The role of ATP-binding cassette transporters in neuro-inflammation: relevance for bioactive lipids

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ATP-binding cassette (ABC) transporters are highly expressed by brain endothelial cells that form the blood–brain barrier (BBB). These efflux pumps play an important role in maintaining brain homeostasis as they actively hinder the entry of unwanted blood-derived compounds into the central nervous system (CNS). Consequently, their high activity at the BBB has been a major hurdle for the treatment of several brain diseases, as they prevent numerous drugs to reach their site of action within the brain. Importantly, recent data indicate that endogenous substrates for ABC transporters may include inflammatory mediators, such as prostaglandins, leukotrienes, cytokines, chemokines, and bioactive lipids, suggesting a potential role for ABC transporters in immunological responses, and more specifically in inflammatory brain disorders, such as multiple sclerosis (MS). In this review, we will give a comprehensive overview of recent findings that illustrate this novel role for ABC transporters in neuro-inflammatory processes. Moreover, we will provide first insights into underlying mechanisms and focus on the importance for bioactive lipids, in particular platelet-activating factor, herein. A thorough understanding of these events may form the basis for the development for selective treatment modalities to dampen the neuro-inflammatory attack in MS and thereby reducing tissue damage.

Keywords: ATP-binding cassette transporters, blood–brain barrier, multiple sclerosis, astrocytes, bioactive lipids, platelet-activating factor, chemokines

ABC TRANSPORTERS AT THE BLOOD–BRAIN BARRIER

The blood–brain barrier (BBB) protects the central nervous system (CNS) from the entry of unwanted compounds and leukocytes, thereby maintaining brain homeostasis. However, during neuro-inflammatory diseases like multiple sclerosis (MS), immune cells traverse the endothelial barrier and accumulate within the brain parenchyma where they cause extensive tissue damage leading to neurological deficits (Frohman et al., 2006; Comabella and Khoury, 2012). The BBB is in essence formed by specialized brain endothelial cells, however surrounding cells, like astrocytes and pericytes also play a key role in maintenance of barrier features (Abbott et al., 2006). Next to the presence of complex tight junctions, barrier properties of the BBB are instated by the presence of specific endothelial ATP-binding cassette (ABC) transporters, which actively remove unwanted compounds from the brain. The ABC transporter family consists of a variety of efflux pumps (Loscher and Potschka, 2005a), of which a few are generally regarded as key BBB transporters, including P-glycoprotein (P-gp), breast-cancer resistance protein (BCRP), and the multi-drug

resistance-associated proteins-1 and -2 (MRP-1, -2). In general, ABC transporters 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). Expression and function of these transporters can be regulated by a broad variety of endogenous and exogenous factors, including nuclear receptors like steroid and xenobiotic receptors (Loscher and Potschka, 2005b) or a variety of inflammatory molecules (see review Miller, 2010).

ABC TRANSPORTERS AND NEURO-INFLAMMATION

Most data on the function of ABC transporters originates from oncology research and is based on their capacity to cause multi-drug resistance (MDR; Lage, 2008). To date, evidence is accumulating that ABC transporters may also contribute to immunological processes based on their expression on various immune cells like antigen presenting cells (Randolph et al., 1998; van de Ven et al., 2006) and their potential to secrete inflammatory molecules like leukotrienes and prostaglandins (see review van de Ven et al., 2009). Together with their high expression on the BBB, ABC transporters may modulate inflammatory processes that occur in CNS disorders, including MS, a chronic inflammatory disorder of the CNS leading to severe neurological deficits. Pathologically, active MS lesions contain abundant cellular infiltrates, which mainly consist of T-cells and macrophages with a foamy appearance (Bruck

et al., 1996). The latter 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 contribute to myelin sheaths damage, resulting in neuronal dysfunction. In the course of lesion progression, enlarged proliferative astrocytes become the most predominant cell type. These reactive astrocytes secrete neurotrophic factors for neuronal survival, but also contribute to pathology by producing pro-inflammatory cytokines and chemokines (Tani et al., 1996; Speth et al., 2005; Sofroniew, 2009), which subsequently attract leukocytes into MS lesions.

