Chapter 5

ABC transporters are involved in multiple sclerosis pathology

Short title: ABC transporters in multiple sclerosis lesions

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Abstract

ATP-binding cassette (ABC) transporters are normally highly expressed at the blood-brain barrier (BBB) and actively hinder passage of harmful compounds, thereby creating multidrug resistance of the brain. Since ABC transporters drive cellular exclusion of potential neurotoxic compounds, alterations in their expression and function at the BBB may contribute to the pathogenesis of neuroinflammatory disorders, such as multiple sclerosis (MS). We therefore investigated the expression pattern of different ABC efflux transporters, including P-glycoprotein (P-gp), multidrug resistance-associated proteins-1 and -2 (MRP-1, -2) and breast cancer resistance protein (BCRP) in various well-characterized human MS lesions. Cerebrovascular expression of P-gp was decreased in both active and chronic inactive MS lesions, whereas infiltrated foamy monocyte-derived macrophages in active MS lesions show enhanced expression of MRP-1 and BCRP, which coincided with increased functionality of these transporters on cultured foamy macrophages. Interestingly, reactive astrocytes display a striking increased expression of P-gp and MRP-1 in both active and inactive MS lesions. To investigate whether ABC transporters on reactive astrocytes can contribute to the inflammatory process, primary cultures of reactive human astrocytes were generated by Toll-like receptor 3 (TLR-3) activation to mimic the phenotype of reactive astrocytes as observed in MS lesions. Notably, using this set-up, we demonstrated for the first time that P-gp and MRP-1 are capable to mediate the secretion of inflammatory agents such as monocyte chemoattractant protein-1 (MCP-1). Together, our data illustrate a novel (patho)physiological role for these ABC transporters on reactive inflammatory astrocytes, suggesting that limiting their activity by dampening astrocyte activation may open therapeutic avenues to diminish tissue damage during MS pathogenesis.
Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) and is neuropathologically characterized by multiple focal demyelinated lesions scattered throughout the CNS (1-5). Active MS lesions contain abundant cellular infiltrates, which mainly consist of T cells and monocyte-derived macrophages (6). The latter are thought to be responsible for causing damage to the myelin sheaths that surround axons, resulting in neuronal dysfunction. In inflammatory demyelinating lesions foamy macrophages are present, which acquire their distinctive morphology by ingestion and accumulation of vast amounts of myelin-derived lipids and cellular debris. Foamy macrophages originate from both resident microglia and infiltrating monocytes (7) and are thought to display an anti-inflammatory phenotype (8). In the course of lesion progression, enlarged proliferative astrocytes become the most predominant cell type. Reactive astrocytes secrete different neurotrophic factors for neuronal survival but also contribute to pathology by production of proinflammatory cytokines and chemokines (9;10).

Loss of blood-brain barrier (BBB) integrity is a key feature of MS pathology (11). Normally, the BBB plays a crucial role in the maintenance of brain homeostasis by protecting the CNS microenvironment from the systemic circulation. The BBB acts as a physical barrier due to the presence of intercellular tight junctions and it regulates the influx and efflux of various compounds and nutrients. Efflux transporters are key molecules in protecting the brain from unwanted compounds, enabling multi-drug resistance (MDR) (12). ATP-binding cassette (ABC) transporters consist of a variety of drug efflux pumps, including P-glycoprotein (P-gp), breast cancer resistant protein (BCRP) and the multidrug resistance-associated proteins (MRPs). These efflux pumps are expressed on different cell types like endothelial cells and immune cells and can drive cellular exclusion of a variety of exogenous compounds and drugs through the cell membrane against a concentration gradient at the cost of ATP hydrolysis (13). Interestingly, several studies using immune cells have suggested that endogenous substrates for efflux pumps on immune cells may include inflammatory mediators, such as steroids, prostaglandins, leukotrienes and cytokines (14-19). Hence it is conceivable that besides actively removing unwanted compounds, ABC transporters at the BBB may also protect the brain from the entry of unwanted inflammatory agents.

