Astrocyte-derived retinoic acid: a novel regulator of blood-brain barrier function during neuroinflammation in multiple sclerosis

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Summary
Multiple sclerosis (MS) lesions are characterized by the presence of activated astrocytes, which are thought to actively take part in the disease process. Reactive astrocytes secrete pro-inflammatory chemokines and cytokines, thereby aggravating disease. Conversely, reactive astrocytes may exert disease dampening effects by the secretion of trophic factors and anti-inflammatory mediators. To date, the protective mediators secreted by reactive astrocytes and the downstream effects on inflammation and MS lesion progression remain largely unknown.

The involvement of blood-brain barrier (BBB) disruption in MS lesion formation is a well-established fact and represents an early event in lesion development. We recently showed that retinoic acid (RA) signalling plays an important role in the development of the BBB. To unravel the potential protective effect of reactive astrocytes on BBB damage we investigated the involvement of RA signalling in MS pathology and in vitro models.

Immunohistochemical analysis of MS lesions revealed that the key enzyme for RA synthesis, retinaldehyde dehydrogenase 2 (RALDH2), was highly expressed by reactive astrocytes in active and inactive white matter lesions, whereas only low expression was observed in control or normal appearing white matter. By mimicking reactive astrocytes in vitro we show that RALDH2 gene expression is increased by pro-inflammatory stimuli, leading to the synthesis and secretion of RA.

RA prevented inflammation-induced loss of different BBB-aspects such as barrier function in brain endothelial cells and induced immune quiescence in vitro. Deep sequencing analysis furthermore demonstrated the increase of an immune-quiescent repertoire of genes in RA-treated brain endothelial cells. As a potential mechanism, we provide evidence that RA decreases the production of reactive oxygen species by inflamed brain endothelial cells, through the activation of anti-oxidant transcription factor nuclear factor (erythroid derived 2)-like 2 (Nrf2). All data combined, we provide evidence that retinoic acid synthesis by reactive astrocytes represents an endogenous protective response to pro-inflammatory stimuli and that astrocyte-derived retinoic acid might protect the BBB against inflammatory insult. A further understanding of the key mediators of RA signalling may lead to the development of novel targets to halt disease progression in MS.
Introduction
Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS), which is pathologically characterized by the presence of multiple focal demyelinated lesions throughout the brain and spinal cord[1,2]. Active demyelinating lesions are marked by the presence of immune cell infiltrates, consisting of T cells and monocyte-derived macrophages[3]. This initial phase of lesion formation is thought to result in severe damage to axonal myelin, and subsequently axons, eventually resulting in neuronal dysfunction. During and after active lesion formation, astrocytes become reactive and are abundantly present inside the lesion and to a lesser extent in the surrounding normal appearing white matter (NAWM). Reactive astrocytes are able to produce and secrete a vast array of trophic factors and cytokines, suggestive of a protective as well as a detrimental role for these cells during, and after neuroinflammation[4,5]. One of the early hallmarks of MS lesion development is the disruption and subsequent functional loss of blood-brain barrier (BBB) integrity. The BBB is comprised of highly specialized endothelial cells supported by astrocytes, which protects the CNS against the entry of immune cells and harmful components from the systemic circulation. Identification of factors that repair or protect the BBB can be of use in halting MS lesion progression during an inflammatory insult. Main cellular candidates for the production of these protective and reparative factors are the abundantly present reactive astrocytes. Identification of astrocyte-derived factors that limit damage caused to or by a defective BBB might lead to new insights in halting lesion progression.

