do not show increased adhesiveness in brain venules in vivo.**

We investigated rolling and arrest of CD4+ and CD8+ cells in brain venules by using a novel method of intravital microscopy in mouse brain microcirculation. It has been previously shown that human adhesion molecules expressed by leukocytes are able to efficiently interact with their endothelial ligands expressed by mouse endothelium in intravital microscopy settings. Our intravital microscopy experiments allowed us to study for the first time the behavior of MS lymphocyte subpopulations under physiological conditions. Normal brain vessels were not able to mediate rolling and arrest of CD4+ or CD8+ cells from healthy or MS donors (data not shown). In fact, we have
previously shown that brain endothelium requires activation in order to recruit lymphocytes from the blood. It is now widely accepted that murine and human brain endothelium express P- and E-selectin, VCAM-1 and ICAM-1 during acute and subacute inflammation. Moreover, brain endothelium expresses endothelial selectins, VCAM-1 and ICAM-1 during EAE and MS. Thus, we next studied the interaction of human CD4 and CD8 T cells by using a novel, well-characterized in vivo model of subacute inflammation in which brain endothelium expresses P-selectin, E-selectin, VCAM-1, and ICAM-1.

We first compared rolling and sticking of CD4+ T cells isolated from healthy subjects versus CD4+ cells obtained from acute MS patients (Fig. 2 A, B, C). The comparison between cells derived from healthy and MS subjects was performed in the same venules, thus in similar hemodynamic conditions and in the presence of equivalent expression of endothelial ligands. As fluctuations in blood flow may occur in brain vessels, hemodynamic parameters were measured during the injection of cells derived from healthy subjects and MS patients. Notably, microvascular hemodynamics were similar during the injection of control cells or after administration of MS-derived cells, supporting the accuracy of our in vivo results (Fig. 2A). No statistically significant differences were observed between the percentage of rolling of CD4+ cells obtained from healthy donors and acute MS patients (Mean ± SD of 10.1 ± 4.4, versus 9.9 ± 3.5%) (Fig 2A). In order to search for potential differences between healthy subjects and MS patients we next analyzed the quality and strength of rolling by measuring the rolling velocities (Vroll) of CD4+ lymphocytes. The median rolling velocity of CD4 lymphocytes from healthy subjects was 12.6 µm/s, while the median rolling velocity of MS CD4 cells was 11 µm/s. Vroll from different experiments were pooled in velocity classes in order to better analyze potential rolling differences between CD4+ cells from healthy donors and acute MS patients. We observed no significant differences between the velocity classes of healthy and MS subjects (Fig.2B).

We also analyzed the arrest of lymphocytes in brain vessels. The percentage of CD4+ cells that firmly adhered was 3.6 ± 2 (mean ± SD) for healthy donors, and 3 ± 1.5 for MS patients (Fig. 2A, C).
Thus, as also shown for rolling fractions, no statistically significant differences were observed between healthy donors and MS patients in the percentages of CD4⁺ T cells undergoing firm arrest. This suggests that CD4⁺ lymphocytes from acute MS patients do not show increased adhesiveness in murine brain venules in vivo.

**CD8⁺ T cells from acute MS patients display increased rolling and sticking in murine brain venules**

In contrast to the results obtained with CD4⁺ T cells, the rolling fraction of MS-derived CD8⁺ T cells was two times higher than for CD8⁺ T cells from healthy controls (Mean ± SD of 14.6 ± 7.9, versus 7.9 ± 7%, P<0.04) (Fig. 3A). The comparison was performed in the same venules, and the hemodynamic parameters were measured during the injection of cells derived from healthy subjects and MS patients. We next measured and analyzed the rolling velocities of CD8⁺ lymphocytes (Fig. 3B). The median rolling velocity for CD8⁺ cells from healthy controls was 15.5µm/s, whereas the median rolling velocity of CD8⁺ cells from acute MS patients was 8µm/s, suggesting an increase of the fraction of slow rolling cells. V_roll from different experiments was pooled in velocity classes (Fig 3B, See also online Supplemental Material, Fig3video.mov). Considering the pool of cells that display a V_roll lower than 10µms, the difference between CD8⁺ cells from healthy controls and CD8⁺ cells from MS patients lymphocytes was striking: in healthy donors the percentage of CD8⁺ cells with low rolling velocities was 14.6%, while the percentage of slow-rolling CD8⁺ cells from MS was 52.4% (P<0.01). Moreover, in healthy controls, the percentage of CD8⁺ T cells rolling at velocities higher than 40µm/s was two-fold higher than that of CD8⁺ T cells derived from MS patients, suggesting different molecular mechanisms mediating rolling interactions in the two CD8⁺ lymphocyte populations.
We next analyzed sticking of CD8\(^+\) cells (Fig. 3A, C). The percentage of CD8\(^+\) cells that firmly adhered was 1.1 ± 1 (mean ± SD) for healthy donors. In contrast, the mean percentage (± SD) of adherent cells was 3.2 ± 2.2 for MS CD8\(^+\) T cells (P<0.04). This data clearly shows that CD8\(^+\) cells from MS patients display increased adhesive capabilities in brain venules when compared with CD8\(^+\) cells from healthy controls.