Malfunction of ABC transporters at the BBB may therefore result to an increased exposure of the vulnerable CNS cells to toxic inflammatory compounds, which subsequently leads to tissue damage as observed during MS. We therefore investigated the expression pattern of different ABC transporters (P-gp, MRP-1, MRP-2 and BCRP) in well-characterized MS lesions using post-mortem tissue. We here demonstrate that various CNS cell types, like endothelial cells, microglia and astrocytes express ABC transporter proteins and striking differences in their expression were observed in both active and inactive MS lesions, which coincided with functional alterations under neuroinflammatory conditions in vitro.
Moreover, we here demonstrate that P-gp and MRP-1 are involved in the secretion of monocyte chemoattractant protein-1 (MCP-1) from reactive astrocytes, which may amplify the neuroinflammatory attack during MS lesion formation. Together, our findings provide novel insights into the expression and function of ABC transporters during MS pathology and illustrate a potential detrimental role of P-gp and MRP-1 on reactive astrocytes under pathological conditions.
ABC transporters in multiple sclerosis lesions

Materials and Methods

Brain tissue
Brain tissue from 10 patients with clinically diagnosed and neuropathologically confirmed MS was obtained at rapid autopsy and immediately frozen in liquid nitrogen (in collaboration 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 4 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. Relevant clinical information was retrieved from the medical records and is summarized in Table 1.

Immunohistochemistry
For immunohistochemical stainings, 5 μm cryosections were air-dried and fixed in acetone for 10 minutes. Sections were incubated overnight at 4°C with primary antibodies (see Table 1).

Table 1. Clinical information of MS and control patient material

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Type of MS</th>
<th>Sex</th>
<th>Post-mortem delay (hr)</th>
<th>Disease duration (years)</th>
<th>Cause of death</th>
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<tbody>
<tr>
<td>Patient 1</td>
<td>52</td>
<td>SP</td>
<td>F</td>
<td>8:25</td>
<td>n.k.</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>Patient 2</td>
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<td>PP</td>
<td>F</td>
<td>10:15</td>
<td>8</td>
<td>Decompensation</td>
</tr>
<tr>
<td>Patient 3</td>
<td>63</td>
<td>PP</td>
<td>M</td>
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<td>24</td>
<td>Cardiac arrest</td>
</tr>
<tr>
<td>Patient 4</td>
<td>56</td>
<td>SP</td>
<td>M</td>
<td>8:00</td>
<td>27</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>Patient 5</td>
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<td>M</td>
<td>7:45</td>
<td>n.k.</td>
<td>Sepsis</td>
</tr>
<tr>
<td>Patient 6</td>
<td>47</td>
<td>SP</td>
<td>M</td>
<td>7:15</td>
<td>7</td>
<td>Urosepsis</td>
</tr>
<tr>
<td>Patient 7</td>
<td>79</td>
<td>SP</td>
<td>F</td>
<td>14:00</td>
<td>39</td>
<td>CVA</td>
</tr>
<tr>
<td>Patient 8</td>
<td>48</td>
<td>PP</td>
<td>F</td>
<td>4:50</td>
<td>25</td>
<td>Euthanasia</td>
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<td>Patient 9</td>
<td>77</td>
<td>SP</td>
<td>M</td>
<td>4:15</td>
<td>26</td>
<td>CVA</td>
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<tr>
<td>Patient 10</td>
<td>41</td>
<td>PP</td>
<td>M</td>
<td>7:20</td>
<td>n.k.</td>
<td>Pneumonia</td>
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<tr>
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<td>M</td>
<td>4:40</td>
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<td>Pneumonia</td>
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<tr>
<td>Control 2</td>
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<td>-</td>
<td>F</td>
<td>5:10</td>
<td>-</td>
<td>Pneumonia by hemothorax</td>
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<tr>
<td>Control 3</td>
<td>57</td>
<td>-</td>
<td>M</td>
<td>6:00</td>
<td>-</td>
<td>Cardiac arrest</td>
</tr>
<tr>
<td>Control 4</td>
<td>77</td>
<td>-</td>
<td>F</td>
<td>8:00</td>
<td>-</td>
<td>Pneumonia</td>
</tr>
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SP: secondary progressive; PP: primary progressive; MS: MS subtype not determined; F: female; M: male; n.k.: not known; CVA: cerebral vascular accident.
For the detection of PLP, MHC class II, P-gp, MRP-2 and BCRP, slides were incubated with EnVision Kit rabbit/mouse-labeled horseradish peroxidase (DAKO, Glostrup, Denmark) for 30 minutes at room temperature. For the detection of MRP-1, sections were incubated with biotin-labeled rabbit anti-rat antibody (DAKO, Glostrup, Denmark) for 30 minutes at room temperature and with avidin biotin complex (ABC; DAKO, Glostrup, Denmark) according to the manufacturer’s description. Peroxidase activity was demonstrated with 0.5 mg/ml 3,3’-diaminobenzidine tetrachloride (DAB; Sigma, St Louis, MO, USA) in PBS containing 0.02% H₂O₂. Between incubation steps, sections were thoroughly washed with phosphate-buffered saline (PBS). 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 xylol and mounted with Entellan (Merck, Darmstadt, Germany). All antibodies were diluted in PBS containing 0.1% bovine serum albumin (BSA, Boehringer-Mannheim, Germany), which also served as a negative control.

