Lack of Junctional Adhesion Molecule (JAM)-B ameliorates experimental autoimmune encephalomyelitis

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Abstract

In multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) autoaggressive $CD4^+$ T cells cross the blood-brain barrier (BBB) and cause neuroinflammation. Therapeutic targeting of $CD4^+$T-cell trafficking into the CNS by blocking $\alpha 4$-integrins has proven beneficial for the treatment of MS but comes with associated risks, probably due to blocking $CD8^+$ T cell mediated CNS immune surveillance. Our recent observations show that $CD8^+$ T cells also rely on $\alpha 4\beta 1$-integrins to cross the BBB. Besides vascular cell adhesion molecule-1 (VCAM-1), we identified junctional adhesion molecule -B (JAM-B) as a novel vascular $\alpha 4\beta 1$-integrin ligand involved in $CD8^+$ T-cell migration across the BBB. This prompted us to investigate, if JAM-B also mediates $CD4^+$ T-cell migration across the BBB. We first ensured that encephalitogenic T cells can bind to JAM-B *in vitro* and next compared EAE pathogenesis in JAM-$B^{-/-}$ C57BL/6J mice and their wild-type littermates. Following immunization with $MOG_{aa35-55}$ peptide, JAM-$B^{-/-}$ mice developed ameliorated EAE compared to their wild-type littermates. At the same time, we isolated higher numbers of $CD45^+$ infiltrating immune cells from the CNS of JAM-$B^{-/-}$ C57BL/6J mice suffering from EAE. Immunofluorescence staining revealed that the majority of $CD45^+$ inflammatory cells accumulated in the leptomeningeal and perivascular spaces of the CNS behind the BBB but do not gain access to the CNS parenchyma. Trapping of CNS inflammatory cells was not due to increased inflammatory cell proliferation. Neither a loss of BBB integrity or BBB polarity potentially affecting local chemokine gradients nor a lack of focal gelatinase activation required for CNS parenchymal immune cell entry across the glia limitans could be detected in JAM-$B^{-/-}$ mice. Lack of a role for JAM-B in the effector phase of EAE was supported by the observation that we did not detect any role for JAM-B in EAE pathogenesis, when EAE was elicited by *in vitro* activated $MOG_{aa35-55}$-specific $CD4^+$effector T cells. On the other hand, we also failed to demonstrate any role of JAM-B in *in vivo* priming, proliferation or polarization of $MOG_{aa35-55}$-specific $CD4^+$ T cells in peripheral immune organs. Finally, our study excludes expression of and thus a role for JAM-B on peripheral and CNS infiltrating myeloid cells. Taken together, although endothelial JAM-B is not required for immune cell trafficking across the BBB in EAE, in its absence accumulation of inflammatory cells mainly in CNS leptomeningeal spaces leads to amelioration of EAE.

Highlights

- CNS expression of the tight junction molecule JAM-B is restricted to endothelial cells but not

correlated to barrier characteristics of CNS endothelial cells

- JAM-B does not play an essential role in establishing BBB integrity at the level of tight junctions
- Absence of JAM-B does not inhibit immune cell migration across the BBB but ameliorates clinical signs of actively induced EAE
- JAM-B is not expressed on mouse myeloid cells
- Ameliorated EAE in JAM-B$^{-/-}$ C57BL/6 mice seems to be due to the trapping of immune cells mainly in leptomeningeal and to a lower degree in perivascular CNS spaces

Keywords: Junctional adhesion molecules, experimental autoimmune encephalomyelitis, glia limitans, gelatinases, tight junctions, blood-brain barrier

1 Introduction

The central nervous system (CNS) coordinates all body and mental functions in vertebrates and proper communication of its neurons requires CNS homeostasis. Protection from the changing milieu of the blood stream is provided by the endothelial blood-brain barrier (BBB). The BBB is established by biochemically and ultrastructurally unique microvascular endothelium that prohibits free diffusion of molecules into the CNS by its low pinocytotic activity and the complex and continuous tight junctions sealing the inter-endothelial space. At the same time, the BBB ensures transport of nutrients into and toxic metabolites out of the CNS by expression of carriers and transporters and efflux pumps (Abbott et al., 2010; Campos-Bedolla et al., 2014; Engelhardt and Coisne, 2011). In its barrier function, the BBB also controls the migration of immune cells across the BBB, which occurs at the level of post-capillary venules. The CNS is an immune privileged organ, in which foreign antigens fail to elicit a rapid adaptive immune response (Engelhardt et al., 2017). During immune surveillance, low numbers of activated T cells are able to cross the endothelial BBB and the endothelial basement membrane to reach the cerebrospinal fluid (CSF) drained subarachnoid and perivascular spaces and the ventricles (Kivisakk et al., 2003). These T cells are separated from the CNS parenchyma proper by the glia limitans, which is composed of the parenchymal basement membrane and astrocyte endfeet and surrounds the entire CNS parenchyma (Engelhardt and Sorokin, 2009). These morphological features allow for CNS immune surveillance in CSF filled spaces without endangering CNS homeostasis (Engelhardt and Coisne, 2011; Engelhardt and Ransohoff, 2012).

During neurological disorders, such as multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE), a large number of circulating immune cells cross the CNS barriers and cause neuroinflammation after entering the CNS parenchyma, as well as demyelination and focal loss of BBB integrity, which all contribute to EAE pathogenesis. BBB breakdown is an early hallmark of MS as indicated by the leakage of gadolinium contrast agent across the BBB visualized by magnetic resonance imaging (MRI) (Tommasin et al., 2017).

Immune cell trafficking into the CNS in autoimmune neuroinflammation is distinct from that in peripheral organs as it requires the immune cells to first breach the endothelial BBB and subsequently the glia limitans in order to reach the CNS parenchyma. T-cell migration across the BBB is a multi-step process initiated by $\alpha 4\beta 1$-integrin-mediated capture and adhesion strengthening of the T cells via binding to vascular cell adhesion molecule (VCAM)-1 on the BBB (Coisne et al., 2009; Vajkoczy et al.,

2001). Subsequently the T cells crawl on endothelial ICAM-1 and ICAM-2 to paracellular or transcellular sites permissive for diapedesis (Abadier et al., 2015; Steiner et al., 2010). The precise mechanisms involved in T-cell diapedesis across the BBB remain to be explored. Once they have crossed the BBB, T cells need to encounter their cognate antigen on macrophages or dendritic cells strategically localized in the leptomeningeal or perivascular spaces to be stimulated to produce pro-inflammatory mediators including TNF-α, IFN-γ, and IL-17 that trigger further mechanisms required for subsequent CNS tissue invasion (Bartholomaus et al., 2009). Focal activation of matrix-metalloproteinases (MMP) -2 and MMP-9 alter chemokine activation and induce degradation of extracellular matrix receptors on astrocyte end-feed, which is prerequisite for inflammatory cell migration across the glia limitans and onset of clinical EAE (Agrawal et al., 2006; Song et al., 2015). There is *in vitro* and *in vivo* evidence demonstrating that activated $CD4^+$ T cells can cross the BBB via a transcellular route that involves pore formation through the endothelial cell body or via a paracellular route, which requires displacement of the junctional complexes between adjacent endothelial cells (Abadier et al., 2015; Lutz et al., 2017; Winger et al., 2014). The complex BBB TJs are composed of the transmembrane proteins occludin and members of the claudin family (Furuse et al., 1993; Nitta et al., 2003; Tietz and Engelhardt, 2015). In addition, the classical junctional adhesion molecules (JAM) -A, JAM-B, and JAM-C are localized in the BBB TJs (Arrate et al., 2001; Aurrand-Lions et al., 2001a; Aurrand-Lions et al., 2001b; Padden et al., 2007; Tietz and Engelhardt, 2015; Vorbrodt and Dobrogowska, 2004; Wyss et al., 2012). These classical JAMs belong to the immunoglobulin (Ig) superfamily and comprise a subfamily of three more closely related cortical thymocyte marker for Xenopus (CTX) proteins. Classical JAMs are type I transmembrane proteins composed of two extracellular Ig domains and a short cytoplasmic domain containing a PDZ-binding motive (Garrido-Urbani et al., 2014; Martin-Padura et al., 1998). JAMs engage in homophilic and heterophilic interactions amongst their family members. Homophilic interaction of JAM-A regulates cell polarity by intracellular association with the cell-polarity PAR (partitioning-defective)-3/atypical protein kinase C (aPKC)/PAR-6 complex (Ebnet et al., 2001). Interestingly, loss of vascular JAM-A immunostaining correlates with BBB leakiness in human brain tissue from MS patients (Padden et al., 2007). Homophilic JAM-C interactions might contribute to brain barriers junction integrity as loss of function mutations of JAM-C lead to hydrocephalus in mice and man (Mochida et al., 2010; Wyss et al., 2012).

Besides their potential role in tight junction integrity, JAMs contribute to leukocyte trafficking across vascular walls during inflammation by interacting with leukocyte integrins. While endothelial JAM-A binds to αLβ2-integrin (LFA-1) on T cells and contributes to their migration across endothelial monolayers *in vitro* (Ostermann et al., 2002), vascular JAM-C can interact with αMβ2-integrin (Mac-1) on myeloid cells and mediate their diapedesis *in vitro* and *in vivo* (Chavakis et al., 2004). JAM-B is the least investigated member of the classical JAMs with its expression restricted to vascular endothelial cells and bone marrow stromal cells (Arcangeli et al., 2011; Aurrand-Lions et al., 2001b; Martin-Blondel et al., 2015; Palmeri et al., 2000). When interacting with JAM-C, JAM-B was shown to regulate the migration of immune cells across the endothelium (Arrate et al., 2001). In addition, JAM-B can engage α4β1-integrin on human T cells following prior interaction of JAM-B with JAM-C co-expressed on these T cells (Cunningham et al., 2002). Furthermore JAM-B was shown to mediate α4β1-integrin dependent T-cell rolling and adhesion to endothelial cells *in vitro* independent of JAM-C (Ludwig et al., 2009).

α4β1-integrins play a dominant role in the migration of T cells across the BBB, which is a critical step in the pathogenesis of autoimmune CNS inflammation such as MS or its animal model EAE (Engelhardt and Kappos, 2008). Therapeutic targeting of α4-integrin mediated T-cell migration across the BBB has proven very efficient in the treatment of MS, does however have the risk of progressive multifocal leukoencephalopathy (PML) (Engelhardt et al., 2005), an opportunistic infection of oligodendrocytes by the JC virus. It has been speculated that this is due to interfering with $CD8^+$ T cell mediated CNS immune surveillance. In fact, we could recently show that α4β1-integrins are essential for $CD8^+$ T-cell entry into the CNS in a $CD8^+$ T-cell mediated mouse model of autoimmune neuroinflammation (Martin-Blondel et al., 2015). Interestingly, although blocking of its vascular ligand VCAM-1 partially reduced $CD8^+$ T-cell infiltration into the CNS it failed to reduce clinical disease. Rather, antibody mediated inhibition of JAM-B reduced the number of CNS-infiltrating $CD8^+$ T cells and ameliorated clinical disease.

This prompted us to explore, if vascular JAM-B is also involved in mediating $CD4^+$ T cell trafficking across the BBB in neuroinflammation. To this end, we studied EAE pathogenesis as a $CD4^+$ T-cell driven autoimmune neuroinflammatory model in C57BL/6J mice with a constitutive lack of JAM-B.

2 Material and Methods

2.1 Mice

Production of JAM-B deficient mice has been described before (Arcangeli et al., 2011). JAM-B$^{-/-}$ mice used in the present study were back-crossed for more than 12 generations on the C57BL/6J background. Mice were housed in individual ventilated cages under specific pathogen free conditions. All animal procedures were performed in accordance with the Swiss legislation on the protection of animals and were approved by the Veterinary Office of the Canton of Bern (permission numbers: BE 42/14, BE 72/15, and BE 31/17).

2.2 Experimental autoimmune encephalomyelitis

Active EAE was induced in 8-12 weeks old female JAM-B$^{-/-}$ C57BL/6J and wild-type littermates using the $MOG_{aa35-55}$-peptide as described before (Coisne et al., 2009). Pertussis toxin was applied intraperitoneally either at the days 0 (day of immunization) and 2 or alternatively at days 1 and 3 post immunization. Adoptive transfer EAE was induced by injection of purified *in vitro* stimulated polyclonal CD4$^+$ T cells from either C57BL/6J mice or JAM-B$^{-/-}$ littermates as described previously (Abadier et al., 2015). 2.5 x 10^6 T cells were injected in 500 µl PBS intraperitoneally into syngeneic recipient wild-type or JAM-B$^{-/-}$ mice. Assessment of clinical disease activity was performed twice daily as described before (Engelhardt et al., 2005; Tietz et al., 2016) as follows: 0, healthy; 0.5, limp tail; 1, hind leg paraparesis; 2, hind leg paraplegia; 3, hind leg paraplegia with incontinence.

2.3 FACS analysis of CD45$^+$ inflammatory cells isolated from the brain and spinal cord

Inflammatory cells from the CNS were isolated as described previously (Doring et al., 2007).
For surface staining, cells were resuspended in FACS-buffer (DPBS, 2.5 % FBS, and 0.1% NaN_3) and distributed into wells of a 96-well U-bottom plate. After Fc-block (in house, clone 2.4G2) for 15 min on ice, inflammatory cells were incubated with primary antibody (supplementary table 1) mixes for 30 min on ice. After two washing steps samples were fixed with 1 % formaldehyde in FACS buffer. Blood and bone marrow samples were fixed with the BD FACS™ Lysing Solution (BD Biosciences) and erythrocytes were lysed in parallel. For intracellular cytokine staining, inflammatory cells were stimulated with 50 ng/ml phorbol myristate acetate (PMA; Alexis Biochemicals), 1 mg/ml Ionomycin (BioVision, Inc.) and 3.3 µl/10 ml Golgi stop (BD Biosciences) for 5 h at 37 °C in 5 ml RPMI1640 (Gibco) supplemented with 10 % FBS, 2 % L-glutamine (Gibco), 1 % NEAA (Gibco), 1 % sodium

pyruvate (Gibco), 1 % PenStrep (Gibco), and 0.4 % 14.3 mM β-Mercaptoethanol (Merck). After the stimulation cells were collected and fixed and permeabilized for 20 min. on ice (BD Biosciences; Cytofix/Cytoperm™). After a washing step, 50 µl of antibody master mixes diluted in Perm/Wash™ solution (BD Biosciences) were added to the cells and incubated for 30 min. on ice. Cells were washed twice after the staining and resuspended in FACS buffer.

