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

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Adenosine triphosphate-binding cassette efflux transporters are highly expressed at the blood–brain barrier and actively hinder passage of harmful compounds, thereby maintaining brain homeostasis. Since, adenosine triphosphate-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. Therefore, we investigated the expression pattern of different adenosine triphosphate-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 showed 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 adenosine triphosphate-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 adenosine triphosphate-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 (patho)physiological role for adenosine triphosphate-binding cassette transporters, suggesting that limiting their activity by dampening astrocyte activation may open therapeutic avenues to diminish tissue damage during multiple sclerosis pathogenesis.

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Introduction

Multiple sclerosis is a chronic demyelinating disease of the CNS and is neuropathologically characterized by multiple focal demyelinated lesions scattered throughout the CNS (Ewing and Bernard, 1998; Lassmann et al., 2001; Lucchinetti et al., 2001; Frohman et al., 2006; Polman et al., 2006). Active multiple sclerosis lesions contain abundant cellular infiltrates, which mainly consist of T cells and monocyte-derived macrophages (Bruck et al., 1996). 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 (Li et al., 1996) and are thought to display an anti-inflammatory phenotype (Boven et al., 2006). 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 (Tani et al., 1996; Speth et al., 2005; Sofroniew, 2009).

The CNS 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 (Loscher and Potschka, 2005a). The ATP-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, such as 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 ATP hydrolysis (Loscher and Potschka, 2005b). Importantly, several studies have suggested that endogenous substrates for ABC transporters may include inflammatory mediators, such as steroids, prostaglandins, leucotrienes and cytokines (Drach et al., 1996; Meijer et al., 1998; Ernste and Bello-Reuss, 1999; Frank et al., 2001; Raggards et al., 2001; Choudhuri and Klaassen, 2006; van de Ven et al., 2009). 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, MRP-2 and BCRP) in well-characterized multiple sclerosis lesions. We demonstrate here that various CNS 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 astrocitic 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 Centre, Amsterdam, The Netherlands. Tissue samples from four 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 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 staining, 5μm cryosections were air-dried and fixed in acetone for 10 min. Sections were incubated overnight at 4°C with primary antibodies (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-labelled horseradish peroxidase (DAKO, Glostrup, Denmark) for 30 min at room temperature. For the detection of MRP-1, sections were incubated with biotin-labelled rabbit anti-rat antibody (DAKO, Glostrup, Denmark) for 30 min at room temperature and with avidin

**Keywords:** adenosine triphosphate-binding cassette transporter; multiple sclerosis; astrocytes; chemokine (C-C motif) ligand 2; blood–brain barrier

**Abbreviations:** ABC = adenosine triphosphate-binding cassette; BCRP = breast cancer resistance protein; CCL2 = chemokine (C-C motif) ligand 2; GFAP = glial fibrillary acidic protein; MRP = multidrug resistance-associated protein; NAWM = normal appearing white matter

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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 haematoxylin for 1 min and extensively washed with tap water for 10 min. 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 staining, sections were incubated for 30 min with 20% normal goat serum. Sections were then incubated overnight at 4°C with primary antibodies for all four transporters (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 subsequently labelled with Alexa-488 coupled goat anti-mouse antibody (for MRP-1, MRP-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 1 Clinical information of multiple sclerosis and control patient material

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Type of multiple sclerosis</th>
<th>Sex</th>
<th>Post-mortem delay (h)</th>
<th>Disease duration (years)</th>
<th>Cause of death</th>
</tr>
</thead>
<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>
<td>44</td>
<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>
<td>7:05</td>
<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>
<td>66</td>
<td>MS</td>
<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>
</tr>
<tr>
<td>Patient 9</td>
<td>77</td>
<td>SP</td>
<td>M</td>
<td>4:15</td>
<td>26</td>
<td>CVA</td>
</tr>
<tr>
<td>Patient 10</td>
<td>41</td>
<td>PP</td>
<td>M</td>
<td>7:20</td>
<td>n.k.</td>
<td>Pneumonia urosepsis</td>
</tr>
<tr>
<td>Control 1</td>
<td>78</td>
<td>–</td>
<td>M</td>
<td>4:40</td>
<td>–</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>Control 2</td>
<td>82</td>
<td>–</td>
<td>F</td>
<td>5:10</td>
<td>–</td>
<td>Pneumonia by haemothorax</td>
</tr>
<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>
</tbody>
</table>