For double immunofluorescence stainings, sections were incubated for 30 minutes with 20% normal goat serum. Then, sections were incubated overnight at 4°C with primary antibodies for all four transporters (see Table 2). To distinguish between different cell types, sections were co-incubated with antibodies directed against glial fibrillary acidic protein (GFAP, astrocytes), and CD11b (microglia/macrophages) (Table 2), and labelled subsequently with Alexa-488 coupled goat anti-mouse antibody (for MRP-1, -2, BCRP, and P-gp), Alexa-633 coupled goat anti-rabbit antibody (for GFAP) and Alexa-647 coupled goat anti-rat antibody.

<table>
<thead>
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<th>Primary antibody</th>
<th>Dilution</th>
<th>Company</th>
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<tr>
<td>CD11b</td>
<td>1:100</td>
<td>Abcam</td>
</tr>
<tr>
<td>Proteolipid protein (clone plpc1)</td>
<td>1:500</td>
<td>Serotec Ltd, Oxford, UK</td>
</tr>
<tr>
<td>Major histocompatibility complex class II</td>
<td>1:100</td>
<td>DAKO</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein</td>
<td>1:20</td>
<td>DAKO</td>
</tr>
<tr>
<td>MDR1 P-gp (clone 15D3)</td>
<td>1:10</td>
<td>Department of Pathology (VUmc, Amsterdam)</td>
</tr>
<tr>
<td>MRP1 (clone MRP1)</td>
<td>1:50</td>
<td>Department of Pathology (VUmc)</td>
</tr>
<tr>
<td>MRP-1 (clone MRPm5) (fluorescence)</td>
<td>1:25</td>
<td>Department of Pathology (VUmc)</td>
</tr>
<tr>
<td>MRP2 (clone M2III-6)</td>
<td>1:50</td>
<td>Department of Pathology (VUmc)</td>
</tr>
<tr>
<td>BCRP (clone BXP-21)</td>
<td>1:50</td>
<td>Department of Pathology (VUmc)</td>
</tr>
</tbody>
</table>
ABC transporters in multiple sclerosis lesions

(for CD11b) (all secondary antibodies from Molecular Probes, Leiden, The Netherlands). After washing, slides were covered with Vectashield (Vector laboratories, Burlington, CA, USA) supplemented with 0.4% DAPI to stain nuclei. Fluorescence analysis was performed with a Leica DM6000 microscope (Leica Microsystems, Heidelberg, Germany).

Cell cultures
Primary astrocytes from control human brain tissue or MS lesions and primary monocytes were isolated and cultured as described previously (20;21). P-gp (CEM/VBL), MRP-1 (2008/MRP-1) and BCRP (MCF7) overexpressing cells, and their control cell lines were a kind gift of dr. G. Scheffer (Dept. of Pathology, VUMC, Amsterdam, the Netherlands) and were cultured as described previously (22).

Cell treatments
Primary human astrocytes and primary human monocytes were cultured in 24-well plates or 96-well plates. Subsequently, astrocytes were incubated with tumor necrosis factor alpha (TNF-α; 5 ng/ml; Peprotech, UK) or the Toll-like receptor 3 ligand polyinosinic-cytidylic acid (poly I:C; 50 μg/ml, Amersham Pharmacia Biotech, Piscataway, NJ) for 6 or 24 hr in the presence or absence of the specific P-gp inhibitor reversin 121 (10 μM; Alexis) or the specific MRP-1 inhibitor MK-571 (25 μM; Dept of Pathology, VUMC, Amsterdam, the Netherlands). Primary monocytes were either incubated with human myelin derived from control white matter (23) or latex beads (Polysciences) for different time points (24 or 48 hr).