ATP-binding cassette transporters have been implicated in various neurological disorders, including Alzheimer's disease (Vogelgesang et al., 2002), HIV encephalitis (Langford et al., 2004), Parkinson's disease (Bartels et al., 2008), and epilepsy (Sisodiya et al., 2002; Aronica et al., 2011). However, as these disorders lack a profound inflammatory component, the potential involvement for ABC transporters in neuro-inflammation has been long overseen. Conversely, we recently identified an important role for ABC transporters in neuro-inflammatory processes underlying MS pathogenesis (Kooij et al., 2009, 2010, 2011). We demonstrated that P-gp, MRP-1, and -2 are absent on healthy brain astrocytes, but their expression is highly increased on reactive astrocytes in MS lesions. In contrast, the expression of P-gp on endothelial cells of the BBB was slightly reduced in active lesions (Kooij et al., 2010), whereas the expression of endothelial BCRP, MRP-1 and -2 remained unaffected. Moreover, we showed that the astrocytic ABC transporters P-gp and MRP-1 mediated the secretion of chemokine (C–C motif) ligand 2 (CCL2), which in turn promotes immune cell migration in an *in vitro* model of the BBB (Kooij et al., 2011). *In vivo* evidence supporting a role for P-gp in immunomodulation was obtained using animals that lack P-gp ($mdr1a/1b^{-/-}$ animals), as they experience a significant reduction in clinical symptoms of experimental autoimmune encephalomyelitis (EAE; Kooij et al., 2009), a validated animal model for MS. Overall, these results strengthen the hypothesis that in addition to exporting unwanted compounds from cells, ABC transporters can actively interfere in immune processes by secreting inflammatory molecules, thereby illustrating a novel (patho)physiological role for these transporters in neuro-inflammatory disorders.

BIOACTIVE LIPIDS

A controversial issue remains whether ABC transporters themselves are capable to transport inflammatory molecules as suggested by some groups (Gsur et al., 1996; Frank et al., 2001) or that ABC transporters mediate the secretion of other relevant physiological substrates, such as bioactive lipids (Raggers et al., 2001) that in turn affect cytokine secretion as a secondary effect (Huang et al., 1999). The latter seems plausible as a large group of ABC transporter substrates have a lipophilic nature (Lagas et al., 2008). To date, the ABC transporter mediated secretome of endogenous molecules remains to be determined, but likely include various bioactive lipids, which are known to regulate a variety of cellular signaling events through biophysical interactions with proteins that modify location, scaffolding, and signal transduction (Wiegmann et al., 1994; Ballou et al., 1996; Bradshaw et al., 1996;

dam-Klages et al., 1998; Kronke, 1999). For example, rapid and transient alterations in the ceramide content of neuronal membranes have been shown to regulate neuronal excitability, synaptic transmitter release, plasma membrane insertion, and removal of transmembrane receptors (Inokuchi et al., 1997; Rohrbough et al., 2004; Kajimoto et al., 2007; Wheeler et al., 2009; Norman et al., 2010). Similarly, ceramide metabolism has been shown to direct immune activation through regulating the formation and function of the immunological synapse, T-cell activation, and cell death (Ballou et al., 1996; D'Souza et al., 1996; Solomon et al., 2003; Falcone et al., 2004; Detre et al., 2006). Other lipids such as sphingosine-1-phosphate, prostaglandins, arachidonic acid, and platelet-activating factor (PAF) act directly as second messengers. Many of these lipid metabolites are released from cells and act as ligands to regulate function through specific receptor interactions. Although many of these lipid metabolites are released from cells in a regulated manner, the exact underlying mechanisms are currently not well defined. Understanding how these lipid-derived second messengers are exported from cells will further increase our understanding of immune regulation and could identify specific therapeutic targets to dampen aberrant immune function in disease settings such as MS.

MECHANISM OF ACTION: PLATELET-ACTIVATING FACTOR

Although the etiology of MS remains elusive, a crucial role has been established for the immune system, including a broad repertoire of inflammatory agents (Steinman, 2001; Hohlfeld and Wekerle, 2004; Comabella and Khoury, 2012). One of these agents is PAF, a potent pro-inflammatory phospholipid mediator with a wide range of biological activities (Stafforini et al., 2003). Several lines of evidence suggest a potential role for PAF in MS and EAE pathogenesis (Desai and Barton, 1990; Pedotti et al., 2003). Bioactivities of PAF are elicited by binding to the PAF receptor (PAFR), which belongs to the family of G-protein-coupled receptors (Honda et al., 1991). Interestingly, animals that lack the PAFR displayed lower incidence of disease and less severe clinical symptoms in the chronic phase of EAE compared to control mice (Kihara et al., 2005). Strikingly, no differences were observed in peripheral immune parameters that are associated with EAE, highlighting an important role for the PAFR in the neuropathology of EAE. Importantly, it has been demonstrated that PAF levels are elevated in the cerebrospinal fluid and plasma of MS patients (Callea et al., 1999). Together, these findings suggest that PAF likely contributes to MS pathogenesis; however, its cellular source in the CNS is yet unknown and data on the expression of PAFR in MS brain tissue remains elusive.