For counting of the absolute cell numbers isolated from the CNS of mice fluorescent bead counting (BD Trucount™; BD Biosciences or PKH26 Reference Microbeads; Sigma-Aldrich) was performed. Flow cytometry was performed using either the FACSCalibur (BD Bioscience), or the LSR-II (BD Bioscience) and data were analyzed with FlowJo software (FlowJo version 10).

2.4 FACS analysis of inguinal lymph node lymphocytes

Lymphocytes were isolated from PBS perfused mice at the peak of the EAE using a Wheaten homogenizer. Subsequently cells were filtered though a 100 µm nylon mesh and washed with wash buffer (DMEM supplemented with 5 % calf serum and 25 mM HEPES). After Fc-blockage (home made anti CD16/32; clone 2.4G2) for 15 min cells were resuspended in primary antibody solution for 30 min in FACS buffer. Flow cytometry analysis was performed using the Attune NxT (Thermofisher scientific). Data analysis was performed with FlowJo software.

2.5 Analysis of *in vivo* cell proliferation

Leukocyte cell proliferation was assessed using 5-ethynyl-2'-deoxyuridine (EdU). Mice suffering from EAE were injected with 0.4 mg EdU (Click-iT™ EdU AlexaFluor™ 488 Kit; Thermofisher scientific) i.p. at day 15 after EAE induction and brains and spinal cords were harvested 24 h later. $CD45^+$ infiltrating immune cells were isolated as described before (Doring et al., 2007) and stained with anti-CD45-APC antibody. Subsequently EdU incorporation was fluorescently labelled by the click reaction following the manufacturers instructions. EdU AlexaFluor™ positive leukocytes were identified using the Attune NxT (Thermofisher scientific). Data analysis was performed with FlowJo software.

2.6 FACS sorting of $CD45^{hi}$ and $CD45^{inter}$ CNS-infiltrating cells

CNS-infiltrating cells were isolated from the brain and spinal cord as explained above. After Fc-block (in house, clone 2.4G2) for 15 min on ice, inflammatory cells were incubated with anti-CD45 antibody

for 45 min on ice and washed two times with DPBS. Cells were then incubated with 200 µl Fixable Viability Dye (FVD) eFluor™ 506 following the manufactures instructions (Thermo Fisher Scientific). Cells were washed two times with FACS-buffer and resuspended in FACS-sorting buffer (DPBS, 2 % FBS, 25 mM HEPES). Single FVD eFluor™506^{-}CD45^{inter}SSC^{hi} and single FVD eFluor™506^{-}CD45^{hi} cells were sorted using FACS Aria (BD Bioscience) and RNA was isolated as described under 2.7.

2.7 Real-time PCR

Sample RNA was isolated following the manufacturers instruction of the High Pure RNA Isolation Kit (Hoffman-La Roche, Basel). Sample cDNA was synthetized from 500 ng sample RNA following the manufacturer's instructions of the SuperScript® III First-Stand Synthesis System for RT-PCR (Invitrogen). mRNA expression was tested using Takyon™ Low Rox SYBR® MasterMix dTTP Blue (Eurogentec). Quantitative real-time PCR was run in a volume of 20 µL per reaction: 10 µL of Takyo™ Low Rox SYBR® MasterMix dTTP Blue, 0.4 µL of each primer (supplementary Table 2), 4.2 µL H_2O and 0.25 ng/µl cDNA. The PCR was performed according to the manufacturer's protocol using MicroAmp Fast Optical 96-Well Reaction Plates (Thermo Fisher Scientific). For each PCR run, a negative control was used with the normal mix without the addition of the cDNA and with H_2O instead. S16 ribosomal protein mRNA levels were used as an endogenous control. All qPCR reactions were performed using the Viia7 Real-Time PCR System (Thermo Fisher Scientific). Relative expression values were calculated according to the comparative ΔC_T method (ΔC_T value = average C_T value of target gene - average C_T value of endogenous reference). Each sample's expression was assayed three times, in triplicate per run.

2.8 Immunofluorescence staining

Mouse tissue preparations and immunofluorescence staining was performed as described previously (Martin-Blondel et al., 2015). Briefly, to stain for CD45, 6 µm cryosections were fixed with acetone at 4 °C for 20 min, followed by treatment with blocking buffer (5 % skimmed milk, 0.3 % TritonX100, 0.04 % NaN_3 in DPBS). Primary rat anti-mouse CD45 (supernatant, in house, hybridoma clone M1/9) or biotinylated goat anti-mouse IgG antibody (10 µg/ml; Vector Laboratories) and rabbit anti-mouse laminin (0.95 µg/ml; DAKO, Agilent) antibodies were incubated with the section for 1 h. For staining of JAM-B, 6 µm cryostat sections were fixed with ice-cold methanol for 20 s, followed by treatment with

blocking buffer (2% BSA, 1% FCS, 1% donkey serum in DPBS). Subsequently, primary rabbit anti-JAM-B antibody (αJB829 1:250, Beat Imhof) and rat anti-mouse PECAM-1 (MEC13.3, 20 µg/ml) was incubated with the sections for 1 h. Antibody incubation was performed at room temperature and in between and after antibody incubation, sections were washed with TBS. Streptavidin-Alexa488 (2.5 µg/ml; Biolegend) was incubated for 1 h and second stage antibody staining was performed as described previously (Martin-Blondel et al., 2015) and immunofluorescent staining was analyzed using a Nikon Eclipse 600 fluorescence microscope and a Zeiss microscope-based 3D Histech Pannoramic MIDI II slide scanner.

For detection of glucose transporter-1 (Glut-1) and P-glycoprotine (Pgp) mice were perfused under deep anesthesia with 10 ml PBS followed by 2 % formaldehyde (PFA; Merck) in DPBS through the left ventricle of the heart and dissected tissues were post-fixed in 2 % PFA over night. 100 µm coronal vibratom sections were cut using a MICROM HM 650V microtome equipped with a vibrating blade (MICROM International GmbH). Free floating sections were blocked for two hours with blocking buffer and primary antibodies (anti-Glut-1 10 µg/ml, Millipore; anti-Pgp 1:25 Enzo Life Science) was incubated overnight at 4 °C. Sections were then washed 3 times with TBS followed by a 2 hour incubation at room temperature with secondary antibodies AMCA-conjugated AffiniPure goat anti-rabbit IgG (10 µg/ml; Jackson ImmunoResearch Laboratories, Inc.) and Cy3 goat anti-mouse (3 µg/ml; Jackson ImmunoResearch Laboratories, Inc.) and mounted on SuperFrost® Plus cover slips (Thermo Scientific) with Aqua-Poly/Mount (Polysciences) mounting media and coverslipped. Sections were imaged using a Leica TCS SP8 Confocal Microscope with Leica LAS X software.

2.9 *In situ* zymography and immunofluorescence counterstaining

In situ zymography and immunostaining was performed according to (Agrawal et al., 2006). Brains from PBS-perfused mice were snap frozen and MMP activity was localized in 6 µm non-fixed brain cyrosections using in situ zymography. 10 µg/ml DQ-gelatinase and 20 µg/ml cold gelatine (Thermo Fisher Scientific; EnzCheck™) in reaction buffer was applied CNS cryosections, in the presence or absence of 50 µM phenanthroline. Slides were incubated for 4 h in a humid chamber at 37°C, washed in ddH_2O, and fixed 5 min in −20°C methanol before immunofluorescence counterstaining.

For counterstaining of CD45 and laminin, sections were blocked with 1 % BSA in PBS for 20 min. Sections were incubated with primary rat anti-mouse CD45 (10 µg/ml; BD Pharmingen; clone 30F11)

and rabbit anti-mouse laminin (0.95 µg/ml; DAKO, Agilent) antibodies for 1 h. Sections were then incubated with secondary goat anti-rat Cy3 (7.5 µg/ml; Jackson ImmunoResearch Laboratories, Inc.) and AMCA anti-rabbit (15 µg/ml; Jackson ImmunoResearch Laboratories, Inc.) for 1 h and mounted with Mowiol (Sigma-Aldrich). Antibody incubation was performed at room temperature and in between and after antibody incubation, sections were washed with DPBS. Sections were examined using a Nikon Eclipse 600 fluorescence microscope.

2.10 *In vivo* permeability assay

To analyze the permeability of the BBB *in vivo*, 2 mg Albumin-fluorescein isothiocyanate conjugate (Sigma-Aldrich) in 100 µl 0.9 % NaCl solution (Mini-Plasco; B. Braun) were injected intravenously into 39-49 weeks old wild-type and JAM-B$^{-/-}$ mice. The tracer was allowed to circulate for 30 min. Subsequently mice were perfused under deep anesthesia with 10 ml PBS followed by 2 % formaldehyde (PFA; Merck) in DPBS through the left ventricle of the heart. Dissected tissues were post-fixed in 2 % PFA over night. 100 µm vibratom sections were cut using a MICROM HM 650V microtome equipped with a vibrating blade (MICROM International GmbH). Free floating sections were counterstained for PECAM-1 for detection of blood vessel endothelial. Sections were blocked for two hours with blocking buffer (5 % skimmed milk, 0.3 % TritonX100, 0.04 % NaN_3 in DPBS) primary antibody (10 µg/ml; PECAM-1 in house clone MEC13.3) was incubated overnight at 4 °C. Sections were then washed 3 times with TBS followed by a 2 hour incubation at room temperature with goat anti-rat (H+L) Cy3 (3 µg/ml; Jackson ImmunoResearch Laboratories, Inc.) and then mounted on SuperFrost® Plus cover slips (Thermo Scientific) with Aqua-Poly/Mount (Polysciences) mounting media and coverslipped. Sections were imaged using a Leica TCS SP8 Confocal Microscope with Leica LAS X software.

To analyze the permeability of the BBB during EAE *in vivo*, 10 mg of fluorescent tracers (10 kDa dextran Alexa fluor 488 (Invitrogen, Molecular Probes) and 3 kDa dextran Texas Red (Invitrogen) were intravenously retro-orbital injected in 100 µl 0.9 % NaCl solution (Mini-Plasco; B. Braun) into Isoflurane (Provet AG) anesthetised mice suffering from EAE. Traces were allowed to circulate for 15 min. Subsequently mice were perfused under deep anesthesia with 10 ml PBS followed by 4 % PFA (Merck) in DPBS through the left ventricle of the heart. Dissected tissues were embedded in Tissue-

Tek (Sakura Finetek). 6 µm cryostat sections were analyzed using a Nikon Eclipse 600 fluorescence microscope.

2.11 Preparation of primary 2D2 T cells

Primary naïve 2D2 T cells were collected from the lymph nodes and the spleen of 2D2 TCR MOG transgenic mice obtained from V. K. Kruchoo (Boston, MA, USA) (Bettelli et al., 2003). $CD4^+$ T cells were purified by $CD4^+$ negative selection using Dynal beads (Invitrogen). $CD4^+$ TCR tg T cells were adjusted to 1 x 10^6 cells/ml in restimulation medium (RPMI1640 supplemented with 10 % FBS, 2 % L-glutamine, 1 % NEAA, 1 % sodium pyruvate, 1 % PenStrep, and 0.4 % 14.3 mM β-Mercaptoethanol) and were mixed together with bone marrow derived dendritic cells for *in vitro* proliferation assy.

2.12 Preparation of bone marrow derived dendritic cells and macrophages

To differentiate cells from the bone marrow, femurs and tibias were removed from wild-type mice and JAM-$B^{-/-}$ C57BL/6J mice. After cutting off the bone ends, bone marrow cells were collected by centrifugation at 4000 rpm for 4 min in homemade collection tubes (a 1.5 ml Eppendorf tube equipped with 0.5 ml Eppendorf tube without a lid containing the lower fifth of 1 ml pipette tip). To obtain dendritic cells, bone marrow cells were cultured in RPMI1640 supplemented with 10 % FBS, 2 % L-glutamine, 1 % NEAA, 1 % sodium pyruvate, 1 % PenStrep, 0.4 % 14.3 mM β-Mercaptoethanol, and 10 % Flt3-Ligand supernatant (in house, produced from SP2/0 transfectants secreting mouse recombinant Flt-3L (Dehlin et al., 2008)) for 7 days. For maturation of DCs 1 µg/ml lipopolysaccharide (LPS from Salmonella; Sigma-Aldrich) was added on day 7 overnight.

For differentiation of macrophages, bone marrow cells were cultured in DMEM/F12 + GlutaMAX medium (Gibco) supplemented with 10 % FCS, 1 % PenStrep, 2 % L-glutamine, and 1 µg/ml M-CSF (PeproTech, Inc.) in non-cell culture treated PD10. Medium was exchanged after 3 days and macrophages were matured at day 7 with 100 ng/ml LPS overnight.

2.13 *In vitro* proliferation assay

For *in vitro* proliferation of primary 2D2 T cells, LPS matured bone marrow derived dendritic cells (BMDCs) were collected from petri dishes using 3 ml 2 mM EDTA/PBS. 1 x 10^4 BMDCs were added in 100 µl RPMI1640 supplemented with 10 % FBS, 2 % L-glutamine, 1 % NEAA, 1 % sodium pyruvate, 1

% PenStrep, and 0.4 % 14.3 mM β-mercaptoethanol into wells of a 96-well U-bottom plate. BMDCs were loaded with different concentrations of $MOG_{aa35-55}$ peptide (1, 2, 10, 20, 100, and 200 µg/ml) or of 0.1 µg/ml anti-CD3- and anti-CD28-antibodies (positive control) or 10 µg/ml purified peptide derivative of tuberculin (PPD, negative control; Statens Serum Institute) for 1 h at 37 °C. After peptide loading of BMDCs 100 µl of naïve 2D2 T cells (1 x 10^5 cells) were added to the BMDCs and cultured for three days. Cells were labeled with 10 µCi/ml ^{3}H-thymidine (PerkinElmer). After 16 h cells were harvested on filters. Filters were dissolved in 2 ml scintillation fluid and the cpm/min was analyzed using a beta-counter as described in (Doring et al., 2007; Engelhardt et al., 2005).

2.14 *In vitro binding assay*

Wells of epoxy slides (12 well slides; Thermo Fisher Scientific) were coated with recombinant proteins (recombinant DNER-Fc chimera, R&D 2254-DN; recombinant ICAM-1-Fc chimera, R&D 796-IC; recombinant VCAM-1-Fc chimera, R&D 643-VM; recombinant MAdCAM-1-Fc chimera, R&D 993-MC; recombinant JAM-B-Fc chimera, R&D 988-VJ; recombinant JAM-C-Fc-chimera, R&D 1213-J3-050; R&D systems, Inc.) as described previously (Martin-Blondel et al., 2015). To perform the binding assay, *in vitro* MOG_{35-55} activated 2D2 T cells were collected at 1 x 10^7 cells/ml in binding buffer (TBS + 1 mM $MnCl_2$). 20 µl cell suspension (2 x 10^5 cells/well) were added and incubated on recombinant proteins on the diagnostic microscopy slide for 90 min at 37 °C. Slides were washed and the number of adherent cells per field of view (FOV) was quantified as previously described (Martin-Blondel et al., 2015).