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

### Table 2 Primary antibodies

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<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-glycoprotein (clone 15D3)</td>
<td>1:10</td>
<td>Department of Pathology (VUmc, Amsterdam)</td>
</tr>
<tr>
<td>MRP1 (clone MRPr1)</td>
<td>1:50</td>
<td>Department of Pathology (VUmc, Amsterdam)</td>
</tr>
<tr>
<td>MRP-1 (clone MRPrm5) (fluorescence)</td>
<td>1:25</td>
<td>Department of Pathology (VUmc, Amsterdam)</td>
</tr>
<tr>
<td>MRP2 (clone M2III-6)</td>
<td>1:50</td>
<td>Department of Pathology (VUmc, Amsterdam)</td>
</tr>
<tr>
<td>BCRP (clone BXP-21)</td>
<td>1:50</td>
<td>Department of Pathology (VUmc, Amsterdam)</td>
</tr>
</tbody>
</table>

Quantification of immunohistochemistry

Relative changes in immunoreactivity were quantified by using ImageJ software (v. 1.37c, NIH, USA) as described previously (Wang et al., 2010). In short, three donors encompassing both active and inactive multiple sclerosis lesions within one section, as well as a considerable area of normal appearing white matter (NAWM), were selected to correct for inter-donor variability. Various ×40 RGB micrographs were recorded for each donor, staining and white matter location (active lesion, inactive lesion and NAWM). Next, images were subjected to colour deconvolution to exclude haematoxylin staining from analysis. The relative area of specific 3,3'-diaminobenzidine deposition to total area was determined by using threshold segmentation. The threshold was separately determined for each ABC transporter and 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 Fig. 2.

Cell cultures

Primary astrocytes from control human brain tissue or multiple sclerosis lesions and primary monocytes were isolated and cultured as described previously (De Groot et al., 1997; Elkord et al., 2005). P-glycoprotein (CEM/VBL), MRP-1 (2008/MPR-1) and BCRP (MCF7) overexpressing cells and their control cell lines were obtained from and cultured as described by Oerlemans et al. (2006). The human brain endothelial cell line hCMEC/D3 was cultured as described previously (Wekslar et al., 2005).

Cell treatments

Primary human astrocytes and primary human monocytes were cultured in 24- or 96-well plates. Astrocytes were subsequently incubated with tumour necrosis factor-a (5 ng/ml; Peprotech, UK) or the Toll-like receptor-3 ligand polynosinic-cytidylic acid (50 μg/ml; Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 6 or 24 h 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 aforementioned treatments were washed away and conditioned media was collected after 24 h. Primary monocytes were either incubated with human myelin derived from control white matter (Van der Goes et al., 2005) or latex beads (Polysciences) for different time points (24 or 48 h).

In vitro assays for ATP-binding cassette transporter function

P-glycoprotein, MRP1 and BCRP function was determined as described previously (van der Pol et al., 2003) with minor modifications. Briefly, after treatment, astrocytes or macrophages were washed three times with phosphate-buffered saline and subsequently incubated for 45 min 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 a 45 min 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 and Dickinson, San Jose, CA, USA). Fluorescence-activated cell sorting 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/MPR1) 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 Fig. 1 for additional information.

Enzyme-linked immunosorbent assay

CCL2 or interleukin 1β protein was measured in culture supernatants of control or polynosinic–cytidylic acid stimulated astrocytes (24 h) 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 1 pg/ml (interleukin 1β) as described previously (Tekstra et al., 1996).

RNA isolation and real-time quantitative polymerase chain reaction

Messenger RNA was isolated from control or polynosinic–cytidylic acid stimulated astrocytes using a messenger RNA capture kit (Roche) according to the manufacturer’s instructions. Complementary DNA was synthesized with the Reverse Transcription System kit (Promega, USA) following the manufacturer’s guidelines and real-time quantitative polymerase chain reaction was performed as described previously (Garcia-Vallejo et al., 2004). All primer sequences are listed in Supplementary Table 1 and expression levels of transcripts obtained with real-time polymerase chain reaction were normalized to glyceraldehyde 3-phosphate dehydrogenase expression levels.

