CHAPTER 4

ATP-binding cassette transporters mediate chemokine (C-C motif) ligand 2 secretion from reactive astrocytes: relevance to multiple sclerosis pathogenesis

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Chapter 4

Summary
ATP-binding cassette efflux transporters are highly expressed at the blood-brain barrier and actively hinder passage of harmful compounds, thereby maintaining brain homeostasis. Since ATP-binding cassette transporters drive cellular exclusion of potential neurotoxic compounds or inflammatory molecules, alterations in their expression and function at the blood-brain barrier may contribute to the pathogenesis of neuroinflammatory disorders, such as multiple sclerosis. We therefore investigated the expression pattern of different ATP-binding cassette efflux transporters, including P-glycoprotein, multidrug resistance-associated proteins-1 and –2 and breast cancer resistance protein in various well-characterized human multiple sclerosis lesions. Cerebrovascular expression of P-glycoprotein was decreased in both active and chronic inactive multiple sclerosis lesions. Interestingly, foamy macrophages in active multiple sclerosis lesions show enhanced expression of multidrug resistance-associated protein-1 and breast cancer resistance protein, which coincided with their increased function of cultured foamy macrophages. Strikingly, reactive astrocytes display an increased expression of P-glycoprotein and multidrug resistance-associated protein-1 in both active and inactive multiple sclerosis lesions, which correlated with their enhanced in vitro activity on astrocytes derived from multiple sclerosis lesions. To investigate whether ATP-binding cassette transporters on reactive astrocytes can contribute to the inflammatory process, primary cultures of reactive human astrocytes were generated through activation of Toll-like receptor-3 to mimic the astrocytic phenotype as observed in multiple sclerosis lesions. Notably, blocking ATP-binding cassette transporter activity on reactive astrocytes inhibited immune cell migration across a blood-brain barrier model in vitro, which was due to the reduction of astrocytic release of the chemokine (C-C motif) ligand 2. Our data point towards a novel (pathoph)physiological role for ATP-binding cassette transporters, suggesting that limiting their activity by dampening astrocyte activation may open therapeutic avenues to diminish tissue damage during multiple sclerosis pathogenesis.
Introduction
Multiple sclerosis is a chronic demyelinating disease of the central nervous system and is neuropathologically characterized by multiple focal demyelinated lesions scattered throughout the central nervous system. Active multiple sclerosis lesions contain abundant cellular infiltrates, which mainly consist of T cells and monocyte-derived macrophages. 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 and are thought to display an anti-inflammatory phenotype. In the course of lesion progression, enlarged proliferative astrocytes become the most predominant cell type. These reactive astrocytes secrete different neurotrophic factors for neuronal survival but also contribute to pathology by production of proinflammatory cytokines and chemokines.

The central nervous system microenvironment is well protected from the infiltration of inflammatory cells by the blood-brain barrier. One of the key protective features of the blood-brain barrier is that it strictly regulates the efflux of various toxic compounds through specialized membrane pumps, thereby maintaining brain homeostasis. Efflux transporters are therefore regarded as key molecules in protecting the brain from unwanted compounds, enabling multi-drug resistance. The adenosine triphosphate-binding cassette (ABC) transporter family consist of a variety of drug efflux pumps, including P-glycoprotein, breast cancer resistant protein (BCRP) and the multidrug resistance-associated proteins-1 and -2 (MRP-1, MRP-2). Interestingly, ABC efflux pumps are expressed on different cell types like brain 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 adenosine triphosphate hydrolysis. Importantly, several studies have suggested that endogenous substrates for ABC transporters may include inflammatory mediators, such as steroids, prostaglandins, leukotrienes and cytokines. Hence, it is conceivable that besides actively removing unwanted compounds, ABC transporters at the blood-brain barrier also mediate the release of inflammatory agents during (neuro) inflammatory processes, highlighting a potential new role in multiple sclerosis pathology. To study this, we first investigated the expression pattern of different ABC transporters (P-glycoprotein, MRP-1, -2 and BCRP) in well-characterized multiple sclerosis lesions. We here demonstrate that various central nervous system cell types, including endothelial cells, microglia and astrocytes express ABC transporter proteins. Importantly, striking differences in their expression were observed in both active and inactive multiple sclerosis lesions, which coincided with functional alterations under neuroinflammatory conditions in vitro. These results implicate a potential novel role for ABC transporters in multiple sclerosis pathology. Notably, blocking astrocytic P-glycoprotein or MRP-1 activity severely impairs monocyte migration across an in vitro model of the blood-brain barrier and we here demonstrate that P-glycoprotein and MRP-1 are involved in the secretion of chemokine (C-C motif) ligand 2 (CCL2) by reactive astrocytes. Together, our findings provide novel insights into the expression and function of ABC transporters during multiple sclerosis pathology and illustrate a potential detrimental role of P-glycoprotein and MRP-1 in reactive astrocytes.
Materials and Methods