2.15 Immunofluorescent staining for JAM-B on cultured cells

Cultured Madin-Darby Canine Kidney (MDCK) cells and MDCK cells transfected with murine JAM-B (Aurrand-Lions et al., 2001b; Lamagna et al., 2005) were fixed with ice-cold methanol for 20 sec. After treatment with blocking buffer (5 % skimmed milk, 0.3 % TritonX100, 0.04 % NaN_3 in DPBS) rabbit anti-mouse JAM-B anti-serum (JAM-B784) was incubated with the cells for 30 min. Cells were washed twice with PBS and incubated with secondary goat anti-rabbit (H+L) Alexa488 (5 µg/ml; Invitrogen) for 30 min. Cells were washed twice, mounted with Mowiol and JAM-B staining was analyzed using a Nikon Eclipse 600 fluorescence microscope.

2.16 Primary mouse brain microvascular endothelial cells

Primary mouse brain microvascular endothelial cells (pMBMECs) isolated from wild-type or JAM-B$^{-/-}$ mice were isolated and cultured exactly as described before (Coisne et al., 2013; Steiner et al., 2010).

2.17 immunofluorescent staining of primary mouse brain microvascular endothelial cells

pMBMECs were fixed with 1 % formaldehyde for 10 min. After treatment with blocking buffer for 20 min (5 % skimmed milk, 0.3 % TritonX100, 0.04 % NaN_3 in DPBS) rabbit anti-ZO-1 (1.25µg/ml; Invitrogen) or rat anti-VCAM-1 (clone 9DB3), or rat anti-ICAM-1 (clone 25ZC7) were incubated with the cells for 30 min. Cells were washed twice with PBS and incubated with secondary goat anti-rabbit (H+L) Alexa488 (5 µg/ml; Invitrogen) or goat anti-rat (H+L) Cy3 (7.5 µg/ml; Jackson ImmunoResearch Laboratories, Inc.) for 30 min. Cells were washed twice, mounted with Mowiol and JAM-B staining was analyzed using a Nikon Eclipse 600 fluorescence microscope.

2.18 *in vitro* transmigration assay

The transmigration of 2D2 T cells across pMBMECs was assessed as described before (Steiner et al., 2011). In brief, pMBMECs were cultured on laminin and Matigel (Corning) coated 0.33 cm^2 filter inserts (5 µm pore size; Transwell® Costar, Corning) for 6 days and stimulated with 10 ng/ml TNF (PromoCell GmbH) and/or 20 ng/ml IL-1β (PeproTech) for 16 h. 1 x 10^5 *in vitro* activated 2D2 cells were allowed to transmigrate for 8 h and the percentage of migrated T cells was calculated referring to the inputs as 100 %.

2.19 Transendothelial permeability

The *in vitro* permeability assay was performed as described previously (Steiner et al., 2011) (Lazarevic and Engelhardt, 2016). Briefly, pMBMECs were cultured of laminin and Matigel (Corning) coated 0.33 cm^2 filter inserts (0.4 µm pore size; Transwell® Costar, Corning) for 6 days and stimulated with 10 ng/ml TNF (PromoCell GmbH) for 16 h. Fluorescent tracer (50 µM Lucifer yellow, 0.457 kDa; Sigma-Aldrich) was provided form the luminal compartment. Fluorescence from different time points was detected in the lower compartment using the Tecan Infinite M1000 Pro (Tecan, Group Ltd) and the clearance of LY (permeability coefficient) was calculated.

2.20 *In vitro* live cell imaging under physiological flow

Dynamic interaction of *in vitro* activated 2D2 T cells with wild-type and JAM-B$^{-/-}$ pMBMECs was investigated by live cell imaging under flow *in vitro* as described before (Coisne et al., 2013; Rudolph et al., 2016; Steiner et al., 2010). Briefly, 1 x 10^6 cells/ml 2D2 T cells were allowed to accumulate on the flow chamber at a low shear (0.1 dyn/cm^2) for 5 min. Dynamic interactions of activated 2D2 T cells with the pMBMECs was recorded under physiological shear flow (1.5 dyn/cm^2) for 20 min (objective EC Plan Neoflur 20X/0.4) with phase or contrast using a monochrome charge-coupled device camera (AxioCam MRm Rev, Carl Zeiss). Image analysis was performed using ImageJ software (ImageJ software, National Institute of Health) using the manual tracking and migration tool plugins.

2.21 Western blotting of JAM-B

Isolated microvessels or cultured primary mouse brain microvascular endothelial cells from wild-type or JAM-B$^{-/-}$ mice were tested for JAM-B expression. After isolation of microvessels red blood cells were lysed for 2 min at room temperature using ammonium chloride Tris-HCl solution (9:1 0.83 % ammonium chloride in Tris-HCl pH 7.5). Freshly isolated brain vessels as well as cultured primary mouse brain microvascular endothelial cells were lysed in RIPA buffer (150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris pH 8) containing protease inhibitor (c0mpete Tablets; Roche). Protein concentration was measured using the Bovine Serum Albumin assay (Thermo Scientific™ Pierce™ Protein Biology). 30 µg of protein were mixed with 2X Sample Buffer (437,5 µl 8M Urea, 5.25 % SDS, 50 mM Tris, 62.5 µl, and 10 µl 0.5 % BPB) and separated by 12 % SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Amersham Protan, GE Healthcare), using a Trans-Blot Turbo transfer system (BioRad Laboratories, Hercules), according to the manufacturer's instructions. Membranes were blocked with 5 % BSA () in TBST (200 mM Tris, 1.5 M NaCl, 0.1 % Tween-20) for 1 hour at room temperature and incubated with rabbit anti-mouse GAPDH (CellSignaling) or rabbit JAM-B784 antiserum overnight at 4 °C. The following day, the membranes were washed with 3 x with TBST and incubated with the secondary antibodies goat anti-rabbit IgG (H+L) IRDye®TM 800 (1:10000, Rockland Immunochemicals), for 1 hour, room temperature. Proteins were detected using the Odyssey software (LI-COR Biotechnology).

3 Results

3.1 JAM-B is expressed in CNS endothelial cells of C57BL/6J mice

We have previously shown that JAM-B is expressed in brain endothelial cells of BALB/c mice (Martin-Blondel et al., 2015). To ensure that JAM-B is also expressed in CNS endothelial cells of C57BL/6J mice we performed immunofluorescence staining of frozen brain and spinal cord sections from healthy C57BL/6J mice (Fig. 1). JAM-B was readily detected in platelet endothelial cell adhesion molecule (PECAM)-1 $^{+}$ blood vessel endothelial cells in the cortex, the cerebellum and the spinal cord. Similarly, in the choroid plexus that lack an endothelial blood-brain barrier (BBB) and rather establishes an epithelial blood-cerebrospinal fluid barrier (BCSFB), JAM-B immunostaining was restricted to PECAM-1^{+} endothelial cells and absent from choroid plexus epithelial cells. Thus, JAM-B protein is expressed by vascular endothelial cells irrespective of their barrier characteristics but not by the epithelial cells of the BCSFB in the choroid plexus.

3.2 JAM-B$^{-/-}$ C57BL/6J mice develop ameliorated EAE

To determine if absence of JAM-B affects pathogenesis of EAE, we immunized JAM-B$^{-/-}$ C57BL/6J mice and their wild-type littermates with $MOG_{aa35-55}$ peptide in CFA. In comparison to wild-type mice, JAM-B$^{-/-}$ mice developed significantly ameliorated disease signs of actively induced EAE (aEAE; Fig. 2A) as determined by the area under the curve (AUC; Fig. 2B). JAM-B$^{-/-}$ mice also showed a less pronounced weight loss (Fig. 2C) and a lower maximal clinical EAE score (Fig. 2D). However, lack of JAM-B did not affect the day of disease onset (11.77 ± 0.5 in wild-type and 13 ± 0.90 in JAM-B$^{-/-}$ mice) nor the disease incidence (incidence 100 % in both, wild-type and JAM-B$^{-/-}$ mice).

3.3 Absence of JAM-B does not affect BBB permeability *in vivo*

As JAM-B localizes to tight junctions, we asked if absence of JAM-B from the BBB tight junctions might lead to BBB breakdown, which is a critical hallmark of EAE pathogenesis. To this end, we first investigated the diffusion of intravenously applied FITC-albumin and endogenous mouse immunoglobulins across brain vessels in aged (39-49 weeks) wild-type and JAM-B$^{-/-}$ mice by immunofluorescence staining. Mouse immunoglobulins were readily detected in the stroma of circumventricular organs or the choroid plexus harboring fenestrated microvessels, but not in the CNS parenchyma or the leptomeninges of wild-type and JAM-B$^{-/-}$ mice (Supplementary Fig. 1).

Furthermore, absence of JAM-B was not associated with diffusion of intravenously applied FITC-albumin beyond PECAM-1$^+$ endothelial cells into the brain and spinal cord parenchyma or leptomeningeal spaces (Supplementary Fig. 1; and data not shown). This underscores that absence of JAM-B even in aged mice does not lead to a compromised BBB. Next we asked if absence of JAM-B may lead to increased BBB dysfunction under neuroinflammatory conditions. We therefore investigated the diffusion of the intravenously applied exogenous fluorescent tracers 3 kDa and 10 kDa Dextran across brain vessels in JAM-B$^{-/-}$ C57BL/6J mice and wild-type littermates at the peak of EAE (Fig. 3; n = 3 wild-type, n = 2 score 2 and n = 1 score 1; n = 3 JAM-B$^{-/-}$ mice, n = 1 score 2, n = 2 score 1). Diffusion of both tracers across the fenestrated vessels of the choroid plexus was readily detectable. Within the brain parenchyma we observed both, leaky as well as non-leaky inflammatory cuffs equally distributed in wild-type and JAM-B$^{-/-}$ mice. Thus, lack of JAM-B did not contribute to a further increase in BBB permeability in EAE inflicted C57BL/6J mice.

As lack of detection of a difference in BBB integrity in JAM-B$^{-/-}$ compared to wild-type mice could be due to the upregulated expression of other tight junction proteins in the absence of JAM-B we isolated brain vessels from wild-type and JAM-B$^{-/-}$ mice and investigated expression of JAM-A, JAM-C as well as additional tight junction molecules e.g. claudin-5, occludin, and ZO-1 by qPCR (Supplementary Fig. 2). We did not find any significant differences in the mRNA expression of any of these tight junction molecules demonstrating that absence of JAM-B does not affect expression of other BBB tight junction molecules *in vivo.*

3.4 Activated CD4$^+$ T cells can bind to recombinant JAM-B *in vitro*

We have previously shown that BBB permeability is regulated independently from T-cell trafficking across the BBB (Abadier et al., 2015). To explore if JAM-B could play a role in the multi-step process of $\alpha4\beta1$-integrin mediated T-cell migration across the BBB, we next investigated if CD4$^+$ MOG_{35-55} reactive 2D2 T cells can engage JAM-B. To this end, we performed *in vitro* binding assays allowing to study 2D2 T cell adhesion to recombinant JAM-B in comparison to other members of the IgCAM superfamily under static conditions (Supplementary Fig. 3). 2D2 T cells were found to readily bind to ICAM-1 and to a lower degree to VCAM-1. A low level of binding of 2D2 T cells to MAdCAM-1 and JAM-B could also be observed. Mixing recombinant JAM-C with JAM-B potentially allowing for prior engagement of JAM-B with JAM-C to facilitate $\alpha4\beta1$-integrin/JAM-B interaction (Cunningham et al.,

2002) did not increase T-cell adhesion to JAM-B *in vitro* (data not shown). Thus, activated 2D2 T cells can engage JAM-B, which may contribute to their migration across the BBB during EAE.

3.5 Lack of JAM-B does not affect T cell transmigration across the BBB *in vitro*

To assess if endothelial JAM-B contributes to the multi-step T-cell migration across the BBB we used primary mouse brain microvascular endothelial cells (pMBMECs) as *in vitro* model of the BBB. We have previously shown that pMBMECs are an excellent model allowing to delineate molecular mechanisms involved in T cell migration across the BBB under static and flow conditions *in vitro* (Steiner et al., 2010; Steiner et al., 2011). pMBMECs have mature tight junctions and express besides occludin, claudin-5, claudin-12 also JAM-A, JAM-B, and, although to a lesser extend, JAM-C (Lyck et al., 2009). Comparing primary mouse brain microvascular endothelial cells (pMBMECs) from wild-type and JAM-B$^{-/-}$ mice we did not observe any differences in morphology, junctional integrity (Supplementary Figure 4A), or in the paracellular diffusion of 0.45 kDa Lucifer yellow (Supplementary Figure 4B) supporting that lack of JAM-B does not impair barrier characteristics of pMBMECs *in vitro*. To our surprise we failed however to detect a positive immunostaining for JAM-B on pMBMECs isolated from wild-type mice, although JAM-B was readily detected on JAM-B transfected Madin-Darby Canine Kidney (MDCK) cells (Aurrand-Lions et al., 2001b; Lamagna et al., 2005) using the same staining protocol (Supplementary Figure 4C). Taking into account that JAM-B protein may be expressed at low levels we nevertheless searched for potential differences in T-cell interaction with pMBMECs from wild-type and JAM-B$^{-/-}$ mice. We could first confirm that cytokine stimulation of pMBMECs with TNF-α or IL-1β induced comparable cell surface expression levels of endothelial ICAM-1 and VCAM-1 on pMBMECs from wild-type and JAM-B$^{-/-}$ mice (data not shown). Comparing the migration of *in vitro* activated encephalitogenic 2D2 T cells across cytokine- stimulated and non-stimulated pMBMECs isolated from wild-type or JAM-B$^{-/-}$ mice in a static two chamber assay we found not differences (Supplementary Fig. 4D). To investigate the potential role of JAM-B in mediating $\alpha 4\beta 1$-integrin mediated T cell rolling or arrest we finally investigated the multi-step T-cell migration across wild-type and JAM-B$^{-/-}$ pMBMEC monolayers under physiological flow by means *of in vitro* live cell imaging as reported before (Abadier et al., 2015; Steiner et al., 2010). We did not observe any difference in the ability of the 2D2 T cells to arrest on wild-type and JAM-B$^{-/-}$ pMBMECs under physiological flow (Supplementary Figure 4E). Also, post-arrest T-cell behaviour on pMBMEC

monolayers was found comparable in the absence or presence of JAM-B (Supplementary Figure 4E). To finally exclude that the *in vitro* studies overlooked a potential role of JAM-B in mediating T-cell interaction with the BBB due to very low JAM-B protein expression levels in pMBMECs we compared JAM-B protein expression in freshly isolated brain microvessels and pMBMECs derived from wild-type and JAM-B$^{-/-}$ mice by Western blotting. While JAM-B was as expected detectable in freshly isolated microvessels from wild-type mice but not in microvessels from JAM-B$^{-/-}$ mice, we could not detect JAM-B in cell lysates from wild-type and JAM-B$^{-/-}$ pMBMECs (Supplementary Fig. 4F). Taken together, these data suggest that pMBMECs do not maintain protein expression levels of JAM-B as observed at the BBB *in vivo*. As protein expression levels in pMBMECs are below the detection level of immunofluorescence stainings and Western blots, *in vitro* studies employing pMBMECs are not suitable to study a role for JAM-B in BBB integrity and T cell migration across the BBB.