**VCAM-1 has an important role in the recruitment of CD4\(^+\) cells from acute MS patients**

We have previously observed that rapid adhesion of human T lymphocytes on murine purified VCAM-1 triggered by SDF-1 is blocked by the anti-human alpha4 antibody (HP2/1) and by the anti-murine VCAM-1 antibody (M.K. 2.7), indicating that human alpha4 integrins efficiently interact with murine VCAM-1 (Laudanna C., unpublished observation). Human PSGL-1 is also able to interact with endothelial selectins expressed by the murine endothelium.\(^{15}\) Taking advantage of the fact that human adhesion molecules expressed by leukocytes are able to efficiently interact with their endothelial ligands expressed by murine endothelium, we next endeavored to identify the molecular mechanisms involved in rolling of CD4\(^+\) T cells derived from acute-MS patients. Although these CD4\(^+\) cells express PSGL-1 at higher levels than CD4\(^+\) cells from healthy donors, a mAb to PSGL-1 had no significant effect on rolling (Fig 4A). In contrast, 65% of rolling was blocked by an anti-VCAM-1 antibody (P<0.01) (Fig. 4A).

We then compared \(V_{\text{roll}}\) before and after treatment with anti-PSGL-1 and anti-VCAM 1 antibodies. No significant effect using anti-PSGL-1 antibody could be ascertained, thus, PSGL-1 does not participate in the strength of rolling interactions (Fig. 4B). \(V_{\text{roll}}\) pooled in velocity classes after VCAM-1 mAb treatment show an increase (between 30-100%) of the number of cells in the velocity classes >30\(\mu\)m/s, and a decrease of 45% of the cells with slow rolling (<10\(\mu\)m/s) (Fig.4C).
Importantly, firm arrest was strongly blocked by treatment with anti-VCAM-1 mAb (inhibition of ~ 92%, p<0.001), but not by anti-PSGL-1 antibody (Fig. 4A). These findings suggest that VCAM-1/α4 integrins represent central molecules in the recruitment of CD4⁺ lymphocytes from acute MS patients.

**PSGL-1 is critical for the recruitment of CD8⁺ cells from acute MS patients**

Flow cytometric data showed that in acute MS patients CD8⁺ T cells present a statistically significant increase in MFI of PSGL-1 when compared to CD4⁺ T cells (P<0.0002). Anti-PSGL-1 antibody blocked 85% of CD8⁺ T cells rolling (P<0.001) (Fig.5A). Moreover, anti-VCAM-1 antibody blocked only 27% of rolling (P>0.05) (Fig.5A). Similarly, the anti-alpha4 antibody HP2/1 did not show a statistically significant effect on rolling (data not shown). These results clearly contrast with data obtained using CD4⁺ cells derived from acute-MS patients, and show that PSGL-1 expression on CD8⁺ T cells is critical for capture and rolling.

V_roll after PSGL-1 treatment was not determined because the number of cells that rolled was very low. We asked whether there might be a significant contribution by α4 integrins to the strength of rolling CD8⁺ cells in brain microcirculation. We observed a two fold increase (from 11.3 to 22.9%) of the percentage of cells with a V_roll >50 µm/s. Thus, VCAM-1/-α4 integrins seems to be involved in the strength of rolling of a small subpopulation of CD8⁺ cells (Fig. 5B).

