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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; α-4 integrin; migration

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

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MCP = monocyte chemotactic protein; MHC = major histocompatibility complex; MHV = mouse hepatitis virus; MOG = myelin oligodendrocyte glycoprotein; VCAM = vascular cell adhesion molecule; VCAM = vascular cell adhesion molecule; VLA = very late antigen

Introduction

Multiple sclerosis is a CNS-directed inflammatory disease characterized by destruction of the myelin sheath, axonal loss and immune cell infiltration. Perivascular 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_H1 and T_H17 $CD4^+$ 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, oligoclonally 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 $protein_{79–87}$- or myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$-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 overexpressing 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 T_H2 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 $MOG_{35–55}$-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_H1 and T_H17 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; Biernacki *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^{hi}] 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 ($\pm$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 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 (BSH Thompson). Filtrate was then passed twice through a 112 µm pore size mesh, collected and treated with collagenase type IV (2 mg/ml; Sigma) for 15 min at 37°C. After inactivation with foetal bovine serum (Sigma), filtrate was spun down and plated on 0.5% (v/v) gelatin-coated six-well plates in endothelial cell culture media composed of M199 cell culture media (Invitrogen) supplemented with 10% (v/v) foetal bovine serum, 20% (v/v) human normal serum, insulin–transferrin–selenium and endothelial cells growth supplement (5 µg/ml) (all from Sigma) at 37°C in 5% CO_2 atmosphere. Two days after initial plating, wells were washed twice with PBS to remove non-adherent cells, and fresh media was added. By days 5–7, colonies of endothelial cells were visible; by day 20, human brain endothelial cells from the microvessel fraction (<112 µm) could be expanded in tissue culture flasks. As previously demonstrated, these cells express factor VIII, von Willebrand factor, Ulex Agglutenens Europaensis-1 binding sites, endothelial antigen HT-7; and are susceptible to tumour necrosis factor (TNF)-induced ICAM-1 and VCAM-1 upregulation. Immunoreactivity for glial fibrillary acidic protein and α-myosin was undetectable, confirming the absence of contaminating astrocytes and smooth muscle cells, respectively (Prat *et al.*, 2000, 2001; Biernacki *et al.*, 2001; Wosik *et al.*, 2007).

For astrocyte culture, human cerebral hemispheres from foetuses of 17–23 weeks of gestation were obtained from the Human Foetal Tissue Repository (Albert Einstein College of Medicine) following approved guidelines from the Canadian Institutes of Health Research. Astrocytes were cultured as previously described (Ifergan *et al.*, 2008) in complete Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% (v/v) foetal bovine serum and express glial fibrillary acidic protein. Astrocyte-conditioned media was harvested once a week from confluent flasks and added to the BBB-endothelial cells culture media when specified.

$CD8^+$, $CD8^+$ $CD62L^+$ 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-phycoerythrin (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 2.5×10^4 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 105 901, R&D Systems), anti-platelet endothelial cell adhesion molecule (PECAM)-1/CD31 (10 µg/ml, clone WM59, Biolegend), anti-MHC-I (15 µg/ml; clone W6/32, eBioscience), anti-IL-8 (10 µg/

ml; clone 6217, R&D Systems), anti-monocyte chemotactic protein (MCP)-1, (10 μg/ml; clone 23007, R&D Systems), anti-αL/CD11a (10 μg/ml, clone Hl111, Biolegend) and anti-α4/CD49d (20 μg/ml, clone 2B4, R&D Systems). All antibodies were applied to the upper chamber 30 min prior to the addition of immune cells. After 24 h, cells that had completely migrated through the BBB and cells remaining in the upper chamber were collected, counted and their profile was analysed by flow cytometry. Migration experiments were carried out with 10 different blood donors, performed in triplicate on seven distinct BBB-endothelial cell preparations.

Endothelial cell death was assessed by flow cytometry on two different preparations. In brief, 7-aminoactinomycin D (0.25 μg; BD Biosciences) was added for 10 min to endothelial cells that had been exposed or not to purified CD8$^+$ T cells for 24 h. The frequency of 7-aminoactinomycin D-positive cells was assessed by gating on CD8$^-$ cells.

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) NaN_3 (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 LSRII 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 (PMA) 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 $MOG_{35–55}$ 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 6, 9, 12, 15 and 18 with anti-α4 integrin (75 μg; clone R1-2, monoclonal; BD Biosciences) or isotype control antibody (75 μg of IgG2b clone A95-1, BD Biosciences). Two independent experiments were performed, one with 10 mice per group and another with 12 mice per group. Clinical signs of EAE were assessed daily according to the following scores: 0, no clinical sign of disease; 1, limp tail; 2, hind limp weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, hind and fore limb paralysis. On days 8, 12 and 18 post immunization, three to four mice of each group were perfused with PBS. Following rapid intra-cardiac PBS perfusion, the spleen, spinal cord and brain were removed and kept on ice. The organs were separately mashed (except for brain and spinal cord) 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).