Recently, we showed that the astrocyte-derived vitamin A (retinol) metabolite retinoic acid (RA) plays a key role in BBB formation during brain development[6]. Retinol is oxidized to retinaldehyde and further processed by retinaldehyde dehydrogenase (RALDH) enzymes, which are expressed by radial glial cells, leading to freely diffusible RA. Target cells that express the nuclear RA receptors (RAR) and the scaffolding Retinoïd X receptors (RXR) react to RA by the heterodimerization of RAR and RXR and subsequent transcription of genes with upstream RARE-sequences (reviewed in[7]). Interestingly, recent evidence indicates that developmental programs involved in the formation of the BBB are reinstated upon injury[8], ensuring the correct patterning and differentiation cues necessary for regeneration or repair. We therefore investigated whether the developmental RA-signaling pathway is involved MS pathology. Immunohistochemical analysis revealed that RALDH2, one of the key enzymes involved in RA production, is highly increased in reactive astrocytes in both active and chronic MS lesions. Moreover, we provide evidence for the production and release of RA by reactive astrocytes in vitro and we demonstrate that RA is able to restore BBB integrity and function under inflammatory conditions. Finally, our findings point to a novel mechanism in which RA exerts its anti-inflammatory effects through activation of the anti-oxidant transcription factor Nuclear factor erythroid 2-related factor (Nrf2) 2. Together, the present study describes a new protective and anti-inflammatory function of reactive astrocytes, which is mediated by RA and prevents BBB dysfunction in inflammatory MS lesions.

Materials and methods
Brain tissue
Brain tissue from 5 patients with clinically diagnosed and neuropathologically confirmed MS was obtained at rapid autopsy and immediately frozen in liquid nitrogen (in collaboration
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with The Netherlands Brain Bank, coordinator Dr. Huitinga). The Netherlands Brain Bank received permission to perform autopsies, for the use of tissue and for access to medical records for research purposes from the Ethical Committee of the VU University medical center (VUMC), Amsterdam, The Netherlands. Tissue samples from 3 control cases without neurological disease were taken from the subcortical white matter and corpus callosum. White matter MS tissue samples were selected on the basis of post-mortem MRI. All patients and controls, or their next of kin, had given informed consent for autopsy and use of their brain tissue for research purposes.

Immunohistochemistry

For single labelling of RALDH1/2, PLP, and MHCII in human tissue sections, sections were deparaffinised and treated with 0.3% H₂O₂ in methanol for 20' to reduce endogenous peroxidase activity. Antigen retrieval was achieved by incubating the sections at 100°C in Tris-EDTA buffer (10mM-0.5mM, pH 9.0) for 10'. After washing with phosphate-buffered saline (PBS), sections were treated with 0.1% saponin in PBS, washed, and subsequently incubated with primary antibody, rabbit αRALDH (Abcam, Cambridge, UK), mouse αProteolipid protein (Serotec Ltd, Oxford, UK), or mouse αMHC II (DAKO, Glostrup, Denmark) in PBS overnight at 4°C. Slides were then washed and incubated with Envision+ Dual link reagent (DAKO, Glostrup, Denmark) for 30' followed by visualization with the peroxidase substrate diaminobenzidine (DAB) (DAKO, Glostrup, Denmark). After a short rinse in tap water, sections were incubated with hematoxylin for 1 minute and extensively washed with tap water for 10 minutes. Finally, sections were dehydrated with ethanol followed by xylene and mounted with Entellan (Merck, Darmstadt, Germany). Fluorescent immunohistochemistry for RALDH was performed using polyclonal rabbit αRALDH (Abcam, Cambridge, UK) and monoclonal mouse αGFAP-Cy3 (Sigma, St. Louis, MO, USA). Tissue sections were stained as described above for single labelling; only peroxidase activity quenching was omitted and all incubation steps were performed in the presence of 1% normal serum of the secondary antibody host (donkey). Secondary antibody used was donkey aradiaG-alexa488 (Invitrogen Carlsbad, CA, USA). Slides were washed and embedded in vinal mounting medium supplemented with 0.4% 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, Leiden, The Netherlands) to stain nuclei. Fluorescent images were recorded with a Leica DM6000 microscope (Leica Microsystems, Heidelberg, Germany).

Cell cultures

Primary astrocytes from post-mortem human brain tissue were isolated and cultured as described previously³. To harvest reactive astrocyte conditioned media, astrocytes were stimulated with TNFα and IFNy for 24 hours, washed thoroughly and conditioned media were collected after 24 hours. The human brain endothelial cell line hCMEC/D3 was cultured as described previously⁴.