3.6 Increased numbers of inflammatory cells accumulate in the CNS of JAM-B$^{-/-}$ C57BL/6J mice suffering from EAE

To investigate if endothelial JAM-B contributes to immune cell entry into the CNS during EAE *in vivo* we next isolated inflammatory cells from the brains and spinal cords of JAM-B$^{-/-}$ C57BL/6J mice and wild-type littermates at the peak of the disease. Multi-colour flow cytometry analysis revealed comparable percentages of CD45^{hi} CD3^{+}CD4^{+} T cells and CD45^{hi}CD3^{+}CD8^{+} T cells, CD45^{hi} B220^{+} B cells and CD45^{hi}Ly6C^{+}Ly6G^{-}F4/80^{-} recently immigrated monocytes in the brains and spinal cords of wild-type and JAM-B$^{-/-}$ mice at (Fig. 4). Similarly, the cytokine profiles of brain and spinal cord infiltrating CD45^{hi} CD3^{+}CD4^{+} T cells and CD45^{hi}CD3^{+}CD8^{+} T cells positive for the Th1 signature cytokine interferon-γ (IFN-γ), the Th17 signature cytokine interleukin -17 (IL-17) and the encephalitogenic cytokine GM-CSF were comparable in JAM-B$^{-/-}$ mice and wild-type littermates at the peak of the disease (Supplementary Fig. 5). At the same time, Th2-associated cytokines were not increased in JAM-B$^{-/-}$ mice. A small and comparable subset of wild-type as well as JAM-B$^{-/-}$ CNS infiltrating CD45^{hi} CD3^{+}CD4^{+} T cells and CD45^{hi}CD3^{+}CD8^{+} T cells stained positive for IL-4 (Supplementary Figure 5), but not IL-10 (data not shown).

To our big surprise however, we detected significantly higher numbers of CD45^{hi} brain infiltrating immune cells in JAM-B$^{-/-}$ mice, when compared to wild-type littermates (Fig. 4D). In fact, we isolated almost double as many CD45^{hi} CD3^{+}CD4^{+} T cells and CD45^{hi}CD3^{+}CD8^{+} T cells, as well as B220^{+} B

cells and Ly6C^{+}Ly6G^{-}F4/80^{-} recently immigrated monocytes from the brains of JAM-B$^{-/-}$ mice compared to wild-type littermates at the peak of EAE (Fig. 4D). Similarly, in the spinal cord we detected increased numbers of infiltrating CD45^{hi} CD3^{+}CD4^{+} T cells and CD45^{hi}CD3^{+}CD8^{+} T cells and B220^{+} B cells, but not of CD45^{hi} Ly6C^{+}Ly6G^{-}F4/80^{-} recently immigrated monocytes (Fig. 4E). Isolation of higher numbers of CNS inflammatory cells from JAM-B$^{-/-}$ C57BL/6J mice compared to wild-type littermates was thus in apparent contrast to the observed ameliorated disease course observed in the absence of JAM-B.

3.7 CNS infiltrating immune cells are trapped in leptomeningeal and perivascular spaces in JAM-B$^{-/-}$ C57BL/6J mice suffering from EAE

To explore how the presence of increased numbers of CNS inflammatory cells in JAM-B$^{-/-}$ C57BL/6J mice compared to wild-type littermates could conform with the observed ameliorated EAE severity in JAM-B$^{-/-}$ mice versus wild-type littermates, we decided to determine the precise localization of the CNS infiltrating CD45^{+} immune cells by immunofluorescence staining of brain and spinal cord cryosections from JAM-B$^{-/-}$ mice and wild-type littermates at peak of EAE. Prior studies have shown that clinical EAE requires immune cell entry across the glia limitans (Agrawal et al., 2006). To distinguish CNS parenchymal from perivascular/leptomeningeal localization of infiltrating immune cells we performed multi-colour immunofluorescence analysis combining the staining for CD45^{+} infiltrating cells with that for laminin^{+} endothelial and parenchymal basement membranes and for nuclei as a general tissue landmarks. Microscopic analysis of the immunofluorescence stainings confirmed the presence of higher numbers of CD45^{+} immune cells in the brains of JAM-B$^{-/-}$ mice when compared to wild-type littermates (Fig.5). Strikingly however, in brain and spinal cord sections of all JAM-B$^{-/-}$ mice the majority of CD45^{+} immune cells was found to be trapped in the leptomeningeal and in some JAM-B$^{-/-}$ mice also in the perivascular spaces bordered by laminin-specific immunostaining (Fig. 5 and Supplementary Figures 6 and 7). Furthermore JAM-B$^{-/-}$ mice displayed massive leptomeningeal and perivascular accumulations of CD45^{+} inflammatory cells in the cerebrum and brain stem (Figure 5) while in EAE in wild-type mice accumulation of inflammatory infiltrates typically localizes to the cerebellum and the spinal cord (Fig. 5, Supplementary Figures 6 and 7).

3.8 Potential causes for CNS infiltrating immune cell trapping in leptomeningeal and perivascular spaces in JAM-B$^{-/-}$ C57BL/6J mice with EAE

It is well known that besides adhesion molecules chemokines are critically involved in medating T-cell trafficking into the CNS during EAE. To determine if absence of JAM-B affects expression of chemokine receptors or integrins required to engage inflammatory chemokines or the parenchymal basement membranes, respectively, we investigated cell surface expression of chemokine receptors including CXCR4, CCR6, and CXCR7/ACKR3 on CNS-infiltrating T cells by flow cytometry. Furthermore, cell surface expression of integrin chains including α4-, α6-, and αV-integrin, as well as β1- and β3-integrin, and $\alpha4\beta7$-integrin was determined on CNS-infiltrating T cells by flow cytometry. We failed to detect any significant difference in integrin or chemokine receptor cell surface expression phenotypes of CNS-infiltrating T cells in wild-type and JAM-B$^{-/-}$ C57BL/6J mice suffering from EAE (data not shown).

Next we hypothesized that loss of BBB polarity in JAM-B$^{-/-}$ mice may lead to a loss of leptomeningeal or perivascular chemokine gradients and thus slow down inflammatory cell entry into the CNS parenchyma during EAE. Upregulated expression of the scavenging receptor CXCR7/ACKR3 on the BBB during EAE was previously shown to mediate internalization of CXCL12 into brain endothelial cells lowering its concentration in the leptomeningeal and perivascular spaces and allowing inflammatory cells to move towards cues provided by inflammatory chemokines produced by glial cells (McCandless et al., 2006) (Cruz-Orengo et al., 2011). We therefore performed immunofluorescent stainings of CXCL12 on brain tissues from healthy wild-type and JAM-B$^{-/-}$ mice as well as on brain tissues from mice suffering from MOG$_{aa35-55}$-induced EAE and adoptive transfer EAE. In accordance to these previous studies we detected CXCL12 immunostaining on the basolateral aspects of brain vessels and in the leptomeninges in healthy wild-type and JAM-B$^{-/-}$ mice and this immunostaining was reduced in wild-type and JAM-B$^{-/-}$ mice suffering from EAE (data not shown). However, we did not observe any difference in the staining patterns between wild-type and JAM-B$^{-/-}$ mice.

We next asked if we found any evidence for an altered BBB polarity in the absence of JAM-B. To this end we performed double-immunofluorescent stainings for the glucose transporter-1 (Glut-1), enriched at the abluminal membrane of BBB endothelial cells and P-glycoprotein (Pgp), enriched at the luminal

membrane of BBB endothelial cells. We indeed found vascular immunostaining for Pgp to be surrounded by the staining for Glut-1 in the cortex and cerebellum of wild-type and JAM-B$^{-/-}$ mice (Supplementary Fig. 8), confirming the polarized localization of these membrane transporters at the BBB. We did not observe any obvious differences in the vascular localization of Glut-1 and Pgp between wild-type and JAM-B$^{-/-}$ mice suggesting that lack of JAM-B does not lead to a loss of BBB polarity.

Finally, pronounced accumulation of CNS infiltrating immune cells in the leptomeningeal and perivascular spaces could be due to a locally increased proliferation. We therefore investigated inflammatory cell proliferation *in vivo* by intraperitoneally injecting 0.4 mg 5-ethynyl-2´-deoxyuridine (EdU) into mice during the peak of EAE and analysing the proliferation of CNS infiltrating immune cells 24 h later by flow cytometry. We found comparable fractions (12-16 %) of EdU^{+}CD45^{hi} infiltrating immune cells in the brains and spinal cords of wild-type and JAM-B$^{-/-}$ mice during the peak of EAE (Supplementary Fig. 9). This suggests that the increased accumulation of inflammatory cells observed in the leptomeningeal and perivascular spaces in JAM-B$^{-/-}$ mice is not due to an increased ratio of immune cell proliferation.

Taking together all these observations, we concluded that JAM-B is not required for immune cell trafficking across the BBB and does not affect the trafficking phenotype of CNS infiltrating T cells. Also, accumulation of inflammatory cells in leptomeningeal and perivascular spaces in JAM-B$^{-/-}$ mice during EAE can not be assigned their increased local proliferation or to a loss of BBB polarity and increased CXCL12 concentrations eventually slowing down immune cell trafficking into the CNS parenchyma. Rather, its absence seems to reduce subsequent re-activation of encephalitogenic T cells in the leptomeningeal and perivascular spaces as a prerequisite for their crossing the glia limitans.

3.9 Lack of JAM-B does not affect focal CNS activation of matrix-matalloproteinases (MMPs)

It has been shown that focal activation of the gelatinases matrix metalloproteinase (MMP)-2 and MMP-9 in perivascular and leptomeningeal spaces is crucial for inflammatory cell penetration of the parenchymal basement membrane and the onset of clinical EAE (Agrawal et al., 2006; Song et al.,

2015). To study if absence of JAM-B interferes with focal MMP-2 and MMP-9 activation in the CNS, we performed *in situ* zymography on brain sections of JAM-B$^{-/-}$ C57BL/6J mice and wild-type littermates suffering from EAE using fluorescein-conjugated gelatine. Combining this technique with immunofluorescence staining for laminin and CD45 on brain sections allowed to determine the precise localization of gelatinase activity with respect to endothelial and parenchymal basement membranes and sites of CD45^{+} immune cell infiltrates. Microscopic inspection of the brain sections revealed comparable gelatinase activity around perivascular and meningeal inflammatory cuffs in brain sections of wild-type mice and JAM-B$^{-/-}$ littermates (Fig.6). This activity could be abolished by the MMP inhibitor phenanthroline (data not shown). These findings indicate that mechanisms other than MMP-2/-9 activity cause the accumulation of inflammatory cells in the perivascular and leptomeningeal spaces in JAM-B$^{-/-}$ C57BL/6J mice during EAE (Fig. 6).

3.10 JAM-B$^{-/-}$ C57BL/6J mice do not display altered release of bone marrow myeloid cells into the blood stream

As perivascular and leptomeningeal accumulation of CNS infiltrating immune cells in JAM-B$^{-/-}$ C57BL/6J mice suffering from EAE could not be assigned to the lack of any of the known molecular mechanisms required for immune cell exit from these compartments, we next asked if an alteration in the immigration of circulating immune cells across the BBB the CNS could be an alternative explanation for our observations.

JAM-B$^{-/-}$ mice display defects in the regulation of their hematopoietic stem cell pool leading to a 2 - 4 fold increase in the release of myeloid cells into the blood stream upon application of hematopoietic stem cell mobilizing agents (Arcangeli et al., 2014; Arcangeli et al., 2011).

We therefore asked if absence of JAM-B in C57BL/6J mice also influences mobilization of myeloid cells from the bone marrow into the circulation upon immunization with CFA. To this end JAM-B$^{-/-}$ mice and wild-type littermates were immunized with CFA and the cellular composition of their blood was analyzed at days 2, 4 and 10 after CFA immunization by flow cytometry and using a VetABC™ hematology analyzer. We first quantified the absolute numbers of circulating white blood cells in JAM-B$^{-/-}$ mice compared to wild-type littermates in response to the CFA stimulus, employing the VetABC™ hematology analyzer. On days 2 and 4 after CFA injection we counted on average 1 x 10^4/µm^3 white blood cells in the blood of both, wild-type and JAM-B$^{-/-}$ mice. This number increased to 2 x 10^4/µm^3

white blood cells on day 10 after CFA challenge in both, wild-type and and JAM-B$^{-/-}$ mice (Fig. 7). Subsequent flow cytometry analysis for circulating CD45^{-}CD117^{+} hematopoietic precursor cells, CD45^{+}Ly6C^{hi}Ly6G^{-}CD11b^{+}CD115^{+} myeloid cells and CD45^{+}Ly6C^{lo}Ly6G^{+}CD11b^{+}CD115^{-} granulocytes confirmed an increased number of these immune cell subsets in the blood stream from day 4 post immunization, yet, again we did not observe any differences in wild-type and JAM-B$^{-/-}$ C57BL/6J mice (Supplementary Fig. 10 and data not shown).

Amongst white blood cells, lymphocytes increased form 0.5 x 10^4/µm^3 at days 2 and 4 after the CFA challenge to 0.8 x 10^4/µm^3 at day 10 and monocytes increased from 0.05 x 10^4/µm^3 at days 2 and 4 to 0.15 x 10^4/µm^3 at day 10 in both, wild-type and JAM-B$^{-/-}$ mice (Fig. 7). Granulocytes increased the most from 0.25 x 10^4/µm^3 at days 2 and 4 after the CFA challenge to 1 x 10^4/µm^3 at day 10 (Fig. 7). These data underscore the mobilization of immune cells from the bone marrow into the blood stream upon immunization with CFA, however, they do not support a role of JAM-B in this increased release of immune cells or precursors from the bone marrow into the blood stream after CFA immunization.