Monocyte migration

We used two established protocols for the measurement of human monocyte migration using the 48-well chemotaxis chamber (Frevert et al., 1998) and/or a Transwell system with cultured brain endothelial cells (Viegas et al., 2006) 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 and top plates of the chamber assembly and the monocytes (50 μl) were added to the top wells at a cell density of 4 × 10⁵ 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 haematoxylin/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 four days. At the start of the experiment, 600 μl of conditioned media from control or reactive astrocytes was added to the bottom wells. Monocytes (100 μl) were then added to the top wells at a cell density of 1 × 10⁶ monocytes/ml. Monocytes were allowed to migrate for 8 h (37°C and 5% CO₂). After 8 h, 400 μl was collected from the lower chamber and 20,000 beads (Beckman Coulter, USA) were added to each sample. Samples were then analysed using a FACScan flow cytometer (Becton and 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 with the total number of monocytes added in the upper chamber.

**Statistical analysis**

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

**Results**

**Multiple sclerosis lesion classification**

Classification of multiple sclerosis lesions was based on standard immunohistochemical staining for inflammatory cells (anti-major histocompatibility complex class II) and myelin (proteolipid protein) as described previously (van der Valk and De Groot, 2000; van Horssen J. et al., 2006a, b). Based on these findings, 12 lesions sampled in this study were classified as active with myelin loss (Figs 1A and 3A) and abundant phagocytic perivascular and parenchymal macrophages containing myelin degradation products (Figs 1B and 3B) and seven lesions as chronic inactive with demyelinated areas (Figs 2A and 4A) containing few major histocompatibility complex class II-positive cells (Figs 2B and 4B).

Enhanced multidrug resistance-associated protein-1 and -2 expression in multiple sclerosis lesions and increased multidrug resistance-associated protein-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 and 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 staining, 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 and I) and CD11b-positive macrophages (Fig. 1H and J). To study whether foamy macrophages are capable of actively removing substrates for MRP-1, we performed an in vitro functional MRP-1 assay. First, in vitro foamy macrophages were generated (Van der Goes et al., 2005; Boven et al., 2006) by adding myelin to human monocytes at different time points (24 and 48 h), 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 and 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-1 and MRP-2 in control or multiple sclerosis brain tissue (Figs 1 and 2), which coincided with low MRP-1 activity in cultured human brain endothelial cells using an in vitro efflux assay (Supplementary Fig. 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 Fig. 2 for detailed description). In line with the immunohistochemical staining, we observed increased transporter immunoreactivity in active lesions (MRP-1 and MRP-2) and chronic inactive lesions (MRP-2) (Fig. 2E).

**P-glycoprotein and breast cancer resistance protein expression in control white matter and multiple sclerosis lesions and increased breast cancer resistance protein expression 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, F and G) and chronic inactive lesions (Fig. 4C, arrow), 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 (Figs 3E, 3G and 4C, arrowheads). Quantification of relative P-glycoprotein immunoreactivity showed a significant increase in active and chronic inactive multiple sclerosis lesions as compared with 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 increase in BCRP staining is observed on CD11b-positive foamy macrophages (Fig. 3F and H), 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 BCRP activity in human brain endothelial cells in vitro (Supplementary Fig. 1). However, in contrast...
Figure 1. MRP-1 and MRP-2 expression in normal appearing white matter and multiple sclerosis 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 major histocompatibility complex class II (MHCII) (magnification × 10). Boxed sites are representative areas of the × 40 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 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