In vitro assays for ABC transporter function
P-gp, MRP1 and BCRP function was determined as described previously (24). Briefly, after treatment, astrocytes or macrophages were washed three times with PBS and subsequently incubated for 45 minutes at 37°C with specific substrates in the presence or absence of specific inhibitors (P-gp substrate Rhodamine 123 (2 μM; Sigma), inhibitor reversin 121 (10 μM; Alexis); MRP-1 substrate Calc-AM (500 nM; Molecular Probes), inhibitor MK-571 (25 μM; Dept of Pathology, VUMC); BCRP substrate Bodipy (100 nM; Dept of Pathology, VUMC), inhibitor KO143 (200 nM; Dept of Pathology, VUMC). After 45 minutes of incubation, cells were washed three times with PBS and fluorescence intensity was measured using a FLUOstar Galaxy microplate reader (BMG Labtechnologies, Offenburg, Germany), excitation 485 nm, emission 520 nm or by a FACScan flow cytometer (Becton & Dickinson, San Jose, CA, USA). FACS analysis was performed on 10,000 viable cells, selected by 7AAD exclusion. ABC transporter activities are expressed as ratios of drug fluorescence with inhibitor and drug fluorescence without inhibitor after subtraction of the fluorescence of the control. Overexpressing cell lines for P-gp (CEM/VBL), MRP1 (2008/MRP1) and BCRP (MCF7) were used for optimizing the functional ABC transporter assays.
MCP-1 ELISA
MCP-1 was measured in culture supernatants of control or poly I:C stimulated astrocytes (24 hr) using an enzyme-linked immunosorbent assay (ELISA) with a lowest detection level of 30 pg/ml (R&D Systems) as described previously (25).

RNA isolation and real-time quantitative PCR
Messenger RNA was isolated from control or poly I:C stimulated astrocytes using an mRNA capture kit (Roche) according to the manufacturer's instructions. cDNA was synthesized with the Reverse Transcription System kit (Promega, USA) following manufacturer's guidelines and RT-PCR was performed as described previously (26). All primer sequences are listed in supplementary table 1 and expression levels of transcripts obtained with real time PCR were normalized to GAPDH expression levels.

Statistical analysis
Data were analyzed statistically by means of a single-column t-test. Statistical significance was defined as * p < 0.05, ** p < 0.01, ***p < 0.001.

Results

MS lesion classification
Classification of MS lesions was based on standard immunohistochemical stainings for inflammatory cells (anti-MHC class II) and myelin (PLP) as described previously (27-29). Based on these findings 12 lesions sampled in this study were classified as active with myelin loss (Figure 1A, 3A) and abundant phagocytic perivascular and parenchymal macrophages containing myelin degradation products (Figure 1B, 3B) and 7 lesions as chronic inactive with demyelinated areas (Figure 2A, 4A) containing few MHC class II-positive cells (Figure 2B, 4B).

Enhanced MRP-1 and MRP-2 expression in MS lesions and MRP-1 function in foamy macrophages
In white matter from non-neurological control brain tissue (data not shown) and normal appearing white matter (NAWM) MRP-1 (Fig. 1C, arrows) and MRP-2 (Fig 1D, arrows) immunoreactivity is mainly restricted to glial cells, whereas endothelial cells that line the cerebral vasculature only weakly express MRP-1 and MRP-2. In active demyelinating MS lesions (Fig 1A, B) enhanced MRP-1 (Fig 1E) and MRP-2 (Fig 1F) staining is observed in foamy macrophages (arrows) and hypertrophic astrocytes (arrowheads). Using double immunofluorescence stainings we confirmed the cellular localization of these efflux pumps.
Figure 1. MRP-1 and MRP-2 expression in normal appearing white matter and MS lesions and increased MRP-1 function on foamy macrophages

(A) Loss of proteolipid protein (PLP) immunoreactivity in a subcortical lesion, with (B) enhanced expression of MHC class II (magnification: 10x). Boxed sites are representative areas of the 40x magnification of adjacent sections stained for MRP-1 and MRP-2. In normal appearing white matter (NAWM) MRP-1 (C) and MRP-2 (D) immunoreactivity is observed in microglial cells (arrowheads), and faintly in endothelial cells. Within an active demyelinating lesion, MRP-1 (E) and MRP-2 (F) immunoreactivity is highly increased in hypertrophic astrocytes and astrocyte processes (arrowheads) and foamy macrophages (arrows). Co-localization of MRP-1 and MRP-2 immunoreactivity (in green) with GFAP (G,J) and CD11b (H,J) immunoreactivity (in red) confirms the morphological observations. Myelin phagocytosis for 24 or 48 hours by primary human monocytes results in increased MRP-1 function (K), whereas the phagocytosis of latex beads did not affect MRP-1 functionality. 100% corresponds to a ratio of 1.07 +/- 0.04. Experiments were performed in triplicate using 3 different human donors and were presented as the mean +/- SEM. ** p< 0.01 by Students t test.
in active MS lesions and showed that MRP-1 and MRP-2 are expressed by GFAP-positive astrocytes (Fig 1G,I) and CD11b-positive macrophages (Fig 1H,J). To study whether foamy macrophages are capable to actively remove substrates for MRP-1, we performed an in vitro functional MRP-1 assay. First in vitro foamy macrophages were generated (8;23), by adding myelin to human monocytes for different time points (24 and 48 hr), resulting in their characteristic foamy appearance (data not shown). We next determined MRP-1 function on control untreated macrophages and myelin-laden macrophages. Notably, MRP-1 function was strikingly enhanced upon addition of myelin at different time points (Fig 1K). In contrast, phagocytosis of latex beads by cultured macrophages did not result in increased functionality of MRP-1 (Fig 1K), indicating that myelin specifically induces MRP-1 efflux transporter activity of macrophages, which correlates with the increased expression levels of MRP-1 on foamy macrophages in active MS lesions. In chronic inactive MS lesions