Transfer of myelin oligodendrocyte glycoprotein-reactive CD8$^+$ T lymphocytes into naive animals

Spleen and inguinal lymph nodes were extracted on day 8 after immunization and passed through a 70-μm cell strainer (BD Biosciences). The cell suspension was treated with erythrocyte lysing solution (0.83% ammonium chloride) and resuspended in RPMI 1640 supplemented with 10% foetal bovine serum. Cells were cultured *in vitro* for 3 days without any stimulation or in presence of 20 μg/ml $MOG_{35–55}$, recombinant mouse IL-2 (50 U/ml) and recombinant mouse IL-15 (1 ng/ml). Levels of IFN-γ production by CD8$^+$ T lymphocytes was evaluated by flow cytometry to assess their activation. Cells were harvested and washed in Hank's Balanced Salt Solution. CD8$^+$ T lymphocytes were purified using magnetic cell sorting (Miltenyi Biotec) according to the manufacturer's instructions. CD8$^+$ T lymphocyte purity was >90% as assessed by flow cytometry. Purified CD8$^+$ T lymphocytes were injected intraperitoneally into 12 naïve recipient mice (10×10^6 cells/mouse). On days 0 and 2 after the transfer, 500 ng of pertussis toxin were administered intraperitoneally. Mice were then injected intraperitoneally on days 1, 3, 5, 7 and 9 with either anti-α4 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 CNS.

Coronavirus-infected mice

Four- to 5-week-old female C57BL/6 mice (Charles River) were infected intracerebrally (i.c.) by the A59 neurotropic, neuroinvasive and neurovirulent strain of mouse hepatitis virus (MHV) (Gruslin *et al.*, 2005). The highest non-lethal dose of 450 plaque forming units was administered in a maximal volume of 50 μl (Gruslin *et al.*, 2005). A group of mice was inoculated with sterile media (mock). On day 4 post infection, mice were injected intraperitoneally with anti-α4 integrin (75 μg; monoclonal; BD Biosciences) or isotype control antibody (75 μg of rat IgG2b; BD Biosciences). On day 7, five mice of each group were perfused with PBS and immune cells from the CNS were isolated. CD3$^+$ CD4$^+$ and CD3$^+$ CD8$^+$ T lymphocyte numbers were determined following the same method as for EAE. Two independent experiments were performed, both with five mice per group. The animal protocol was approved by the Comité Institutionnel de Protection des Animaux de l'INRS-Institut Armand-Frappier (CIPA #0412-05).

