Uptake, processing and presentation of myelin-derived antigens by brain endothelial cells facilitates antigen-specific T cell migration

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Submitted for publication
**Abstract**

Immune cell traffic into the central nervous system (CNS) is tightly controlled by the blood-brain barrier (BBB). Under inflammatory conditions, such as present in multiple sclerosis (MS), circulating T-lymphocytes and monocytes/macrophages cross the BBB and infiltrate into the brain. During disease progression, inflammatory processes result in BBB dysfunction as well as up-regulation of molecules involved in cell migration. However, what drives the migration of antigen-specific effector T cells to the inflamed areas of the brain is unclear. Since some studies suggested that endothelial cells can act as non-professional antigen-presenting cells (APCs) we hypothesize that brain endothelial cells (BECs) can take up and process myelin antigens and that antigen presentation by inflamed BECs drives the migration of myelin-specific T lymphocytes into the brain. In this study we demonstrate that inflamed BECs express de novo MHC-II molecules and internalize myelin via the endo-lysosomal compartment. Both resting and activated BECs process myelin in a time- and concentration-dependent manner. Importantly, we show that these myelin-derived antigens are presented on the surface in MHC-II molecules, driving the migration of antigen-specific T cells. Blocking MHC-II prevented T cell transmigration, confirming the necessity of antigen presentation by the brain endothelium for efficient antigen-specific T cell migration. These results demonstrate that BECs are capable of antigen processing and presentation and that this phenomenon may contribute to the pathogenesis and lesion development in MS.
**Introduction**

In neuroinflammatory disorders of the central nervous system (CNS), such as in multiple sclerosis (MS), the trafficking of immune cells into the brain is exacerbated and detrimental for lesion formation and disease progression\(^1\). MS has long been seen as an autoimmune disorder, where autoreactive T cells infiltrate the brain, contributing to neuronal and tissue damage and degeneration\(^2\). Although evidence suggests that this phenomenon also occurs in the absence of leukocyte infiltrates\(^3\), it is well accepted that immune cell migration into the brain has a detrimental effect in MS pathogenesis. Therefore, the etiology of the disease is still not clearly understood.

The blood-brain barrier (BBB) is composed of brain endothelial cells (BECs) which are highly specialized structures composed of tightly regulated junctions that limit the entry of immune cells into the brain, have low endocytic capacity and low fenestrations\(^4,5\). They are therefore regarded as an important barrier in MS. However, inflammatory processes in the brain also affect the BBB which in turn secrete pro-inflammatory cytokines and chemokines, further attracting leukocytes to the lesion area\(^6-8\). One of the issues that remain unresolved is what drives T cell infiltration into specific areas of the brain and how new lesions are formed.

Many studies suggested a role for BECs as non-professional antigen presenting cells (APCs)\(^9-12\). This immune function is related to the upregulation of specific molecules by inflamed BECs such as MHC class II and co-stimulatory molecules such as CD40, ICOSL and PD-L1 and PD-L2 but not CD80 or CD86\(^13,14\). Interestingly, the importance of antigen presentation by BECs in regulating immune cell migration is controversial. Some studies suggest that antigen presentation of cognate antigens by BECs helps the migration of antigen-specific T cells across the brain endothelium\(^15-17\) while others showing no influence of such process in T cell migration\(^18-20\). Since most of these studies have been done by analyzing migration across meningeal vessels of the spinal cord, it still remains unclear whether T cells need an antigen-specific signal to traffic to the brain parenchyma.

The potential for antigen presentation by BECs is dependent on antigen uptake and processing. Although it has been shown that BECs can take up soluble antigens by macropinocytosis and clathrin-coated pits\(^14\), not much is known about their processing capacity of internalized antigens. Therefore, in this study we explored the potential of BECs as antigen-presenting cells and determined how this process contributes to migration of antigen-specific T cells. Here we demonstrate that inflamed BECs upregulate MHC-II molecules, which are important for antigen presentation and T cell activation. Furthermore, BECs can take up and process myelin via the lysosomal
degradation pathway in a time and concentration-dependent manner. Importantly, these myelin-derived antigens are presented in MHC-II and facilitate the migration of antigen-specific T cells through the brain endothelium.