Firm arrest was strongly blocked by treatment with anti-PSGL-1 mAb (inhibition of ~ 90%, p<0.001). In contrast with the data obtained using CD4⁺ T cells, the percentage of CD8⁺ T cells that arrested after treatment with anti-VCAM-1 mAb was 68 ± 14 (mean ± SEM; P>0.05) (Fig. 5A). Similarly, the anti-alpha4 antibody HP2/1 did not show a statistically significant effect on sticking (data not shown). These findings show that VCAM-1 does not significantly contribute to sticking of CD8⁺ cells from MS patients, suggesting that integrins other than VLA-4 are involved in this step.
**CD4⁺ and CD8⁺ T cell recruitment on P-selectin and VCAM-1 in vitro under flow**

Finally, we studied tethering and rolling capacity of CD8⁺ and CD4⁺ lymphocytes in a reduced, well-controlled system in capillary tubes coated with human P-selectin or VCAM-1 under a physiologic shear of 2 dyne/cm² (Fig. 6). The number of CD4⁺ lymphocytes from healthy controls that rolled on P-selectin was comparable to the number of CD4⁺ T cells derived from MS patients (mean ± SD of 25 ± 11 versus 22 ± 9) (Fig. 6A). In contrast, CD8⁺ cells from MS donors displayed a significant increase of rolling on P-selectin when compared with CD8⁺ cells from healthy donors (mean ± SD of 68 ± 17 versus 26 ± 10; P<0.01) (Fig. 6B). Importantly, MS-derived CD8⁺ cells exhibited a 3 fold increase in tethering and rolling on P-selectin when compared with MS-derived CD4⁺ cells (68 ± 17 versus 22 ± 9, P<0.01) (Fig. 6C). These results strongly indicate that CD8⁺, but not CD4⁺, cells from acute MS have a higher capacity to roll on P-selectin, a ligand expressed by acute and subacute inflamed endothelium.¹²,²⁸ VCAM-1 supported no rolling of either CD4⁺ or CD8⁺ cells derived from controls and MS patients (Fig. 6A, B). This is in agreement with previous data showing that VLA-4/VCAM-1 alone are not able to mediate rolling, as VLA-4/VCAM-1 mediated rolling requires a G-protein-linked signaling induced by a chemokine coexpressed with VCAM-1.²⁹
Discussion

We show here that CD8+ T cells from patients with RRMS in the acute phase of the disease have increased adhesion capabilities in brain vessels when compared to cells from healthy donors. We also show that a large fraction of CD8+ T cells from acute MS patients express surface markers characteristic of memory effector cells. Furthermore, we document a fundamental dichotomy in the molecular mechanisms controlling the adhesion of CD8+ versus CD4+ lymphocytes from acute MS patients, indicating the way for a potential selective blocking of the recruitment of lymphocyte subpopulations in RRMS.

The present international guidelines for RRMS therapy recommend early treatment, thus recruitment of patients not undergoing immunomodulatory therapy is becoming increasingly difficult. To our knowledge, we are the first to study the adhesiveness of lymphocyte subpopulations at the onset of a MS clinical attack under physiological conditions. The patients with RRMS used in this study had never previously undergone immuno-modulating therapy and donated blood within 24 hours after the onset of the first clinical symptom of neurological dysfunction. Consequently, the results obtained with CD4+ and CD8+ cells isolated from the blood of these patients have high relevance for the study of early inflammation in RRMS.