To first gain insight into the PAFR expression profile we performed a comprehensive immunohistochemical analysis to examine the distribution pattern of PAFR in various MS lesion stages. Classification of MS lesions was based on standard immunohistochemical analysis for myelin (proteolipid protein, PLP, **Figure 1A**). Active demyelination was demonstrated by the presence of phagocytic perivascular and parenchymal macrophages containing myelin degradation products (**Figure 1A**, insert) as described previously (van der Valk and De Groot, 2000; van Horssen et al., 2006a,b). In white matter from non-neurological control brain tissue (data not shown) and normal appearing
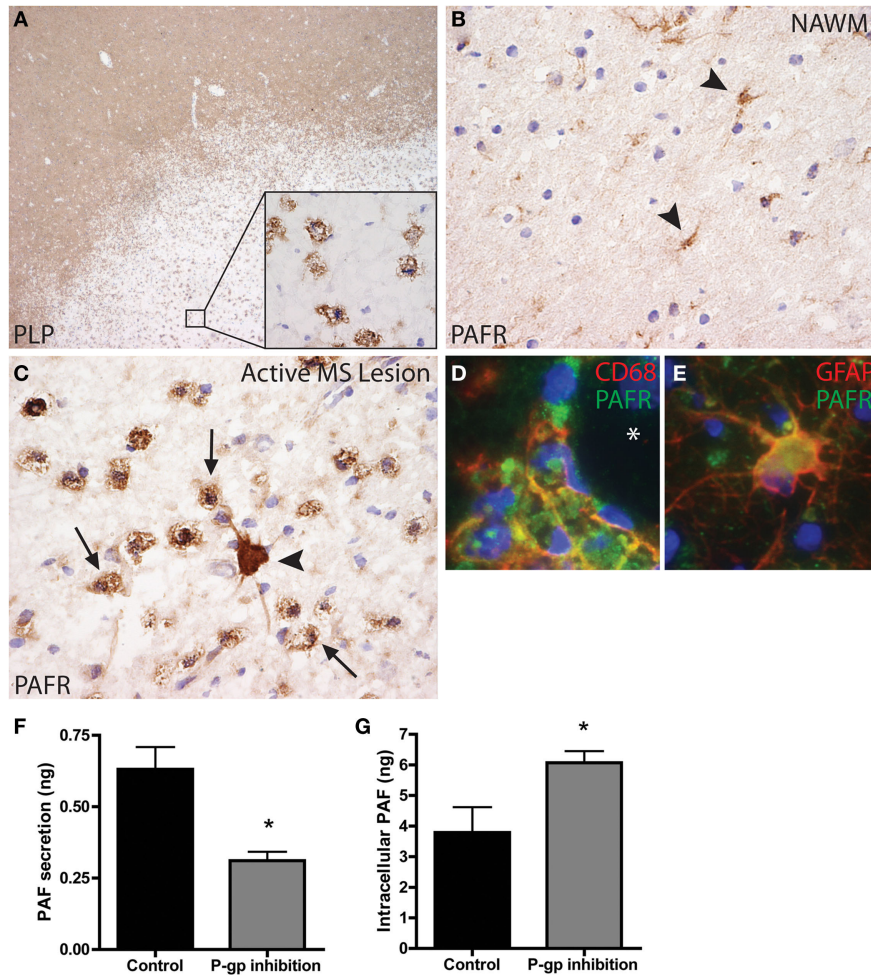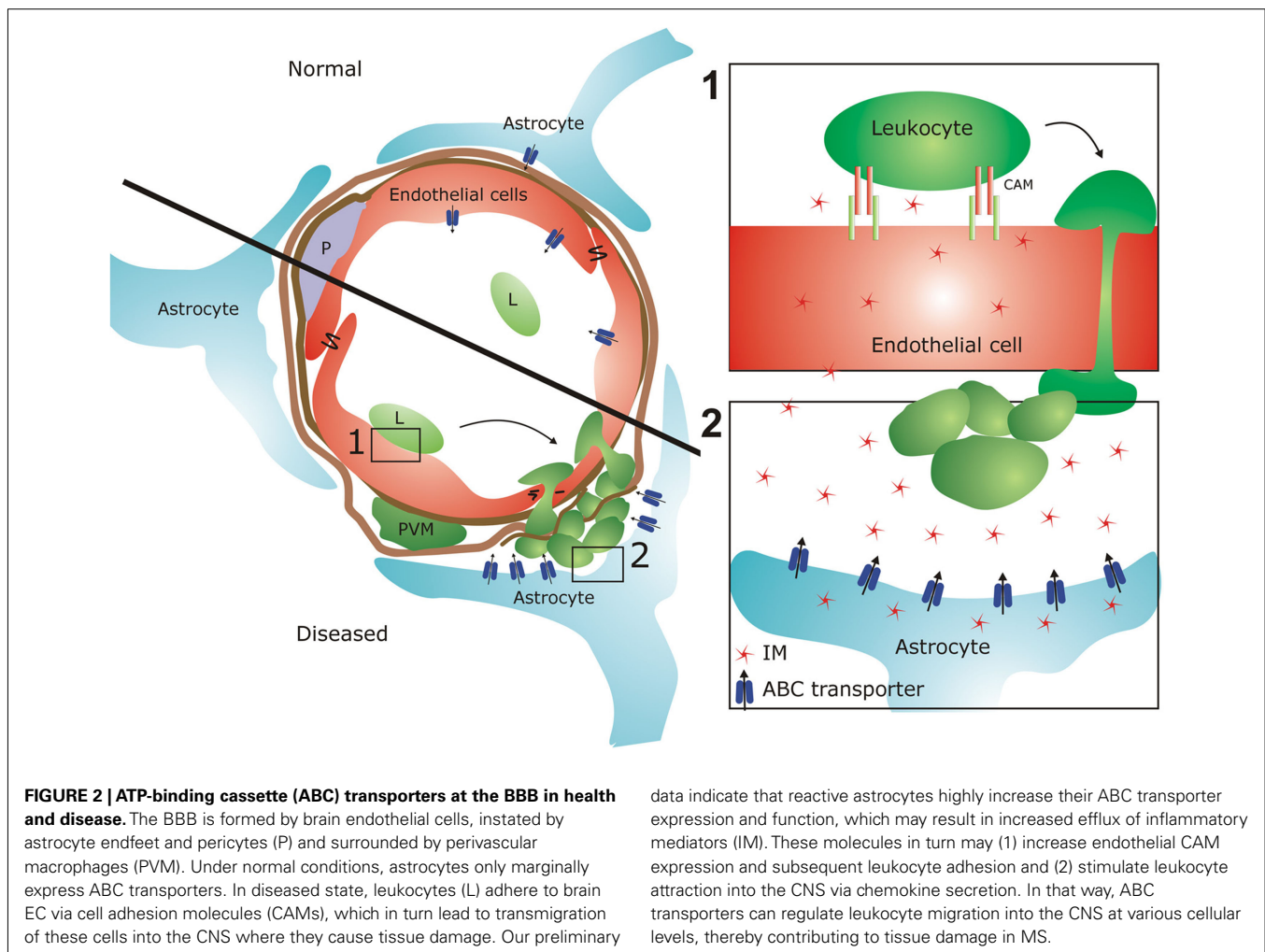
FIGURE 1 | Platelet-activating factor receptor (PAFR) expression in active MS lesions. (A) Active MS lesions are characterized by a loss of myelin (proteolipid protein, PLP), which is ingested by foamy macrophages (insert). **(B)** In normal appearing white matter (NAWM), PAFR expression was mainly restricted to glial cells (arrowheads). **(C)** In active lesions PAFR immunostaining was intense in reactive astrocytes (arrowhead) and foamy macrophages (arrows). Co-localization of PAFR immunoreactivity (green) with CD68 **(D)** and GFAP **(E)** immunoreactivity (in red) confirms the morphological observations. Original magnification: **(A)** 20×, **(B,C)** 200×, **(D,E)** 250×. PAF C16:0 levels were determined in supernatants and cell pellets derived from primary human reactive astrocytes from MS lesions using liquid chromatography electro spray ionization mass spectrometry (LC/ESI/MS/MS). P-gp inhibition reduced PAF secretion from reactive astrocytes **(F)**, whereas intracellular levels were increased **(G)**. $*p < 0.05$ by Student's t-test.

