Central nervous system recruitment of effector memory CD8⁺ T lymphocytes during neuroinflammation is dependent on α4 integrin

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Clonally expanded CD8⁺ T lymphocytes are present in multiple sclerosis lesions, as well as in the cerebrospinal fluid of patients with multiple sclerosis. In experimental autoimmune encephalomyelitis, CD8⁺ T lymphocytes are found in spinal cord and brainstem lesions. However, the exact phenotype of central nervous system-infiltrating CD8⁺ T lymphocytes and the mechanism by which these cells cross the blood–brain barrier remain largely unknown. Using cerebrospinal fluid from patients with multiple sclerosis, spinal cord from experimental autoimmune encephalomyelitis and coronavirus-induced encephalitis, we demonstrate that central nervous system-infiltrating CD8⁺ T lymphocytes are mostly of the effector memory phenotype (CD62L⁻/CCR7⁻/GranzymeB hi). We further show that purified human effector memory CD8⁺ T lymphocytes transmigrate more readily across blood-brain barrier-endothelial cells than non-effector memory CD8⁺ T lymphocytes, and that blood-brain barrier endothelium promotes the selective recruitment of effector memory CD8⁺ T lymphocytes. Furthermore, we provide evidence for the recruitment of interferon-γ- and interleukin-17-secreting CD8⁺ T lymphocytes by human and mouse blood-brain barrier endothelium. Finally, we show that in vitro migration of CD8⁺ T lymphocytes across blood-brain barrier-endothelial cells is dependent on α4 integrin, but independent of intercellular adhesion molecule-1/leucocyte function-associated antigen-1, activated leucocyte cell adhesion molecule/CD6 and the chemokine monocyte chemotactic protein-1/CCL2. We also demonstrate that in vivo neutralization of very late antigen-4 restricts central nervous system infiltration of CD8⁺ T lymphocytes in active immunization and adoptive transfer experimental autoimmune encephalomyelitis, and in coronavirus-induced encephalitis. Our study thus demonstrates an active role of the blood-brain barrier in the recruitment of effector memory CD8⁺ T lymphocytes to the CNS compartment and defines α4 integrin as a major contributor of CD8⁺ T lymphocyte entry into the brain.

Keywords: multiple sclerosis; blood–brain barrier; CD8⁺ T lymphocytes; α4 integrin; migration

Abbreviations: ALCAM = activated leucocyte cell adhesion molecule; BBB = blood–brain barrier; EAE = experimental autoimmune encephalomyelitis; ICAM = intercellular adhesion molecule; IFN = interferon; LFA = leucocyte function-associated antigen;
CD8+ T lymphocytes in neuroinflammation

**Introduction**

Multiple sclerosis is a CNS-directed inflammatory disease characterized by destruction of the myelin sheath, axonal loss and immune cell infiltration. Pervascular immune cell infiltrates found in active multiple sclerosis lesions are dominated by CD4+ T lymphocytes, antigen-presenting cells and CD8+ T lymphocytes (Sospedra and Martin, 2005; McFarland and Martin, 2007). The pathogenic contribution of CD4+ T lymphocytes in multiple sclerosis has been extensively documented and is supported by the association between susceptibility to multiple sclerosis and major histocompatibility complex (MHC)-II genes (Ebers et al., 1996; Haines et al., 1996; Sawcer et al., 1996; Oksenberg and Hauser, 2005), as well as by data demonstrating transfer of disease by myelin-reactive CD4+ T lymphocytes in the animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). In that regard, studies in EAE have shown a crucial role for both T helper 1 (Th1) and T helper 17 (Th17) CD4+ T lymphocytes in the initiation and progression of EAE (Becher et al., 2003; Langrish et al., 2005; Kebir et al., 2007; Kroenke et al., 2008; Stromnes et al., 2008). Meanwhile, very little is known about the phenotype, the origin and the route of entry of CD8+ T lymphocytes found in multiple sclerosis or EAE lesions.

The exact role of CD8+ T lymphocytes in autoimmune CNS inflammation remains controversial, and recent evidence supports both pathogenic and protective roles for CD8+ T lymphocytes in multiple sclerosis and in EAE. In favour of the pathogenic contribution of CD8+ T lymphocytes, oligoclones expanded CD8+ T cells were detected in demyelinated multiple sclerosis tissue (Babbe et al., 2000), suggesting CNS antigen reactivity and CD8-dependent lytic functions. CD8+ T cells have also been found closely associated with demyelinated axons in multiple sclerosis brain tissue (Neumann et al., 2002) and these cells were shown to interact with neural cells in an NKG2D-MICA-dependent manner (Saikali et al., 2007). Recently, several groups have also developed models of CD8+ T lymphocyte-dependent EAE-like disease (Huseby et al., 2001; Sun et al., 2001; Cabarrocas et al., 2003; Zehntner et al., 2003; Saxena et al., 2008), including those induced by transfer of myelin basic protein (MBP)-specific CD8+ T lymphocytes in C3H and in C57BL/6 or Rag1-/- animals, respectively (Huseby et al., 2001; Sun et al., 2001). Moreover, Cabarrocas et al. (2003) were able to induce EAE-like disease by transferring haemagglutinin-specific CD8+ T lymphocytes into animals over-expressing haemagglutinin on oligodendrocytes, demonstrating the lytic capacity of CNS-infiltrating CD8+ T lymphocytes. Conversely, several reports indicate that CD8+ T lymphocytes could play a regulatory role and protect against disease development (Jiang et al., 1992, 2001, 2003; Koh et al., 1992; Friese and Fugger, 2005; Linker et al., 2005; York et al., 2010). These include the findings that β-2 microglobulin-deficient mice exhibit a significantly more severe EAE than wild-type mice (Linker et al., 2005). Moreover, Jiang et al. (2001) demonstrated that CD8+ T lymphocytes are able to dictate the phenotype of CD4+ T lymphocytes in the periphery of EAE mice by inducing more Th17 cells. In addition, in a myelin basic protein-induced EAE model, CD8+ T lymphocytes downregulated pathogenic myelin basic protein-reactive CD4+ T lymphocyte clones (Jiang et al., 2003). Also, CD8+ T lymphocytes have been shown to prevent disease relapses (Jiang et al., 1992; Koh et al., 1992). Finally, the transfer of MOG35-55-specific CD8+ T lymphocytes in mice suppresses the induction of EAE and inhibits ongoing EAE by a cytotoxic/suppressor mechanism (York et al., 2010). Therefore, the exact contribution of CD8+ T lymphocytes to the pathology of multiple sclerosis and EAE remains unresolved.