3.11 Absence of JAM-B does not ameliorate adoptive transfer EAE

JAM-B is expressed on vascular endothelial cells including those of the high endothelial venules (HEVs) in lymph nodes (Pfeiffer et al., 2008) and may therefore contribute to the homing of lymphocytes and thus eventually to the *in vivo* priming of encephalitogenic T cells upon induction of active EAE by subcutaneous immunization with MOG$_{aa35-55}$ peptide in CFA. We therefore decided to further study the role of JAM-B in EAE pathogenesis by employing the passive transfer EAE model, in which freshly *in vitro* MOG$_{aa35-55}$ activated CD4^{+} effector T cells are transferred into syngeneic recipients. To this end, lymphocytes and splenocytes were harvested from JAM-B$^{-/-}$ C57BL/6J mice and wild-type littermates at day 10 after immunization with MOG$_{aa35-55}$/CFA and restimulated *in vitro* with MOG$_{aa35-55}$. The cytokine profile for MOG$_{aa35-55}$ activated T cells derived from wild-type and JAM-B$^{-/-}$ mice was found to be indistinguishable by flow cytometry (data not shown). EAE was then induced in wild-type and JAM-B$^{-/-}$ mice by transfer of 2.5 x 10^6 purified freshly activated CD4^{+} T cells from wild-type or JAM-B$^{-/-}$ mice (Fig. 8). Interestingly, we did not observe any significant differences in the development of clinical EAE between wild-type and JAM-B$^{-/-}$ recipients irrespective of induction of EAE by MOG$_{aa35-55}$ specific CD4^{+} T cells obtained from wild-type or JAM-B$^{-/-}$ mice (Fig. 8). Also, unlike in actively induced EAE we did not observe any differences in the size of the leptomeningeal or

perivascular inflammatory cuffs in the brains of wild-type or JAM-B$^{-/-}$ recipients suffering from EAE upon transfer of encephalitogenic T cells derived from either wild-type nor in JAM-B$^{-/-}$ mice (Supplementary Figure 11). These observations suggest that JAM-B is not required for the reactivation of encephalitogenic effector T cells in the leptomeningeal or perivascular spaces. As amelioration of EAE in the absence of JAM-B is thus solely observed upon disease induction immunization with MOG$_{aa35-55}$ in CFA, JAM-B may rather play a role in peripheral *in vivo* priming of encephalitogenic T cells outside of the CNS.

3.12 Lack of JAM-B does not impair MOG$_{aa35-55}$ specific CD4$^+$ T cell activation and proliferation

To investigate a potential role for JAM-B in priming of encephalitogenic T cells *in vivo*, we next addressed if JAM-B plays a role in *in vivo* T cell activation and proliferation or polarization after immunization with MOG$_{aa35-55}$ in CFA and prior to manifestation of clinical EAE.

In light of our previous observations that JAM-B is expressed in vascular endothelial cells of lymph nodes (Pfeiffer et al., 2008) we first searched for potential differences in lymph node architecture of wild-type and JAM-B$^{-/-}$ C57BL/6J mice suffering from EAE. To this end, we performed immunofluorescent staining for Lyve-1$^+$ lymphatic vessels, VE-cadherin$^+$ endothelial cells, PNAd$^+$ high endothelial venules and laminin$^+$ basement membranes (Supplementary Fig. 12). We did not observe any significant differences in the architecture of the lymph nodes between JAM-B$^{-/-}$ mice and wild-type littermates thus excluding altered lymph node structure as a potential cause of differential encephalitogenic T-cell priming in wild-type versus JAM-B$^{-/-}$ C57BL/6J mice.

Next we investigated if absence of JAM-B affects the composition of antigen presenting cells (APCs) in the draining lymph nodes in mice suffering from EAE. To this end we performed flow cytometric analysis of immune cell suspensions of inguinal lymph nodes of mice suffering from EAE. We found comparable subsets of B220$^+$ as well as MHC-class II$^+$ APCs and F4/80$^+$ macrophages within the CD45$^+$ immune cells of wild-type and JAM-B$^{-/-}$ mice (Supplementary Fig. 13). Similarly, analysis of the myeloid subsets present in those lymph nodes showed comparable fractions of CD45$^+$CD11b$^+$Ly6C$^+$CD11c$^+$ dendritic cells, as well as comparable low fractions of CD45$^+$CD11b$^+$Ly6C$^+$Ly6G$^+$ neutrophils to be present in the inguinal lymph nodes of wild-type and JAM-B$^{-/-}$ mice (Supplementary Fig. 13).

To determine if absence of JAM-B has a direct influence on the number of or the cytokine profile of T cells in draining lymph nodes after immunization with $MOG_{aa35-55}$ in CFA, we next harvested the draining inguinal and axillary lymph nodes at days 6, 8 and 10 after immunization of JAM-$B^{-/-}$ C57BL/6J mice and wild-type littermates. Performing flow cytometry we did not reveal any significant differences in the percentage of CD4 and CD8 T cells within the $CD45^+$ immune cell subsets isolated from the different lymph nodes at the investigated days after immunization between JAM-$B^{-/-}$ mice and wild-type littermates (data not shown and Supplementary Figure 14). Similarly, the cytokine profiles (IFN-γ, IL-17, GM-CSF) of $CD4^+$ and $CD8^+$ T cells from the different lymph nodes did not show any significant differences between JAM-$B^{-/-}$ mice and wild-type littermates at the investigated time points after immunization (data not shown and Supplementary Figure 14). Thus absence of JAM-B does not affect T cell priming and polarization.

To study if absence of JAM-B influences T-cell proliferation in response to the $MOG_{aa35-55}$ peptide, we next prepared bone marrow derived dendritic cells (BMDCs) from wild-type and JAM-$B^{-/-}$ C57BL/6J mice and matured them by overnight incubation with LPS. BMDCs derived from either wild-type or JAM-$B^{-/-}$ mice did not show any differences in the expression of MHC-II, CD80 and CD86 (Supplementary Figure 15). BMDCs from wild-type and JAM-$B^{-/-}$ mice were then pulsed with $MOG_{aa35-55}$ and cultured together with naïve $CD4^+$ 2D2 T cells for four days and antigen-specific T-cell proliferation was measured during the last 16 hours of co-culture by incorporation of H^3-thymidine (Fig. 9A). BMDCs from wild-type and JAM-$B^{-/-}$ mice comparably induced $MOG_{aa35-55}$ -specific proliferation of 2D2 T cells in a dose dependent manner (Fig. 9B).

Taken together these experiments failed to show any role of JAM-B in peripheral T cell priming, polarization and proliferation *in vivo* following immunization with $MOG_{aa35-55}$ in CFA.

3.13 JAM-B is not expressed by brain infiltrating immune cells nor by bone marrow derived dendritic cells and bone marrow derived macrophages

Finally, we speculated that focal upregulation of JAM-B expression on CNS infiltrating myeloid cells may contribute to immune cell progression across the glia limitans. To this end, we studied if CNS-infiltrating immune cells are able to upregulate JAM-B and compared this to the potential of BMDCs or bone marrow derived macrophages (BMM) to upregulated JAM-B. To this end, we isolated brain-infiltrating immune cells and sorted them into $CD45^{hi}$ infiltrating immune cells and $CD45^{lo}$ myeloid cells

by flow cytometry. Performing real-time PCR did not allow to detect expression of JAM-B on any of the CNS isolated immune cell subsets (Fig. 10 and data not shown). Furthermore, we did not detect expression of JAM-B mRNA in LPS-matured BMDC or BMM from wild-type C57BL/6J mice and JAM-B$^{-/-}$ mice (Fig. 10) while expression of JAM-B was readily detectable in Madin-Darby Canine Kidney (MDCK) cells transfected with mouse JAM-B (Fig. 10). These data underscore that in the mouse JAM-B is not expressed on hematopoietic cells. Thus, trapping of immune cells in perivascular and leptomeningeal spaces is not due to a lack of JAM-B/$\alpha4\beta1$-integrin mediated interaction of antigen-presenting cells with autoaggressive T cells.

Taken together, our study shows that if encephalitogenic T cells are primed *in vivo* lack of JAM-B results in the amelioration of EAE due to the fact that inflammatory cells are trapped mainly in the leptomeningeal and to a certain degree in perivascular spaces by an unknown mechanism.

4 Discussion

Junctional adhesion molecule B (JAM-B) has been described to localize to vascular endothelial tight junctions and to function as a vascular ligand for $\alpha4\beta1$-integrin on immune cells (Cunningham et al., 2002) (Ludwig et al., 2009). Blood-brain barrier (BBB) breakdown and immune cell trafficking into the central nervous system are early hallmarks of multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). In MS and EAE, tight junction architecture changes and $\alpha4\beta1$-integrins play a prominent role in the migration of auto-aggressive immune cells into the CNS during autoimmune neuroinflammation. This prompted us to study the role of the tight junction protein JAM-B in the pathogenesis of EAE and MS.

In accordance to previous studies pointing to an endothelial cell specific expression of JAM-B in peripheral organs (Boscacci et al., 2010) we here confirm endothelial cell specific expression of JAM-B in the CNS. While we found no expression of JAM-B in choroid plexus epithelial cells forming the BCSFB, JAM-B was detected on BBB forming endothelial cells in the parenchyma and leptomeninges but also in endothelial cells of the fenestrated microvessels in the choroid plexus. Endothelial JAM-B expression in the CNS thus does not correlate with endothelial barrier characteristics. This is in

contrast to other tight junction proteins, e.g. claudin-5, which is highly expressed in plasmalemmal vesicle-associated protein (PLVAP)-negative barrier forming endothelial cells, but absent from the PLVAP-positive fenestrated microvascular endothelial cells in the choroid plexus (Zhou et al., 2014).

Lack of a role for JAM-B in establishing or maintaining a functional BBB is confirmed by our present observation that JAM-B$^{-/-}$ C57BL/6J mice display a functional BBB during health also in ageing and do not show any increased diffusion of different sized endogenous and exogenous vascular tracers across the BBB during EAE. Our present study shows that maintenance of barrier integrity in the absence of JAM-B is not due to the compensatory upregulated expression of other tight junction proteins. Thus, at least homophilic interactions of JAM-B are not essential for BBB integrity. In addition, heterophilic interactions of JAM-B with JAM-C need to be considered as JAM-B has been shown to stabilize JAM-C localization in endothelial tight junctions (Arcangeli et al., 2014). Although JAM-C expression in the CNS is not restricted to endothelial cells, its expression at the vascular level overlaps with that of JAM-B observed in the present study, namely to barrier forming and non-barrier forming CNS vascular endothelium (Wyss et al., 2012). JAM-C$^{-/-}$ C57BL/6J mice develop a hydrocephalus which is however not due to the vascular function of JAM-C as endothelial re-expression of JAM-C fails to rescue the hydrocephalus phenotype of JAM-C$^{-/-}$ mice (Wyss et al., 2012). Thus, also a potential loss of JAM-C from JAM-B-deficient endothelial junctions will not lead to BBB disruption, underscoring that neither homophilic JAM-B/JAM-B nor heterophilic JAM-B/JAM-C interactions are essential in establishing BBB integrity.

Next, we considered the role of JAM-B in mediating immune cell entry into the CNS in an $\alpha 4\beta 1$-integrin dependent fashion. Immune cell trafficking across the vascular wall is a multi-step process, in which upon its initial interaction with the inner lining of the vessels the immune cell rolls along the endothelium allowing it to reduce its velocity and to interact with vascular ligands that in a subsequent step trigger the additional activation of immune cell integrins, which leads to the integrin mediated immune cell arrest on the endothelium. Following crawling on the endothelial surface, the immune cell eventually crosses the endothelial barrier at paracellular or transcellular sites of diapedesis. JAM-B has been suggested mediate different steps of T-cell migration across the vascular wall. *In vitro* and *in vivo* live cell imaging studies demonstrated a role for JAM-B in mediating $\alpha 4\beta 1$-integrin mediated

immune cell rolling and arrest to the vascular wall independent of JAM-C (Ludwig et al., 2009). Additional studies showed that following interaction with JAM-C, endothelial JAM-B can engage $\alpha4\beta1$-integrin and mediate T-cell diapedesis across the vascular wall (Cunningham et al., 2002). First evidence for a potential role of JAM-B in mediating T-cell trafficking to the CNS was provided in our previous study in a CD8 T-cell mediated model of CNS autoimmune inflammation in BALB/c mice, in which we found an essential role for $\alpha4\beta1$-integrin in mediating $CD8^+$ T cell entry into the CNS (Martin-Blondel et al., 2015). Injection of function blocking antibodies identified JAM-B in addition to VCAM-1, as vascular ligands involved in $\alpha4\beta1$-integrin mediated $CD8^+$ T cell migration across the BBB (Martin-Blondel et al., 2015). More importantly, we observed that blocking of JAM-B rather than VCAM-1 reduced clinical signs of $CD8^+$ T cell mediated autoimmune neuroinflammation.

These experimental observations combined with the known prominent role of $\alpha4\beta1$-integrins in mediating immune cell entry into the CNS in EAE and also MS suggest a potential role for vascular JAM-B in the pathogenesis of these diseases. Observing ameliorated EAE in JAM-$B^{-/-}$ C57BL/6J mice compared to wild-type littermates thus at a first glance supported the notion of a role of vascular JAM-B in T-cell migration across the BBB. However, the isolation of significantly higher numbers of immune cells from the brains and spinal cords of JAM-$B^{-/-}$ mice when compared to wild-type littermates at the peak of the disease contradicted this assumption. We found no difference in the composition of the isolated immune cell infiltrates with respect to the presence of immune cell subsets between wild-type and JAM-$B^{-/-}$ C57BL/6J mice during the peak of EAE. Furthermore, analyzing the cytokine profile of CNS infiltrating T cells showed no difference in the expression of the Th1 and Th17 signature cytokines IFN-γ, IL-17 and GM-CSF or the Th2 signature cytokine IL-4. Thus lack of JAM-B does neither inhibit nor facilitate the migration of a given immune cell subset across the BBB.

Employing an *in vitro* model of the BBB that has previously allowed to define the precise roles of endothelial VCAM-1 and ICAM-1 and ICAM-2 in the multi-step T-cell extravasation across the BBB by means of live cell imaging (Abadier et al., 2015; Steiner et al., 2010) failed to show any involvement of endothelial JAM-B in T-cell migration across the BBB. These *in vitro* investigations were, however, hampered by lower expression levels of JAM-B in brain endothelial cells that despite the detection of JAM-B mRNA expression did not allow for detection of JAM-B protein.