Retinoic acid response element and antioxidant response element promoter-reporter constructs

The lentiviral vector pRRL-cPPT-CMV-X2-PRE-SIN (kindly provided by Dr. J. Seppen, Department of Experimental Hepatology, Academic Medical Center, Amsterdam, The
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Netherlands) was modified for use as a carrier of promoter-reporter cassettes as described previously. The reporter plasmid pGL-RARE-luciferase containing trimered Retinoic Acid Receptor Response Elements in front of the firefly luciferase gene was previously described. The pARE reporter plasmid hQR28 was also previously described and used in a similar fashion. The resulting lentiviral RARE and ARE promoter-firefly luciferase reporter vectors were then packaged, in the presence of a small amount (5% wt/wt) of the herpes simplex virus thymidine kinase Renilla luciferase lentiviral vector, using HEK-293 T cells. Virus-containing supernatants were used for transductions, and luciferase activities were measured after at least 2 days, following ACM incubations or RA/vehicle pre-treated TNFα/INFγ stimulations, using the dual luciferase system (Promega, Madison, WI, USA). Renilla luciferase levels were used to normalize firefly luciferase activity.

Flow cytometric analysis
Flow cytometric analysis of VCAM-1 expression was performed on brain endothelial cells incubated with or without TNFα and IFNγ for 24 hours, following 24 hours of pre-treatment with 5μM RA or vehicle. Cells were detached from 24-well culture plates by collagenase type I treatment (1 mg/ml, Sigma). Washed cells were incubated with monoclonal mouse anti-rat antibodies directed against VCAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at 4 °C. Binding was detected using FITC-conjugated goat anti-mouse antibodies (1 μg/ml; Invitrogen, Carlsbad, CA, USA). Omission of primary antibodies served as negative control. Fluorescence intensity was measured using a FACS-Calibur flow cytometer (Becton & Dickinson, San Jose, CA). The mean fluorescence intensity of 10,000 viable cells, selected by 7-amino actinomycin D exclusion (7AAD, Molecular Probes), was used as a measure for the expression of VCAM-1.
ROS production was assessed using CM-H₂DCFDA (Invitrogen, Carlsbad, CA, USA) in a similar fashion, following the manufacturer’s guidelines.

Electric cell-substrate impedance sensing (ECIS) assay
ECISSTM Model 1600R (Applied Biophysics, Troy, NY, USA) was used to measure the transendothelial electric resistance of human hCMEC/D3 cell monolayers. A measure previously described to have a close inverse correlation with small molecule-permeability. 1x10⁴ cells were seeded onto each well of an 8W10+ ECIS array (Ibidi GmbH, Munchen, Germany) coated with collagen and impedance was measured at multiple frequencies in real time. Cells were treated with 5μM RA (Sigma, St Louis, MO, USA) or vehicle (100% DMSO) directly after seeding. Upon reaching maximal barrier resistance, TNFα and IFNγ were added to the culture medium. All ECIS measurements were furthermore subjected to a mathematical model to calculate the component of resistance attributed to cell-cell interactions, called barrier resistance (Rb) as described previously.

Permeability assay
Paracellular permeability of human brain endothelial cell monolayers was analyzed as described previously. hCMEC/D3 were seeded at confluency onto collagen-coated Costar Transwell filter (pore-size 0.4 μm; Corning Incorporated) in growth medium containing 2.5% FCS and grown for 4 days. Paracellular permeability to FITC-dextran (150 kDa, 500 μg/ml in culture medium; Sigma-Aldrich) in the apical to basolateral direction was assayed...
in the presence or absence of TNFα and IFNγ. After 24 hours of pre-treatment with 5μM RA or vehicle. Samples were collected from the acceptor chambers for measurement of fluorescence intensity using a FLUOstar Galaxy microplate reader (BMG Labtechnologies), excitation 485 nm and emission 520 nm.