Seven-color FACS analysis was used for the first time for the characterization of the CD4+ and CD8+ lymphocytes derived from patients with RRMS at the beginning of the relapse phase. We showed that CD8+, but not CD4+, T cells from acute MS patients present lower levels of L-selectin when compared to healthy donors. Moreover, we observed that in patients in the acute phase of MS, expression of L-selectin by CD8+ T cells is significantly lower than that of CD4+ T cells. It is known
that L-selectin is downregulated on T cells with the memory-effector phenotype, and that cells that downregulate L-selectin lose the ability to migrate into lymphoid organs, but acquire the capacity to migrate into peripheral tissues and sites of inflammation.\textsuperscript{30,33} Thus, our data suggest that CD8\textsuperscript{+}, but not CD4\textsuperscript{+}, T cells from acute MS patients have an increased ability to migrate into sites of inflammation. Both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells from acute MS patients expressed increased levels of PSGL-1 when compared to healthy donors. PSGL-1 is constitutively expressed by almost all lymphocytes, but it was shown that only memory/activated T cells express the functional PSGL-1 that is able to bind P-selectin.\textsuperscript{34-36} PSGL-1 becomes functional following post-translational modifications by glycosyltransferases that are upregulated in activated lymphocytes.\textsuperscript{36} We have previously shown that in vitro antigen-activated CD4\textsuperscript{+} T cells, in contrast with naïve cells, express functional PSGL-1 which has a critical role in tethering and rolling on endothelial selectins in inflamed brain venules.\textsuperscript{12} However, increased expression of PSGL-1 on CD4\textsuperscript{+} T cells derived from acute MS patients was not functional as shown by lymphocyte rolling in capillary tubes coated with P-selectin and by the lack of effect of anti-PSGL-1 antibodies on the recruitment in inflamed brain venules expressing P-selectin. In contrast, CD8\textsuperscript{+} T cells from acute MS patients have increased expression of PSGL-1 and display a striking increase of rolling on P-selectin in vitro under flow. This data suggest that higher expression of PSGL-1 and improved post-translational modifications might contribute to the increased recruitment of CD8\textsuperscript{+} T cells in inflamed brain venules in vivo. Recently, Weninger et al., explored the migratory capacities of naïve, central memory and effector memory CD8\textsuperscript{+} T cells in vivo.\textsuperscript{33} The study has shown that memory effector CD8\textsuperscript{+} T cells are L-selectin\textsuperscript{7/low}, possess high binding capacity to P-selectin and efficiently migrate into sites of inflammation, but not into lymphoid organs.\textsuperscript{33} Consistent with this data, our results show a decrease in the percentage of L-selectin positive cells and a three-fold increase in the capacity of cells to roll on P-selectin under flow suggesting an increase of the memory effector phenotype of CD8\textsuperscript{+} T cells in acute MS patients. We also observed that CD8\textsuperscript{+} cells negative for both L-selectin and CCR7...
are responsible for the increased MFI of PSGL-1, suggesting that PSGL-1 expression is increased on memory effector cells (data not shown). Thus, taken together our results support the hypothesis that at the beginning of a clinical relapse in patients with RRMS, CD8⁺, but not CD4⁺, T cells represent the effector arm of the immune response.

No study has yet distinguished the adhesive capacity of lymphocyte subpopulations in MS patients. Here we studied for the first time rolling and arrest of CD4⁺ and CD8⁺ T cells from acute, untreated MS patients under physiological conditions by using: i) a novel method of intravital microscopy in mouse brain microcirculation and ii) in vitro under flow assays to study rolling in capillary tubes coated with human P-selectin or VCAM-1. We first compared the adhesive capacities of T cells from acute MS patients versus healthy donors in intravital microscopy experiments performed in inflamed mouse brain venules. In our experimental model, brain endothelium expresses P- and E-selectin, ICAM-1 and VCAM-1, thus displaying the endothelial adhesion molecules involved in lymphocyte extravasation during inflammation.¹²,32,33 We observed a striking difference in adhesiveness between CD4⁺ and CD8⁺ T cells from acute MS patients: MS-derived CD4⁺ T cells showed rolling and arrest fractions comparable to those of CD4⁺ T cells from healthy donors. Moreover, analysis of rolling velocities showed no significant differences between velocity classes of CD4⁺ T cells from MS patients and control subjects. In contrast, the percentage of CD8⁺ T cells that rolled and arrested was significantly higher in acute MS patients than in healthy subjects. Analysis of rolling velocities revealed a significant increase in the percentage of MS-derived CD8⁺ T cells with slow, "inflammatory" rolling. Taken together, these results indicate that CD8⁺, but not CD4⁺, T cells isolated at the beginning of a clinical relapse are more activated and have increased adhesion capacities in inflamed brain venules.