white matter (NAWM) PAFR (**Figure 1B**) immunoreactivity was mainly restricted to glial cells (arrowheads). In active demyelinating lesions (**Figure 1C**) abundant PAFR staining was observed in foamy macrophages (arrows) and hypertrophic astrocytes (arrowhead), whereas endothelial cells only weakly express PAFR. In chronic inactive MS lesions, PAFR was consistently upregulated in reactive astrocytes (data not shown). Using double immunofluorescence analysis we confirmed the cellular localization of PAFR in active MS lesions and showed that it is expressed by CD86-positive macrophages (**Figure 1D**) and GFAP-positive astrocytes (**Figure 1E**). These results are in line with microarray data showing increased levels of PAFR transcripts in MS lesions (Lock et al., 2002), but illustrate for the first time the cellular origin of PAFR. Kihara and co-workers (Kihara et al., 2005) have shown that binding of PAF to its receptor increases the phagocytic activity of macrophages, a process that may likely occur in MS lesion

development. However, the cellular source of PAF in the CNS during neuro-inflammation is to date unknown.

In general, PAF is produced by a variety of cells, including neutrophils, eosinophils, monocytes, macrophages, and vascular endothelial cells (Montrucchio et al., 2000). Although it is currently unknown how PAF is secreted from these cells, an important role has been suggested for ABC transporters like P-gp (Raggers et al., 2001). Our findings suggest an important role for P-gp on reactive astrocytes, as P-gp expression and function are highly increased early in the course of MS lesion formation (Kooij et al., 2011). Astrocytic P-gp may mediate the release of leuko-cyte attracting chemokines, a process that might be mediated by PAF. We isolated human primary reactive astrocytes directly from active MS lesions (as described previously by De Groot et al., 1997; Elkord et al., 2005) and cultured them in the presence or absence of the specific P-gp inhibitor reversin 121 for 6 h. Supernatants

FIGURE 2 | ATP-binding cassette (ABC) transporters at the BBB in health and disease. The BBB is formed by brain endothelial cells, instated by astrocyte endfeet and pericytes (P) and surrounded by perivascular macrophages (PVM). Under normal conditions, astrocytes only marginally express ABC transporters. In diseased state, leukocytes (L) adhere to brain EC via cell adhesion molecules (CAMs), which in turn lead to transmigration of these cells into the CNS where they cause tissue damage. Our preliminary data indicate that reactive astrocytes highly increase their ABC transporter expression and function, which may result in increased efflux of inflammatory mediators (IM). These molecules in turn may (1) increase endothelial CAM expression and subsequent leukocyte adhesion and (2) stimulate leukocyte attraction into the CNS via chemokine secretion. In that way, ABC transporters can regulate leukocyte migration into the CNS at various cellular levels, thereby contributing to tissue damage in MS.

and cell pellets were harvested and PAF C16:0 levels were determined using liquid chromatography electro spray ionization mass spectrometry (LC/ESI/MS/MS; Wheeler et al., 2008). Notably, PAF secretion was significantly reduced in the presence of P-gp inhibition, whereas intracellular levels were increased (**Figures 1F,G**). These data strongly suggest a direct role for P-gp in the release of PAF form reactive astrocytes, and provide the first mechanistic insights in the role of bioactive lipids and ABC transporters in MS lesion formation.

FUTURE PERSPECTIVES

Over the last decade, a novel role for ABC transporters in regulating immune responses is emerging. These new findings are challenging the current view of ABC transporters as gatekeepers of the BBB, but instead indicate that these transporters are able to contribute to degenerative and inflammatory responses under pathological conditions (**Figure 2**, model). Hence, further research is warranted to gain insight into the ABC transporter secretome under normal and inflammatory conditions to define a set of bioactive lipids that regulate the release of inflammatory mediators from cells. Moreover, future experiments need to define the exact mechanisms by which bioactive lipids induce cytokine/chemokine release from cells, or if they act as chemoattractants themselves (i.e., G-protein-coupled PAFR has many similarities with chemokine receptors). From a therapeutic point of view, targeting cell-specific ABC transporter substrates may be an attractive approach to dampen inflammation, as systemic inhibition of ABC transporters will likely cause major side effects. Next to that, more research is warranted to delineate the contribution of bioactive lipids in immune responses and neuro-inflammatory diseases like MS, using specific knock-out animals and/or selective inhibitors. Ultimately, this will open new therapeutic avenues to limit the neuro-inflammatory attack and prevent brain tissue damage.

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