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

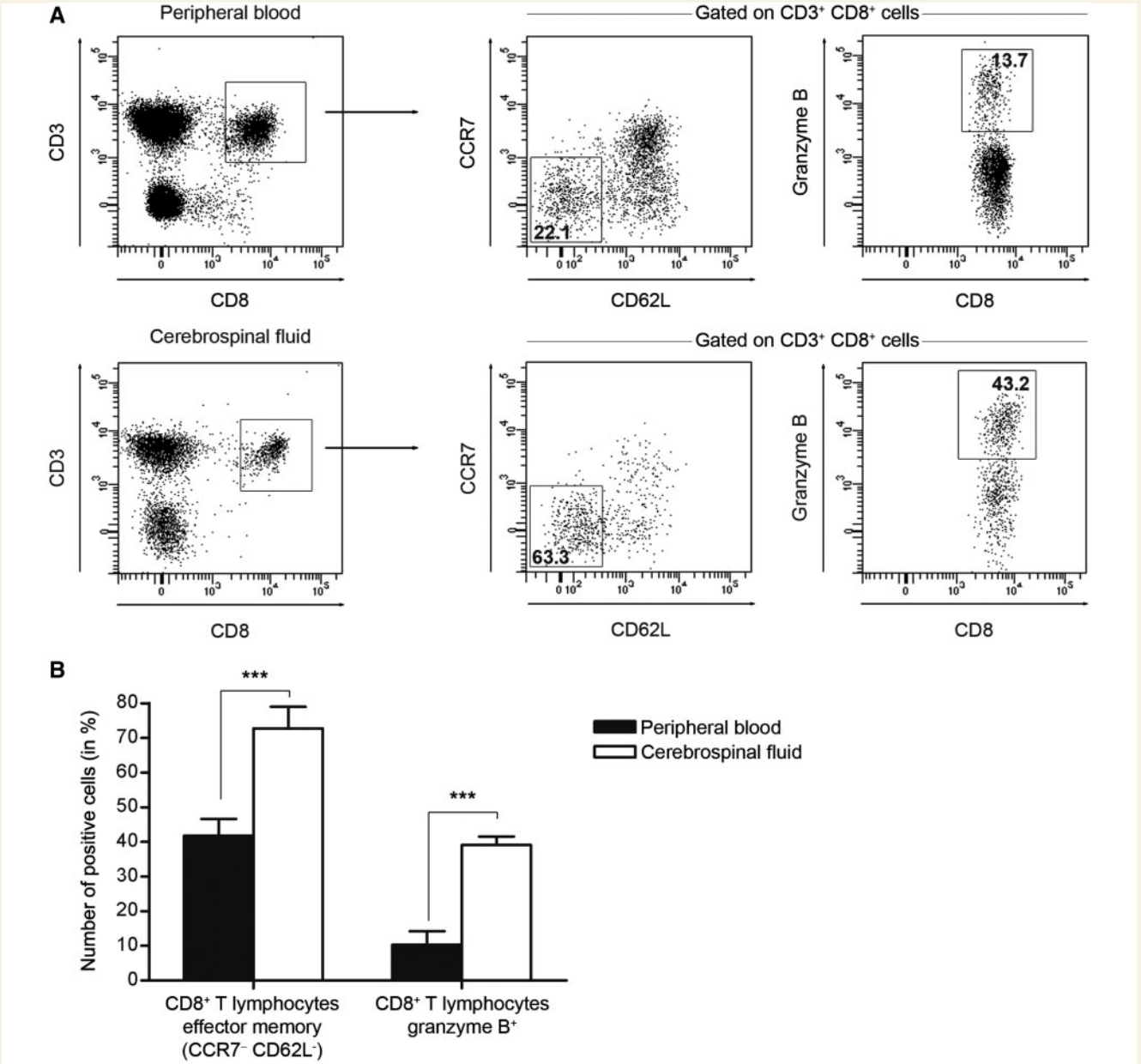

Figure 1 CSF of patients with multiple sclerosis contains predominantly effector memory CD8$^+$ T lymphocytes. (**A**) Flow cytometry profile of peripheral blood and CSF of one representative patient with relapsing-remitting multiple sclerosis reveals that CCR7$^-$ CD62L$^-$ effector memory CD8$^+$ T lymphocytes are enriched in the CSF (*lower middle* panel) compared to the peripheral blood (*upper middle* panel). Also, CD8$^+$ T lymphocytes in the CSF express higher levels of granzyme B (*lower right* panel) than CD8$^+$ T lymphocytes isolated from the blood (*upper right* panel) of the same patient. Data shown are representative of 17 independent experiments using 17 distinct multiple sclerosis donors. (**B**) Percentage of effector memory and granzyme B-expressing CD8$^+$ T lymphocytes in the CSF and in the blood of 17 patients with relapsing-remitting multiple sclerosis. The CSF (white bars) showed significantly more effector memory and granzyme B$^+$ CD8$^+$ T lymphocytes compared to the peripheral blood (black bars). Results are expressed as the mean ± SEM of the 17 patients with multiple sclerosis (***$P < 0.001$).