Brain tissue

Brain tissue from 10 patients with clinically diagnosed and neuropathologically confirmed multiple sclerosis 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, Amsterdam, The Netherlands. Tissue samples from 4 control cases without neurological disease were taken from the subcortical white matter and corpus callosum. White matter multiple sclerosis tissue samples were selected on the basis of post-mortem magnetic resonance imaging. 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.

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<th>Case</th>
<th>Age</th>
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<td>8.00</td>
<td></td>
<td>Pneumonia</td>
</tr>
</tbody>
</table>

SP: secondary progressive; PP: primary progressive; MS: MS subtype not determined; F: female; M: male; NK: not known; CVA: cerebral vascular accident.

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 2). For the detection of proteolipid protein, major histocompatibility complex class II, P-glycoprotein, 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 (DAKO, Glostrup, Denmark) according to the manufacturer’s
description. Peroxidase activity was demonstrated with 0.5 mg/ml 3,3'-diaminobenzidine tetrachloride (Sigma, St Louis, MO, USA) in phosphate-buffered saline containing 0.02% hydrogen peroxide. Between incubation steps, sections were thoroughly washed with phosphate-buffered saline. 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 phosphate-buffered saline containing 0.1% bovine serum albumin (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-glycoprotein), Alexa-633 coupled goat anti-rabbit antibody (for GFAP) and Alexa-647 coupled goat anti-rat antibody (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% 4',6-diamidino-2-phenylindole to stain nuclei. Fluorescence analysis was performed with a Leica DM6000 microscope (Leica Microsystems, Heidelberg, Germany).

Table 2. Primary antibodies used in this study

<table>
<thead>
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<th>Antibody</th>
<th>Dilution</th>
<th>Manufacturer</th>
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<td>CD11b</td>
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<td>Abcam</td>
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<tr>
<td>Proteolipid protein (clone plpc1)</td>
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<td>Serotec</td>
</tr>
<tr>
<td>MHC class II</td>
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<td>DAKO</td>
</tr>
<tr>
<td>GFAP</td>
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<td>DAKO</td>
</tr>
<tr>
<td>MDR1 P-glycoprotein (clone 15D3)</td>
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<tr>
<td>MRP-1 (clone MRPr1)</td>
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<td>MRP-2 (clone M2,II6)</td>
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<tr>
<td>BCRP (clone BXP-21)</td>
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Quantification of immunohistochemistry

Relative changes in immunoreactivity were quantified by using ImageJ software v. 1.37c (NIH, USA) as described previously. In short, 3 donors encompassing both active and inactive multiple sclerosis lesions within 1 section, as well as a considerable area of NAWM, were selected to correct for inter donor variability. Various 40x RGB micrographs were recorded for each donor, staining, and white matter location (AL, IL, and NAWM). Next, images were subjected to color deconvolution to exclude hematoxlin staining from analysis. The relative area of specific DAB deposition to total area was determined by using threshold segmentation. The threshold was separately determined for each ABC transporter, and
for each donor in NAWM micrographs, to include specific immunoreactivity and exclude background immunoreactivity. The same threshold was applied to active and inactive multiple sclerosis lesion micrographs to assess the relative increase in immunoreactivity. An example of the segmentation procedure is shown in supplementary figure 2.

**Cell cultures**

Primary astrocytes from control human brain tissue or multiple sclerosis lesions and primary monocytes were isolated and cultured as described previously\(^2\), \(^3\). P-glycoprotein (CEM/VBL), MRP-1 (2008/MRP-1) and BCRP (MCF7) overexpressing cells, and their control cell lines were obtained from and cultured as described by\(^4\). The human brain endothelial cell line hCMEC/D3 was cultured as described previously\(^5\).