(continued)
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 with 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 multidrug resistance-associated protein-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 messenger RNA expression level of the (reactive) astrocyte marker GFAP (Pekny and Nilsson, 2005) by means of quantitative polymerase chain reaction. 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 and C), which correlates with the enhanced expression levels of astrocytic P-glycoprotein and MRP-1 in human multiple sclerosis lesions (Figs 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 tumour necrosis factor-α and/or polynosinic-cytidylic acid, a double-stranded RNA mimetic ligand for Toll-like receptor-3, to mimic a pro-inflammatory environment as observed during neuroinflammation (Obata et al., 2008). Interestingly, both inflammatory mediators increased MRP-1 and P-glycoprotein efflux capacity by astrocytes (Fig. 5B and C), with Toll-like receptor-3 activation being the most potent inducer (Fig. 5B and C). To determine whether Toll-like receptor-3 activation on astrocytes leads to a reactive astrocyte phenotype, we verified messenger RNA expression levels of various reactive astrocytic markers, such as GFAP, S100β, vimentin and interleukin 6 (Ridet et al., 1997) and the ABC transporters P-glycoprotein (MDR1) and MRP-1 by means of real-time quantitative polymerase chain reaction. Notably, polynosinic-cytidylic acid-treated astrocytes display increased transcription levels of GFAP, interleukin 6, S100β, vimentin and interleukin 6 (Ridet et al., 1997) and the ABC transporters P-glycoprotein (MDR1) and MRP-1 by means of real-time quantitative polymerase chain reaction. Notably, polynosinic-cytidylic acid-treated astrocytes display increased transcription levels of GFAP (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 multidrug resistance-associated protein-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 (Tani et al., 1996; Speth et al., 2005). In particular chemokines, such as chemokine (C-C motif) ligand 2 (CCL2), are known to attract leucocytes and monocyte-derived macrophages into multiple sclerosis lesions, which in turn results in severe tissue damage (Tani and Ransohoff, 1994). As ABC transporters have been suggested to be involved in the secretion of inflammatory mediators, we investigated whether P-glycoprotein and MRP-1 are capable of regulating the efflux of the astrocyte-derived chemokine CCL2. Our results show that polynosinic-cytidylic acid-treated astrocytes secrete (Fig. 6A) and produce (Fig. 6B) high levels of CCL2 compared with control astrocytes. Notably, blocking P-glycoprotein or MRP-1 activity with specific inhibitors (see Supplementary Fig. 1) such as reversin 121 (Koubeissi et al., 2006) and MK-571 (van de Ven et al., 2006), respectively, significantly reduced CCL2 secretion from reactive astrocytes (Fig. 6C), whereas CCL2 messenger RNA expression levels remained unaffected (Fig. 6D). Moreover, P-glycoprotein and MRP-1 on reactive astrocytes did not affect the secretion of the proinflammatory cytokine interleukin 1β (Fig. 6E) or interferon-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 assay. Conditioned media from polynosinic-cytidylic acid-treated astrocytes or multiple sclerosis lesion-derived astrocytes significantly increased monocyte migration across filters (Fig. 7A) compared with 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 in leukocyte migration processes. To mimic the blood–brain barrier in
more detail, we next used a Transwell system using human brain endothelial cells cultured on Transwell filters. Notably, conditioned media from reactive astrocytes significantly enhanced monocyte migration across brain endothelial cells (Fig. 7B), which could be blocked by using specific P-glycoprotein or MRP-1 inhibitors (Fig. 7B). Together these results indicate that reactive astrocytes are actively involved in the inflammation process during multiple sclerosis lesion formation and point to a novel role of ABC transporters on reactive astrocytes in inducing monocyte migration across brain endothelial cells under pathological conditions.

**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 the potential contribution of ABC transporters to neuroinflammation. The 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.
Figure 3  P-glycoprotein and BCRP expression in normal appearing white matter and multiple sclerosis lesions and increased BCRP function on foamy macrophages. (A) Loss of proteolipid protein (PLP) immunoreactivity in a subcortical lesion, with (B) enhanced expression of major histocompatibility complex class II positivity (magnification ×10). Boxed sites are representative areas of the ×40 magnification of adjacent sections stained for P-glycoprotein (P-gp) and BCRP. In NAWM, P-glycoprotein immunoreactivity (C) is observed on endothelium
conditions. Moreover, we show here that Toll-like receptor-3 activation in astrocytes induces enhanced expression and function of P-glycoprotein and MRP-1. We provide evidence that the astrocytic ABC transporters may play a role in the neuroinflammatory 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, such as 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 patients with epilepsy (Sisodiya et al., 2002; Marroni et al., 2003), 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, MRP-1 and MRP-2 are weakly expressed by brain endothelial cells. A value of 100% corresponds to a ratio of 1.19