Figure 2. MRP-1 and MRP-2 expression in chronic inactive MS lesions

(A) Loss of PLP immunoreactivity in a subcortical lesion, with (B) a low number of MHC class II positive cells (magnification 10x). Boxed site is a representative area of the 40x magnification of adjacent sections stained for MRP-1 and MRP-2. In chronic inactive MS lesions, MRP-1 (C) and MRP-2 (D) immunoreactivity is observed in hypertrophic astrocytes (arrowheads) and resting microglia (arrows).
ABC transporters in multiple sclerosis lesions

Figure 3. P-gp and BCRP expression in normal appearing white matter and MS lesions and increased MRP-1 function on foamy macrophages

(A) Loss of PLP immunoreactivity in a subcortical lesion, with (B) enhanced expression of MHC class II positivity (magnification 10x). Boxed sites are representative areas of the 40x magnification of adjacent sections stained for P-gp and BCRP. In NAWM P-gp immunoreactivity (C) is observed in endothelium (arrows), and faintly in astrocytes (arrowhead). BCRP immunoreactivity in NAWM (D) is prominent on endothelial cells (arrows) as well as in resting microglial cells (arrowheads). Within an active demyelinating lesion, P-gp immunoreactivity (E) is highly increased in hypertrophic astrocytes (arrowheads) and is decreased in endothelium (arrow). BCRP immunoreactivity in within an active lesion is unaltered on endothelium (arrow) and highly present in foamy macrophages (arrowheads). Co-localization of P-gp immunoreactivity (in green) with GFAP (in red) (G) and BCRP immunoreactivity (in green) with CD11b (in red) (H) confirms the morphological observations. Myelin phagocytosis for 24 or 48 hours by primary human monocytes results in increased BCRP function (I), whereas the phagocytosis of latex beads did not affect BCRP functionality. 100% corresponds to a ratio of 1.19 +/- 0.13. Experiments were performed in triplicate using 3 different human donors and were presented as the mean +/- SEM. ** p < 0.01 by Students t test.
(Fig 2A,B), hypertrophic astrocytes express MRP-1 (Fig 2C, arrowheads) and MRP-2 (Fig 2D, arrowheads), whereas microglia also express MRP-2 (arrows) to the same level as under control conditions (Fig 1D). Brain endothelial cells express relatively low amounts of MRP-1 and MRP-2 in control or MS brain tissue (Fig 1-2), which coincided with low MRP-1 activity on cultured human brain endothelial cells using an in vitro efflux assay (data not shown). Moreover, no striking differences in vascular MRP-1 and MRP-2 expression are observed between MS lesions and NAWM.

P-gp and BCRP expression in control white matter and MS lesions and increased BCRP function in foamy macrophages.

Figure 4. P-gp and BCRP expression in chronic inactive MS lesions
(A) Loss of PLP immunoreactivity in a subcortical lesion, with (B) a low number of MHC class II positive cells (magnification 10x). Boxed site is a representative area of the 40x magnification of adjacent sections stained for P-gp and BCRP. In chronic inactive lesions P-gp immunoreactivity (C) is prominent in hypertrophic astrocytes (arrowheads) and faintly present in endothelium (arrow). BCRP immunoreactivity (D) in chronic inactive lesions is present in endothelium (arrow) and resting microglial cells (arrowheads).
P-gp immunoreactivity is predominantly localized to the cerebral microvasculature in NAWM (Fig 3C, arrows) and control brain tissue (data not shown) and only weakly expressed on astrocytes (arrowheads). Notably, in active demyelinated MS lesions (Fig 3E, arrows) and chronic inactive lesions (Fig 4C, arrow) a decreased vascular P-gp immunoreactivity was observed. Interestingly, hypertrophic GFAP-positive astrocytes were markedly decorated with anti-P-gp in active and chronic inactive MS lesions (Fig. 3E, 3G, 4C, arrowheads). BCRP expression is restricted to the brain microvasculature (arrows) and microglial cells (arrowheads) in NAWM (Fig 3D) and control brain tissue (data not shown). In active demyelinating MS lesions a markedly increased BCRP staining is observed in CD11b-positive foamy macrophages (Fig 3F, 3H). Increased BCRP expression correlated with enhanced BCRP functionality upon myelin phagocytosis of human monocytes at different time points (Fig 3I). In line with MRP-1 (Fig 1K), enhanced BCRP functionality on foamy macrophages appeared to be a myelin specific effect, as phagocytosis of latex beads did not alter its efflux capacity (Fig 3I). Brain endothelial cells express high amounts of P-gp and BCRP in control tissue, which coincides with high P-gp and BCRP activity on human brain endothelial cells in vitro (data not shown). However, in contrast to P-gp, no striking differences in endothelial BCRP expression were observed between MS lesions and normal appearing white matter.