**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 polynosinic-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-glycoprotein inhibitor reversin 121 (10 µM; Alexis) or the specific MRP-1 inhibitor MK-571 (25 µM; Merck Frosst Canada). Multiple sclerosis lesion reactive astrocytes were only cultured in the presence or absence of P-glycoprotein or MRP-1 inhibitors. Subsequently, supernatants were harvested for enzyme-linked immunosorbent assay. For migration experiments, all of the above mentioned treatments were washed away and conditioned media was collected after 24h. Primary monocytes were either incubated with human myelin derived from control white matter\(^6\) or latex beads (Polysciences) for different time points (24 of 48 hr).

**In vitro assays for ABC transporter function**

P-glycoprotein, MRP1 and BCRP function was determined as described previously\(^7\) with minor modifications. Briefly, after treatment, astrocytes or macrophages were washed three times with phosphate-buffered saline and subsequently incubated for 45 minutes at 37°C with specific substrates in the presence or absence of specific inhibitors (P-glycoprotein 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; Merck Frosst Canada); BCRP substrate Bodipy (100 nM; kind gift from dr. G. Scheffer, VUMC, Amsterdam, the Netherlands), inhibitor KO143 (200 nM; kind gift from dr. G. Scheffer, VUMC, Amsterdam, the Netherlands). After 45 minutes of incubation, cells were washed three times with phosphate-buffered saline 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-glycoprotein (CEM/VBL), MRP1 (2008/MRP1) and BCRP (MCF7) were used for optimizing the functional ABC transporter assays and indicated high selectivity of all different inhibitors used in our assays. See supplementary figure 1 for
additional information.

**Enzyme-linked immunosorbent assay**
CCL2 or IL-1β protein was measured in culture supernatants of control or poly I:C stimulated astrocytes (24 hr) or multiple sclerosis lesion derived astrocytes using an enzyme-linked immunosorbent assay (R&D Systems) with a lowest detection level of 30 pg/ml (CCL2) or 1pg/ml (IL-1β) as described previously²⁸.

**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 real-time quantitative PCR was performed as described previously ²⁸. 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.

**Monocyte migration**
We used two established protocols for the measurement of human monocyte migration using the 48-well chemotaxis chamber³⁰ and/or a Transwell system with cultured brain endothelial cells ³¹, with minor modifications. Briefly, chemotaxis chamber migration was measured using 48-well chambers with nitrocellulose filters. Conditioned media from control, in vitro generated or multiple sclerosis lesion derived reactive astrocytes was added to the bottom wells (25 μl) of the 48-well plate. Nitrocellulose filters (Neuro Probe) with a 5 μm pore size were placed between the bottom plate and top plate of the chamber assembly and the monocytes (50 μl) were added to the top wells at a cell density of 4×10⁴ monocytes/ml. The chamber was incubated for 1.5 h (37°C and 5% CO₂) and the non-migrating cells on top of the filter were removed by gentle scraping. The filter was air dried, fixed and stained with a modified hematoxylin/eosin stain. Filters were mounted on glass slides and monocyte migration was measured visually by counting the number of cells at the leading front of migration in 10 high-powered fields (×450).

To investigate the influence of conditioned media on the capacity of monocytes to cross a monolayer of brain endothelial cells, Transwell migration experiments were performed using human brain endothelial hCMEC/D3 cells, which were cultured onto collagen (upper side, Sigma, St. Louis, USA) coated Costar Transwell filters (pore-size 5 μm; Corning Incorporated, Corning, NY, USA) for 4 days. At the start of the experiment, 600 μl of conditioned media from control or reactive astrocytes was added to the bottom wells. Next, monocytes (100 μl) were added to the top wells at a cell density of 1×10⁶ monocytes/ml. Monocytes were allowed to migrate for 8hr (37°C and 5% CO₂). After 8hr, 400 μl was collected from the lower chamber and 20,000 beads (Beckman Coulter, USA) were added to each sample. Samples were then analyzed using a FACSscan flow cytometer (Becton & Dickinson, San Jose, CA, USA) and based on 5000 gated beads, the number of migrated monocytes was determined. In both assays, monocyte migration was presented as the absolute number of migrated monocytes compared to the total number of monocytes added in the upper chamber.
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
Multiple sclerosis lesion classification.
Classification of multiple sclerosis lesions was based on standard immunohistochemical
stained for MRP-1 and MRP-2. In normal appearing white matter (NAWM) MRP-1 (G) and MRP-2 (D) immunoreactivity is observed on microglial cells (arrows), and faintly on endothelial cells. Within an active demyelinating multiple sclerosis lesion, MRP-1 (E) and MRP-2 (F) immunoreactivity is highly increased on hypertrophic astrocytes and astrocyte processes (arrowheads) and foamy macrophages (arrows). These figures show representative images observed in all patient material. 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.

stainings for inflammatory cells (anti-major histocompatibility complex class II) and myelin (proteolipid protein) as described previously. 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 major histocompatibility complex class II-positive cells (Figure 2B, 4B).