Under physiological conditions, a very limited number of peripheral blood immune cells cross the endothelial cells of the blood–brain barrier (BBB) and the meningeal vessels, in a process called immune surveillance of the CNS (Sospedra and Martin, 2005). During an inflammatory process, meningeal or BBB-endothelial cells amplify the migration of immune cells to the CNS parenchyma, in a multi-step process that involves selectins, chemokines (and their receptors) and cell adhesion molecules (Springer, 1994). Leucocytes first undergo E- and P-selectin-mediated rolling along the surface of endothelial cells (Kubes and Ward, 2000), followed by chemokine-mediated activation and firm adhesion to the endothelium (Engelhardt and Ransohoff, 2005). BBB-endothelial cells and glial cells are an important source of the pro-inflammatory chemokines CCL2/MCP-1, RANTES and CXCL10/IP-10 (Ifergan et al., 2006), which are required for T helper 1 (Th1) and T helper 17 lymphocyte and monocyte recruitment to the CNS (Ransohoff et al., 2003; Engelhardt and Ransohoff, 2005; Mahad et al., 2006). Lastly and most importantly, upon activation with pro-inflammatory cytokines, BBB-endothelial cells express cell adhesion molecules such as intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and activated leucocyte cell adhesion molecule (ALCAM), which mediate at least in part, the adhesion process and the transmigration of leucocytes to the CNS through their interaction with integrin αLβ2 [leucocyte function-associated antigen (LFA)-1], α4β1 [very late antigen (VLA)-4] and CD6, respectively (Greenwood et al., 1995; Wong et al., 1999; Biemacki et al., 2001; Prat et al., 2002; Cayrol et al., 2008). Despite the ongoing controversy on the role of CD8+ T lymphocytes in multiple sclerosis and EAE, the exact phenotype of CD8+ T lymphocytes found in the inflamed brain and the molecular mechanism used by these CD8+ T lymphocytes to access the target organ remain incompletely understood.

In the current study, we demonstrate that human and mouse CD8+ T lymphocytes in the inflamed CNS compartment are mostly CD62L+ CCR7+ effector memory lymphocytes. We provide evidence that effector memory CD8+ T lymphocytes have a better propensity to migrate across human BBB-endothelial cells.
than non-effector memory cells. We further show that early in EAE, CD8+ T lymphocytes infiltrating the CNS display more aggressive functions [granzyme B^+ interferon (IFN)-γ^+] than CD4^+ T lymphocytes and that IFN-γ- and IL-17-secreting CD8^+ T lymphocytes migrate faster across human and mouse BBB-endothelial cells, in vitro and in vivo, respectively. Finally, we show that the migration of CD8^+ T lymphocytes across the BBB is mainly dependent on α4 integrin, in vitro and in vivo.

Materials and methods

Patients

Seventeen untreated patients with relapsing–remitting multiple sclerosis diagnosed according to McDonald’s criteria (McDonald et al., 2001) and 10 healthy volunteers were included in the study. Forty millilitres of blood and 5 ml of CSF were collected from patients with multiple sclerosis for cell phenotype analysis. Informed consent, as approved by the local ethics committee, was given by each patient before blood and CSF collection (local ethic approval SL05.022 and 023). Mean age of patients with multiple sclerosis and healthy controls (±SD) was 36.9 ± 9.5 years and 34.3 ± 10.2 years, respectively. For patients with multiple sclerosis, disease duration and last year relapse rate were 5.22 years and 1.1 ± 0.3, respectively.

Isolation and culture of blood–brain barrier endothelial cells and astrocytes

CNS tissue was obtained from temporal lobe resection specimens from young adults undergoing surgery for the treatment of intractable epilepsy. Informed consent and ethics approval were given prior to surgery (ethics approval number BH 07.001). BBB-endothelial cells were isolated from non-epileptic material according to our published protocol (Ifergan et al., 2008; Kebir et al., 2009; Cayrol et al., 2011). In brief, meninges were removed and cortical and subcortical white matter material was minced (3 mm^3), resuspended in pH 7.2 phosphate-buffered saline (PBS) and washed several times to remove matter material was minced (3 mm^3), resuspended in pH 7.2 phosphate-buffered saline (PBS) and washed several times to remove blood. CNS material was then homogenized using five strokes of loose-fitting Dounce homogenizer at 40 g and filtered on a 350 μm pore size mesh. The homogenate was snap-frozen in liquid nitrogen and stored at −80°C until further use.

CD8^+, CD8^+ CD62L^+ and CD8^+ CD62L^- T lymphocyte isolation

Eighty millilitres of venous blood samples were obtained from 10 consenting healthy donors, in accordance with institutional guidelines. Peripheral blood mononuclear cells were isolated from EDTA anti-coagulated blood using standard Ficoll-Hypaque™ (Amersham Pharmacia Biotech) density gradient centrifugation. CD8^+ T lymphocytes were purified from peripheral blood mononuclear cells using magnetic cell sorting (Miltenyi Biotec) according to the manufacturer’s instructions. CD8^+ T lymphocytes purity was >97% as assessed by flow cytometry using anti-CD8-fluorescein isothiocyanate (FITC), anti-CD4-phycocerythrin (PE) and anti-CD3-allophycocyanin (APC) (BD Biosciences).

CD62L^- and CD62L^+ cells were purified from CD8^+ T lymphocyte populations from three consenting healthy donors (obtained from a negative selection) using anti-CD62L-PE antibodies and anti-PE immunobeads (magnetic cell sorting; Miltenyi Biotec). Purity of positively selected cells was consistently >95% as confirmed by flow cytometry.

Migration across blood–brain barrier endothelial cells

BBB-endothelial cells grown in primary cultures were used to generate an in vitro model of the human BBB as previously published (Ifergan et al., 2006; Kebir et al., 2009). BBB-endothelial cells were plated on gelatin-coated 3 μm pore size Boyden chambers (Collaborative Biomedical Products) at a density of 2.5 × 10^5 cells/well, in endothelial cell culture media supplemented with 40% (v/v) astrocyte-conditioned media for 96 h, in order to allow them to reach confluency. The formation of a confluent monolayer was confirmed by haematoxylin–eosin staining, as well as soluble tracer diffusion (Ifergan et al., 2006). Media were removed, replaced with fresh endothelial cell media supplemented with 40% (v/v) astrocyte-conditioned media. Freshly purified human CD8^+, CD8^+ CD62L^- or CD8^+ CD62L^+ T lymphocytes were then added to the upper chamber and allowed to migrate for 24 h across BBB-endothelial cells either untreated or pre-activated for 24 h with 100 U/ml IFN-γ (1 U = 2 ng/ml) and 100 U/ml TNF-α (1 U = 0.05 ng/ml) (BioSource-Invitrogen). When applicable, migration experiments were performed in the presence of blocking antibodies or the corresponding isotype. We used anti-ICAM-1 (10 μg/ml; clone BBIG-I1, R&D Systems), anti-VCAM-1 (10 μg/ml; clone BBIG-V1, R&D Systems), anti-ALCAM (30 μg/ml; Clone 105, R&D Systems), anti-platelet endothelial cell adhesion molecule (PECAM)-1/CD31 (10 μg/ml, clone WM59, Biologend), anti-MHC-I (15 μg/ml; clone W6/32, ebioscience), anti-IL-8 (10 μg/
Flow cytometric analyses