**Increased expression and function of P-gp and MRP-1 in reactive astrocytes in vitro**

Increased expression of P-gp and MRP-1 on reactive astrocytes in MS lesions suggests an altered function of these efflux pumps under neuroinflammatory conditions. To study this, we first isolated astrocytes from MS lesions and NAWM and determined the expression level of the reactive astrocyte marker GFAP (30) by means of quantitative PCR (qPCR). Interestingly, MS lesion-derived astrocytes display increased expression levels of GFAP (Fig. 5A), illustrating a reactive phenotype. Next, in vitro functional assays for P-gp and MRP1 were performed on NAWM (control) or MS lesion-derived astrocytes. Notably, reactive astrocytes isolated from MS lesions display increased functionality of both efflux pumps (Fig 5B,C), which correlates with the enhanced expression levels of astrocytic P-gp and MRP-1 in human MS lesions (figure 1–4). To investigate whether inflammatory mediators could affect astrocytic P-gp and MRP-1 functionality, we treated primary human astrocytes with inflammatory mediators like tumor necrosis factor alpha (TNF-α) and/or polyinosinic-cytidylcic acid (poly I:C), a dsRNA mimetic ligand for Toll-like receptor 3 (TLR-3), to mimic a pro-inflammatory environment as observed during neuroinflammation (31). Interestingly, both inflammatory mediators strikingly increased MRP-1 and P-gp efflux capacity by astrocytes (Fig 5B,C), with TLR-3 activation being the most potent inducer (Fig 5B,C). To determine whether TLR-3 activation on astrocytes leads to a reactive astrocyte phenotype, we verified expression levels of various reactive astrocytic markers like GFAP, S100B, vimentin and IL-6 (32) and the ABC transporters P-gp and MRP-1 by means of qPCR. Notably,
TLR-3-induced astrocytes display increased expression levels of GFAP, IL-6, S100β, P-gp and MRP-1 compared to control astrocytes (Fig 5D), whereas vimentin levels remained unaltered. These results illustrate an in vitro model for the generation of reactive astrocytes by TLR-3 activation. Together, these results show that P-gp and MRP-1 expression and function are highly increased on inflammatory reactive astrocytes.

Figure 5. Increased P-gp and MRP-1 expression and function in reactive astrocytes

(A) Increased GFAP expression on primary human astrocytes isolated from active MS lesions compared to astrocytes isolated from control white matter. GFAP expression was determined by RT-PCR and presented as relative expression compared to GAPDH. Enhanced MRP-1 (B) and P-gp (C) functionality in primary human astrocytes isolated from active MS lesions (gray bars) and in control astrocytes stimulated with either TNF-α (5 ng/ml) or poly I:C (50 μg/ml) for 6 hours, compared to untreated astrocytes. Control astrocytes have a ratio of 1.03 +/- 0.18 (P-gp) or 1.15 +/- 0.07 (MRP-1). GFAP, IL-6, S100β, vimentin, P-gp and MRP-1 transcripts from control or poly I:C-treated (24 hr) astrocytes (D) were detected by RT-PCR and presented as relative expression compared to GAPDH. Experiments were performed in triplicate using 3 different human donors and were presented as the mean +/- SEM. *p<0.05, **p<0.01, *** p< 0.001 by Students t test.
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P-gp and MRP-1 regulate MCP-1 secretion from reactive astrocytes

Reactive astrocytes can contribute to the inflammatory process by the production and secretion of proinflammatory cytokines and chemokines (9;10). In particular chemokines, like monocyte chemoattractant protein-1 (MCP-1) are known to attract leukocytes and monocyte-derived macrophages into MS lesions, which in turn results in severe tissue damage (33). As ABC transporters are suggested to be involved in the secretion of inflammatory mediators, we next investigated whether P-gp and MRP-1 are involved in the efflux of the astrocyte-derived chemokine MCP-1. TLR-3-activated astrocytes secrete (Fig 6A) and produce (Fig 6B) high levels of MCP-1 compared to control astrocytes. Moreover, blocking P-gp or MRP-1 activity significantly reduced MCP-1 secretion from reactive astrocytes (Fig 6C), whereas MCP-1 mRNA expression levels remained unaffected (Fig 6D). Together, these results show that both P-gp and MRP-1 are involved in the efflux but not the production of MCP-1 from reactive astrocytes.