Enhanced MRP-1 and MRP-2 expression in multiple sclerosis lesions and increased 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 multiple sclerosis 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 in active multiple sclerosis 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.
(D) Immunoreactivity is observed on hypertrophic astrocytes (arrowheads) and resting microglia (arrows). These figures show representative images observed in all patient material. (E) Quantification of the relative difference in immunoreactivity for both MRP-1 and MRP-2 in NAWM, active lesions, and inactive lesions. Presented as mean fold change from NAWM, +/-SEM. * p<0.05, *** p<0.001 by Students t test.

by adding myelin to human monocytes for different time points (24 and 48 hr), resulting in their characteristic foamy appearance (data not shown). Subsequently, we determined MRP-1 activity in untreated or myelin-laden macrophages. Notably, MRP-1 function was 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 on macrophages, which correlates with the increased expression levels of MRP-1 on foamy macrophages in active multiple sclerosis lesions. In chronic inactive multiple sclerosis lesions (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 seen in control white matter (Fig 1D). Brain endothelial cells express relatively low amounts of MRP-
thinity on astrocytes (arrowheads). BCRP immunoreactivity in NAWM (D) is prominent on endothelial cells (arrows) as well as on resting microglial cells (arrowheads). Within an active demyelinating multiple sclerosis lesion, P-glycoprotein immunoreactivity (E) is highly increased on hypertrophic astrocytes (arrowheads) and is decreased on the endothelium (arrow). BCRP immunoreactivity within an active lesion is unaltered on the endothelium (arrow) and highly present on foamy macrophages (arrowheads). These figures show representative images observed in all patient material. Co-localization of P-glycoprotein 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.

1 and MRP-2 in control or multiple sclerosis brain tissue (Fig 1-2), which coincided with low MRP-1 activity in cultured human brain endothelial cells using an in vitro efflux assay (supplementary figure 1). Moreover, no differences in vascular MRP-1 and MRP-2 expression are observed between multiple sclerosis lesions and NAWM. To quantitatively assess MRP-1 and MRP-2 expression in multiple sclerosis lesions, we quantified MRP-1 and MRP-2 immunoreactivity in NAWM and different multiple sclerosis lesions (see supplementary figure 2 for detailed description). In line with the immunohistochemical stainings, we observed increased transporter immunoreactivity in active lesions (MRP-1 and MRP-2) and chronic inactive lesions (MRP-2) (Fig 2E).

P-glycoprotein and BCRP expression in control white matter and multiple sclerosis lesions and increased BCRP function in foamy macrophages.

P-glycoprotein immunoreactivity is predominantly localized to the cerebral microvasculature in NAWM (Fig 3C, arrows) and control brain tissue (data not shown) and only weakly expressed by astrocytes (arrowheads). Notably, in active demyelinated multiple sclerosis lesions (Fig 3E, arrows) and chronic inactive lesions (Fig 4C, arrow) a decreased vascular P-glycoprotein immunoreactivity was observed. Surprisingly, hypertrophic GFAP-positive astrocytes were markedly decorated with anti-P-glycoprotein in active and chronic inactive multiple sclerosis lesions (Fig. 3E, 3G, 4C, arrowheads). Quantification of relative P-glycoprotein immunoreactivity showed a significant increase in active and chronic inactive multiple sclerosis lesions as compared to NAWM (Fig 4E). 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 multiple sclerosis lesions a marked increased BCRP staining is observed on CD11b-positive foamy macrophages (Fig 3F,
3H), which correlated with enhanced BCRP functionality upon myelin phagocytosis of human macrophages at different time points (Fig 3I). In line with MRP-1 (Fig 1K), enhanced BCRP activity in 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-glycoprotein and BCRP in control tissue, which coincides with high P-glycoprotein and

![image]

Figure 4. P-glycoprotein and BCRP expression in chronic inactive multiple sclerosis lesions.