**Materials and Methods**

**Cell culture**

The human brain endothelial cell (BEC) line hCMEC/D3\(^2\) was kindly provided by Dr. P.-O. Couraud (Institut Cochin, Université Paris Descartes, Paris, France). BECs were grown in EBM-2 medium supplemented with hEGF, hydrocortisone, GA-1000, VEGF, hFGF-B, R3-IGF-1, ascorbic acid and 2.5% fetal calf serum (Lonza, Basel, Switzerland).

**Flow cytometry**

For antigen internalization experiments, resting or 24hrrhTNFα activated (5ng/ml, Peprotech, UK) BECs were seeded in collagen-coated plates and when confluent, incubated with 10µg/ml labeled myelin (myelin-AF555) for 4h or 24h. Subsequently, cells were extensively washed with PBS to remove external myelin and fluorescence intensity was measured using a FACS Calibur flow cytometer (Becton & Dickinson, San Jose, CA, USA).

The following antibodies were used to detect the presence of MHC and costimulatory molecules on resting or TNFα activated BECs: FITC-conjugated anti-HLA-ABC (clone DX-17) and -VCAM-1 (clone STA); PE-conjugated anti-HLA-DR (clone G46-6); -CD80 (clone L307.4). Binding of unconjugated anti-CD40 (clone TRAP-1) was detected using goat-anti-mouse IgG1-A488 (Life Technologies). All antibodies were obtained from BD Pharmingen, except anti-VCAM which was obtained from eBiosciences.

**Western blot**

For processing experiments, 10µg/ml, 25µg/ml or 50µg/ml of human myelin was added to confluent BECs for 24h. Alternatively, BECs were pre-stimulated with 5ng/ml rhTNFα for 24h before addition of 20µg/ml of myelin for either 4h or 24h. After incubation with myelin, cells were thoroughly washed with ice-cold PBS and lysed on ice with lysis buffer (Cell Lysis buffer, Cell Signaling Technology, Boston, MA, USA) containing protease inhibitor cocktail (Roche, Almere, the Netherlands) for 30min following manufacturer's instructions. Protein lysates were quantified (Pierce BCA Protein kit, Thermo Fisher, USA) and 50µg of protein was taken up in sample buffer (1M Tris-HCl pH 6.8, 20% SDS, 50% glycerol, 0.2% bromophenol blue) and heated to 95°C for 3min.
Lysates were analyzed on a 10% gel by SDS-PAGE, proteins were transferred to a PVDF membrane and blocked for 1h at room temperature (RT) with blocking buffer (LI-COR, Lincoln, USA). Subsequently, membranes were incubated with the primary antibodies against myelin basic protein (MBP) (Serotec, Uden, the Netherlands) and actin (clone AC-15, Sigma Aldrich). Primary antibodies were detected with IRDye antibodies and using the Odyssey infrared imaging system (LI-COR, Lincoln, USA). Actin quantification was used to correct for protein loading.

**Imaging flow cytometry**

Confluent BECs were seeded in 6-well plates (Corning, Amsterdam, The Netherlands) and stimulated with 5ng/ml rhTNFα for 24h. 10µg/ml of fluorescently-labeled human myelin was added to BECs for 4h or 24h. Cells were then extensively washed with ice-cold PBS, detached with trypsin and fixated with 4% formaldehyde. Cells were then permeabilized with 0.05% saponin for 30min at RT and subsequently blocked with 10% goat serum in PBS/BSA. Cells were labeled with EEA1-FITC (BD Bioscience), LAMP1 (BD Pharmingen) and goat anti-mouse Alexa 488 (Molecular Probes, Eugene, OR). Cells were analyzed on the ImageStream X100 (Amnis-Merck Millipore) imaging flow cytometer as previously described. A minimum of 15000 cells were acquired per sample. For standard acquisition, the 488 nm laser line (for EEA-1 and LAMP-1) was set at 10mW and the 642nm laser line (for myelin) was set at 5mW. For FRET, only the 488 nm laser line was used and set at 10mW. Internalization and co-localization scores were calculated as previously described. Briefly, cells were acquired on the basis of their area. Analysis was performed with single cells after compensation (with a minimum of 5000 cells). Firstly, a mask was designed based on the surface of BECs in the brightfield image. This mask was then eroded to exclude the cell membrane. Finally, the resulting mask was applied to the fluorescence channel. The internalization score was then calculated on this mask using the Internalization feature provided in the Ideas v6.0 software (Amnis-Merck Millipore). Internalization can be interpreted as a log-scaled ratio of the intensity of the intracellular space versus the intensity of the entire cell. Cells that have internalized antigen typically have positive scores, while cells that show the antigen still on the membrane have negative scores. Cells with scores around 0 have similar amounts of antigen on the membrane and in intracellular compartments (supplementary fig. 1A). Co-localization is calculated using the bright detail similarity R3 feature in the Ideas software. This feature corresponds to the logarithmic transformation of Pearson’s correlation coefficient of the localized bright spots with a radius of 3 pixels or less within the whole cell area in the two input images.
particle counts were calculated using the peak mask in combination with the spot count feature as previously described\textsuperscript{24}.