Human cells were phenotyped using antibodies specific for human CD3, CD4, CD8, CCR7, CD62L, granzyme B and perforin (all from BD Biosciences). Mouse cells were phenotyped using specific antibodies for mouse CD3ε, CD4, CD8, CCR7, CD62L and granzyme B (all from BD Biosciences). Cells were stained for 30 min at 4°C, washed with fluorescence-activated cell sorting (FACS) buffer containing PBS containing 1% (v/v) foetal bovine serum and 0.1% (w/v) Na2S (Sigma). Cells were then fixed and permeabilized in 4% (w/v) paraformaldehyde (Sigma) with 0.1% (w/v) saponin in Hank’s Balanced Salt Solution for 10 min at room temperature. Intracellular staining was performed by incubating cells with antibodies against granzyme B and perforin for 30 min on ice in FACS buffer containing 0.1% (w/v) saponin, followed by two washes and resuspended in FACS buffer. Cells were acquired on a BD LSR II and analysed using BD FACSDiva software.

For IFN-γ (clone B27, BD Biosciences) and IL-17 (clone eBio64DEC17, eBioscience) staining, cells were activated for 18 h with 1 μg/ml ionomycin and 20 ng/ml phorbol 12-myristate 13-acetate (PMAA) in the presence of 2 μg/ml brefeldin A (Sigma) for the last 6 h of co-culture. Cells were stained for surface markers and an intracellular staining was performed.

Experimental allergic encephalomyelitis induction and scoring

Eight- to 9-week-old female C57BL/6 mice (Charles) were used to induce EAE by active immunization. Mice were injected subcutaneously with 200 μg of MOG35–55 peptide (Alpha Diagnostic International) emulsified in complete Freund’s adjuvant supplemented with 600 μg Mycobacterium tuberculosis (Difco). On days 0 and 2 after immunization, 500 ng pertussis toxin were administered intraperitoneally. Mice were then injected intraperitoneally on days 1, 3, 5, 7 and 9 with either anti-CD4 integrin (75 μg; clone R1-2, monoclonal; BD Biosciences) or isotype control antibody (75 μg of IgG2b clone A95-1, BD Biosciences). On day 18 post transfer, three mice of each group were perfused with PBS and their organs were recovered. CNS-immune cells were isolated by Percoll gradient centrifugation. The number of CD3+ CD4+ T lymphocytes and CD3+ CD8+ T lymphocytes in the CNS were determined by multiplying the percentage of lineage marker-positive cells by the total number of mononuclear cells isolated from the organ. The animal protocol was approved by the Comité Institutionnel de Protection des Animaux du CRCHUM (N07027PAs).

Transfer of myelin oligodendrocyte glycoprotein-reactive CD8+ T lymphocytes into naïve animals

Spleen and inguinal lymph nodes were extracted on day 8 after immunization and passed through a 70-μm cell strainer to make single cell suspensions. Splenocytes were treated with 0.83% ammonium chloride for 3 min at room temperature to lyse red blood cells. Cells were then passed again through another 70-μm cell strainer. CNS cells were isolated by density gradient centrifugation using Percoll (Amersham Biosciences AB). The number of CD3+ CD4+ T lymphocytes and CD3+ CD8+ T lymphocytes were determined by multiplying the percentage of lineage marker-positive cells by the total number of mononuclear cells isolated from the organ. The animal protocol was approved by the Comité Institutionnel de Protection des Animaux du CRCHUM (N07027PAs).

Coronavirus-infected mice

Four- to 5-week-old female C57BL/6 mice (Charles River) were infected intracerebrally (i.c.) with the A99 neurotropic, neuroinvasive and neurovirulent strain of mouse hepatitis virus (MHV) (Guslin et al., 2005). The highest non-lethal dose of 450 plaque forming units was administered in a maximal volume of 50 μl (Guslin et al., 2005). A group of mice was inoculated with sterile media (mock). On day 4 post infection, mice were infected intraperitoneally with anti-CD4 integrin (75 μg; clone R1-2, monoclonal; BD Biosciences) or isotype control antibody (75 μg of IgG2b clone A95-1, BD Biosciences). On day 7, five mice of each group were perfused with PBS and their organs were recovered. CNS-immune cells were isolated by Percoll gradient centrifugation. The number of CD3+ CD4+ T lymphocytes and CD3+ CD8+ T lymphocytes in the CNS were determined by multiplying the percentage of lineage marker-positive cells by the total number of mononuclear cells isolated from the CNS.

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Immunostaining of human and mouse central nervous system material

Luxol fast blue and haematoxylin–eosin stainings were performed on human brain tissue specimens obtained from three patients with multiple sclerosis (autopsy) and on mouse spinal cord specimens as previously described (Kebir et al., 2009). Sections showing acute demyelinated lesions and active perivascular mononuclear cell infiltration were selected (three donors: 8–12 blocks per donor). Mean age was 49 ± 6 years and disease duration ranged from 3 to 23 years. The causes of death were pneumonia (n = 1), urosepsis (n = 1) and barbiturate intoxication (n = 1). CNS material from EAE animals was collected following rapid intra-cardiac PBS perfusion and snap-frozen in liquid nitrogen. Human and murine tissues were cryosectioned (7-μm thick), mounted on superfrost slides (Thermo Scientific), fixed in −20°C acetone for 10 min and hydrated in PBS. Endogenous biotin was blocked with the Avidin/Biotin blocking kit (Invitrogen) when required. Non-specific immunoglobulin binding was blocked with serum for 30 min. Sections were then incubated for 1 h with the primary antibody diluted in serum. Slides were washed five times for 3 min with PBS Tween-20 0.05% (v/v) after each incubation. This was followed by 1 h incubation with the secondary antibody. All incubations were done at room temperature. Corresponding isotypes were used as controls for the immunostains. Sections were then mounted with Gelvatol containing TOPRO-3 (Invitrogen) for nuclear staining. Fluorescence acquisition was carried out on a Leica SP5 confocal microscope (Leica Microsystems). Imaging processing and analysis were performed with Leica LAS AF software.

Statistical analysis

Statistical analyses were performed using PRISM Graphpad™ software and data are presented as the mean ± SEM. One-way ANOVA was performed followed by Bonferroni multiple comparison post hoc test for all experiments except for the migration across the BBB, which was done using two-way ANOVA without post hoc test. Only P < 0.05 were considered significant.