(A) Loss of proteolipid protein immunoreactivity in a subcortical lesion, with (B) a low number of major histocompatibility complex class II positive cells (magnification 10x). Boxed site is a representative area of the 40x magnification of adjacent sections stained for P-glycoprotein and BCRP. In chronic inactive lesions P-glycoprotein immunoreactivity (C) is prominent on hypertrophic astrocytes (arrowheads) and faintly present on the endothelium (arrow). BCRP immunoreactivity (D) in chronic inactive lesions is present on the endothelium (arrow) and resting microglial cells (arrowheads). These figures show representative images observed in all patient material. (E) Quantification of the relative difference in immunoreactivity for both P-glycoprotein and BCRP in NAWM, active lesions, and inactive lesions. Presented as mean fold change from NAWM, +/-SEM. * p<0.05 by Students t test.
Figure 5. Increased P-glycoprotein and MRP-1 expression and function in reactive astrocytes. 
(A) Increased GFAP mRNA expression on primary human astrocytes isolated from active multiple sclerosis lesions compared to astrocytes isolated from control white matter. GFAP expression was determined by real-time quantitative PCR and presented as relative expression compared to GAPDH. Enhanced MRP-1 (B) and P-glycoprotein (C) functionality in primary human astrocytes isolated from active multiple sclerosis 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-glycoprotein) or 1.15 +/- 0.07 (MRP-1). GFAP, IL-6, S100β, vimentin, MDR1 (P-glycoprotein) and MRP-1 transcripts from control or poly I:C-treated (24 hr) astrocytes (D) were detected by real-time quantitative 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 Student's t test.

BCRP activity in human brain endothelial cells in vitro (supplementary figure 1). However, in contrast to P-glycoprotein, no differences in endothelial BCRP expression were observed between multiple sclerosis lesions and normal appearing white matter. The increase in relative BCRP immunoreactivity observed in active multiple sclerosis lesions as compared to NAWM (Fig 4E) can be explained by the presence of BCRP positive macrophages, which are absent in chronic inactive multiple sclerosis lesions.

Increased expression and function of P-glycoprotein and MRP-1 in reactive astrocytes in vitro.

Increased protein expression of P-glycoprotein and MRP-1 on reactive astrocytes in multiple sclerosis lesions suggests an altered function of these efflux pumps under neuroinflammatory conditions. To study this, we first isolated astrocytes from multiple
sclerosis lesions and control white matter and determined the mRNA expression level of the (reactive) astrocyte marker GFAP by means of quantitative PCR (qPCR). Interestingly, multiple sclerosis lesion-derived astrocytes display increased transcription levels of GFAP (Fig. 5A), illustrating a reactive phenotype. Next, in vitro functional assays for P-glycoprotein and MRP1 were performed on control and multiple sclerosis lesion-derived astrocytes. Notably, reactive astrocytes isolated from multiple sclerosis lesions display increased functionality of both efflux pumps (Fig 5B,C), which correlates with the enhanced expression levels of astrocytic P-glycoprotein and MRP-1 in human multiple sclerosis lesions (figure 1-4). To investigate whether inflammatory mediators could affect astrocytic P-glycoprotein and MRP-1 activity, we treated primary human astrocytes with inflammatory mediators like TNF-α and/or poly I:C, a dsRNA mimic ligand for Toll-like receptor 3, to mimic a pro-inflammatory environment as observed during neuroinflammation. Interestingly, both inflammatory mediators increased MRP-1 and P-glycoprotein efflux capacity by astrocytes (Fig 5B,C), with Toll-like receptor-3 activation being the most potent inducer (Fig 5B,C). To determine whether Toll-like receptor-3 activation on astrocytes leads to a reactive astrocyte phenotype, we verified mRNA expression levels of various reactive astrocytic markers like GFAP, S100β, vimentin and IL-6 and the ABC transporters P-glycoprotein (MDR1) and MRP-1 by means of real-time quantitative PCR. Notably, Poly I:C-treated astrocytes display increased transcription levels of GFAP, IL-6, S100β, MDR1 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 Toll-like receptor-3 activation. Together, these results show that P-glycoprotein and MRP-1 expression and function are highly increased in inflammatory reactive astrocytes.