**Western blotting**

Protein levels of Nrf2 in nuclear fractions were investigated in confluent monolayers of hCMEC/D3 cells grown in 6 well microplates (Corning, Lowell, MA, USA), treated with indicated concentrations of RA or vehicle for 24 hours. Cells were washed with ice-cold PBS and lysed and fractioned using the NE-PER extraction kit following the manufacturer’s guidelines (Thermo Fischer scientific, Rockford, IL, USA). Nuclear fractions were then taken up in sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris–HCl pH 6.8), 4% SDS, 20% glycerol and 5%β-mercaptoethanol) and heated to 95°C for 5 min. Lysates were then resolved on SDS–polyacrylamide gel electrophoresis in triplicate, blotted and incubated over night with the primary antibodies rabbit-αNrf2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat-αLamin B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in Odyssey blocking buffer (LI-COR, Lincoln, AK, USA) diluted 1:1 in PBS, after initial blocking with blocking buffer for 1 hour at RT. Primary antibodies were detected and quantified by incubation with appropriate IRDye secondary antibodies (RT 1hour in blocking buffer) and the Odyssey infrared imaging system (LI-COR, Lincoln, AK, USA). Lamin B quantification was used to correct for total protein loading.

**Real-time quantitative PCR**

Gene expression analysis was performed on confluent monolayers of hCMEC/D3 cells in 24 well microplates (Corning, Lowell, MA, USA). Cells were incubated with or without TNFα and IFNγ for 24 hours, following 24 hours of pre-treatment with 5μM RA or vehicle. Messenger RNA was isolated using Trizol (Invitrogen Carlsbad, CA, USA). cDNA was synthesized with the Reverse Transcription System kit (Promega, Madison, WI, USA) following manufacturer’s guidelines and RT-PCR was performed as described previously23. Primer sequences used are described in table 1. Primers for CCL-2 and IL-6 were kindly provided by J.J. Garcia-Vallejo, VU University medical center, Amsterdam, The Netherlands.

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Statistical analysis
Data analysis was performed using Graphpad Prism software (v5.01 Graphpad Software, La Jolla, CA, USA). Results are shown as mean with standard error of the mean, and statistical analysis was performed with either unpaired, two-tailed Student t-test or with one-way ANOVA and subsequent posttest correction. The applied test for each calculated value described in the legends.

Results
Reactive astrocytes in active and chronic active MS lesions express the RA-producing enzyme RALDH2.
We first assessed the cellular localization of RALDH1 and -2 in well-characterized MS lesions and non-neurological controls (Figure 1). Chronic active MS lesions are characterized by loss of myelin immunoreactivity (PLP, Fig 1A) and a rim of MHCII-positive cells near the edge of the lesion (Fig 1B). A concentrated line of intensely stained RALDH1/2-positive cells was detected in the vicinity of the active edge of the lesion (Fig 1C). Furthermore, in active lesions, characterized by a loss of myelin and MHCII-positive cells throughout the lesion (Fig 1 D-E), RALDH1/2-immunoreactive cells decorated the entire lesion area. Higher magnification micrographs of NAWM, the active rim of a chronic active lesion (CAL), and the inactive center of a CAL (Fig 1G-I) indicated that the RALDH1/2-positive cells are present in both the active rim and the inactive center of MS lesions and resemble reactive astrocytes. These cells were not found in NAWM although some RALDH1/2-positive structures are present. Fluorescent immunohistochemistry revealed co-localization of the astrocytic marker glial fibrillary acidic protein (GFAP) with RALDH1/2-positive cells in the active rim of a CAL, confirming the astrocytic expression of RALDH1/2. Together, the immunohistochemical analysis demonstrated a marked increase of astrocytic RALDH1/2 expression during the active phase of MS lesion formation, which is maintained in the inactive phase of the lesion. As the antibody we used recognizes both RALDH-1 and -2 we next assessed which RALDH family member was specifically upregulated in MS lesions. Here, we analyzed the mRNA expression levels of the genes responsible for RALDH protein expression, aldehyde dehydrogenase 1 family, members (ALDH1A) 1-3, in chronic MS lesion tissue compared to NAWM from the same donors. ALDH1A2 mRNA levels are significantly increased in lesion tissue compared to NAWM (736.3% ± 211.8 of control; Fig2A), whereas both ALDH1A1 and ALDH1A3 mRNA levels are decreased in lesion vs. NAWM tissue (resp. 49.3% ± 9.5 of control and 58.4% ± 5.3 of control; Fig 2A).