![Figure 6. P-gp and MRP-1 regulate MCP-1 secretion from reactive astrocytes](image-url)

Primary human astrocytes were treated with or without poly I:C (50 μg/ml) for 24 hr and MCP-1 secretion was determined in cell supernatants by ELISA (A) and MCP-1 transcripts were determined by RTPCR and presented as relative expression compared to GAPDH (B). Astrocytes were treated with poly I:C (50 μg/ml) for 24 in the presence or absence of the P-gp inhibitor reversin 121 (10 uM) or the MRP-1 inhibitor MK-571 (25 uM), after which MCP-1 secretion (C) and expression (D) was determined by ELISA or RTPCR respectively. 100% corresponds to 18.0 +/- 0.46 μg/ml MCP-1 (C) or 0.032 +/- 0.003 MCP-1 expression relative to GAPDH (D). Experiments were performed in triplicate using 3 different human donors and were presented as the mean +/- SEM. *p<0.05, **p<0.01, *** p< 0.001 by Students t test.
Chapter 5

Discussion

In this study we provide for the first time a detailed overview of ABC transporter expression in MS brain tissue and we illustrate their potential contribution to neuroinflammation. Predominant cell types involved in MS pathology, including brain endothelial cells, reactive astrocytes and infiltrated foamy macrophages, display striking alterations in their ABC transporter expression, which coincides with functional changes in vitro under inflammatory conditions. Moreover, we here define an in vitro model for the generation of reactive astrocytes by TLR-3 activation, which closely mimics the reactive phenotype of astrocytes isolated from MS lesions. These reactive astrocytes are known to exacerbate the inflammatory response by the secretion of inflammatory molecules like MCP-1, which we here show to be mediated by the ABC transporters P-gp and MRP-1, indicating a novel role for ABC transporters in the pathogenesis of neuroinflammatory disorders such as MS.

In different stages of MS lesions we observed an altered expression pattern of various ABC transporter proteins like P-gp, MRP-1, MRP-2 and BCRP. In particular hypertrophic astrocytes, which are abundantly present in MS lesions, display enhanced expression of P-gp, MRP-1 and MRP-2. We are the first to show increased astrocytic ABC transporter expression under neuroinflammatory conditions in human brain material. So far, enhanced astrocytic expression of P-gp and MRP-1 has been reported in brain tissue of epilepsy patients 34, 35, which has been suggested to be a result of seizures or drug treatment. Notably, we here demonstrate that increased astrocytic expression in MS lesions correlated with enhanced P-gp and MRP-1 activity of lesion-derived astrocytes as compared to astrocytes isolated from non-affected white matter. Activation of astrocytes has been implicated in the pathogenesis of a variety of neurodegenerative diseases, including Alzheimer’s disease, inflammatory demyelinating diseases and human immunodeficiency virus (HIV)-associated dementia 36. Upon activation, reactive astrocytes secrete neurotrophic factors for neuronal survival, however, it is believed that severe activation augments an inflammatory response, leading to neuronal death and brain injury 10. In our study, reactive astrocytes were generated in vitro by TLR-3 activation of primary human astrocyte cultures, which resulted in high expression levels of the reactive astrocyte marker GFAP to a similar extent as observed in astrocytes isolated from MS lesions. Furthermore, TLR-3 activation enhanced the expression of S100b and IL-6, which are well-known markers for astrocyte activation 32, indicating that TLR-3 activation is a suitable in vitro model for the generation of reactive astrocytes. Interestingly, TNF-α treatment and TLR-3 activation of astrocytes led to an increased P-gp and MRP-1 function, indicating that these inflammatory agents are involved in the regulation of ABC transporter expression and function in astrocytes. It has been described that at the transcripational level ABC transporters are under control of the orphan nuclear receptors such as steroid and xenobiotic receptor (SXR in human; or pregnane
X receptor (PXR) in rodents. Furthermore, expression and function are regulated by environmental stimuli that evoke stress responses, like the excitatory neurotransmitter glutamate or the inflammatory cytokines. So far, only immune regulation of brain endothelial P-gp expression and function has been reported. We here extend these results by demonstrating that inflammatory mediators can affect both MRP-1 and P-gp expression and function on human astrocytes.