**P-glycoprotein and MRP-1 on reactive astrocytes mediate monocyte migration across a blood-brain barrier model.**

Reactive astrocytes contribute to the inflammatory process by the production and secretion of proinflammatory cytokines and chemokines. In particular chemokines, like chemokine (C-C motif) ligand 2 (CCL2) are known to attract leukocytes and monocyte-derived macrophages into multiple sclerosis lesions, which in turn results in severe tissue damage. As ABC transporters are suggested to be involved in the secretion of inflammatory mediators, we investigated whether P-glycoprotein and MRP-1 are capable to regulate the efflux of the astrocyte-derived chemokine CCL2. Our results show that Poly I:C-treated astrocytes secrete (Fig 6A) and produce (Fig 6B) high levels of CCL2 compared to control astrocytes. Notably, blocking P-glycoprotein or MRP-1 activity with specific inhibitors (see supplementary figure 1) like reversin 121 and MK-571 respectively, significantly reduced CCL2 secretion from reactive astrocytes (Fig 6C), whereas CCL2 mRNA expression levels remained unaffected (Fig 6D). Moreover, P-glycoprotein and MRP-1 on reactive astrocytes did not affect the secretion of the proinflammatory cytokines IL-1β (Fig 6E) or IFN-γ (data not shown), indicating that these transporters are selectively involved in CCL2 secretion, but not the production of CCL2 from reactive astrocytes. As CCL2 is a potent chemokine involved in leukocyte migration, we hypothesize that P-glycoprotein and MRP-1 on reactive astrocytes contribute to the inflammatory process by mediating CCL2 efflux and induce immune cell migration. To assess this, we determined the potential role for these transporters in mediating monocyte migration in a chemotaxis...
Chapter 4

Figure 6. P-glycoprotein and MRP-1 mediate CCL2 secretion from reactive astrocytes

Primary human astrocytes were treated with or without poly I:C (50 μg/ml) for 24 hr and CCL2 secretion was determined in cell supernatants by enzyme-linked immunosorbent assay (A) and CCL2 transcripts were determined by real-time quantitative PCR and presented as relative expression (F: fold induction) compared to GAPDH (B). Astrocytes were treated with poly I:C (50 μg/ml) for 24 hr in the presence or absence of the P-glycoprotein inhibitor verapamil 121 (10 μM) or the MRP-1 inhibitor MK-571 (25 μM), after which CCL2 secretion (C) and expression (D) or IL-1β secretion (E) was determined by enzyme-linked immunosorbent assay (C,D,E) or real-time quantitative PCR (D). 100% corresponds to 18.0 ± 0.46 μg/ml CCL2 (C) or 0.032 ± 0.003 CCL2 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.

assay. Conditioned media from Poly I:C-treated astrocytes or multiple sclerosis lesion-derived astrocytes significantly increased monocyte migration across filters (Fig 7A) compared to conditioned media from control astrocytes. Interestingly, blocking MRP-1 and in particular P-glycoprotein activity strikingly reduced the migration capacity of monocytes (Fig 7A), indicating that both P-glycoprotein and MRP-1 on reactive astrocytes are involved
ABC transporters in multiple sclerosis lesions

Figure 7. P-glycoprotein and MRP-1 on reactive astrocytes mediate monocyte migration.
Conditioned media from control astrocytes, poly I:C treated astrocytes or multiple sclerosis lesion derived astrocytes (cultured in the presence or absence of specific P-glycoprotein or MRP-1 inhibitors) was used to assess its capacity to attract monocytes in a chemotaxis assay (A) or in a Transwell migration assay (B), which represents an in vitro model of the blood-brain barrier. Data is presented as absolute numbers of migrated monocytes compared to the total number of monocytes added in each well. Experiments were performed in triplicate using 3 different human donors (both control and multiple sclerosis donors) and were presented as the mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001 compared to vehicle treated control astrocytes; "p<0.001 compared to vehicle poly I:C treated astrocytes; "p<0.05, #p<0.001 compared to vehicle multiple sclerosis lesion astrocytes as determined by Students t test.

Discussion
In this study we provide for the first time a comprehensive overview of ABC transporter expression in multiple sclerosis brain tissue and we illustrate their potential contribution to neuroinflammation. Predominant cell types involved in multiple sclerosis pathology, including brain endothelial cells, reactive astrocytes and infiltrated foamy macrophages, display marked alterations in their ABC transporter expression, which coincides with functional changes in vitro under inflammatory conditions. Moreover, we here show that Toll-like receptor-3 activation in astrocytes induces enhanced expression and function of P-glycoprotein and MRP-1. We here provide evidence that the astrocytic ABC transporters may play a role in the neuro-inflammatory process by mediating the efflux of the inflammatory molecule CCL2, thereby promoting immune cell migration across brain endothelial cells.