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$^+$

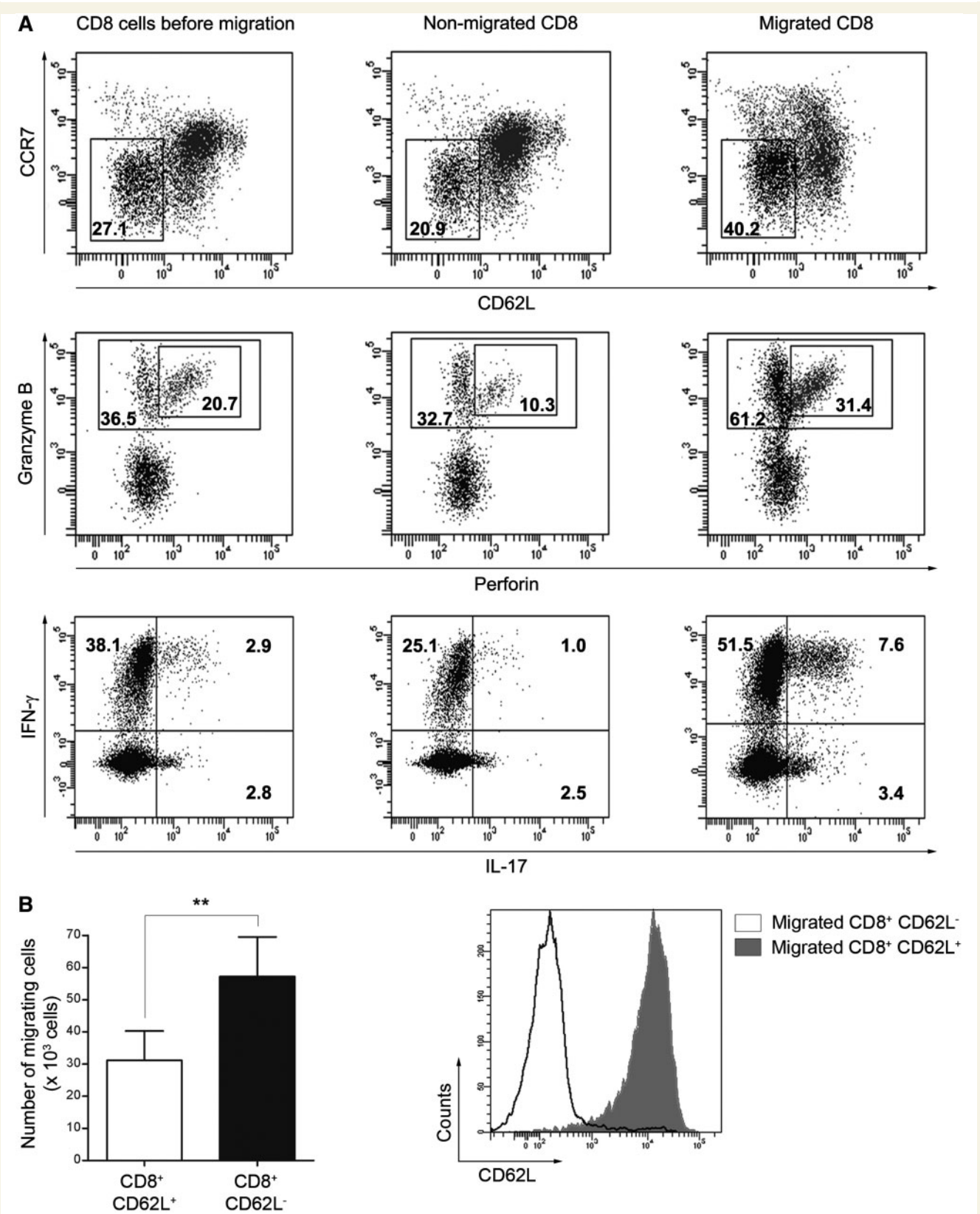

Figure 2 Increased migration of *ex vivo* effector memory CD8$^+$ T lymphocytes across blood–brain barrier (BBB) endothelial cells. (**A**) Human *ex vivo* CD8$^+$ T lymphocytes were allowed to migrate for 24 h across activated human BBB-endothelial cells in a modified Boyden chamber and then analysed 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

(continued)

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$^+$ lymphocyte population in the CNS of $MOG_{35–55}$-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 $\geqslant 2.5$) 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 (Kebir *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

Figure 2 Continued
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 $\pm$ 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.