We showed that anti-PSGL-1 antibodies blocked almost 90% of rolling of MS-derived CD8⁺ T cells, suggesting that PSGL-1 is critical for the recruitment of CD8⁺ T cells in inflamed brain vessels. In agreement with intravital microscopy data, CD8⁺ T cells showed a significant increase of rolling on P-
selectin in vitro when compared with cells from healthy subjects. Importantly, we documented that CD8⁺ T cells from acute MS patients have a 3-fold increase in rolling on P-selectin in capillary tubes when compared to CD4⁺ T cells from the same patients. Thus, CD8⁺ T cells present an important characteristic required for the effector function, that is the capacity to bind P-selectin. Recent results obtained by Carrithers et al., suggest that P-selectin, a PSGL-1 ligand, may be involved in the early recruitment of encephalitogenic lymphocytes into the brain. Moreover, Keerfoot and Kubes 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. Moreover, E-selectin has been previously described in vessels from acute plaques of multiple sclerosis patients. Antibodies to PSGL-1 inhibit interactions of leukocytes to areas of inflammation in other animal models. Furthermore, recombinant soluble forms of PSGL-1 inhibit selectin-mediated inflammatory responses in models of inflammation and thrombosis in vivo. Thus, PSGL-1, the molecule responsible for the preferential recruitment of CD8⁺ T cells from acute MS patients, possibly represents a new pharmaceutical target which may be exploited to block the selective entrance of activated CD8⁺ cells into the brain in the early phases of brain inflammation.

Flow cytometry results showed an increased expression of PSGL-1 also on CD4⁺ T cells from acute MS patients. In contrast with data obtained with CD8⁺ T cells, anti-PSGL-1 antibodies had no inhibitory effect on rolling of MS-derived CD4⁺ T cells, suggesting that PSGL-1 expressed on CD4⁺ T cells is not functional and is not exposing the carbohydrate epitopes required for interaction with endothelial selectins. However, anti-VCAM-1 antibodies blocked ~ 65% of CD4⁺ cells rolling in brain venules. These results show that VCAM-1, but not PSGL-1, has an important role for capture and rolling, and that expression α4 integrins on CD4⁺ cells from acute MS is required for efficient primary adhesion in brain venules. However, ~35% of CD4⁺ T cells rolling was not blocked by antibodies anti-VCAM-1, suggesting that other molecules, such as L-selectin might participate in rolling. We have
recently shown that PSGL-1 is critical for the rolling of antigen-stimulated mouse CD4+ T cells in inflamed brain venules. Flow cytometry results showed that CD4+ T cells have no increased capacity to bind P-selectin and display equivalent levels of L-selectin expression when compared with healthy donors. Taken together, these results suggest that patients with RRMS have no increase of CD4+ cells in the effector phase at the beginning of a clinical relapse.

Anti-VCAM-1 antibodies dramatically blocked the arrest of MS-derived CD4+ T cells, showing that α4 integrins/VCAM-1 control sticking of CD4+ T cells. In contrast, anti-VCAM-1 antibodies inhibited only ~30% of MS patient-derived CD8+ T cells arrest, leading us to speculate that LFA-1 may be critical in this step of extravasation. The diversity in the recruitment mechanisms of CD4+ and CD8+ lymphocytes from MS patients suggest that quantitative and/or qualitative expression of adhesion molecules on brain endothelium might orchestrate the preferential extravasation of lymphocyte subpopulations. Moreover, the notion that distinct adhesion mechanisms exist for CD8+ and CD4+ lymphocytes raises the possibility to achieve potential selective blocking of the recruitment of lymphocyte subpopulations in MS.

There is now convincing evidence in the literature that autoreactive CD8+ T cells contribute to the pathogenesis of organ-specific autoimmune diseases. A role for CD8+ T cells in MS is supported by evidence of over-expression of CD8+ T cells in the leading edge of MS plaques, by the oligoclonal expansion of CD8+ T cells in patients with MS, and by the presence of MHC class I, but not class II on the surface of oligodendrocytes. The myelinating cells of the CNS. Moreover, CD8+ T cell clones specific for myelin antigens have been isolated from the periphery of MS patients and myelin-specific CD8+ T cells lyse human HLA-matched oligodendrocytes. Recent evidence from two independent labs strongly supports a major role for CD8+ T cells in autoimmune demyelination in EAE, the animal model of MS. Interestingly, CD8-induced EAE explains some important results obtained in clinical
trials of MS better than the CD4-induced models of EAE.\textsuperscript{7} It is widely known that the principal function of CD8\textsuperscript{+} T effector cells is the defence against viral infections. Various data point to a viral pathogenesis of MS: geographic association of disease susceptibility with evidence of MS clustering, abnormal response to a variety of viruses, an increase of disease exacerbations with viral infection, and an analogy with animal models and other human diseases in which viruses can cause demyelinating diseases.\textsuperscript{46} Thus, given the central role of CD8\textsuperscript{+} T cells in viral infections, our finding of an increase in the frequency of cells displaying the effector phenotype within the CD8\textsuperscript{+} population and the display of an increased adhesive capacity in brain vessels by this cell subset further substantiates the hypothesis that viral infections or reactivations may trigger clinical exacerbations in RRMS.