Results

Effector memory CD8⁺ T lymphocytes are enriched in the cerebrospinal fluid of patients with multiple sclerosis

CD8⁺ T lymphocytes have been reported to be a significant cellular constituent of active multiple sclerosis lesions (Traugott et al., 1983; Hauser et al., 1986; Babbe et al., 2000; Saikali et al., 2007) and to be found in the CSF of patients with multiple sclerosis (Jacobsen et al., 2002). We thus elected to compare the phenotype of CD8⁺ T lymphocytes present in the CSF to those in the peripheral blood of patients with multiple sclerosis. Peripheral blood mononuclear cells and CSF cells of untreated patients with relapsing–remitting multiple sclerosis were immunostained and analysed by flow cytometry. We noted an enrichment of effector memory CCR7⁻ CD62L⁻ CD3⁺ CD8⁺ T lymphocytes in the CSF when compared with the peripheral blood of the same patients (Fig. 1A). To establish whether these lymphocytes carry effector lytic molecule(s) typically ascribed to the CD8 effector memory phenotype, we evaluated their expression of granzyme B. We detected an increased number of granzyme B-expressing CD8⁺ T cells in the CSF when compared with the peripheral blood collected from the same patients (Fig. 1A). We repeated the phenotypic analysis on cells obtained from 17 patients with relapsing–remitting multiple sclerosis and confirmed the significant increase of CD62L⁻ CCR7⁻ granzyme B⁺ effector memory CD8⁺ T lymphocytes in the CSF, relative to the peripheral blood (Fig. 1B; P < 0.001). Collectively, these data demonstrate the presence, accumulation and enrichment of potentially lytic effector memory CD8⁺ T lymphocytes in the CSF of multiple sclerosis patients with active disease.

Human blood–brain barrier endothelial cells favour the recruitment of effector memory CD8⁺ T lymphocytes

In order to gain access to the CSF and accumulate in the CNS compartment, peripheral blood CD8⁺ T lymphocytes need to cross microvascular structures, including BBB-endothelial cells, astrocytic end-feet, pericytes and basement membranes. To establish whether the enrichment of effector memory CD8⁺ T lymphocytes observed in the CSF of patients with relapsing multiple sclerosis reflects a preferential migration of peripheral blood effector memory subsets, or whether the effector memory phenotype is induced during the transmigration process, we used a well-established in vitro model of the BBB consisting of primary cultures of human CNS microvascular endothelial cells grown in the presence of astrocytic factors (Ifergan et al., 2006; Kebir et al., 2009). Upon reaching confluence in the Boyden chamber, endothelial cells were stimulated with TNF and IFN-γ (both at 100 U/ml), two cytokines reported to partake in multiple sclerosis pathogenesis (Sospedra and Martin, 2005) and known to activate endothelial cells (Calabresi et al., 2001; Biernacki et al., 2004). After several washes to remove residual cytokines, ex vivo CD8⁺ T lymphocytes isolated from the peripheral blood of healthy donors were allowed to migrate for 24 h across human activated BBB-endothelial cells. Migrated and non-migrated CD8⁺ T lymphocytes were collected and their phenotype was analysed by flow cytometry. CD8⁺ T lymphocytes recovered from the lower chamber (migrated cells) were consistently enriched in CCR7⁻ CD62L⁻ cells, as compared with ex vivo CD8⁺ T lymphocytes (Fig. 2A and Supplementary Fig. 1). Consistent with these findings, we detected higher proportions of CD3⁺ CD8⁺ granzyme B⁺ T lymphocytes and perforin⁺ cells (Fig. 2A and Supplementary Fig. 1) in the migrated population than in non-migrated and in ex vivo (peripheral blood) cells. We did not observe any difference in endothelial cell death whether endothelial cells were cultured in the presence or absence of CD8⁺ T cells (n = 4; Supplementary Fig. 2), ruling out the possibility that the migration was the result of a damaged endothelial monolayer caused by exposure to cytotoxic CD8⁺ T lymphocytes.

Activated CD8⁺ T lymphocytes are characterized by their production of IFN-γ (Kaech et al., 2002). It is also well-established that IL-17 secreted by T lymphocytes can disrupt endothelial and epithelial barriers (Kebir et al., 2007). In humans, these two
pro-inflammatory cytokines are known to be expressed by memory rather than by naive CD4+ and CD8+ T lymphocytes (Kebir et al., 2007, 2009). Therefore, we analysed the capacity of activated human BBB-endothelial cells to promote the recruitment of cytokine-secreting CD8+ T lymphocytes. While the migrated cell population was significantly enriched in CD3+ CD8+ T lymphocytes expressing IFN-γ alone or both IFN-γ and IL-17 (Fig. 2A and Supplementary Fig. 1), the percentage of IFN-γ- or IL-17-expressing cells recovered from the upper chamber (non-migrated cells) did not significantly differ from the one in ex vivo peripheral blood cells.

To rule out the possibility that the effector memory phenotype was induced on CD8+ T lymphocytes following their transmigration across the activated endothelium, CD8+ CD62L+ and CD8+
Increased migration of ex vivo effector memory CD8\(^+\) T lymphocytes across blood–brain barrier (BBB) endothelial cells. (A) Human ex vivo CD8\(^+\) T lymphocytes were allowed to migrate for 24 h across activated human BBB-endothelial cells in a modified Boyden chamber and then analysed by flow cytometry. The migrated population (upper right panel) contained an enriched effector memory phenotype (CCR7\(^{-}\) CD62L\(^{-}\)) compared to the non-migrated (upper middle panel) and to the ex vivo (upper left panel) populations. Also, migrated cells were enriched in the cytolytic enzymes granzyme B (large boxes) and perforin (small boxes) (centre right panel) compared to the non-migrated (centre left panel) and to the ex vivo (centre middle panel) groups. (B) Graph showing the number of migrating cells (x10^3 cells) from CD8\(^+\) CD62L\(^{+}\) and CD8\(^+\) CD62L\(^{-}\) populations with error bars indicating standard deviation. The migrated CD8\(^+\) CD62L\(^{-}\) group had a significantly higher number of migrating cells compared to the migrated CD8\(^+\) CD62L\(^{+}\) group (***p < 0.001).
CD8+ CD62L− populations were sorted and allowed to migrate separately across BBB-endothelial cells. Interestingly, we found that CD8+ CD62L− cells migrated more avidly across activated BBB-endothelial cells than CD8+ CD62L+ cells (Fig. 2B). Similar results were obtained using non-activated BBB-endothelial cells (data not shown). Furthermore, only a very small percentage (<2%) of CD62L−-sorted CD8+ T lymphocytes lost the surface expression marker CD62L upon migration across BBB-endothelial cells (Fig. 2C). This confirms that the enrichment of effector memory CD8+ T lymphocytes following in vitro migration of CD8+ T lymphocytes results from their preferential recruitment across BBB-endothelial cells rather than induction of an effector phenotype from non-effector memory CD8 populations. Collectively, our findings demonstrate that activated human BBB-endothelial cells promote the recruitment of effector memory CD8+ T lymphocytes with an increased propensity to secrete granzyme B, perforin, IFN-γ and IL-17.