Reactive astrocytes in vitro express the RA-producing enzyme RALDH2 and produce and secrete RA.
To mimic MS lesion astrocytes in vitro, primary human astrocytes from non-neurological control donors were exposed to TNF-α and IFN-γ, two pro-inflammatory cytokines described to be abundantly present during active demyelination in MS lesions. Our analysis showed that cultured reactive astrocytes closely resembles those found in MS lesions as evidenced by increased gene expression levels of pro-inflammatory mediators interleukin (IL) 6 (2667% ±165 of control) and chemokine C-C motif ligand (CCL) 2 (370.4% ±87.2 of control). Importantly, exposure to pro-inflammatory mediators also leads to significantly increased gene expression levels of ALDH1A2 (1605% ±439.5 of control) (Fig 2B) as was
Figure 1. Reactive astrocytes in MS lesions express RALDH.

A-C: Immunohistochemistry for myelin (PLP) and HLA-DR (MHCII) shows a chronic active lesion with increased immunoreactivity for RALDH along the active rim of the lesion, which appears to be mainly astrocytic (H), as well as in the active center (I), compared to NAWM (G). D-F: Immunohistochemistry for PLP and MHCII shows an active demyelinating lesion with increased RALDH immunoreactivity throughout the lesion area. J-L: Immunohistochemical analysis of RALDH (green) and GFAP (red) in the active rim of a chronic active lesion (CAL) shows GFAP-positive astrocytes with high immunoreactivity for RALDH.

shown in inflammatory MS lesions. ALDH1A1 and ALDH1A3 gene expression levels remained unchanged upon pro-inflammatory stimulation. Finally, to investigate the functional
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Figure 2. Reactive astrocytes in MS lesions express RALDH2 and can be functionally mimicked in vitro.

A. Expression levels of ALDH1A2, but not ALDH1A1 and 3, are increased in MS lesion tissue compared to NAWM (student T-test, n=3). B. Primary human astrocytes show increased ALDH1A2, IL-6, and CCL2 gene expression levels after 24 hours of TNFα and IFNγ stimulation (student T-test, n=4). C. Human brain endothelial cells stably transfected with a RARE-luciferase reporter increase luciferase expression in response to reactive astrocyte conditioned medium (T/I ACM), but not after incubation with astrocyte conditioned medium (ACM) (* compared to medium, # compared to ACM; one-way ANOVA, n=4). */#p-value < 0.05 **p-value < 0.01 ***p-value < 0.001.

The extent of increased ALDH1A2 gene expression by reactive astrocytes in vitro, we collected supernatants of TNFα/IFNγ-treated astrocytes to assess the secretion of RA into the culture medium. Using human cerebral microvascular endothelial cells (hCMEC/D3) harbouring RA response elements (RARE) coupled to the luciferase gene, we observed a modest increase of endothelial luciferase activity induced by normal astrocyte conditioned medium (ACM) compared to unconditioned medium (119.3% ± 2.7 of control, Fig 2C). Treatment with ACM harvested from TNFα/IFNγ-treated astrocytes (T/I ACM) significantly enhanced RA-mediated reporter gene expression in brain endothelial cells (176.3% ± 27 of control, Fig 2C). Taken together, these data strongly suggest that increased ALDH1A2 gene expression in reactive astrocytes underlies the enhanced production and secretion of RA. Of note, once released into the extracellular space, RA is able to activate an RA-response in brain endothelial cells.

Retinoic acid prevents inflammation-induced barrier loss and immune activation of brain endothelial cells.