Taken together, these data suggest that in this $CD4^+$ T cell driven CNS autoimmune disease other vascular $\alpha4\beta1$-integrin ligands, namely VCAM-1 and fibronectin, rather than JAM-B mediate immune cell migration across the BBB (Engelhardt et al., 1998; Man et al., 2009).

To examine how the presence of increased numbers of immune cells in the CNS of JAM-$B^{-/-}$ C57BL/6J mice suffering from EAE can be correlated to the ameliorated disease course observed when compared to wild-type littermates, we next performed immunostainings of brain and spinal cord sections and analyzed the localization of the immune cell infiltrates. While in the CNS of wild-type mice perivascular cuffs and inflammatory cell infiltrates were observed mainly in the spinal cord and cerebellum in a pattern typical for EAE in C57BL/6 mice, in the brains and spinal cords of JAM-$B^{-/-}$ C57Bl/6J mice the majority of immune cells was found to accumulate within the leptomeninges and in some mice also in perivascular spaces leading to a massive enlargement of these compartments. Absence of JAM-B was also associated with the leptomeningeal and perivascular accumulation of cellular infiltrates in the cerebrum and brain stem, which is not typically seen in EAE in C57BL/6 mice.

Thus, although JAM-B does not impair immune cell migration across the BBB its absence causes accumulation of inflammatory cells mainly in the leptomeningeal but also in perivascular spaces bordered by the endothelial and parenchymal basement membranes. Thus also in the absence of JAM-B the molecular mechanisms, e.g. interaction of T cell $\alpha4\beta1$- and $\alpha L\beta2$-integrins with endothelial VCAM-1 and ICAM-1/ ICAM-2 respectively, mediating the initial T-cell migration across leptomeningeal vessels as visualized by intravital microscopy (Bartholomaus et al., 2009; Schlager et al., 2016; Vajkoczy et al., 2001) and subsequently across the BBB in parenchymal microvessels are intact.

Leptomeningeal and perivascular trapping of CNS infiltrating cells in EAE has been observed before in L-selectin KO mice (Grewal et al., 2001), macrophage-depleted mice (Tran et al., 1998), in TNF-α KO mice (Korner et al., 1997), and in MMP2/MMP9 double KO mice (Agrawal et al., 2006). Lack of the respective cell or molecule was found to be involved in the mechanisms mediating immune cell migration across the glia limitans underscoring the concept that immune cell migration into the CNS requires sequential crossing of two distinct barriers (Owens et al., 2008). Passage across the

endothelial BBB is the first step allowing for immune surveillance of the CNS without affecting CNS function, while the second step, in which immune cells cross the glia limitans and enter the CNS parenchyma correlates with the onset of clinical EAE (Sixt et al., 2001). These previous observations have thus provided evidence that the molecular mechanisms involved in immune cell penetration across the glia limitans differ from those involved in crossing the endothelial component of the BBB. In fact, it has been shown that T cell penetration across the glia limitans in EAE requires re-activation of encephalitogenic T cells in the perivascular and leptomeningeal spaces by recognition of their cognate antigen on the local antigen-presenting cells (Bartholomaus et al., 2009). Increased expression of TNF-α by the antigen-presenting cells triggered by the T-cell interaction induces localized induction of astrocyte-derived gelatinases MMP2 and MMP9. The local activity of these gelatinases leads to the cleavage of the astrocyte extracellular matrix receptor β-dystroglycan (Agrawal et al., 2006) and modulates the local activity of chemokines at this CNS border (Song et al., 2015) allowing in a final step immune cell exit from perivascular and leptomeningeal spaces across the glia limitans into the CNS parenchyma.

In a series of experiments we aimed to define a potential role for JAM-B in the above outlined cascade of molecular mechanism promoting immune cell migration across the glia limitans in EAE. We excluded a potential contribution of upregulation of JAM-B on myeloid cells in CNS leptomeningeal and perivascular spaces demonstrating lack of JAM-B expression by this immune cell subset. The trafficking phenotype (chemokine receptors, integrins) of T cells isolated from the CNS of JAM-B$^{-/-}$ mice and wild-type littermates during the peak of the disease was comparable. Absence of JAM-B also did not affect immune cell proliferation in the CNS of mice during the peak of EAE. Furthermore, in situ zymographies and immunofluorescence stainings confirmed gelatinase activity in the brains of JAM-B$^{-/-}$ C57BL/6J mice during the peak of EAE to be indistinguishable from that observed in their wild-type littermates. Thus, the accumulation of immune cells in perivascular and leptomeningeal spaces and their inability to exit from these compartments in the absence of JAM-B is not due to alterations in the T-cell characteristics or their local reactivation.

We next hypothesized that lack of JAM-B may affect BBB polarity and subsequent loss of chemokine gradients required for immune cell entry into the CNS parenchyma during EAE. High concentrations of the chemokine CXCL12 have been suggested to maintain localization of CXCR4 expressing

effector/memory T cells in perivascular and leptomeningeal spaces during CNS immunosurveillance (McCandless et al., 2006). During EAE expression of the CXCL12 scavenging receptor CXCR7/ACKR3 is increased leading to internalization and inactivation of CXCL12 (Cruz-Orengo et al., 2011). Reducing the levels of CXCL12 have been correlated with increased migration of infammatory cells across the glia limitans probably due to facilitated migration towards gradients of inflammatory chemokines produced by astrocytes (summarized in (Huang et al., 2000)). Based on these previous observations we investigated localization of CXCL12 by immunofluorescent stainings on brain tissues from wild-type and JAM-B$^{-/-}$ mice. Although we could confirm loss of perivascular staining for CXCL12 in mice suffering from EAE we did not detect any difference in CXCL12 immunostaining in wild-type and JAM-B$^{-/-}$ mice. In accordance to these findings we also did not find any loss of BBB polarity based on immunostaining for Glut-1 and Pgp in mice lacking JAM-B. Taken together these data exclude a role of altered chemokine gradients responsible for immune cell accumulation in CNS leptomeningeal and perivascular spaces in JAM-B$^{-/-}$ mice during EAE.

Increased numbers of immune cells in perivascular and leptomeningeal compartments could theoretically also be due to an increased immigration of circulating immune cells across the BBB. Indeed, it is known that besides VCAM-1 and CXCL12, also JAM-B expressed by bone marrow stromal cells contributes to the maintenance of the hematopoietic stem cell niche (Arcangeli et al., 2011). We therefore also addressed, if JAM-B$^{-/-}$ C57BL/6J mice release an increased number of (immature) bone marrow derived cells into the blood stream following an inflammatory stimulus. Analysis of the bone marrow and the blood of wild-type and JAM-B$^{-/-}$ mice did not provide any evidence for increased release of bone marrow derived cells into the blood stream in response to the massive CFA stimulus employed to induce EAE as a potential increased source of immune cells crossing the BBB of JAM-B deficient mice during EAE.

Finally, we considered a potential role of JAM-B in the activation of encephalitogenic T cells in peripheral immune organs after induction of EAE by immunization with the MOG$_{35-55}$ peptide in CFA. JAM-B is expressed in lymphatic and vascular endothelial cells of peripheral lymph nodes (Pfeiffer et al., 2008) allowing for its potential involvement in $\alpha4\beta1$-integrin mediated T-cell priming. Indeed, $\alpha4\beta1$-integrins have been proposed to be involved in the activation of T cells (Engelhardt et al., 1998). To

circumvent *in vivo* priming of encephalitogenic T cells and to focus on the effector phase of EAE, we therefore studied EAE pathogenesis upon transfer of *in vitro* antingen-activated encephalitogenic T cells derived from wild-type or JAM-B$^{-/-}$ C57BL/6J mice into either wild-type or JAM-B$^{-/-}$ C57BL/6J mice. We did not observe any difference in disease development in any of the recipient mice, irrespective of the absence or presence of JAM-B that could have supported a role of JAM-B in T cell priming in the peripheral immune system. Furthermore, at the same time *in vitro* antigen -activated T cells derived from JAM-B$^{-/-}$ mice did not show any impairment in transferring EAE when compared to antigen -activated T cells derived from wild-type mice. Interestingly, we did not observe accumulation of inflammatory cells in leptomeningeal and perivascular spaces in transfer EAE underscoring that JAM-B is not required for focal reactivation of T cells prior to their migration across the glia limitans. It has been reported that $\alpha 4\beta 1$-integrins are recruited to the immunological synapse, where they can control T-cell priming and proliferation suggesting a potential role for $\alpha 4\beta 1$-integrin ligands including JAM-B in this process (Mittelbrunn et al., 2004). Although we did not detect expression of JAM-B in hematopoietic cells in the mouse, we confirmed that lack of JAM-B does not affect availability of antigen-presenting cells in peripheral lymph nodes and verified that JAM-B is not required for *in vivo* activation of $MOG_{aa35-55}$ –specific encephalitogenic T cells. The comparable T-cell cytokine profiles for IFN-γ, IL-17, GM-CSF and IL-4 in T cell subsets from wild-type and JAM-B$^{-/-}$ mice corroborated the notion that JAM-B does not play a critical role in *in vivo* priming, proliferation or polarization of encephalitogenic CD4$^+$ T cells in peripheral immune organs. This is in accordance with our previous observations that lack of $\beta 1$-integrins on encephalitogenic T cells and thus of the JAM-B ligand $\alpha 4\beta 1$-integrin, does not affect their priming and proliferation (Bauer et al., 2009).

5 Conclusion

Taken together, our data demonstrate that JAM-B is neither required for peripheral T-cell activation nor for immune cell trafficking across the BBB in EAE. Rather, its absence causes the trapping of inflammatory cells mainly in CNS leptomeningeal and to a lesser degree in perivascular spaces and thus amelioration of EAE by mechanisms distinct from those described to date that have lead to similar phenotypes. Further studies will have to address, if endothelial JAM-B could play a role in triggering integrin activation of immune cells crossing the BBB as previously described for PECAM-1 in regulating the activation of neutrophil integrins during endothelial diapedesis in peripheral tissues

(Dangerfield et al., 2002). In this context absence of PECAM-1 was found to lead to the trapping of neutrophils in the vascular basement membranes due to their failure to activate $\alpha 6\beta 1$-integrins, which is required for neutrophils to cross the basement membrane. In the present context, $\alpha 4\beta 1$-mediated interaction of T cells with vascular JAM-B may contribute to the subsequent activation of $\alpha 6\beta 1$-integrins required to engage the parenchymal basement membrane of the glia limitans and thus facilitate exit of the perivascular space.

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Conflict of interest disclosure

The authors Silvia Tietz, Therese Périnat, Gretchen Greene, Gaby Enzmann, Urban Deutsch, Ralf Adams, Beat Imhof, Michel Aurrand-Lions, and Britta Engelhardt declare no conflict of interest.

References

Abadier, M., N. Haghayegh Jahromi, L. Cardoso Alves, R. Boscacci, D. Vestweber, S. Barnum, U. Deutsch, B. Engelhardt, and R. Lyck. 2015. Cell surface levels of endothelial ICAM-1 influence the transcellular or paracellular T-cell diapedesis across the blood-brain barrier. *Eur J Immunol*. 45:1043-1058.

Abbott, N.J., A.A. Patabendige, D.E. Dolman, S.R. Yusof, and D.J. Begley. 2010. Structure and function of the blood-brain barrier. *Neurobiol Dis*. 37:13-25.

Agrawal, S., P. Anderson, M. Durbeej, N. van Rooijen, F. Ivars, G. Opdenakker, and L.M. Sorokin. 2006. Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. *J Exp Med*. 203:1007-1019.

Arcangeli, M.L., F. Bardin, V. Frontera, G. Bidaut, E. Obrados, R.H. Adams, C. Chabannon, and M. Aurrand-Lions. 2014. Function of Jam-B/Jam-C interaction in homing and mobilization of human and mouse hematopoietic stem and progenitor cells. *Stem Cells*. 32:1043-1054.

Arcangeli, M.L., V. Frontera, F. Bardin, E. Obrados, S. Adams, C. Chabannon, C. Schiff, S.J. Mancini, R.H. Adams, and M. Aurrand-Lions. 2011. JAM-B regulates maintenance of hematopoietic stem cells in the bone marrow. *Blood*. 118:4609-4619.

Arrate, M.P., J.M. Rodriguez, T.M. Tran, T.A. Brock, and S.A. Cunningham. 2001. Cloning of human junctional adhesion molecule 3 (JAM3) and its identification as the JAM2 counter-receptor. *J Biol Chem*. 276:45826-45832.

Aurrand-Lions, M., L. Duncan, C. Ballestrem, and B.A. Imhof. 2001a. JAM-2, a novel immunoglobulin superfamily molecule, expressed by endothelial and lymphatic cells. *J Biol Chem*. 276:2733-2741.

Aurrand-Lions, M., C. Johnson-Leger, C. Wong, L. Du Pasquier, and B.A. Imhof. 2001b. Heterogeneity of endothelial junctions is reflected by differential expression and specific subcellular localization of the three JAM family members. *Blood*. 98:3699-3707.

Bartholomaus, I., N. Kawakami, F. Odoardi, C. Schlager, D. Miljkovic, J.W. Ellwart, W.E. Klinkert, C. Flugel-Koch, T.B. Issekutz, H. Wekerle, and A. Flugel. 2009. Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature*. 462:94-98.

Bauer, M., C. Brakebusch, C. Coisne, M. Sixt, H. Wekerle, B. Engelhardt, and R. Fassler. 2009. Beta1 integrins differentially control extravasation of inflammatory cell subsets into the CNS during autoimmunity. *Proc Natl Acad Sci U S A*. 106:1920-1925.

Bettelli, E., M. Pagany, H.L. Weiner, C. Linington, R.A. Sobel, and V.K. Kuchroo. 2003. Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J Exp Med*. 197:1073-1081.

Boscacci, R.T., F. Pfeiffer, K. Gollmer, A.I. Sevilla, A.M. Martin, S.F. Soriano, D. Natale, S. Henrickson, U.H. von Andrian, Y. Fukui, M. Mellado, U. Deutsch, B. Engelhardt, and J.V. Stein. 2010. Comprehensive analysis of lymph node stroma-expressed Ig superfamily members reveals redundant and nonredundant roles for ICAM-1, ICAM-2, and VCAM-1 in lymphocyte homing. *Blood*. 116:915-925.

Campos-Bedolla, P., F.R. Walter, S. Veszelka, and M.A. Deli. 2014. Role of the blood-brain barrier in the nutrition of the central nervous system. *Arch Med Res*. 45:610-638.