**Recruitment of effector memory CD8+ T lymphocytes in the early phase of experimental autoimmune encephalomyelitis**

In order to validate our human in vitro findings, we evaluated the temporal profile of CD8+ and CD4+ T lymphocyte recruitment to the CNS in experimental models of neuroinflammation. Overall, we found a predominance of CD4+ over CD8+ T lymphocytes within the CD3+ T lymphocyte population in the CNS of MOG35–55− induced EAE mice (Fig. 3A). However, at the preclinical phase of EAE (day 8 post immunization, score <1.0) most CD8+ T lymphocytes found in the CNS were CCR7− CD62L− (effector memory), while CD4+ T lymphocytes were CCR7+ CD62L+ (non-effector memory) (Fig. 3A). During the symptomatic phase of the disease, the proportion of effector memory CD4+ T lymphocytes increased significantly (day 12, EAE scores 1–2.5, Fig. 3A) and by day 18 (EAE score >2.5) was equivalent to that of effector memory CD8+ T lymphocytes (Fig. 3A). The production of cytokines and granzyme B by CD8+ T lymphocytes correlated with their effector memory phenotype. At day 8 post immunization, 94.8% of all CD8+ T lymphocytes in the CNS expressed granzyme B (Fig. 3B) and 40.5% were IFN-γ+ (Fig. 3B). At days 12 and 18, we observed a marked reduction in the proportion of granzyme B- and of IFN-γ+ expressing CD8+ T lymphocytes infiltrating the CNS of EAE mice (Fig. 3B).

Throughout the course of the disease, IL-17-expressing CD8+ T lymphocytes represented a negligible population of CNS infiltrating cells. These *in vivo* data corroborate our *in vitro* observations in human, suggesting a preferential recruitment of effector memory CD8+ T lymphocytes to the CNS compartment, in the early stage of autoimmune CNS inflammation. Moreover, these results support the notion that in EAE, specific populations of CCR7− CD62L− effector memory CD8+ T lymphocytes access the CNS at least 10 days prior to the recruitment or the development of a significant population of CCR7+ CD62L+ effector memory CD4+ T lymphocytes. FACS analysis of CNS homogenates does not allow us to identify the exact location (perivascular versus parenchymal white or grey matter) of these effector memory CD8+ T lymphocytes, within the CNS compartment.

**Presence of effector memory CD8+ T lymphocytes in multiple sclerosis and experimental autoimmune encephalomyelitis lesions**

To determine whether effector memory CD8+ T lymphocytes found in the CSF of patients with multiple sclerosis were also present in brain tissue, we performed *in situ* immunostaining for CD8, granzyme B and IFN-γ on >20 distinct post-mortem specimens obtained from three subjects with multiple sclerosis. Since effector memory populations are defined by the absence or low expression of CD62L and CCR7, these markers could not be used for immunostainings. We focused the histological examination (Luxol fast blue and haematoxylin–eosin staining) on lesions characterized by perivascular infiltration within areas of demyelination, as previously shown (Keber et al., 2007, 2009; Cayrol et al., 2008; Ifergan et al., 2008). The infiltration of CD8+ T lymphocytes was relatively limited in these lesions and most CD8+ T lymphocytes were scattered through the parenchyma or found in leptomeninges, as previously shown (Hayashi et al., 1988; Saikali et al., 2007). Triple immunofluorescent staining demonstrated co-expression of IFN-γ and granzyme B in CD8+ T lymphocytes within white matter infiltrates (Fig. 4A) and in leptomeninges, with at least 20 ± 3 cells per lesion analysed. Overall, we found to the non-migrated populations (centre middle and left panels). Inflammatory cytokines IFN-γ and IL-17 were also detected at higher levels in migrated CD8+ T lymphocytes (lower right panel) compared to the non-migrated CD8+ T lymphocytes (lower middle panel) and to the CD8+ T lymphocytes before migration (lower left panel). Data shown are representative of 10 independent experiments using 10 distinct blood donors on seven distinct BBB-endothelial cell preparations. (B) Human *ex vivo* CD8+ CD62L+ or CD8+ CD62L− T lymphocytes were sorted and allowed to migrate for 24 h across inflamed human BBB-endothelial cells. Sorted CD8+ CD62L+ T lymphocytes (black bar) had an enhanced migratory capacity when compared to CD8+ CD62L− T lymphocytes (white bar). Results are expressed as mean ± SEM of three independent experiments, performed in triplicate on two distinct BBB-endothelial cell preparations (**P < 0.01). (C) CD8+ CD62L+ and CD8+ CD62L− T lymphocytes were allowed to migrate for 24 h across inflamed human BBB-endothelial cells, collected from the lower chamber and analysed for CD62L expression by flow cytometry. Sorted CD8+ CD62L− T lymphocytes (shaded histogram) did not lose CD62L expression after migration and CD8+ CD62L− T lymphocytes (open histogram) did not acquire CD62L expression after migration. Data shown are representative of three independent experiments.

**Figure 2 Continued**
Figure 3  Effector memory CD8+ T lymphocytes accumulate in the CNS of EAE mice. EAE was induced by active immunization of C57BL/6 animals with MOG35-55/complete Freund’s adjuvant. At days 8, 12 and 18 after induction of the disease, the brain and spinal cord were homogenized and immune cells were isolated by Percoll gradient centrifugation and analysed by flow cytometry. (A) Cells were first gated on CD3 (upper panels), and expression of CCR7 and CD62L was assessed on CD3+ CD4+ CD8- (middle panels) and CD3+ CD4- CD8+ (lower panels). (continued)
that the ratio of CD4+ to CD8+ T lymphocytes in active multiple sclerosis lesions was of 8:1 (n = 12 lesions, from three donors).

We next sought to confirm these findings in MOG-immunized C57BL/6 EAE animals. Histological examination of frozen spinal cord sections obtained from four EAE mice showed that 41.8 ± 7.8% infiltrating CD8+ T lymphocytes were positive for IFN-γ (Fig. 4B and C) and 78.1 ± 13.4% were positive for granzyme B (Fig. 4B, lower panels; Fig. 4C, right panel). These data support the notion that effector memory CD8+ T lymphocytes infiltrating multiple sclerosis and EAE lesions carry a cytolytic potential.

The migration of CD8+ T lymphocytes to the central nervous system compartment is mediated by α4 integrin

We next studied the mechanism of CD8+ T lymphocyte recruitment to the CNS compartment, using the in vitro model of the BBB and blocking antibodies directed against several adhesion molecules, chemokines and integrins known to be involved in the leucocyte transmigration process. We found that specific blockade of ICAM-1, VCAM-1, ALCAM, PECAM-1, MHC-I, IL-8, MCP-1 and integrin αL did not alter the migration of human CD8+ T lymphocytes across resting (data not shown) or across TNF and IFN-γ-activated BBB-endothelial cells (Fig. 5A). However, specific blockade of α4 integrin using the 2B4 monoclonal antibody significantly reduced the migration of CD8+ T lymphocytes across BBB-endothelial cells, when compared with appropriately matched isotype monoclonal antibody (Fig. 5A; P < 0.001). This suggests that the migration of human CD8+ T lymphocytes across human BBB-endothelial cells is dependent on α4 integrin, but independent of ICAM-1/LFA-1, ALCAM/CD6, PECAM-1/PECAM-1 interactions.