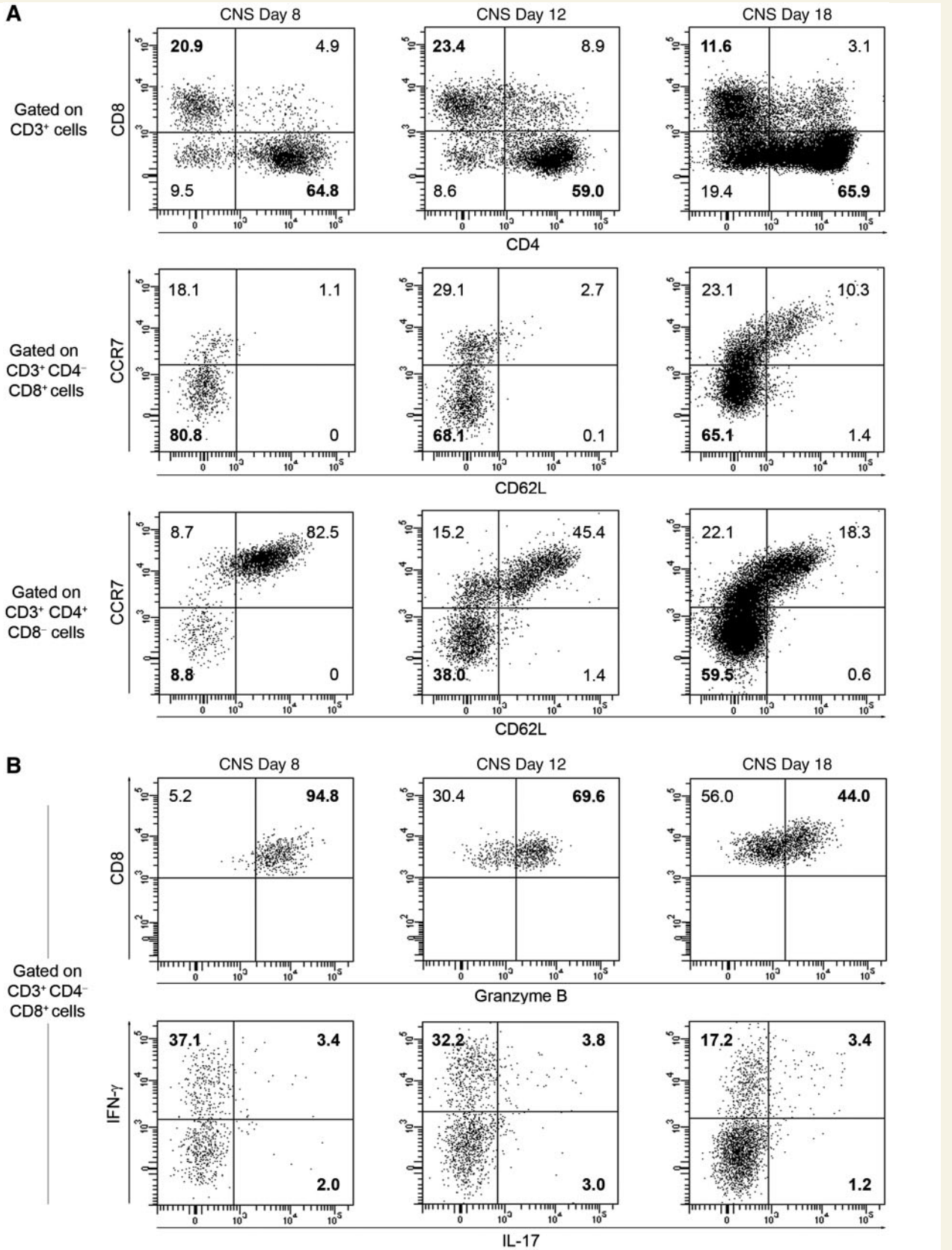

Figure 3 Effector memory $CD8^+$ T lymphocytes accumulate in the CNS of EAE mice. EAE was induced by active immunization of C57BL/6 animals with MOG_{35-55}/complete Freund's adjuvant. At days 8, 12 and 18 after induction of the disease, the brain and spinal cord were homogenized and immune cells were 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 $CD3^+$ $CD4^+$ $CD8^-$

(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 control 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 $MOG_{35–55}$-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 naive 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 $MOG_{35–55}$, IL-2 and IL-15 before transfer (Supplementary Fig. 4A and B). While isotype control-treated animals developed a mild disease (average score of 0.70 ± 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 (46 500 ± 5500) and the CD8 adoptive transfer groups (44 860 ± 11 320), suggesting that activated MOG-reactive CD8$^+$ lymphocytes from the donor animal can attract bystander naive 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

Figure 3 Continued
(*lower* panels) T lymphocytes. The frequency of effector memory CD8$^+$ T lymphocytes decreased from 80.8% at day 8 (*upper left* panel) to 65.1% at day 18 (*upper right* panel), whereas the frequency of effector memory CD4$^+$ T lymphocytes increased from 8.8% at day 8 (*lower left* panel) to 59.5% at day 18 (*lower right* panel). (**B**) Expression of granzyme B (*upper* panels), IFN-γ and IL-17 (*lower* panels) were assessed by flow cytometry on CD3$^+$ CD8$^+$ T lymphocytes found in the CNS of EAE mice. The frequency of granzyme B$^+$ cells decreased from 94.8% at day 8 (*upper left* panel) to 44.0% at day 18 (*upper right* panel). The percentage of CD8$^+$ IFN-γ^+ cells decreased from 40.5% at day 8 (*lower left* panel) to 20.6% at day 18 (*lower right* panel) and that of CD8$^+$ IL-17$^+$ cells did not change (*lower right* panel). Data shown are representative of two independent EAE experiments performed on 3–4 mice per time point.