One of the hallmarks of an active demyelinating lesion is disruption of BBB integrity. In order to assess the effects of RA secretion by reactive astrocytes on BBB function during neuroinflammation, we investigated the effect of RA on different aspects of inflammation-induced BBB damage. Using electrical cell impedance sensing (ECIS) we were able to determine paracellular resistance (Rb) over time under the influence of pro-inflammatory mediators. TNFα/IFNγ-treatment significantly decreases barrier resistance (-30.64% ± 3.3 at t=72, Fig 3A). Pre-treatment of brain endothelial cells with 5µM RA abolished the loss of Rb (+12.20% ± 2.3 at t=72, Fig 3A). As a correlate of electrical resistance, permeability of brain endothelial cells to FITC-labeled 70 kDa Dextran is increased after pro-inflammatory stimulation (131% ± 3.5 of control at t=24, Fig 3B). Pre-treatment of brain endothelial cells with RA prevented the inflammation-induced permeability to FITC-Dextran (118.3% ± 5 of control at t=24, Fig 3B).

Immune activation of brain endothelial cells is another important aspect of BBB disruption in MS. We therefore investigated the expression levels of pro-inflammatory genes IL-6 and...
CCL2, as well as the key immune cell adhesion molecule vascular cell adhesion molecule (VCAM)-1 in immune activated brain endothelial cells. Gene expression levels of CCL2, IL-6, and VCAM-1 are highly increased upon pro-inflammatory treatment with TNFα/IFNγ (resp. % of control: 193.1% ± 19.5; 418.8 ± 28.2, 1898 ± 64.6, Fig 3C). Pre-treatment with RA prevented the inflammation-induced expression of these immune-related genes (resp. % of control: 21 ± 4.9, 165.1 ± 24.4, 949.0 ± 46.1, Fig 3C). The prevention of inflammation-induced VCAM-1 protein expression was further validated by FACS-analysis (-297% ± 2.2 of increase from control, Fig 3D). A broader perspective of the immune quiescence induced by RA in brain endothelial cells was acquired by genome-wide profiling of gene expression. RA treatment of naïve brain endothelial cells resulted in down regulation of a large number of genes related to endothelial inflammation, listed in supplemental table 2. Products of genes that are downregulated include those involved in pro-inflammatory cytokine and chemokine signaling, immune cell adhesion, and permeability-promoting factors.
Together, these results suggest that RA induces a general immune quiescent state in the CNS endothelium.

Retinoic acid induces protective anti-oxidant pathways via Nrf2 translocation.

Reactive oxygen species (ROS), produced during neuroinflammation can damage the BBB and promote leukocyte adhesion and infiltration. In order to protect endothelial cells from ROS-induced damage, anti-oxidant pathways lead to the expression of protective enzymes that can neutralize ROS. To investigate the role of RA on anti-oxidant pathways in brain endothelial cells, we analyzed endothelial ROS production after pro-inflammatory stimulation. TNFα/IFNγ-treatment leads to a significant increase in endothelial ROS-production (155.4% ± 3.4 of control, Fig 4A), which was diminished by RA pre-treatment (-26.3% ± 6.3, Fig 4A). RA stimulation does not alter endothelial ROS-production in the absence of TNFα/IFNγ. These findings show that RA enhances the endogenous anti-oxidant potential of BBB endothelium to efficiently neutralize ROS in response to TNFα/IFNγ.

To investigate the activation of anti-oxidant pathways by RA we made use of brain endothelial cells harbouring a luciferase expression construct driven by an antioxidant response element (ARE) promoter. Whereas TNFα/IFNγ-stimulation did not affect ARE-mediated luciferase expression, RA stimulation highly enhanced ARE-driven luciferase activity (388% of control).
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± 100.8, Fig 4B), which reflects an increased activation of the anti-oxidant pathway by anti-oxidant transcription factors. A well known anti-oxidant transcription factor is nuclear factor (erythroid-derived 2)-like (Nrf) 2. Translocation of Nrf2 from the cytoplasm to the nucleus allows Nrf2 to bind to ARE leading to subsequent transcription of anti-oxidant enzymes. To establish whether RA can mediate nuclear accumulation of Nrf2, nuclear fractions of brain endothelial cells treated with RA were analyzed by western blot. 24 hours of RA-treatment resulted in increased nuclear Nrf2 levels (160.1% ± 11.9 of control, Fig 4C). Although Nrf2 is the predominant ARE-binding transcription factor, multiple transcription factors can bind ARE and mediate anti-oxidant activity. We therefore specifically silenced mRNA expression of NFE2L2, the gene responsible for Nrf2 protein expression, in brain endothelial cells and analyzed RA-induced ARE luciferase expression. In brain endothelial cells transfected with a control siRNA construct (siCtrl), RA-treatment induced normal ARE activation (Fig 4D). However, in the NFE2L2-silenced brain endothelial cells, ARE-luciferase expression is no longer enhanced by RA (siNFE2L2). Taken together, these data show that RA enhances the anti-oxidant capacity of brain endothelial cells through the translocation and activation of anti-oxidant transcription factor Nrf2.