In conclusion, our results support a role for CD8\textsuperscript{+} T cells in early inflammation in relapsing-remitting MS, and together with growing data from the literature suggest that CD8\textsuperscript{+} T cells may represent major effectors in the autoimmune attack of the brain.
References


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Table 1. Expression of adhesion molecules on CD4⁺ and CD8⁺ T cells isolated from healthy and MS donors. Shown are the percentages (mean ± SD) and mean fluorescence intensity (MFI) ± SD of marker positive cells in the total population of four to eight experiments.
Figure 1. Phenotype of CD4$^+$ and CD8$^+$ T cells obtained from the blood of acute MS patients and healthy controls. 

A. Percentage of CD4$^+$ and CD8$^+$ T cells before and after isolation from PBL using MACS MicroBeads. 

B. The cells were phenotyped using 7-color FACS. Gates were set on either CD4$^+$ T cells or CD8$^+$ cells. One representative experiment from a total of eight.
Figure 2. CD4⁺ lymphocytes from acute MS patients do not show increased rolling and sticking in brain venules. CD4⁺ cells were isolated from 4 patients and 4 healthy controls. The experiments were performed in 4 mice. 10 venules were examined. A. Rolling and arrest fractions were compared between healthy and MS donors. Mean and ± SD are presented. D, diameter; Vm, mean blood flow velocity; WSS, wall shear stress. B. Velocity histograms were generated by measuring rolling velocities. Frequency distributions were calculated after cells were assigned to velocity classes from >0μm/s to
10 μm/s; 10 to 20 μm/s; 20 to 30 μm/s; and so on. 61 rolling cells were examined for healthy donors, and 69 rolling cells were considered for acute MS. C. Adhered CD4+ T cells obtained from a healthy donor (Healthy) (CMFDA-labeled) and acute MS patient (MS) (CMTMR-labeled) in the same cerebral venule.
Figure 3. CD8$^+$ lymphocytes from acute MS patients display increased rolling and sticking in brain venules. CD8$^+$ cells were isolated from 4 patients and 4 healthy controls. The experiments were performed in 4 mice. 7 venules were examined. A. Rolling and arrest fractions were compared between healthy and acute MS donors. Mean and ± SD are presented. *$P < 0.04$. D, Vm and WSS as described in Fig.1A. B. Velocity histograms were generated as described for Fig. 1B. 59 rolling cells were examined for healthy donors, and 64 rolling cells were considered for acute MS. C. Adhered CD8$^+$ T cells obtained from a healthy donor (Healthy) (CMFDA-labeled) and acute MS patient (MS) (CMTMR-
labeled) in the same cerebral venule. Note the difference in the number of adhered cells between the two populations.