vascular expression of P-glycoprotein and BCRP, while 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 κ B signalling (Kooij et al., 2010). 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 BCRP (Krishnamurthy and Schuetz, 2006), MRP-2 (Potschka et al., 2003) and to a lesser extent for MRP-1 (Nies et al., 2004). In contrast, other groups did not detect the MRP-1 protein on the microvasculature when analysed by immunohistochemistry (Rao et al., 1999; Aronica et al., 2003), which might be explained by the use 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 with 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 dementia (Eng and Ghirnikar, 1994). Conversely, an equal body of evidence suggest that astrocyte activation can also exert beneficial effects (see Sofroniew, 2009 for a review), as reactive astrocytes can secrete neurotrophic factors. However, severe activation might augment an inflammatory response, leading to neuronal death and brain injury (Tani et al., 1996). In spite of the ubiquitous presence of reactive astrocytes at various sites of CNS 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 receptor-3 activation of primary human astrocyte cultures, which resulted in high messenger RNA 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 interleukin 6, which are well-known markers for...
Astrocyte activation (Ridet et al., 1997), indicating that Toll-like receptor-3 activation is a suitable method for the generation of reactive astrocytes in vitro. Notably, tumour necrosis factor-α 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) (Loscher and Potschka, 2005b). Furthermore, their expression and function are regulated by environmental stimuli that evoke stress responses (Sukhai and Piquette-Miller, 2000), such as the excitatory neurotransmitter glutamate (Zhu and Liu, 2004) or the inflammatory cytokines (Bauer et al., 2007). So far, only regulation of brain endothelial P-glycoprotein expression and function has been reported (Goralski et al., 2003; Bauer et al., 2007). We here extend these results by demonstrating that inflammatory

Figure 4 P-glycoprotein and BCRP expression in chronic inactive multiple sclerosis lesions. (A) Loss of proteolipid protein (PLP) immunoreactivity in a subcortical lesion, with (B) a low number of major histocompatibility complex class II (MHCII) positive cells (magnification × 10). Boxed site is a representative area of the × 40 magnification of adjacent sections stained for P-glycoprotein and BCRP. In chronic inactive lesions P-glycoprotein (P-gp) 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 (P-gp) and BCRP in NAWM, active lesions and inactive lesions. Presented as mean fold change from NAWM ± SEM. *P < 0.05 by Student’s t-test.
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 such as CCL2 play a key role in the attraction of immune cells into these multiple sclerosis lesions (Tani and Ransohoff, 1994). Notably, mice lacking the receptor for CCL2 (CCR2) did not develop experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis (Fife et al., 2000; Izikson et al., 2000), indicating its in vivo relevance. Moreover, CCL2 expression appeared to be restricted to reactive astrocytes in multiple sclerosis lesions (van der Voorn et al., 1999). 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 (Drach et al., 1996; Meijer et al., 1998; Ernest and Bello-Reuss, 1999; Frank et al., 2001; Choudhuri and Klaassen, 2006) and now possibly chemokines such as CCL2. It still remains unknown whether ABC transporters themselves are capable of transporting chemokines or if they are...
involved in the secretion of other relevant more lipophilic inflammatory substrates such as platelet activating factor (Raggers et al., 2001) that in turn may affect CCL2 secretion (Huang et al., 1999) 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.

Figure 6 P-glycoprotein and MRP-1 mediate CCL2 secretion from reactive astrocytes. Primary human astrocytes were treated with or without polyinosinic-cytidylic acid (poly I:C) (50 μg/ml) for 24 h and CCL2 secretion was determined in cell supernatants by enzyme-linked immunosorbent assay (A). CCL2 transcripts were determined by real-time quantitative polymerase chain reaction and presented as relative expression (FI = fold induction) compared with glyceraldehyde 3-phosphate dehydrogenase (B). Astrocytes were treated with polyinosinic-cytidylic acid (50 μg/ml) for 24 h in the presence or absence of the P-glycoprotein inhibitor (P-gp inh) reversin 121 (10 μM) or the MRP-1 inhibitor (MRP-1 inh) MK-571 (25 μM), after which CCL2 secretion (C) and expression (D) or interleukin 1β (IL-1β) secretion (E) was determined by enzyme-linked immunosorbent assay (C and E) or real-time quantitative polymerase chain reaction (D). One hundred percent corresponds to 18.0 ± 0.46 μg/ml CCL2 (C) or 0.032 ± 0.003 CCL2 expression relative to glyceraldehyde 3-phosphate dehydrogenase (D). Experiments were performed in triplicate using three different human donors and were presented as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test.
across brain endothelial cells. Recently, we reported that P-glycoprotein knockout mice developed reduced clinical signs of experimental autoimmune encephalomyelitis (Kooij et al., 2009), illustrating that ABC transporters may be considered as a potential therapeutic target. However, as ABC transporters such as 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 CNS 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 show here 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 coincide 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 pathophysiological conditions, and may open therapeutic avenues to diminish the neuroinflammatory attack during multiple sclerosis pathology.

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Supplementary material
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