Discussion
In this study we describe a novel anti-inflammatory mechanism, utilized by reactive astrocytes during the course of MS-lesion formation, which prevents neuroinflammatory damage to the BBB. First, we show that in active and chronic active MS lesions, reactive astrocytes highly express the enzyme RALDH2, a bottleneck enzyme in the synthesis pathway of RA from retinol. Reactive astrocytes were mimicked in vitro and actively produced and secreted bioactive RA into the extracellular space. Astrocyte-derived RA induced RARE-specific gene transcription in human brain endothelial cells. Moreover, the deleterious effects of neuroinflammation on brain endothelial cells, reflected by the loss of barrier function, increased inflammation-related gene expression, and ROS production, are all prevented by the presence of RA. Finally, our findings point to a key role of the anti-oxidant transcription factor Nrf2 in RA-mediated protection against oxidative stress.

Under control conditions, the functions of astrocytes in the CNS are numerous and include support of neuronal transmission, trophic support of neurons, nutrient storage, and buffering of the extracellular space. To date, astrocytes are considered the predominant cell type to maintain barrier properties within the brain endothelium in adulthood. There is strong evidence (for review see 21) that astrocytes induce key BBB features, leading to complex tight junctions, expression and localization of specific transporters and specialized metabolic systems. Soluble factors secreted by astrocytes are reported to induce barrier properties. Recent evidence indicated that astrocytes-derived sonic hedgehog (Shh)22 and RA10 induce features of a BBB phenotype in endothelial cells during development and promote immune quiescence.

MS lesion formation is characterized by astrogliosis occurs, a process where astrocytes become reactive and have been described to enhance the inflammatory process seen in MS (for an extensive overview see24). In response to stimuli from infiltrating leukocytes and activated microglia, a vast array of cytokines, chemokines, and are produced by reactive astrocytes that drive inflammation, demyelination and neurodegeneration as observed in MS lesions. Pro-inflammatory mediators secreted by reactive astrocytes are able to directly
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... affect the barrier function of endothelial cells, contributing to the neuro-inflammatory process. Subsequent promotion of the immune quiescence of reactive astrocytes by therapeutic agents like Fingolimod resulted in the dampening of the neuroinflammatory response and improvement of BBB function.

We here show that reactive astrocytes in MS lesions highly express RALDH2, and that this response of reactive astrocytes can be mimicked in vitro by pro-inflammatory stimulation. We propose that part of the inflammatory response of astrocytes encompasses the increased expression of RALDH2, subsequently leading to the secretion of anti-inflammatory RA into the extracellular space. Our findings are in line with in vitro studies that indicate that reactive astrocytes maintain the capacity to secrete T-cell suppressive factors, anti-inflammatory cytokines and neurotrophic factors, and most recently, Shh. These studies support the notion that reactive astrocytes, depending on their micro-environment, might switch from an initial pro-inflammatory to a later anti-inflammatory or repair phenotype, although in vivo or post-mortem confirmation for this switch is lacking.