\textbf{Isolation and culture of primary murine BECs}

Primary mouse brain endothelial cells (mBECs) were isolated from brains of C57BL/6 mice. Brains were harvested and superficial blood vessels, meninges and cerebellum were removed. Brains were homogenized in isolation medium (HBSS supplemented with 10mM HEPES and 0.1% BSA) in a potter and centrifuged. Pellet was resuspended in 15% dextran (70kDa) and spun at 3000g for 25min. After centrifugation, pellet was resuspended in 0.2% collagenase/dispase with 10µg DNase in culture medium (DMEM supplemented with 20% FCS, 1% aminoacids, 2% sodium pyruvate and 50µg/ml gentamycin) and incubated for 30min in a 37°C waterbath. Cells were washed and seeded in collagen-coated dishes in culture medium. After 24h of culture, medium was supplemented with 1ng/ml FGF.

\textbf{Immunohistochemistry}

For immunohistochemical analysis, 5µm cryo sections of mice brain were fixed in acetone and incubated with rat anti-mouse-CD31 (ERM-P-12). Subsequently, sections were incubated with an anti-rat-HRP antibody for 1h at RT. Diaminobenzidine tetrachloride (DAB; DAKO, Glostrup, Denmark) was used as the chromogen. Between incubation steps, sections were thoroughly washed with phosphate-buffered saline (PBS). Sections were then stained with biotin-labelled rat anti-mouse MHC-II (BD Pharmingen, Breda, the Netherlands) overnight at 4°C. Staining was visualized with a streptavidin-AP (alkaline phosphatase) (Invitrogen, Bleiswijk, the Netherlands) 1h RT. Sections were then thoroughly washed with Tris-0.1M HCl pH7.6 after which staining was revealed with liquid permanent red (DAKO, Glostrup, Denmark) supplemented with 1mM levamisole. After a short rinse in tap water sections were incubated with haematoxylin for 1min and extensively washed with tap water. Finally, sections were dehydrated with ethanol followed by xylol and embedded in glycergel (DAKO, Glostrup, Denmark). All antibodies were diluted in PBS containing 0.1% bovine serum albumin (BSA, Boehringer-Mannheim, Germany).

\textbf{Generation of MOG-specific T cell subsets}

Single cells suspensions of spleens and lymph nodes from 2D2 Tg mice were depleted of erythrocytes using ACK lysis buffer. Subsequently, CD4\textsuperscript{+} T cells were enriched using the mouse CD4\textsuperscript{+} T cell enrichment kit (eBiosciences) according to manufacturer’s
instructions; stained with anti-CD4-PE and CD62L-APC antibodies and naïve CD4+CD62Lhigh T cells were sorted using a MoFlow (DakoCytomation, Glostrup, Denmark). Naïve T cells (5x10^4) were incubated with MOG\textsubscript{35-55}/LPS loaded BMDCs (1x10^4) to promote Th1 differentiation. Incubation of naïve CD4+ T cells with MOG-loaded BMDCs in the presence of PGN (10µg/ml) or TGFR1 (2.5ng/ml) promoted Th17 or Treg differentiation. Two days later, 10U/ml rmIL-2 (Invitrogen, Bleijswijk, The Netherlands) was added to the Th1 and Treg promoting cultures and another three days later T cells were harvested and used in functional assays.