To confirm the role of integrin α4 in the migration of CD8+ T lymphocytes across BBB-endothelial cells, we evaluated the effect of anti-α4 blockade on CD8+ T lymphocyte recruitment to the CNS during EAE. Using the MOG35–55-induced C57BL/6 EAE model, we tested the ability of anti-α4 integrin antibody (75 μg per injection intraperitoneally on days 6, 9, 12, 15 and 18 post immunization) to impact on the recruitment of CD8+ T lymphocytes into the CNS compartment, which includes the perivascular space, the parenchyma and the leptomeninges. As previously demonstrated (Theien et al., 2001), anti-α4 integrin treatment had a significant impact on the development of clinical signs of EAE (Fig. 5B) and on infiltration of immune cells into the CNS compartment (Fig. 5C and D). At the histopathological level, total counts of CNS-infiltrating CD4+ and CD8+ T lymphocytes isolated at days 8, 12 and 18 revealed a predominance of CD4+ over CD8+ T cells (Fig. 5C and D). However, and in accordance with our in vitro human data, we detected significantly lower numbers of CD3+ CD4+ and of CD3+ CD8+ T lymphocytes in the CNS compartment of EAE mice treated with anti-α4 integrin antibody, when compared with those treated with the isotype monoclonal antibody (Fig. 5C and D).

To demonstrate a direct effect of anti-α4 integrin antibody on the recruitment of CD8+ T lymphocytes, we first evaluated CD49d expression by infiltrating CD3+ CD8+ cells found in the CNS compartment of EAE mice 18 days post immunization. We found that 96.8% of CNS-infiltrating CD8+ T lymphocytes seen in isotype-treated mice expressed CD49d (Supplementary Fig. 3A). We also showed that there are no differences in the number of CD3+ CD8+ T lymphocytes found in the spleen of isotype-treated mice and anti-α4 integrin-treated mice at days 8, 12 and 18 (Supplementary Fig. 3B), demonstrating that the antibody treatment does not deplete CD8+ T lymphocytes in the periphery.

Finally, in order to demonstrate that α4 integrin blockade does not affect CD8+ T lymphocyte migration through CD4+ lymphocyte migration blockade, MOG-reactive CD8+ T lymphocytes were transferred into naïve recipient mice, in the presence or absence of the anti-α4 integrin antibody. CD3+ CD8+ lymphocytes were purified and restimulated in vitro in the presence of MOG35–55, IL-2 and IL-15 before transfer (Supplementary Fig. 4A and B). While isotype control-treated animals developed a mild disease (average score of 0.7 ± 0.26, data not shown), anti-α4-treated animals did not develop clinical signs of EAE (scores 0.1 ± 0.1, one out of six animals developed a score of 0.5). Furthermore, there was significantly less infiltration of both CD4+ and CD8+ T lymphocytes into the CNS compartment of mice treated with the anti-α4 integrin antibody, when compared with those treated with the isotype monoclonal antibody at day 18 post transfer (Fig. 5E; P < 0.01). The number of CD4+ T lymphocytes found in the CNS compartment were similar between the active immunization (46500 ± 5500) and the CD8 adoptive transfer groups (44860 ± 11320), suggesting that activated MOG-reactive CD8+ lymphocytes from the donor animal can attract bystander naïve CD4+ lymphocytes of the recipient animal into the CNS. Analysis of the CD8+ T lymphocyte population found in the CNS of recipient mice revealed a large portion of effector memory cells (62.1%; Supplementary Fig. 4C) carrying the lytic enzyme granzyme B and the inflammatory cytokine IFN-γ (68.2 and 63.3%, respectively; Supplementary Fig. 4C). These results put forward the important contribution of α4 integrin in the recruitment of CD8+ T lymphocytes into the inflamed CNS compartment and suggest that encephalitogenic CD8+ T lymphocytes across human BBB-endothelial cells are dependent on α4 integrin, but independent of ICAM-1/LFA-1, ALCAM/CD6, PECAM-1/PECAM-1 interactions.
Granzyme B and IFN-γ are highly expressed by CD8⁺ T lymphocytes in multiple sclerosis and EAE tissue. (A) Frozen CNS specimens (7-μm sections) from patients with multiple sclerosis were immunostained for CD8 (in blue), granzyme B (in red) and IFN-γ (in green). Immunostainings were acquired by confocal microscopy. The majority of CD8⁺ T lymphocytes found multiple sclerosis brain specimens expressed granzyme B and IFN-γ (arrowheads). Photomicrographs shown are representative of immunostainings performed on 12 brain sections from frozen CNS material of three patients with multiple sclerosis. Scale bar = 10 μm. (B) Frozen CNS sections (7 μm) from EAE mice 12 days post-induction were immunostained for CD8 (in green), TOPRO-3 for nuclear staining (in blue) and either IFN-γ (in red, upper panel) or granzyme B (in red, lower panel). Co-localization is presented in right panels, and CD8⁺ T lymphocytes expressing either IFN-γ or granzyme B are shown by arrowheads. Photomicrographs shown are representative of >20 immunostainings performed on post-mortem material (brain and spinal cord) from four animals. Scale bar = 10 μm. (C) Quantification of IFN-γ⁺ and granzyme B⁺ CD8⁺ T lymphocytes in the CNS of EAE animals revealed that the majority of CNS infiltrating CD8⁺ lymphocytes expressed granzyme B. IL-17-expressing CD8⁺ T lymphocytes could not be detected by microscopy. Data shown represent mean ± SEM from n = 10 CNS sections from four animals (*P < 0.05).
Figure 5 Migration of CD8+ T lymphocytes to the CNS depends on α4 integrin. (A) Human ex vivo CD8+ T lymphocytes isolated from the blood of healthy donors were allowed to migrate across activated human blood–brain barrier (BBB)-endothelial cells in a modified Boyden chamber for 24 h and then analysed by flow cytometry. The migration of CD8+ T lymphocytes was significantly reduced by anti-α4 integrin blockade (clone 2B4), but not by neutralization of ICAM-1, VCAM-1, ALCAM, PECAM-1, MHC-I, IL-8, MCP-1 or αL integrin (CD11a). Individual effect of treatment groups on CD8+ T lymphocyte migration was normalized to its own isotype control, to allow comparison. Results are expressed as mean ± SEM of 10 independent experiments, performed in triplicate on seven distinct BBB-endothelial cell preparations. ***P < 0.001, treatment group compared to its corresponding isotype. (B) In vivo blockade of α4 integrin improves EAE clinical scores. EAE was induced by active immunization of C57BL/6 animals with MOG35–55/complete Freund’s adjuvant. Anti-α4 integrin (clone R1-2, open triangle) or isotype control antibody (IgG2b, filled circle) were injected intraperitoneally (75 μg) on days 6, 9, 12, 15 and 18 post-immunization (arrowheads). Results are expressed as mean ± SEM of at least 10 mice per group per day. Data shown are representative of two independent experiments performed on 22 mice per group (*P < 0.05). (C) On days 8, 12 and 18 after induction of the EAE, the brain and spinal cord were homogenized and immune cells were isolated by Percoll gradient centrifugation. There were significantly less CD8+ (left bar graphs) and CD4+ T lymphocytes (right bar graphs) in the CNS of EAE (continued)
lymphocytes can attract naive bystander CD4+ T lymphocytes into the CNS.