The current study is the first to show that the RA pathway is involved in MS pathology. RALDH1/2 immunoreactivity in NAWM is marginal compared to the intense staining in inflammatory and chronic lesions. RA synthesis by radial glial cells and astrocytes has thus far mainly been described in view of the developing CNS, and in situ analysis, showing that RALDH expression by astrocytes in the human and rodent healthy CNS is almost undetectable, indicative of a RALDH-inhibitory environment in the CNS under normal conditions. Altogether, our current and previous findings show that the RA-synthesis pathway is mainly utilized during CNS development, plays a minor role during adult CNS homeostasis, and is reactivated upon CNS damage or inflammation. Interestingly, enhanced RALDH expression and RA signaling may provide a general protective mechanism in CNS pathology. A recent study described similar findings in an animal model for demyelination. Following cuprizone-induced demyelination, RALDH expression in the corpus callosum increased, as well as nuclear RA receptor expression by astrocytes. Since we also showed that reactive astrocytes in the inactive center of chronic lesions exhibit high levels or RALDH expression, it seems that this response is maintained in the absence of neuroinflammation.

We provide evidence that RA counteracts the deleterious effects of pro-inflammatory cytokines on barrier resistance and permeability. Loss of BBB integrity and the leakage of serum components into the CNS often precede lesion formation in MS patients, and correlates highly with disease severity in the animal model for MS, experimental autoimmune encephalomyelitis. The BBB integrity-restoring function of RA might therefore be an important mechanism to restore BBB integrity in MS lesions. The endothelial expression of extracellular matrix-degrading enzymes like matrix metalloproteinases (MMPs) has been associated with the loss of BBB integrity in vivo, including MMP2. Interestingly, we found that MMP2 gene expression is reduced by RA-treatment, although the involvement of endothelial expression of MMPs and MMP inhibitors during neuroinflammation warrants further investigation. The BBB further maintains immune quiescence of the CNS through the minimal expression of adhesion molecules and chemokines. Under inflammatory conditions however, the expression of these molecules is strongly upregulated which in turn mediates the migration of immune cells into the brain. RA was able to prevent the immune activation status of brain endothelial cells in both inflammatory and normal conditions. Although many current therapies for relapsing-remitting MS rely on anti-inflammatory actions that also
affect the BBB\textsuperscript{16, 37}, little is known about the endogenous anti-inflammatory signals received by brain endothelial cells. It is of interest that a number of the downstream inflammatory mediators influenced by RA described in this study have been shown to underlie MS or EAE symptoms in vivo. For instance, targeting CCL2 expression by endothelial cells suppresses EAE symptoms\textsuperscript{38, 39}, IL-6 deficient mice fail to develop EAE due to loss of IL-6 induced VCAM-1 expression on BBB endothelial cells\textsuperscript{40}, and inhibition of leukocyte binding to VCAM-1 by integrin-blockade is currently used in the clinic to treat MS\textsuperscript{41}.

Finally, we provide evidence that RA can reduce ROS production by brain endothelial cells. We have previously reported that inflammation-driven ROS production by brain endothelial cells is responsible for BBB disruption\textsuperscript{42}, which could be prevented by enhancing anti-oxidant systems like peroxiredoxin-1 expression\textsuperscript{43}. Our current findings show that RA induces translocation of the anti-oxidant transcription factor Nrf2 to the endothelial nucleus, thereby activating anti-oxidant pathways. Furthermore, Nrf2 activation has been shown to dampen immune activation of endothelial cells resulting in a reduction of VCAM-1 expression\textsuperscript{44}. Finally, Nrf2 expression has been described to be highly increased in MS lesion pathology\textsuperscript{45}.

Altogether, our findings point to a potential therapeutic role for RA in brain diseases associated with BBB dysfunction and neuroinflammation. Other have shown before that exogenous RA can exert neuroprotective effects during inflammation in the CNS\textsuperscript{46} and dampens the immune-response of microglia and astrocytes in vitro\textsuperscript{47}. A detailed understanding of RA-specific or other endogenous anti-inflammatory mechanisms in CNS may lead to the discovery of new therapeutic targets to diminish the severity of inflammatory events, as well as boost CNS regeneration.

Reference List


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FC: Fold change compared to vehicle control.