Quantitative PCR
Messenger RNA was isolated from mBECs using the TRIzol® method (Life Technologies, Bleiswijk, the Netherlands) and cDNA was synthesized with the Reverse Transcription System kit (Promega, Leiden, the Netherlands). The following primer sequences were used: IFN-γ FWD: TACTACCTTCTTCAGCAACAGC, IFN-γ REV: AATCAGCAGCGACTCTTTTC, IL-17, IL-10-FWD: GGCGCTGATCGATTCTTC; IL-10 REV: ATGGCCTTGTAGACACCTTGG, T-bet FWD: CAGGGAACCGCTTTATATG, T-bet REV: CTGGCTCTCATCATTCA, RORγt FWD: GGAGCAGAGCTTAAACCC; RORγT REV: TCCCAGATGACTGTCCCA, Foxp3 FWD: TCCCCAGCTCGGTTACAC, Foxp3 REV: CCACTTGACAGATCATTGC, GAPDH FWD: GACAACTCATCAAGATTGTCAGCA; GAPDH REV: TTCATGAGCCCTTCCACAATG. Oligonucleotides were synthesized by Invitrogen (Bleiswijk, the Netherlands). Quantitative PCR (qPCR) reactions were performed in an ABI7900HT sequence detection system using the SYBR Green method (Applied Biosystems, New York, USA). Expression levels were normalized to GAPDH expression levels.

Transwell migration
Ex vivo isolated mBECs were seeded on collagen-coated 5µm pore size Costar transwells (Corning, Amsterdam, The Netherlands) for 5-7 days. mBECs were loaded with 72.5µg/ml myelin, 10µg/ml MOG\textsubscript{35-55} or 10µg/ml OVA in the presence of 25ng/ml TNFα for 24h. Cells were thoroughly washed and 1x10^5 Th1, Th17 or Tregs were added per transwell. Anti-mouse MHC-II blocking antibody (#16-5321-81, eBioscience) was added at 5µg/ml per transwell, 1h prior to addition of T cells. After 3h Th cells were recovered from the lower well and 20 000 beads (Beckman Coulter, USA) were added to each sample. Samples were analyzed by flow cytometry on a FACScalibur (BD, San Jose, USA) and by gating and counting 5000 beads, the number of migrated cells was determined.
Statistical analysis
Results are shown as mean values with standard error of the mean. Statistical analysis was performed using GraphPad Prism software (v5.01 GraphPad Software, La Jolla, CA, USA) using either unpaired Student t test or one-way ANOVA followed by post hoc Bonferroni correction. All statistical tests are described in the figure legends.

Results and Discussion
Activated brain endothelial cells upregulate molecules involved in antigen presentation
To determine if BECs play a role in antigen-specific migration of CD4+ T cells, we first assessed the expression of molecules necessary for antigen presentation. Resting BECs express MHC-I but MHC-II, CD40 and VCAM-1 are expressed at low levels. Upon activation, BECs express high levels of VCAM-1, and significantly increased the levels of HLA-DR with almost 50% of the cells being HLA-DR positive (p<0.01, fig. 1). HLA-ABC/MHC class I was highly expressed in resting as well as in activated BECs. These results confirm what has been previously demonstrated for this BEC cell line14. This led us to conclude that BECs have the adequate machinery for antigen presentation under inflammatory conditions. Interestingly, CD40 engagement to its ligand CD40L present on T cells has been shown to induce the expression of adhesion molecules, cytokines and chemokines by endothelial cells26-28. CD40 has also been associated with increased adhesion of T cells to the brain endothelium via CD40L29,30.

BECs take up myelin particles
A report has demonstrated the availability of extracellular myelin in the perivascular spaces in the brain of MS patients31. Since myelin-derived antigens are the major target of auto-reactive T cells in MS, we investigated if BECs can take up and process myelin. For this, we incubated BECs with fluorescently-labeled myelin for different time-points in resting and inflammatory conditions. Cells were extensively washed, detached and harvested, and the binding/uptake of fluorescent myelin was measured by flow cytometry. As depicted in fig. 2A, a time-dependent increase in the proportion of myelin+ BECs could be observed. Although not significantly, both the percentage as well as the geometric mean fluorescence of myelin+ cells was lower in BECs exposed to inflammatory conditions (fig. 2A). Since standard flow cytometry does not provide spatial resolution, we could not discern with the previous experiment whether the
myelin signal was associated with the membrane (binding) or the intracellular space of BECs (internalization). In order to investigate the localization of myelin in/on myelin-treated BECs we repeated the previous experiment and measured the cells using imaging flow cytometry. This method is based on the acquisition of digitalized images of cells under flow, which allows the combination of high-resolution quantitative morphological analysis of the localization of fluorescent markers in statistically meaningful cell populations.