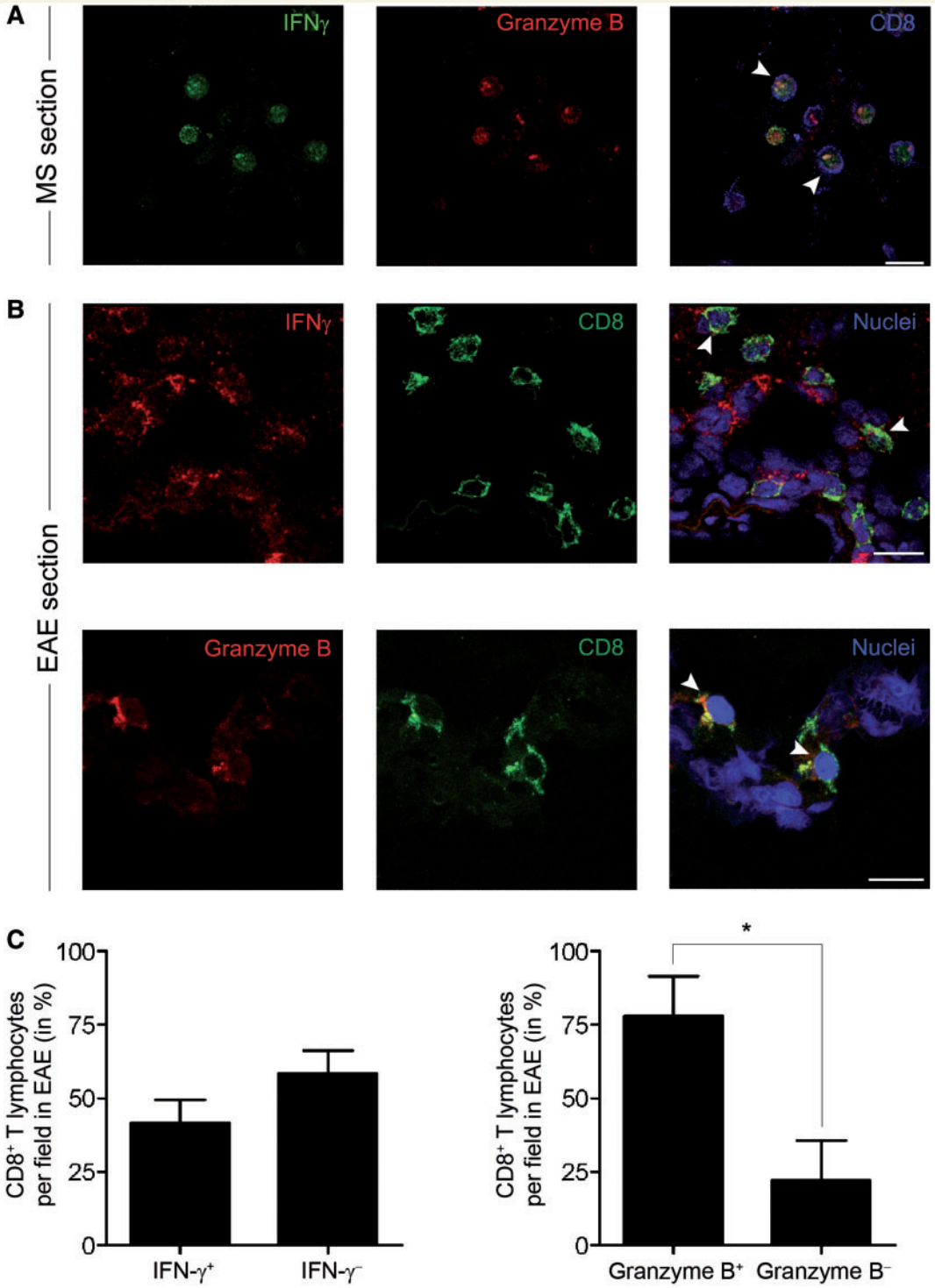

Figure 4 Granzyme B and IFN-γ are highly expressed by CD8$^+$ T lymphocytes in multiple sclerosis and EAE tissue. (**A**) Frozen CNS specimens (7-μm sections) from patients with multiple sclerosis were immunostained for CD8 (in blue), granzyme B (in red) and IFN-γ (in green). Immunostainings were acquired 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 $>$20 immunostainings performed on post-mortem material (brain and spinal cord) from four animals. Scale bar = 10 μm. (**C**) Quantification of IFN-γ and granzyme B-expressing CD8$^+$ 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 (*$P < 0.05$).