**α4 integrin blockade affects the recruitment of CD4+ and CD8+ T lymphocytes into the CNS compartment of coronavirus-infected mouse**

One of the essential roles of CD8+ T lymphocytes is to protect the host against viral infections. To evaluate whether anti-α4 integrin blocking antibody could impact on the ability of CD8+ T lymphocytes to perform anti-viral surveillance of the CNS, we used a mouse model of CNS infection in which the MHV-A59 coronavirus is injected directly into the brain. CNS infection with MHV-A59 induces the migration into the brain of CD8+ T lymphocytes expressing VLA-4 and low levels of CD62L (Stohlman et al., 1998; Bergmann et al., 2006). The peak of CNS immune cell infiltration is observed 7 days after infection, coinciding with the clearance of infectious virus (Gruslin et al., 2005; Hosking and Lane, 2009). As anticipated, and when compared with uninfected animals, CD8+ T lymphocytes were the predominant subset found in the CNS of MHV-A59-infected mice (97.8 ± 10^3 CD8+ T cells versus 38.6 ± 10^3 CD4+ T cells per brain; ratio of three CD8+ T lymphocytes for one CD4+ lymphocyte; Fig. 6A and D). Most infiltrating CD8+ T lymphocytes in the brain of MHV-A59-infected mice had an effector memory phenotype and expressed granzyme B (Fig. 6B and C). Granzyme B expression was higher in infiltrating CD8+ T lymphocytes than in CD4+ T lymphocytes, while the expression of IFN-γ was comparable between the two T lymphocyte subsets (Fig. 6C). These data are compatible with previously published articles on the importance of immune cell response for the clearance and survival of mice following an MHV infection and has been shown in several studies using RAG1−/− and SCID mice (Wang et al., 1990; Houtman and Fleming, 1996; Wu and Perlman, 1999). To evaluate the role of α4 integrin in immune surveillance and defence against CNS viral infection, anti-α4 integrin antibody or IgG2b isotype were injected intraperitoneally on day 4 after infection and animals were sacrificed at day 7. Anti-α4 integrin antibody-treated mice had a worse clinical phenotype (moribund), when compared with animals that received isotype control antibody. We observed a significant reduction in CD4+ and CD8+ T lymphocyte infiltration in the CNS of mice treated with anti-α4 integrin when compared with control animals (Fig. 6D, P < 0.05 and 0.01, respectively). Again, to demonstrate the direct effect of anti-α4 integrin antibody on CD8+ T lymphocyte recruitment to the CNS, we evaluated CD49d expression on CD3+ CD8+ cells found in the CNS of MHV-A59-infected mice 7 days after infection: 95.2% of infiltrating CD8+ T lymphocytes found in isotype-treated mice expressed CD49d (Supplementary Fig. 3A). We also showed that there was no difference in the number of CD3+ CD8+ cells found in the spleen of isotype-treated mice and anti-α4 integrin-treated mice 7 days post infection (Supplementary Fig. 3B), demonstrating that the antibody treatment does not deplete CD8+ T lymphocytes in the periphery. These data confirm the critical role of α4 integrin in the recruitment of both T lymphocyte subsets during viral infection of the CNS compartment.

**Discussion**

The goal of the current study was to characterize the phenotype of CD8+ T lymphocytes found in the CNS during autoimmune and virus-induced neuroinflammatory events, and to identify the molecular determinants involved in the trafficking of CD8+ T lymphocytes to the CNS compartment, the CSF, the brain parenchyma, the perivascular space or the leptomeninges. We demonstrate herein a preferential migration of CCR7−/− CD62L− effector memory CD8+ T lymphocytes to the CNS compartment, early in the course of EAE and MHV-A59 coronavirus infection. Our human data, using ex vivo CSF and peripheral blood samples of patients with multiple sclerosis and in situ immunostainings of multiple sclerosis lesions demonstrate that the majority of CD8+ T lymphocytes found in the CNS compartment of patients with multiple sclerosis (CSF and parenchyma) are CCR7−/− CD62L− effector memory CD8+ T lymphocytes. In addition, data obtained from our in vitro model of the BBB, composed of primary cultures of human endothelial cells and astrocyte-conditioned media, show that CCR7−/− CD62L− effector memory CD8+ T lymphocytes are better suited to migrate through BBB-endothelial cells. While our in vitro human BBB assay does not recapitulate the entire complexity of the neurovascular unit (including pericytes and the parenchymal basement membrane), data presented herein provide strong evidence that effector memory CD8+ T lymphocytes...
Figure 6  Anti-α4 integrin treatment restricts migration of CD8+ T lymphocytes to the CNS in MHV-A59-infected mice. Mice were infected intracerebrally with the A59 neurotropic, neuroinvasive and neurovirulent strain of MHV. On day 7, five mice were sacrificed and immune cells from the CNS were isolated by Percoll gradient centrifugation. (A) Characterization of CNS infiltration by CD3+CD8+ and CD3+CD4+ T lymphocytes in uninfected mice (left panel) and coronavirus-infected mice (right panel). (B) The profile of CD3+CD8+ T lymphocytes was analysed by flow cytometry in infected mice. The proportion of effector memory (CCR7-CD62L-) CD8+ T lymphocytes was 73.9% (lower left panel). The proportion of CD8+ T lymphocytes expressing granzyme B (lower centre panel) and IFN-γ (lower right panel) were 80.2 and 41.6%, respectively. Data shown are representative of five mice in two independent experiments. (C) Effector memory (CCR7-CD62L-) phenotype, granzyme B, IFN-γ and IL-17 expression of CD3+CD8+ (white bars) or CD3+CD4+ T lymphocytes (black bars) collected from the brains of MHV-A59-infected mice. The majority of CD8+ T lymphocytes collected from the CNS had an effector memory phenotype and expressed granzyme B. In comparison, 50% of CD4+ T lymphocytes had an effector memory phenotype. Expression levels of IFN-γ and IL-17 remained similar for both cell types, and IL-17 expression remained barely detectable in the CNS of MHV-A59 infected animals. Results are expressed as mean ± SEM, n = 10 mice. (D) Mice infected intracerebrally with MHV-A59 virus were injected intraperitoneally with anti-α4 integrin or isotype control IgG2b antibody (75 μg) on day 4. On day 7, animals were sacrificed and immune cells from the CNS were isolated. A significant reduction in the number of both CD8+ and CD4+ T lymphocytes was found in the CNS of mice treated with anti-α4 integrin (grey bars) compared to control isotype injected animals (black bars). Results shown are expressed as mean ± SEM of five animals, and representative of two consecutive experiments (*P < 0.05 and **P < 0.01). Ab = antibody.
have a better propensity to migrate through BBB-endothelial cells and vascular basement membrane than other CD8+ T cell subsets. However, our model does not fully recapitulate the two-step concept developed by Bechmann et al. (2007), in which immune cells must migrate through a second layer of CNS perivascular cells (glia limitans) to gain access to the brain parenchyma.