Figure 1: Human brain endothelial cells upregulate surface receptors involved in antigen presentation. Confluent monolayers of brain endothelial cells (BECs) were stimulated with 5ng/ml TNFα for 24h. Expression of MHC-I (HLA-ABC), MHC-II (HLA-DR), CD40 and VCAM-1 was determined by flow cytometry in resting (grey histogram) and activated (black histograms) BECs. Graphs in the lower panel depict percentage of positive cells for the different markers. Data presented are the means of triplicate values ± SEM of at least three independent experiments. Statistical analysis was carried out using student t-test where *p<0.05, **p<0.01, ***p<0.001.

In order to measure whether the localization of the myelin signal was intracellular or membrane-bound, we designed a mask that excludes the cell membrane and calculated a ratio of the amount of fluorescence located in the mask vs the total amount of fluorescence, as previously reported. The results indicate that the majority of the signal was intracellular, demonstrating that BECs are able to efficiently internalize myelin (supplementary fig. 1A). Imaging flow cytometry also allows for the quantification of the number of myelin particles per cell, as previously reported. As shown in fig. 2B, BECs that were able to capture myelin increased the number of myelin...
particles over time to a maximum of 3 myelin particles/cell after a 24 h incubation. Also with imaging flow cytometry we observed an increased percentage of myelin+ BECs over time exposure to myelin (fig. 2C). However, the average amount of myelin particles per cell was the same in both resting and inflammatory conditions, demonstrating that more cells in the resting state take up myelin particles, but the number of particles taken up per cell does not change when cells are activated (fig. 2D).
values ± SEM of at least three independent experiments. Statistical analysis was carried out using student t-test where *p<0.05, **p<0.01, ***p<0.001.

**Internalized myelin particles by BECs are directed to the endo-lysosome compartment**

The endo-lysosomes are the typical antigen-processing compartment of APCs since its low pH enables the activation of specific proteases necessary for antigen proteolysis. This route allows optimal processing and transfer to the MHC-II compartment for loading and subsequent presentation to T cells. In order to determine the route of myelin uptake we stained myelin-treated BECs with antibodies against EEA1 (a marker
of early endosomes) and LAMP1 (a marker of late endosomes and lysosomes) and measured co-localization with the myelin signal using imaging flow cytometry. We observed that myelin co-localized with both EEA1 and LAMP1 (fig. 3A). Moreover, the co-localization was higher at 24h of exposure to myelin compared to 4h (fig. 3A). Importantly, the co-localization of myelin with LAMP1 was higher than with EEA1, suggesting that the majority of myelin was present in late endosomes/lysosomes.

Figure 3: Myelin particles are preferably routed to the endo-lysosomes. Confluent monolayers of brain endothelial cells (BECs) were activated with 5ng/ml TNFα for 24h. Fluorescently labeled myelin was added to resting or activated BECs for 4h or 24h after which cells were extensively washed and stained for the endosomal marker EEA1-FITC and the lysosomal marker LAMP1-FITC. (A) Uptake of myelin and co-localization was analyzed by imaging flow cytometry. Myelin particles co-localized with both EEA1 and LAMP1 in both resting and activated BECs. The co-localization was increased after 24h exposure to myelin. (B) To determine if myelin was present in endosomes (EEA1) or lysosomes (LAMP-1), BECs were analyzed using FRET to excite the myelin fluorochrome (642nm). In this setting, only the 488nm laser line was used, which excited either EEA1 or LAMP1 fluorochrome. Myelin particles co-localized with both cellular compartments as observed with FRET and indicated by the arrows. Data presented are the means of triplicate values ± SEM of at least three independent experiments.
Co-localization was measured using two approaches, either using the Bright Detail Similarity R3 feature incorporated in the Ideas software (see Methods for details) or as a measure of transfer of energy between closely located fluorochromes (FRET). In the latter, cells were acquired with only the 488 nm laser line switched on. With these settings, fluorescently-labeled myelin cannot be imaged (supplementary fig. 1B). However, when cells were stained with the anti-EEA-1 or anti-LAMP-1 antibodies, acquisition with only the 488 nm laser switched on resulted in a clear signal in the myelin channel, indicating that the proximity of the EEA-1/LAMP-1 and myelin fluorochromes was close enough to allow FRET (fig. 3B).