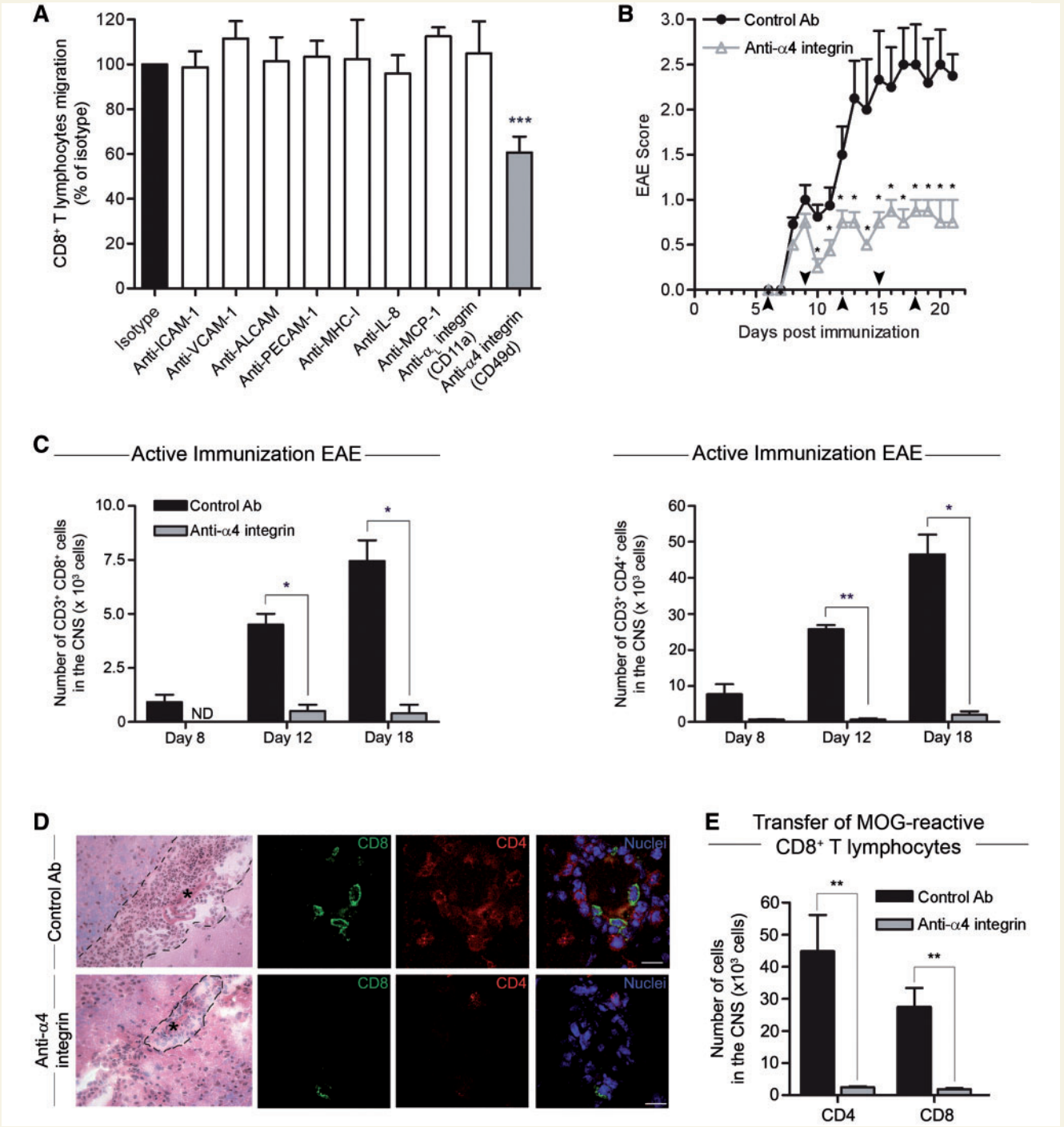

Figure 5 Migration of CD8$^+$ T lymphocytes to the CNS depends on α4 integrin. (**A**) Human *ex vivo* CD8$^+$ T lymphocytes isolated from the blood of healthy donors were allowed to migrate across activated human blood–brain barrier (BBB)-endothelial cells in a modified Boyden chamber for 24 h and then 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 $MOG_{35–55}$/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

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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 5 Continued
mice treated with anti-α4 integrin (grey bars) than in EAE mice treated with the control isotype IgG2b (black bars) at days 12 and 18. Results are expressed as mean ± SEM of two independent EAE experiments performed on 3–4 mice per time point (*$P < 0.05$ and **$P < 0.01$). (**D**) Frozen CNS specimens from EAE mice were stained for Luxol fast blue, haematoxylin and eosin or immunostains for CD8 (in green), CD4 (in red) and TOPRO-3 for nuclear staining (in blue). EAE brains from anti-α4 integrin treated mice (*lower* panel) showed a reduction in immune cell infiltration, demyelination, and in number of infiltrating CD4$^+$ and CD8$^+$ T lymphocytes, as compared to the isotype IgG2b control group (*upper* panel). Photomicrographs shown are representative of >20 stainings performed on four animals. Dotted lines delineate areas of infiltration. Asterisk indicates a vessel. Scale bar = 10 μm. (**E**) *In vivo* blockade of α4 integrin in a CD8$^+$ T lymphocyte transfer model reduces CD4$^+$ and CD8$^+$ T lymphocyte infiltration to the CNS compartment. Ten million MOG-reactivated CD8$^+$ T lymphocytes were injected intraperitoneally into recipient mice. Anti-α4 integrin or isotype control antibody (75 μg) was injected intraperitoneally, on days 1, 3, 5, 7 and 9 post transfer. There were significantly less CD8$^+$ and CD4$^+$ T lymphocytes (day 18 post transfer) in the CNS of mice treated with anti-α4 integrin (grey bars) than in mice treated with the control isotype IgG2b (black bars). Results are expressed as mean ± SEM of three mice (**$P < 0.01$). Ab = antibody.