In different stages of multiple sclerosis lesions we observed an altered expression pattern of various ABC transporter proteins like P-glycoprotein, MRP-1, MRP-2 and BCRP. In particular reactive astrocytes, which are abundantly present in multiple sclerosis lesions, display enhanced expression of P-glycoprotein, MRP-1 and MRP-2. So far, enhanced astrocytic expression of P-glycoprotein and MRP-1 has been reported in brain tissue of epilepsy patients^41, 42, which has been suggested to be a result of seizures or drug treatment.
Moreover, we observed enhanced expression of BCRP, MRP-1 and MRP-2 on infiltrated foamy macrophages in active multiple sclerosis lesions. Notably, enhanced function of BCRP and MRP-1 in 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 ABCG141. Moreover, as both MRP-1 and BCRP participate in cellular detoxification44, 45, it is likely that both 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 treatment41. We here demonstrate that macrophages upon myelin phagocytosis establish increased MRP-1 and BCRP activity, which is in line with the increased expression pattern of these ABC transporters in inflammatory multiple sclerosis lesions.

In control white matter and NAWM, we observed a cerebrovascular expression of P-glycoprotein and BCRP while MRP-1 and MRP-2 are weakly expressed by brain endothelial cells. In vitro functional assays confirmed that both P-glycoprotein and BCRP are highly active on human brain endothelial cells, whereas MRP-1 function is nearly absent. Notably, we detected a decreased endothelial P-glycoprotein expression in multiple sclerosis lesions, whereas no differences in endothelial BCRP, MRP-1 and MRP-2 were observed. We have previously shown that vascular P-glycoprotein expression and function is strongly decreased during multiple sclerosis pathology and identified a crucial role for activated CD4+ T cells in endothelial P-glycoprotein regulation via intracellular adhesion molecule -1 and nuclear factor kappa B signalling47. 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 and further research is warranted to define these underlying differences. Cerebrovascular expression has previously been shown for BCRP44, MRP-248 and to a lesser extent for MRP-149. In contrast, other groups did not detect the MRP1 protein on the microvasculature when analyzed by immunohistochemistry50, 51, which might be explained by the usage of different antibodies to MRP-1. Together, our results demonstrate the expression of P-glycoprotein, BCRP, MRP-1 and MRP-2 in the cerebral vasculature in NAWM, of which P-glycoprotein is selectively affected during multiple sclerosis pathology. In both active and inactive multiple sclerosis lesions, we observed an increased astrocytic P-glycoprotein and MRP-1 expression, which correlated with enhanced P-glycoprotein and MRP-1 activity of lesion-derived astrocytes 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-associated dementia52. Conversely, an equal body of evidence suggest that astrocyte activation can also exert beneficial effects (see review53), as reactive astrocytes can secrete neurotrophic factors. However, severe activation might augment an inflammatory response, leading to neuronal death and brain injury9. In spite of the ubiquitous presence of reactive astrocytes at various sites of central nervous system pathology, their potential contribution to pathology and underlying mechanisms are still poorly understood. To study the potential contribution of reactive astrocytes in multiple sclerosis pathology in more detail, we first generated reactive astrocytes in vitro by Toll-like

90
ABC transporters in multiple sclerosis lesions

receptor-3 activation of primary human astrocyte cultures, which resulted in high mRNA expression levels of the reactive astrocyte marker GFAP to a similar extent as observed in astrocytes isolated from multiple sclerosis lesions. Furthermore, Toll-like receptor-3 activation enhanced the expression of S100b and IL-6, which are well-known markers for astrocyte activation\textsuperscript{37}, indicating that Toll-like receptor-3 activation is a suitable method for the generation of reactive astrocytes in vitro. Notably, TNF-\textalpha\ treatment and Toll-like receptor-3 activation of astrocytes led to an increased P-glycoprotein and MRP-1 activity, 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 transcriptional level ABC transporters are under the control of the orphan nuclear receptors such as steroid and xenobiotic receptor (or pregnane X receptor in rodents)\textsuperscript{31}. Furthermore, their expression and function are regulated by environmental stimuli that evoke stress responses\textsuperscript{33}, like the excitatory neurotransmitter glutamate\textsuperscript{34} or the inflammatory cytokines\textsuperscript{35}. So far, only regulation of brain endothelial P-glycoprotein expression and function has been reported\textsuperscript{35,36}. We here extend these results by demonstrating that inflammatory mediators can affect both MRP-1 and P-glycoprotein expression and function on human astrocytes.