While the purpose of this study was not to prove the encephalitogenic potential of effector memory CD8+ T lymphocytes, we provide evidence that the majority of CNS-infiltrating CD8+ T lymphocytes express granzyme B, perforin and IFN-γ. Moreover, we show that the migration of effector memory CD8+ T lymphocytes into the CNS precedes that of effector memory CD4+ T lymphocytes in EAE induced by active immunization and that the transfer of MOG-reactive CD8+ T lymphocytes into naïve animals promotes the recruitment of bystander CD4+ T lymphocytes of the recipient animal into its own CNS. Finally, we demonstrate that MOG-reactive CD8+ T lymphocytes can induce a clinical form of mild EAE (average score of $0.70 \pm 0.26$), despite the recruitment of an equivalent number of CD4+ T lymphocytes. Our study therefore emphasizes the potential encephalitogenic activity of these CNS-infiltrating CD8+ T lymphocytes, as previously proposed (Saikali et al., 2007; Friese and Fugger, 2009), but does not provide the evidence that CD8+ T lymphocytes can induce EAE, in the absence of MOG-reactive CD4+ T lymphocytes.

Using three distinct animal models of neuroinflammation and a human in vitro transmigration assay, we also clearly demonstrate that blocking α4 integrin leads to a significant reduction in the migration of CD8+ T lymphocytes across CNS vascular structures. Surprisingly, CD8+ T lymphocyte migration was not affected by blocking interactions between αL integrin–ICAM-1, ALCAM–CD6, PECAM-1–PECAM-1 or CCL2/MCP-1–CCR2. Since each of these molecules was previously shown to partake in the recruitment of various subsets of leucocytes to the CNS, including CD4+ T lymphocytes (αL integrin, ALCAM, CCR2) (Greenwood et al., 1995; Prat et al., 2002; Cayrol et al., 2008), monocytes/dendritic cells (αL integrin, ALCAM, CCR2) (Seguin et al., 2003; Cayrol et al., 2008), CD19+ B lymphocytes (αL integrin, ALCAM, CCR2) (Alter et al., 2003; Cayrol et al., 2008), eosinophils (αL integrin) (Gonlugur and Efegolu, 2004), neutrophils (αL integrin, PECAM-1) (Choi et al., 2009) and that none of them affect CD8+ T lymphocyte transmigration, we conclude that CD8+ T lymphocytes use different and more restricted molecular mechanisms to gain access to the CNS. It remains to be established whether, aside from α4 integrin, additional and possibly still unidentified adhesion molecule or chemokine pathways are involved in the trafficking of CD8+ T lymphocytes to the CNS. Nevertheless, our results point to an important and significant effect of α4 integrin in the transendothelial recruitment of CD8+ T lymphocytes to the CNS in mice and in humans, using an experimental in vitro BBB system. Our data also suggest that the considerable redundancy in molecular routes of migration observed for numerous leucocyte subsets (i.e. for CD4+ T lymphocytes and monocytes) might not apply to CD8+ T lymphocytes, and that the recruitment of these cells into target organs might depend on a more restricted array of adhesion molecules.

Natalizumab is a humanized monoclonal antibody directed against α4 integrin (Engelhardt and Kappos, 2008). α4 integrin either associates with β1 integrin to form VLA-4 or with β7 integrin to form LPAM-1. Both heterodimers are expressed by lymphocytes in human and mouse, and are reported to be key mediators for the firm adhesion and migration of peripheral blood lymphocytes and antigen-presenting cells to CNS vascular structures, through interaction with VCAM-1 and/or the CS-1 specialized domain of matrix protein fibronectin (Berlin et al., 1995; von Andrian and Engelhardt, 2003; Luster et al., 2005). In EAE, injection of mice with anti-α4 integrin before the onset of symptoms prevents the development of the disease (Yednock et al., 1992; Theien et al., 2001). In patients with multiple sclerosis, natalizumab treatment decreases the number of lymphocytes in the CSF and has shown beneficial effects on relapses, disability, T2 lesions and gadolinium-positive lesions (Tubridy et al., 1999; Miller et al., 2003; O’Connor et al., 2004; Stuve et al., 2006). Unfortunately, anti-α4 integrin therapy has been linked to an increase in the emergence of progressive multi-focal leucoencephalopathy, a devastating demyelinating disease of the CNS caused by reactivation of the human JC polyomavirus (Kleinschmidt-DeMasters and Tyler, 2005; Langer-Gould et al., 2005; Van et al., 2005), and previously known to occur mainly in patients with HIV/AIDS, haematological malignancies or post-transplantation immunosuppression (Berger, 2000). The cellular immune response mediated by antigen-specific effector memory CD8+ T lymphocytes against JC virus was shown to be crucial in the control of progressive multi-focal leucoencephalopathy (Koralnik et al., 2002; Du Pasquier et al., 2003, 2004; Marzocchetti et al., 2009). Although speculative, it is tempting to suggest that the effects of anti-α4 integrin therapy on effector memory CD8+ T lymphocyte trafficking to the CNS, as observed in our study, could explain the emergence of progressive multi-focal leucoencephalopathy in patients treated with natalizumab. Given that the primary function of effector memory CD8+ T lymphocytes is to defend the organism against acute and persistent or latent viral infections, our data now provide evidence that immune surveillance of the CNS compartment by CD8+ T lymphocytes is significantly hampered by anti-α4 integrin therapy.

Overall, our data demonstrate that effector memory CD8+ T lymphocytes essentially depend on α4 integrin to migrate across BBB-endothelial cells in vitro and in the CNS compartment in vivo. Given that this specific subset of CD8+ T lymphocytes is known to be involved in immune surveillance of target organs and to control acute or persistent infections, we postulate that the emergence of progressive multi-focal leucoencephalopathy in natalizumab-treated patients arises from a deficit in CD8+mediated immunosurveillance of the CNS compartment. Immune cell transmigration across BBB-endothelial cells represents a critical step for initiation of CNS-directed immune reactions. Therefore, a better understanding of the mechanisms involved in the transmigration of specific leucocyte subsets is critically needed to develop therapies aiming to reduce organ-targeted inflammation, without affecting CD8-mediated immune surveillance.

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**Supplementary material**

Supplementary material is available at Brain online.

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