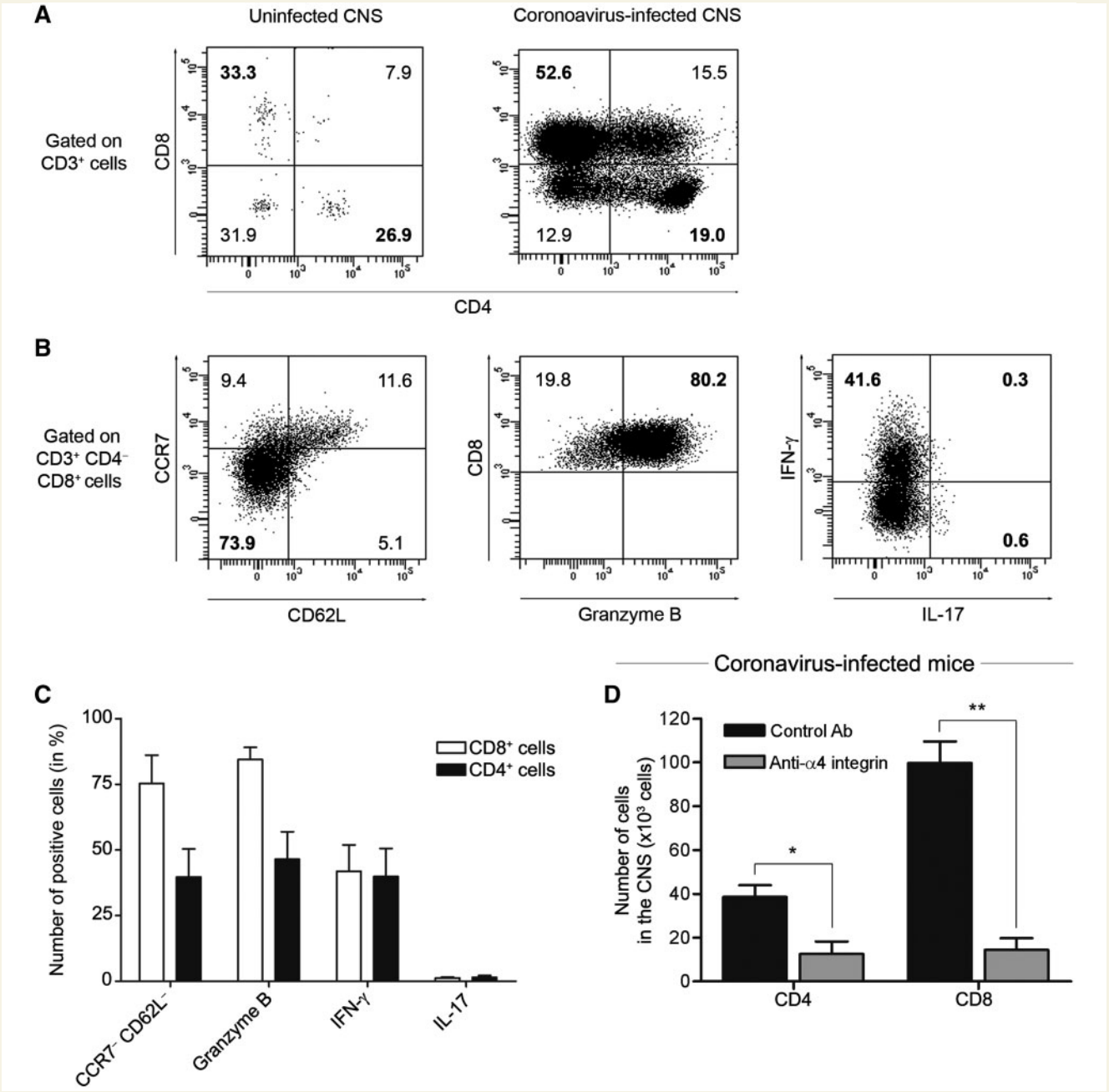

Figure 6 Anti-α4 integrin treatment restricts migration of CD8$^+$ T lymphocytes to the CNS in MHV-A59-infected mice. Mice were infected intracerebrally with the A59 neurotropic, neuroinvasive and neurovirulent strain of MHV. On day 7, five mice were sacrificed and immune cells from the CNS were isolated 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 $\pm$ 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 $\pm$ 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 naive 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 ± 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 Efeoglu, 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 $CD8^+$ lymphocytes to the CNS. Nevertheless, our results point to an important and significant effect of α4 integrin in the *trans*-endothelial recruitment of $CD8^+$ T lymphocytes to the CNS in mice and in human, 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 $CD8^+$ 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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