Chavakis, T., T. Keiper, R. Matz-Westphal, K. Hersemeyer, U.J. Sachs, P.P. Nawroth, K.T. Preissner, and S. Santoso. 2004. The junctional adhesion molecule-C promotes neutrophil transendothelial migration in vitro and in vivo. *J Biol Chem*. 279:55602-55608.

Coisne, C., R. Lyck, and B. Engelhardt. 2013. Live cell imaging techniques to study T cell trafficking across the blood-brain barrier in vitro and in vivo. *Fluids Barriers CNS*. 10:7.

Coisne, C., W. Mao, and B. Engelhardt. 2009. Cutting edge: Natalizumab blocks adhesion but not initial contact of human T cells to the blood-brain barrier in vivo in an animal model of multiple sclerosis. *J Immunol*. 182:5909-5913.

Cruz-Orengo, L., D.W. Holman, D. Dorsey, L. Zhou, P. Zhang, M. Wright, E.E. McCandless, J.R. Patel, G.D. Luker, D.R. Littman, J.H. Russell, and R.S. Klein. 2011. CXCR7 influences leukocyte entry into the CNS parenchyma by controlling abluminal CXCL12 abundance during autoimmunity. *J Exp Med*. 208:327-339.

Cunningham, S.A., J.M. Rodriguez, M.P. Arrate, T.M. Tran, and T.A. Brock. 2002. JAM2 interacts with alpha4beta1. Facilitation by JAM3. *J Biol Chem*. 277:27589-27592.

Dangerfield, J., K.Y. Larbi, M.T. Huang, A. Dewar, and S. Nourshargh. 2002. PECAM-1 (CD31) homophilic interaction up-regulates alpha6beta1 on transmigrated neutrophils in vivo and plays a functional role in the ability of alpha6 integrins to mediate leukocyte migration through the perivascular basement membrane. *J Exp Med*. 196:1201-1211.

Dehlin, M., M. Bokarewa, R. Rottapel, S.J. Foster, M. Magnusson, L.E. Dahlberg, and A. Tarkowski. 2008. Intra-articular fms-like tyrosine kinase 3 ligand expression is a driving force in induction and progression of arthritis. *PLoS One*. 3:e3633.

Doring, A., M. Wild, D. Vestweber, U. Deutsch, and B. Engelhardt. 2007. E- and P-selectin are not required for the development of experimental autoimmune encephalomyelitis in C57BL/6 and SJL mice. *J Immunol*. 179:8470-8479.

Ebnet, K., A. Suzuki, Y. Horikoshi, T. Hirose, M.K. Meyer Zu Brickwedde, S. Ohno, and D. Vestweber. 2001. The cell polarity protein ASIP/PAR-3 directly associates with junctional adhesion molecule (JAM). *EMBO J*. 20:3738-3748.

Engelhardt, B., and C. Coisne. 2011. Fluids and barriers of the CNS establish immune privilege by confining immune surveillance to a two-walled castle moat surrounding the CNS castle. *Fluids Barriers CNS*. 8:4.

Engelhardt, B., and L. Kappos. 2008. Natalizumab: targeting alpha4-integrins in multiple sclerosis. *Neurodegener Dis*. 5:16-22.

Engelhardt, B., B. Kempe, S. Merfeld-Clauss, M. Laschinger, B. Furie, M.K. Wild, and D. Vestweber. 2005. P-selectin glycoprotein ligand 1 is not required for the development of experimental autoimmune encephalomyelitis in SJL and C57BL/6 mice. *J Immunol*. 175:1267-1275.

Engelhardt, B., M. Laschinger, M. Schulz, U. Samulowitz, D. Vestweber, and G. Hoch. 1998. The development of experimental autoimmune encephalomyelitis in the mouse requires alpha4-integrin but not alpha4beta7-integrin. *J Clin Invest*. 102:2096-2105.

Engelhardt, B., and R.M. Ransohoff. 2012. Capture, crawl, cross: the T cell code to breach the blood-brain barriers. *Trends Immunol*. 33:579-589.

Engelhardt, B., and L. Sorokin. 2009. The blood-brain and the blood-cerebrospinal fluid barriers: function and dysfunction. *Semin Immunopathol*. 31:497-511.

Engelhardt, B., P. Vajkoczy, and R.O. Weller. 2017. The movers and shapers in immune privilege of the CNS. *Nat Immunol*. 18:123-131.

Furuse, M., T. Hirase, M. Itoh, A. Nagafuchi, S. Yonemura, S. Tsukita, and S. Tsukita. 1993. Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol*. 123:1777-1788.

Garrido-Urbani, S., P.F. Bradfield, and B.A. Imhof. 2014. Tight junction dynamics: the role of junctional adhesion molecules (JAMs). *Cell Tissue Res*. 355:701-715.

Grewal, I.S., H.G. Foellmer, K.D. Grewal, H. Wang, W.P. Lee, D. Tumas, C.A. Janeway, Jr., and R.A. Flavell. 2001. CD62L is required on effector cells for local interactions in the CNS to cause myelin damage in experimental allergic encephalomyelitis. *Immunity*. 14:291-302.

Huang, D., Y. Han, M.R. Rani, A. Glabinski, C. Trebst, T. Sorensen, M. Tani, J. Wang, P. Chien, S. O'Bryan, B. Bielecki, Z.L. Zhou, S. Majumder, and R.M. Ransohoff. 2000. Chemokines and chemokine receptors in inflammation of the nervous system: manifold roles and exquisite regulation. *Immunol Rev*. 177:52-67.

Kivisakk, P., D.J. Mahad, M.K. Callahan, C. Trebst, B. Tucky, T. Wei, L. Wu, E.S. Baekkevold, H. Lassmann, S.M. Staugaitis, J.J. Campbell, and R.M. Ransohoff. 2003. Human cerebrospinal fluid central memory CD4+ T cells: evidence for trafficking through choroid plexus and meninges via P-selectin. *Proc Natl Acad Sci U S A*. 100:8389-8394.

Korner, H., D.S. Riminton, D.H. Strickland, F.A. Lemckert, J.D. Pollard, and J.D. Sedgwick. 1997. Critical points of tumor necrosis factor action in central nervous system autoimmune inflammation defined by gene targeting. *J Exp Med*. 186:1585-1590.

Lamagna, C., P. Meda, G. Mandicourt, J. Brown, R.J. Gilbert, E.Y. Jones, F. Kiefer, P. Ruga, B.A. Imhof, and M. Aurrand-Lions. 2005. Dual interaction of JAM-C with JAM-B and alpha(M)beta2 integrin: function in junctional complexes and leukocyte adhesion. *Mol Biol Cell*. 16:4992-5003.

Lazarevic, I., and B. Engelhardt. 2016. Modeling immune functions of the mouse blood-cerebrospinal fluid barrier in vitro: primary rather than immortalized mouse choroid plexus epithelial cells are suited to study immune cell migration across this brain barrier. *Fluids Barriers CNS*. 13:2.

Ludwig, R.J., K. Hardt, M. Hatting, R. Bistrian, S. Diehl, H.H. Radeke, M. Podda, M.P. Schon, R. Kaufmann, R. Henschler, J.M. Pfeilschifter, S. Santoso, and W.H. Boehncke. 2009. Junctional adhesion molecule (JAM)-B supports lymphocyte rolling and adhesion through interaction with alpha4beta1 integrin. *Immunology*. 128:196-205.

Lutz, S.E., J.R. Smith, D.H. Kim, C.V.L. Olson, K. Ellefsen, J.M. Bates, S.P. Gandhi, and D. Agalliu. 2017. Caveolin1 Is Required for Th1 Cell Infiltration, but Not Tight Junction Remodeling, at the Blood-Brain Barrier in Autoimmune Neuroinflammation. *Cell Rep*. 21:2104-2117.

Lyck, R., N. Ruderisch, A.G. Moll, O. Steiner, C.D. Cohen, B. Engelhardt, V. Makrides, and F. Verrey. 2009. Culture-induced changes in blood-brain barrier transcriptome: implications for amino-acid transporters in vivo. *J Cereb Blood Flow Metab*. 29:1491-1502.

Man, S., B. Tucky, N. Bagheri, X. Li, R. Kochar, and R.M. Ransohoff. 2009. alpha4 Integrin/FN-CS1 mediated leukocyte adhesion to brain microvascular endothelial cells under flow conditions. *J Neuroimmunol*. 210:92-99.

Martin-Blondel, G., B. Pignolet, S. Tietz, L. Yshii, C. Gebauer, T. Perinat, I. Van Weddingen, C. Blatti, B. Engelhardt, and R. Liblau. 2015. Migration of encephalitogenic CD8 T cells into the central nervous system is dependent on the alpha4beta1-integrin. *Eur J Immunol*. 45:3302-3312.

Martin-Padura, I., S. Lostaglio, M. Schneemann, L. Williams, M. Romano, P. Fruscella, C. Panzeri, A. Stoppacciaro, L. Ruco, A. Villa, D. Simmons, and E. Dejana. 1998. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol*. 142:117-127.

McCandless, E.E., Q. Wang, B.M. Woerner, J.M. Harper, and R.S. Klein. 2006. CXCL12 limits inflammation by localizing mononuclear infiltrates to the perivascular space during experimental autoimmune encephalomyelitis. *J Immunol*. 177:8053-8064.

Mittelbrunn, M., A. Molina, M.M. Escribese, M. Yanez-Mo, E. Escudero, A. Ursa, R. Tejedor, F. Mampaso, and F. Sanchez-Madrid. 2004. VLA-4 integrin concentrates at the peripheral

supramolecular activation complex of the immune synapse and drives T helper 1 responses. *Proc Natl Acad Sci U S A*. 101:11058-11063.

Mochida, G.H., V.S. Ganesh, J.M. Felie, D. Gleason, R.S. Hill, K.R. Clapham, D. Rakiec, W.H. Tan, N. Akawi, M. Al-Saffar, J.N. Partlow, S. Tinschert, A.J. Barkovich, B. Ali, L. Al-Gazali, and C.A. Walsh. 2010. A homozygous mutation in the tight-junction protein JAM3 causes hemorrhagic destruction of the brain, subependymal calcification, and congenital cataracts. *Am J Hum Genet*. 87:882-889.

Nitta, T., M. Hata, S. Gotoh, Y. Seo, H. Sasaki, N. Hashimoto, M. Furuse, and S. Tsukita. 2003. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol*. 161:653-660.

Ostermann, G., K.S. Weber, A. Zernecke, A. Schroder, and C. Weber. 2002. JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. *Nat Immunol*. 3:151-158.

Owens, T., I. Bechmann, and B. Engelhardt. 2008. Perivascular spaces and the two steps to neuroinflammation. *J Neuropathol Exp Neurol*. 67:1113-1121.

Padden, M., S. Leech, B. Craig, J. Kirk, B. Brankin, and S. McQuaid. 2007. Differences in expression of junctional adhesion molecule-A and beta-catenin in multiple sclerosis brain tissue: increasing evidence for the role of tight junction pathology. *Acta Neuropathol*. 113:177-186.

Palmeri, D., A. van Zante, C.C. Huang, S. Hemmerich, and S.D. Rosen. 2000. Vascular endothelial junction-associated molecule, a novel member of the immunoglobulin superfamily, is localized to intercellular boundaries of endothelial cells. *J Biol Chem*. 275:19139-19145.

Pfeiffer, F., V. Kumar, S. Butz, D. Vestweber, B.A. Imhof, J.V. Stein, and B. Engelhardt. 2008. Distinct molecular composition of blood and lymphatic vascular endothelial cell junctions establishes specific functional barriers within the peripheral lymph node. *Eur J Immunol*. 38:2142-2155.

Rudolph, H., A. Klopstein, I. Gruber, C. Blatti, R. Lyck, and B. Engelhardt. 2016. Postarrest stalling rather than crawling favors CD8(+) over CD4(+) T-cell migration across the blood-brain barrier under flow in vitro. *Eur J Immunol*. 46:2187-2203.

Schlager, C., T. Litke, A. Flugel, and F. Odoardi. 2016. In Vivo Visualization of (Auto)Immune Processes in the Central Nervous System of Rodents. *Methods Mol Biol*. 1304:117-129.

Sixt, M., B. Engelhardt, F. Pausch, R. Hallmann, O. Wendler, and L.M. Sorokin. 2001. Endothelial cell laminin isoforms, laminins 8 and 10, play decisive roles in T cell recruitment across the blood-brain barrier in experimental autoimmune encephalomyelitis. *J Cell Biol*. 153:933-946.

Song, J., C. Wu, E. Korpos, X. Zhang, S.M. Agrawal, Y. Wang, C. Faber, M. Schafers, H. Korner, G. Opdenakker, R. Hallmann, and L. Sorokin. 2015. Focal MMP-2 and MMP-9 activity at the blood-brain barrier promotes chemokine-induced leukocyte migration. *Cell Rep*. 10:1040-1054.

Steiner, O., C. Coisne, R. Cecchelli, R. Boscacci, U. Deutsch, B. Engelhardt, and R. Lyck. 2010. Differential roles for endothelial ICAM-1, ICAM-2, and VCAM-1 in shear-resistant T cell arrest, polarization, and directed crawling on blood-brain barrier endothelium. *J Immunol*. 185:4846-4855.

Steiner, O., C. Coisne, B. Engelhardt, and R. Lyck. 2011. Comparison of immortalized bEnd5 and primary mouse brain microvascular endothelial cells as in vitro blood-brain barrier models for the study of T cell extravasation. *J Cereb Blood Flow Metab*. 31:315-327.

Tietz, S., and B. Engelhardt. 2015. Brain barriers: Crosstalk between complex tight junctions and adherens junctions. *J Cell Biol*. 209:493-506.

Tietz, S.M., M. Zwahlen, N. Haghayegh Jahromi, P. Baden, I. Lazarevic, G. Enzmann, and B. Engelhardt. 2016. Refined clinical scoring in comparative EAE studies does not enhance the chance to observe statistically significant differences. *Eur J Immunol*. 46:2481-2483.

Tommasin, S., C. Gianni, L. De Giglio, and P. Pantano. 2017. Neuroimaging techniques to assess inflammation in Multiple Sclerosis. *Neuroscience*.

Tran, E.H., K. Hoekstra, N. van Rooijen, C.D. Dijkstra, and T. Owens. 1998. Immune invasion of the central nervous system parenchyma and experimental allergic encephalomyelitis, but not leukocyte extravasation from blood, are prevented in macrophage-depleted mice. *J Immunol*. 161:3767-3775.