Figure 4. VCAM-1 is required for rolling and arrest of acute MS-derived CD4⁺ T cells. A. To determine the role of PSGL-1, CD4⁺ T cells were isolated from 4 patients with acute MS. Control cells received no treatment, while other cells were pretreated with 100µg/ml anti-PSGL-1 mAb for 15 min at 25°C in a total volume of 300µl and then injected through the right carotid catheter. A supplement of up to 100µg mAb was administered together with anti-PSGL-1 mAb-treated cells. Ten venules were analyzed in total. Venular and hemodynamic parameters (mean ± SD) during the experiments were the following: \(D=40.6 ± 10.1\mu m\), \(V_m=1857 ± 410 \mu m/s\), \(WSS=8.4 ± 2.2 \text{ dyne/cm}^2\) for control cells, and \(D=40.6 ± 10.1\mu m\), \(V_m=2067 ± 393 \mu m/s\), \(WSS=10.2 ± 3.5 \text{ dyne/cm}^2\) for antibody-treated cells. To determine the role of VCAM-1, CD4⁺ T cells were isolated from 3 patients with acute MS. Control cells
were injected before the mAb administration. Then mice received intravenously 100µg mAb anti-VCAM-1 and after 10min., we injected the same number of cells as for control. Six venules were examined. Venular and hemodynamic parameters (mean ± SD) during the experiments were the following: D=46.6 ± 21.8µm, Vm=2125 ± 448 µm/s, WSS=10.5 ± 4.6 dyne/cm² for control cells, and D=46.6 ± 21.8µm, Vm=2743 ± 629 µm/s, WSS=12.5 ± 5.3 dyne/cm² after antibody treatment. Bars depict rolling and arrest fractions as percentage of control cells that rolled and arrested in the same venule. Data are expressed as the mean ± SEM. Groups were compared with control using Kruskall-Wallis test followed by Bonferoni correction of P. *P<0.01; **P<0.001. B. Velocity histograms were generated as described for Fig. 1B. n = number of rolling cells examined.
Figure 5. PSGL-1 is critical for the recruitment of acute MS-derived CD8\(^+\) T cells. A. To determine the role of PSGL-1, CD8\(^+\) T cells were isolated from 3 patients with acute MS. Control cells received no treatment, while other cells were treated as described at Fig. 4A. Seven venules were analyzed in total. Venular and hemodynamic parameters (mean ± SD) during the experiments were the following: D=44.7 ± 10.9 µm, \(V_m=1919 ± 749\) µm/s, \(WSS=8.6 ± 2.5\) dyne/cm² for control cells, and D=44.7 ± 10.9 µm, \(V_m=1905 ± 682\) µm/s, \(WSS=8.6 ± 3.1\) dyne/cm² for anti-PSGL-1 mAb-treated cells. To determine the role of VCAM-1, CD8\(^+\) T cells were isolated from 3 patients with acute MS. Control cells were injected before the mAb administration. Initially mice received 100 µg mAb anti-VCAM 1 intravenously. After 10 min. we injected the same number of cells as for control. Six venules were examined. Venular and hemodynamic parameters (mean ± SD) during the experiments were the following: D=50.8 ± 5.1 µm, \(V_m=2017 ± 964\) µm/s, \(WSS=7.7 ± 3\) dyne/cm² for control cells, and D=50.8 ± 5.1 µm, \(V_m=2256 ± 1022\) µm/s, \(WSS=8.6 ± 3.1\) dyne/cm² after antibody treatment. Bars depict rolling and arrest fractions as percentage of control cells that rolled and arrested in the same venule. Data are expressed as the mean ±
SEM. Groups were compared with control using Kruskall-Wallis test followed by Bonferoni correction of P. *P<0.01. B. Velocity histograms were generated as described for Fig. 1B. n = number of rolling cells examined.
Figure 6. CD8$^+$ cells from acute MS patients exhibit increased rolling on P-selectin in vitro. A. B. C. CD4$^+$ and CD8$^+$ T cells were obtained from the blood of healthy donors and acute MS patients. Three patients were studied. Values are mean percentage of total interacting cells to an area of 0.2 mm$^2$ in 30 seconds from one representative experiment from a total of three. Error bars are standard deviation values from at least 10 separate areas on the same capillary tube. Single areas of 0.2 mm$^2$ were recorded for at least 30 seconds. *P<0.01.
Online supplemental material: Fig3video.mov Intravital microscopy experiment in mouse inflamed brain microcirculation. Brain vessels were labeled by using FITC-Dextran. Brain endothelium was activated by pre-treating the mice with LPS 5h before starting the intravital microscopy experiment. CMFDA-labeled CD8+ T cells from acute MS patients (white moving dots) display slow inflammatory rolling suggesting a role for endothelial selectins and PSGL-1. Video characteristics (QuickTime file): length, 32s; frame size, 320 x 240 pixels; size, 4MB; Sorensen compression.

*Supplemental material is not available with the pre-published paper but will be available online only at the time of final publication.*
CD8⁺ T cells from acute multiple sclerosis patients display selective increase of adhesiveness in brain venules: a critical role for P-selectin glycoprotein ligand-1

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