In this study we revealed a novel pathophysiological role for P-glycoprotein and MRP-1 on reactive astrocytes in mediating immune cell migration across brain endothelial cells, which may aggravate the inflammatory attack during multiple sclerosis lesion progression. Active multiple sclerosis lesions are characterized by the presence of infiltrated leukocytes and chemokines like CCL2 play a key role in the attraction of immune cells into these multiple sclerosis lesions\textsuperscript{38}. Notably, mice lacking the receptor for CCL2 (CCR2) did not develop experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis\textsuperscript{57,58}, indicating its in vivo relevance. Moreover, CCL2 expression appeared to be restricted to reactive astrocytes in multiple sclerosis lesions\textsuperscript{59}. These in vivo observations were confirmed in vitro in our study, as reactive astrocytes produce and secrete high levels of CCL2 upon Toll-like receptor-3 activation. Moreover, we identified a novel role for P-glycoprotein and MRP-1 in the modulation of CCL2 secretion from reactive astrocytes. These results support the hypothesis that endogenous substrates for ABC transporters may include inflammatory mediators, such as prostaglandins, leukotrienes and cytokines as observed in studies using immune cells\textsuperscript{64,68} and now possibly chemokines like CCL2. It yet remains unknown whether ABC transporters themselves are capable to transport chemokines or that they are involved in the secretion of other relevant more lipophilic inflammatory substrates like platelet activating factor\textsuperscript{69} that in turn may affect CCL2 secretion\textsuperscript{60} as a secondary effect. Nevertheless, increased ABC transporter expression and function on reactive astrocytes may result in local enhanced efflux of inflammatory mediators in multiple sclerosis lesions, amplifying the inflammatory response. To reveal such a novel pathophysiological role for the ABC transporters P-glycoprotein and MRP-1 on reactive astrocytes, conditioned media from either in vitro generated or multiple sclerosis lesion-derived reactive astrocytes markedly enhanced monocyte migration across an in vitro model of the blood-brain barrier. Moreover, blocking P-glycoprotein or MRP-1 on these reactive astrocytes severely inhibited this monocyte migration capacity, illustrating a novel detrimental role for these ABC transporters on reactive astrocytes by facilitating immune cell migration across brain endothelial cells. Recently, we reported that P-glycoprotein knockout mice developed reduced clinical signs of experimental autoimmune encephalomyelitis\textsuperscript{61}, illustrating that
ABC transporters may be considered as a potential therapeutic target. However, as ABC transporters like P-glycoprotein or MRP-1 are widely expressed on a variety of cells, including cells of the immune system, it is not feasible to specifically inhibit ABC transporters solely on central nervous system cells, such as astrocytes. Therefore, further research is warranted to generate mice that lack astrocyte-specific ABC transporters to unravel the role of astrocytic ABC transporters in multiple sclerosis pathogenesis in detail.

In conclusion, we here show that ABC transporter expression is markedly altered in multiple sclerosis 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-glycoprotein and MRP-1 were shown to mediate CCL2 secretion from reactive astrocytes, thereby controlling monocyte migration across a blood-brain barrier model. 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 multiple sclerosis pathology.

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Supplementary figure 1: ABC transporter inhibitor specificity.
P-glycoprotein, MRP1 and BCRP function was determined on human brain endothelial cells (A) or primary human astrocytes (B) by using specific transporter substrates (P-glycoprotein: Rhodamine 123; BCRP: Bodipy; MRP-1: Calc-AM) and specific inhibitors (P-glycoprotein: reversin 121; BCRP: KO147; MRP-1: MK571). ABC transporter function is presented as the ratio of drug fluorescence with inhibitor divided by drug fluorescence without inhibitor after subtraction of the fluorescence of the control. A ratio of 1 indicates that the inhibitor does not affect the transporter function. Human brain endothelial cells display P-glycoprotein and BCRP but not MRP-1 activity (A). Primary human astrocytes display P-glycoprotein and MRP-1 but not BCRP activity (B). All ABC transporters used in this assay have normal ratios between 1.3 and 1.5 when their specific inhibitors are used. Results represent three independent experiments performed in triplicate.

Supplementary figure 2: Segmentation analysis of representative DAB micrograph.
The original DAB micrograph of P-glycoprotein immunohistochemistry of an active lesion (A) shows both vascular (arrowhead) and glial (arrow) P-glycoprotein immunoreactivity. After color deconvolution (B), segmentation analysis was performed to measure the total area of specific immunoreactivity above a set threshold (shown in red), immunoreactivity below this threshold was not measured.