Vajkoczy, P., M. Laschinger, and B. Engelhardt. 2001. Alpha4-integrin-VCAM-1 binding mediates G protein-independent capture of encephalitogenic T cell blasts to CNS white matter microvessels. *J Clin Invest*. 108:557-565.

Vorbrodt, A.W., and D.H. Dobrogowska. 2004. Molecular anatomy of interendothelial junctions in human blood-brain barrier microvessels. *Folia Histochem Cytobiol*. 42:67-75.

Winger, R.C., J.E. Koblinski, T. Kanda, R.M. Ransohoff, and W.A. Muller. 2014. Rapid remodeling of tight junctions during paracellular diapedesis in a human model of the blood-brain barrier. *J Immunol*. 193:2427-2437.

Wyss, L., J. Schafer, S. Liebner, M. Mittelbronn, U. Deutsch, G. Enzmann, R.H. Adams, M. Aurrand-Lions, K.H. Plate, B.A. Imhof, and B. Engelhardt. 2012. Junctional adhesion molecule (JAM)-C deficient C57BL/6 mice develop a severe hydrocephalus. *PLoS One*. 7:e45619.

Zhou, Y., Y. Wang, M. Tischfield, J. Williams, P.M. Smallwood, A. Rattner, M.M. Taketo, and J. Nathans. 2014. Canonical WNT signaling components in vascular development and barrier formation. *J Clin Invest*. 124:3825-3846.

Figure legends

Figure 1: Co-localization of JAM-B and PECAM-1 in the CNS of C57BL/6J mice

Immunofluorescence staining for JAM-B in the mouse CNS was evaluated on 6 µm cryosections of brains and spinal cord of healthy wild-type (WT) C57BL/6J mice. Immunofluorescence staining for JAM-B (third vertical row, red) and PECAM-1 (second vertical row, green) show co-localization of JAM-B and PECAM-1 (fourth vertical row, merged) on all vessels in the mouse cortex (first horizontal row), the choroid plexus endothelium (second horizontal row), all vessels in the cerebellum (third horizontal row), and all vessels in the spinal cord (fourth horizontal row). All sections were counter-stained with DAPI to show cell nuclei (first vertical row). Bars are 50 µm. Panel shown above constitutes one representative staining; in total three individual tissues were processed. For all pictures shown in the panel above Brightness and contrast were adjusted (brightness 72; contrast 100) using Photoshop CS6 software.

Figure 2: JAM-B$^{-/-}$ C57BL/6J mice develop ameliorated EAE.

A: The clinical severity of MOG_{35-55} induced EAE as followed over 35 days in wild-type (WT; black dots) and JAM-B$^{-/-}$ C57BL/6J mice (grey squares) is shown. **B:** Overall disease severity as determined by the area under the curve (AUC) and analyzed until day 35 showed a significant reduction in JAM-B$^{-/-}$ mice (median 11) when compared to wild-type (WT) littermates (median 17.25). Bar graphs represent the mean ± SEM of 3 independent experiments including n = 22 WT and n = 20 JAM-B$^{-/-}$ mice. Statistical analysis was performed using the GraphPad Prism 6 software. Differences in between the AUCs were evaluated by Man-Whitney test p > 0.01**. **C:** Body weight of wild-type (WT; black dots) and JAM-B$^{-/-}$ littermates (grey squares) during EAE progression is shown; mean ± SEM. **D:** The maximal clinical score was evaluated as mean of the maximal score of each mouse (n = 22 wild-type (WT), black bar; n = 20 JAM-B $^{-/-}$ mice, grey bar) during the disease progression. The mean maximal clinical score of WT mice was 1.5 ± 0.13 and mean maximal score of JAM-B$^{-/-}$ mice 1 ± 0.09. Differences between the maximal clinical score are evaluated by student's *t*-test; p values > 0.01**.

Figure 3: *In vivo* BBB permeability in C57BL/6J wild-type and JAM-B$^{-/-}$ mice suffering from EAE

A: Representative pictures show 3 kDa Dextran (red) and 10 kDa Dextran (green) in the choroid plexus (3rd ventricle) in the brain of wild-type (WT; upper row; EAE day 18 score 2) and JAM-B$^{-/-}$

C57BL/6J mice (lower row; EAE day 18 score 2) suffering from EAE. **B:** Representative pictures show 3 kDa Dextran (red) and 10 kDa Dextran (green) in blood vessels in the cortex in the brain of wild-type (WT; upper row) and JAM-B$^{-/-}$ mice (lower row) suffering from EAE. C: Representative pictures show 3 kDa Dextran (red) and 10 kDa Dextran (green) in the cerebellum of WT (upper row) and JAM-B$^{-/-}$ mice (lower row) suffering from EAE. 3 kDa Dextran and 10 kDa Dextran is present in the perivascular space of inflammatory cuffs (white arrowheads) WT (upper row) and JAM-B$^{-/-}$ mice (lower row) suffering from EAE. Scale bars = 50 µm. In total three individual tissues per genotype were processed (EAE day 18; n = 3 WT scores: n = 2 score 2, n = 1 score 1; n = 3 JAM-B$^{-/-}$ C57BL/6J scores: n = 1 score 2, n = 2 score 1).

Figure 4: Increased numbers of CD45^{+} inflammatory cells infiltrate the CNS of JAM-B$^{-/-}$ C57BL/6J mice when compared to WT littermates at the peak of EAE

A: Exemplary demonstration of the gating strategy of CD45^{+} immune cells isolated from the spinal cords of n = 4 wild-type (WT) at the peak of EAE (day 16 or day 20 post EAE-induction) is shown. A gate was set to the CD45^{hi}SSC^{lo} population (left graph), which was further gate on CD3 and CD4 (middle graph). Four rectangular gates were set according to the isotype control (right graph) to divide non-overlapping populations using the FlowJo software version 10. WT allocated gates were pasted to the data obtained from JAM-B$^{-/-}$ C57BL/6J mice. **B:** Bar graphs show the mean ± SD percentage of CD45^{hi}SSC^{lo} infiltrating immune cell populations isolated from the brains of n = 12 wild-type (WT) mice (black bars) and n = 12 JAM-B$^{-/-}$ mice (grey bars) at the peak of EAE. Data are plotted as a summary of 3 independent experiments including n = 4 mice (pooled) per genotype and experiment. **C:** Bar graphs show the mean ± SD percentage of CD45^{hi}SSC^{lo} infiltrating immune cell populations isolated from the spinal cords of n = 12 wild-type (WT) mice (black bars) and n = 12 JAM-B$^{-/-}$ mice (grey bars) at the peak of EAE. Data are plotted as a summary of 3 independent experiments including n = 4 mice (pooled) per genotype and experiment. **D:** Absolute numbers of CD45$^{\mathbf{hi}}$SSC$^{\mathbf{lo}}$ brain infiltrating immune cell subsets per mouse were levied by projection to the number of flow cytometry counting beads. Data show the number of brain infiltrating CD45^{hi}SSC^{lo} cells of n = 12 JAM-B$^{-/-}$ mice (grey bars) normalized to n = 12 wild-type (WT) mice (black bars); mean ± SD. Data are plotted as a summary of 3 independent experiments including n = 4 mice (pooled) per genotype and experiment. For statistical analysis the student's *t*-test was used to determine differences between the groups and p values > 0.5

were assigned to be significant*. **E:** Absolute numbers of CD45^{hi}SSC^{lo} brain infiltrating immune cell subsets per mouse were levied by projection to the number of flow cytometry counting beads. Data show the number of brain infiltrating CD45^{hi}SSC^{lo} cells of n = 12 JAM-B$^{-/-}$ mice (grey bars) normalized to n = 12 wild-type (WT) mice (black bars); mean ± SD. Data are plotted as a summary of 3 independent experiments including n = 4 mice (pooled) per genotype and experiment. For statistical analysis the student's *t*-test was used to determine differences between the groups and p values > 0.5 were assigned to be significant*.

Figure 5: CD45^{+} inflammatory cells are trapped in leptomeningeal and perivascular spaces in the CNS of JAM-B$^{-/-}$ C57BL/6J mice at the peak of EAE.

To investigate the localization of CNS infiltrating CD45^{+} immune cells in WT and JAM-B$^{-/-}$ C57BL/6J mice at the peak of EAE, immunofluorescence staining was performed on 6 µm cryosections of brain and spinal cord tissue. Representative pictures from the cerebrum, brain stem and the spinal cord of wild-type (WT) mice and JAM-B$^{-/-}$ mice with clinical EAE (day 16, clinical score 2) are shown. Three mice per genotype were analyzed at the peak of EAE (day 16 after immunization; n = 2 WT and n = 2 JAM-B$^{-/-}$ mice score 2 and n = 1 WT and n = 1 JAM-B$^{-/-}$ mice score 1). Trapping of CD45^{+} immune cells (red) between the endothelial and parenchymal basement membranes (laminin staining in green) and thus widening of these spaces is obvious in tissue sections of JAM-B$^{-/-}$ mice when compared to WT littermates. Blue = nuclear staining with DAPI. Scale bar 100 µm. Representative pictures from the cerebrum show the periventricular space adjacent to the choroid plexus (3rd ventricle). Pictures from the brain stem show trapped immune cells in the leptomeninges. Selected pictures from the spinal cord show meningeal infiltration in the lumbar spinal cord as well as inflammatory cuffs in the white matter.

Figure 6: Analysis of gelatinase (MMP) activity in EAE brains by *in situ* zymography

In situ zymography coupled with pan-laminin (blue) and CD45 (red) immunofluorescence staining on brain sections of wild-type (WT) and JAM-B$^{-/-}$ C57BL/6J mice with EAE (day 16; n = 2 WT, score 2 and score 1; n = 2 JAM-B$^{-/-}$, score 2 and score 1) is shown. Proteolytic digestion of DQ-gelatine unquenches the bright green fluorescence of fluorescein, which allows to localize gelatinase/MMP activity. While patchy areas of green fluorescence on the tissue section have to be considered as

unspecific signal, local MMP activity is observed by the typical granular bright green fluorescence signal, examples of which are highlighted by the arrowheads. Specific MMP activity can therefore be visualized in the cerebellum beyond the parenchymal basement membrane (blue) at parenchymal and leptomeningeal sites of immune cell infiltration (red). Note the large Scale bar = 100 µm. Representative pictures of a total of 3 mice per genotype analyzed. Pictures were taken how and. Brightness and contrast were adjusted (brightness 72; contrast 100) using Photoshop CS6 software.

Figure 7: Analysis of blood of CFA challenged mice

Evaluation for the number of leukocytes (white blood cells) in the blood of CFA immunized C57BL/6J wild-type (WT; black dots, n = 9) and JAM-B$^{-/-}$ mice (grey dots, n = 9) at days 2, 4, and 10 after CFA challenge (left column). Second column: numbers of lymphocytes at days 2, 4, and 10 after CFA challenge of WT (black dots, n = 9) and JAM-B$^{-/-}$ mice (grey dots, n = 9). Third column: numbers of monocytes at days 2, 4, and 10 after CFA challenge of WT (black dots, n = 9) and JAM-B$^{-/-}$ mice (grey dots, n = 9). Right column: numbers of granulocytes at days 2, 4, and 10 after CFA challenge of WT (black dots, n = 9) and JAM-B$^{-/-}$ mice (grey dots, n = 9). Data are displayed as a summary of three independent experiments including 3 mice per genotype; each dot represents 1 mouse

Figure 8: Adoptive transfer EAE induced by polyclonal encephalitogenic CD4^{+} T cell transfer in C57BL/6J wild-type and JAM-B$^{-/-}$ mice

Clinical course of adoptively transferred EAE by intraperitoneal injection of 2.5 x 10^6 MOG$_{aa35-55}$-activated CD4^{+} T cells obtained from wild-type (WT) donor mice into WT recipients (black dots; WT ➔ WT) and JAM-B$^{-/-}$ littermates (grey dots; WT ➔ JAM-B$^{-/-}$) and clinical course of adoptively transferred EAE by intraperitoneal injection of 2.5 x 10^6 MOG$_{aa35-55}$-activated CD4^{+} T cells obtained from JAM-B$^{-/-}$ donor mice into WT (black squares; JAM-B$^{-/-}$ ➔ WT) and JAM-B$^{-/-}$ mice (grey squares; JAM-B$^{-/-}$ ➔ JAM-B$^{-/-}$). Adoptive transfer EAE was followed over 29 days in a total of n = 19 WT and n = 19 JAM-B$^{-/-}$ mice. Data show mean ± SEM as a summary of three independent experiments including n = 5-7 mice per group, respectively. Overall disease severity is shown as area under the curve (AUC) for WT ➔ WT tEAE (black bars), WT ➔ JAM-B$^{-/-}$ mice (grey bars black cross-striped), JAM-B$^{-/-}$ ➔ WT (black bars grey cross-striped), and JAM-B$^{-/-}$ ➔ JAM-B$^{-/-}$). (grey bars); mean ± SEM. Statistical analysis was performed using the GraphPad Prism 6 software; one-way ANOVA; p = 0.6406.

Figure 9: *In vitro* proliferation of MOG_{35-55} T-cell receptor transgenic 2D2 T cells in response to MOG_{35-55} peptide presented by bone marrow derived dendritic cells

A: The graph shows the CPMA values of proliferating naïve 2D2 T cells co-cultured with either BMDCs generated from wild-type (WT, black dots) C57BL/6J mice or JAM-$B^{-/-}$ littermates (grey squares) in the medium control, tuberculin purified protein derivative (PPD) negative control, and anti-CD3/anti-CD28 positive control. **B:** The graph shows increasing CPMA levels correlating to increasing concentrations of MOG in both naïve 2D2 T cells co-cultured with BMDCs generated from wild-type (WT, black dots) C57BL/6J mice and JAM-$B^{-/-}$ littermates (grey squares). The data show 1 experiment as a representative of 3 independent experiments; mean ± SD. All concentrations were set up as triplicates in each experiment.

Figure 10: JAM-B expression on BMDC, BMM, and brain-infiltrating immune cells

The graph shows JAM-B ΔCt values (ΔCt(GOI) = Ct(GOI)-Ct(s16 normalizer)). MDCK Fc-JAM-B (n = 1) positive control; black bar. JAM-B ΔCt values of BMDCs (bright grey bars) as well as JAM-B ΔCt values of BMMs (dark grey bars) from wild-type (WT) and JAM-$B^{-/-}$ C57BL/6J mice are presented as mean ± SD from three independent preparations including 1 mouse per genotype. JAM-B ΔCt form pooled brain-infiltrating $CD45^{hi}$ cells obtained from C57BL/6J mice (n = 9: score 2 n = 6 and score 1 n = 3) at day 16 after EAE induction (blue bar).