Active MS lesions are characterized by the presence of infiltrated leukocytes and monocyte-derived macrophages, which are responsible for tissue destruction and demyelination. Chemokines are believed to be involved in the attraction of these immune cells into MS lesions of which MCP-1 has been linked to participate in the pathogenesis of MS. Mice that lack the receptor for MCP-1 (CCR2) did not develop experimental allergic encephalomyelitis, an animal model for MS, indicating its in vivo relevance. Moreover, MCP-1 expression appeared to be restricted to reactive astrocytes in MS lesions. These in vivo observations were confirmed in vitro in our study, as reactive astrocytes produce and secrete high levels of MCP-1 upon TLR-3 activation. Moreover, we identified a novel role for the ABC transporters P-gp and MRP-1 in the regulation of MCP-1 secretion from reactive astrocytes. These results support the hypothesis that endogenous substrates for ABC transporters may include inflammatory mediators, such as steroids, prostaglandins, leukotrienes and cytokines as observed in studies using immune cells and now also chemokines like MCP-1. Therefore, increased ABC transporter expression and function on reactive astrocytes may result in local efflux of inflammatory mediators in MS lesions, amplifying the inflammatory response.

In active MS lesions, we observed enhanced expression of BCRP, MRP-1 and MRP-2 on infiltrated foamy macrophages. Notably, enhanced function of BCRP and MRP-1 of myelin-laden macrophages was found in our in vitro model. Although the exact role of BCRP- and MRP-1 function in this process is yet unknown it has been described that macrophages rely on cholesterol efflux mechanisms to maintain cellular cholesterol homeostasis by means of ABC transporters ABCA1 and ABCG1. Moreover, as both MRP-1 and BCRP participate in cellular detoxification, we here postulate that these ABC transporters may be involved in the removal of phagocytosed myelin components like cholesterol from foamy macrophages and thereby control cellular homeostasis. Recently, BCRP and MRP-1 expression has been detected on rheumatoid arthritis synovial tissue macrophages, which was suggested to be a result of drug treatment. We here demonstrate that activation of macrophages upon myelin phagocytosis results in increased MRP-1 and BCRP activity, which correlates to the increased expression pattern of these ABC transporters in inflammatory MS lesions.
In control white matter and NAWM, we observed a cerebrovascular expression of P-gp and BCRP and to a lesser extent MRP-1 and MRP-2. Moreover, in vitro functional assays revealed that both P-gp and BCRP are highly active on human brain endothelial cells, whereas MRP-1 function is nearly absent. Interestingly, we detected a decreased endothelial P-gp expression in MS lesions, whereas no striking differences in endothelial BCRP, MRP-1 and MRP-2 were observed. We have previously shown that vascular P-gp expression and function is strongly decreased during MS pathology and identified a crucial role for activated CD4+ T cells in endothelial P-gp regulation via intracellular adhesion molecule -1 and nuclear factor kappa B signalling [Kooij et al, submitted]. Since no changes in vascular expression for BCRP, MRP-1 and MRP-2 were observed, our results indicate differential ABC transporter regulatory mechanisms during pathological conditions, although further research is warranted to define these underlying differences. Cerebrovascular expression has previously been shown for BCRP 45, MRP-2 48 and to a lesser extent for MRP-1 49. In contrast, other groups did not detect the MRP1 protein on the microvasculature when analyzed by immunohistochemistry 50, 51, which might be explained by the usage of different antibodies to MRP-1. Together, our results demonstrate the expression of P-gp, BCRP, MRP-1 and MRP-2 in the cerebral vasculature in NAWM, of which P-gp is selectively affected during MS pathology.

In conclusion, we here demonstrate that ABC transporter expression is strikingly altered in MS brain tissue. In particular, hypertrophic reactive astrocytes and infiltrating foamy macrophages show high expression levels of different ABC transporters, which coincides with increased transporter activity in vitro under inflammatory conditions. Moreover, the ABC transporters P-gp and MRP-1 were shown to mediate MCP-1 secretion from reactive astrocytes, illustrating a novel role for these transporters in MS pathology. Our study provides first evidence for a novel detrimental role of ABC transporters on reactive astrocytes under pathological conditions, and may open therapeutic avenues to diminish the neuroinflammatory attack during MS pathology.

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