**BECs can process internalized myelin in a time and concentration-dependent manner**

We have so far demonstrated that BECs can take up myelin (Fig. 3). To show that BECs can actually process the internalized myelin, BECs were incubated with different concentrations of myelin and the generation of myelin basic protein (MBP) was analyzed. After 24h of myelin exposure, a concentration-dependent increase in MBP was observed in the cells lysates (Fig. 4A). To further study the protein degradation capacity of BECs, treatment with myelin was also performed in resting and activated BECs as well as for different time points. We could show that myelin processing is time-dependent, since more MBP was generated after incubation for 24h when compared to 4h (Fig. 4B). Furthermore, the generation of MBP was not significantly affected by inflammation-induced activation of BECs, since resting as well as activated BECs degraded similar amounts of myelin (Fig. 4B).

The fact that BECs can degrade myelin independently from their activation state is not completely unexpected. Although immature DCs have been regarded as high antigen (internalization and) processing cells, with their mature counterparts being more specialized in antigen presentation\textsuperscript{35,36}, some studies have shown that DCs can still take up and process antigens after their maturation\textsuperscript{37,38}. This could also be the case for BECs, although other regulatory mechanisms may be present in these two different cell types.
Figure 4: Processing of myelin particles by BECs is time- and concentration-dependent. (A) Human myelin was added to confluent monolayers of BECs at different concentrations (10µg/ml, 25µg/ml and 50µg/ml) for 24h. Cells were extensively washed and expression of myelin basic protein (MBP) was detected in cell lysates by western blot. MBP expression increased when BECs were subjected to higher concentrations of myelin. (B) Resting and activated BECs were treated with 20µg/ml of myelin for both 4h and 24h. MBP expression was again assessed by western blot. Myelin degradation increased over time and no significant changes were detected between resting and activated BECs. Data presented are the means of triplicate values ± SEM of at least three independent experiments. Statistical analysis was carried out using ANOVA with Bonferroni correction where *p<0.05, **p<0.01, ***p<0.001.

Myelin-derived peptides presented by mBECs facilitate migration of MOG-specific T cells

Previous studies regarding BECs as non-professional APCs used an allogeneic setting to demonstrate an event that is highly dependent on a matching HLA haplotype\textsuperscript{14}.

Taken into account the HLA-matching issues and due to the lack of human myelin-specific T cell clones, we decided to use murine BECs (mBECs) as a model system and the MOG\textsubscript{35-55}-specific CD4\textsuperscript{+} T cells from T cell receptor transgenic (2D2) mice\textsuperscript{39}. Importantly, we observed an increased expression of MHC-II in cerebral blood vessels of mice in the active phase of the animal model of MS – experimental autoimmune encephalomyelitis (EAE) – when compared to control mice (CFA) (fig. 5). This result demonstrates that mBECs, similar to human counterparts, are properly equipped to present antigens to CD4\textsuperscript{+} T cells.
Importantly, Th1, Th17 and Treg cells have been described in the brain of MS patients as well as in EAE. However, if these particular T cell subsets present different requirements for T cell migration has not been studied. To investigate the specific contribution of antigen presentation by mBECs to the migration capacity of Th1, Th17 and Treg subsets, we isolated naive MOG-specific CD4\(^+\) T cells from 2D2 mice and generated the different subsets in vitro (fig. 6). To test whether antigen presentation by mBECs facilitates T cell migration, mBECs were cultured on transwells and loaded with myelin, MOG or OVA in the presence of TNF\(\alpha\). To allow antigen processing by mBECs, the different T cell subsets were added 24h later and their migration was quantified after 3h. As seen in fig. 7A-C, loading of mBECs with OVA does not induce a significant increase in the migration of MOG-specific T cells. However, when mBECs were loaded with MOG or myelin, a significant increase in migrated Th1, Th17 and Treg cells was observed, demonstrating that presentation of myelin-derived peptides by mBECs leads to an antigen-specific T cell migration. Although it has been suggested that Th1 cells need an antigen-specific signal to infiltrate the brain, it has not been described if Treg cell migration is also antigen-specific. We here show that transmigration of Treg cells across the BBB is antigen-specific/MHC-II-dependent.
Figure 6: Generation of myelin-specific CD4+ Th1, Th17 and Treg subsets from TCR transgenic mice. Naïve CD4+CD62L^high T cells were sorted from 2D2 Tg mice and different T helper (Th) subsets were generated in vitro by co-culture with bone marrow-derived dendritic cells. To assess differentiation, T cells were collected and markers for specific cell subsets were analyzed by qPCR. High expression of T-bet and IFN-γ was observed in Th1-differentiated CD4+ T cells, IL-17 and RORγt in Th17 cells and IL-10 and Foxp3 in Treg cells. Data presented are the means of triplicate values ± SEM of at least three independent experiments.

Migration of antigen-specific T cells is dependent on MHC-II-antigen presentation by mBECs

To demonstrate that presentation of myelin-derived antigens in MHC-II by mBECs is what drives the T cell migration, we added a MHC-II-blocking antibody during the migration period. Blocking MHC-II could reduce migration of all three T cell subsets (fig. 7D-F), providing evidence that presentation of myelin-derived antigens in MHC-II by mBECs facilitates T cell migration. It has been shown that animals deficient in MHC-II expression do not develop the disease and no immune cell infiltration into the brain was observed^{16}. Another study has shown that mice lacking functional class-II restricted antigen processing machinery are resistant to both active and adoptive transfer EAE, suggesting that a proper processing of antigens is essential for disease initiation^{46}. 
Figure 7: Presentation of myelin-derived antigens in MHC-II by mBECs enhances antigen-specific T cell transmigration. Brain endothelial cells were isolated from C57Bl6 mice (mBECs) and seeded onto transwells. After reaching confluence, mBECs were activated with TNFα and loaded with MOG\textsubscript{35-55}, myelin or OVA for 24h. Cells were washed and the different Th subsets were added to the upper compartment at a density of 1x10\textsuperscript{5} Th cells/well for 3h. T cell migration was quantified by flow cytometry using fluorescently labeled beads as reference. (A-C) Antigen-specific Th1, Th17 and Treg cells migration is enhanced when mBECs are loaded with either myelin or MOG. (D-F) MHC-II blocking antibody was added to mBECs one hour prior to the addition of the different Th subsets. Antigen-specific migration was decreased by MHC-II blockade. Statistical analysis was carried out using ANOVA with Bonferroni correction where *p<0.05, **p<0.01, ***p<0.001.
Although these results could be due to the lack of activation of auto-reactive T cells by APCs in the periphery, the lack of a functional antigen processing machinery, including in BECs, could explain the resistance in disease induction by adoptive transfer, since no antigens could be presented by the endothelium and therefore impaired T cell trafficking into the brain.

Taken together, and considering our results, it seems clear that antigen-presentation by the brain endothelium facilitates the entry of CD4+ T cells into the brain. This phenomenon has also been described in other diseases as diabetes\textsuperscript{47,48}. Whether antigen-specific T cell entry is favored over entry of T cells with other specificities is still a matter of debate. However, it has been shown that the entry of encephalitogenic T cells into the brain pave the way to the entry of other non-CNS-specific T cells\textsuperscript{49,50} demonstrating the importance of antigen-specific T cell migration in the pathogenesis of EAE and possibly in MS. Interestingly, presentation of antigens via MHC-I by BECs has been shown important for the migration of antigen-specific CD8+ T cells, reinforcing the role of the brain endothelium in regulating immune cell trafficking into the CNS\textsuperscript{17}. Overall, our results demonstrate that BECs can take up and process myelin particles in a time- and concentration-dependent manner. Furthermore, the upregulation of HLA-DR/MHC-II expression under inflammatory conditions reinforces the idea of a non-professional antigen presenting cell role. Importantly, myelin-derived antigens can be presented in MHC-II to antigen-specific T cell subsets, aiding in the diapedesis of these cells. These findings have major implications in neuroinflammatory disorders such as MS, since increase immune cell trafficking has a detrimental effect in disease progression. Furthermore, these results demonstrate that the brain endothelium is an active contributor to disease pathogenesis.
Supplementary figure 1: Imaging flow cytometry. (A) To quantify internalization scores, a mask was designed based on the surface of BECs in the brightfield image. This mask was then eroded to exclude the cell membrane. The resulting mask was applied to the fluorescence channel. The internalization score, interpreted as a ratio of the intensity of the intracellular space versus the intensity of the whole cell, was calculated on this mask using the internalization feature in the Ideas v6.0 software (AMNIS-Merk Millipore). Cells that have internalized antigen have positive scores, as depicted here for BECs. (B) For FRET, only the 488nm laser line was used. To determine if myelin was present in the same compartment as EEA1 and LAMP1, BECs were analyzed using FRET to excite the myelin fluorochrome (642nm). Single stainings and excitation of the fluorochromes with either 488nm or 488nm together with 642nm settings are shown.
References
