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Experimental Model Systems to Define Mechanisms of Immune-Mediated Blood Brain Barrier Disruption in Acute Disseminated Encephalomyelitis (ADEM) and Acute Hemorrhagic Leukoencephalitis (AHLE)

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1. Introduction

Blood brain barrier (BBB) disruption is an integral feature of numerous neurological diseases with infectious, inflammatory, neoplastic and vascular components. It is of particular interest in the immune mediated neurological diseases multiple sclerosis (MS), acute disseminated encephalomyelitis (ADEM), and acute hemorrhagic leukoencephalitis (AHLE). In particular, AHLE is associated with very high mortality. A fundamental question in these diseases is the extent inflammatory immune cells contribute to CNS vascular permeability. This lack of understanding currently undermines therapeutic approaches to ameliorate uncontrolled BBB disruption. In this review, we highlight the current experimental model systems available to address the contribution of inflammatory cells in BBB disruption. The contribution of T cells and other immune cell subsets in altering blood brain barrier tight junctions in the experimental autoimmune encephalomyelitis (EAE), lipopolysaccharide (LPS), and virus induced CNS vascular permeability model systems is also addressed. Results obtained from the use of these model systems have put forward a defined role for neutrophils, CD4 and CD8 T cells and vascular endothelial growth factor (VEGF) in BBB disruption. Based on these findings, we describe models in which immune cells engage and alter CNS cell types of the neurovascular unit and define future avenues of research.

Acute disseminating encephalomyelitis (ADEM) and acute hemorrhagic leukoencephalitis (AHLE) are demyelinating diseases that usually present shortly after infections or vaccination (Callen et al., 2009; Gibbs et al., 2005). ADEM typically has an overall favorable prognosis, with 60-80% of patients showing a complete recovery (Dale et al., 2000; Gibbs et al., 2005; Rust, 2000; Tenembaum et al., 2002). AHLE, which is also known as Hurst’s disease, has been considered a severe form of ADEM and has a very poor prognosis, usually resulting in death in 2-14 days (Geerts et al., 1991; Hart & Earle 1975; Pinto et al., 2011; Posey et al., 1994; Suchowersky et al., 1983). This disease is characterized by extensive BBB disruption and microhemorrhage formation. Typical acute symptoms of AHLE include fever, malaise, and headache, followed by a rapid progression of multifocal neurologic signs.
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(Posey et al., 1994). The MRIs of AHLE patients present with edema, white matter lesions, and petechial hemorrhage (Gibbs et al., 2005; Fugate et al., 2010; Pinto et al., 2011). Neutrophilic leukocytosis in the CSF has also been shown in cases of AHLE (Pinto et al., 2011). Usually, the patient’s level of consciousness progressively declines, resulting in coma (Posey et al., 1994). AHLE progresses rapidly, causing an accelerated deterioration of the patient. This coupled with its close resemblance to herpes simplex encephalitis (HSVE) may cause this disease to be under diagnosed in addition to demonstrating a probably viral etiology to AHLE (Gibbs et al., 2005). Furthermore, recent clinical cases have shown that this commonly fatal disease may also result from severe H1N1 influenza infection (Fugate et al., 2010; Wang, G., et al., 2010) and Epstein-Barr virus (Befort et al., 2010). There are currently no standard treatments for ADEM or AHLE, and the etiology of these two disorders is still not well understood. Several studies have demonstrated that the neurovascular unit (NVU), which is composed of cerebral endothelial cells (CECs) surrounded by astrocytes, pericytes, axonal endings, and microglia (Begley, 2004), may ultimately control permeability of the BBB through tight junctions between CECs of the vasculature. These tight junctions are composed of the proteins occludin and claudin, which seem to provide the primary seal of the tight junction, JAMs, which may play a role in monocyte and leukocyte adhesion, (Ballabh et al., 2004; Hawkins & Davis 2005) and the zona-occludin proteins, which provide the stability for the formation of tight junctions (Lai et al., 2005). However, the extent inflammatory immune cells contribute to the alteration of the NVU to a permeable state remains largely undefined. This relative lack of knowledge currently undermines effective therapies to treat diseases characterized by uncontrolled disruption of the BBB. Therefore, it is essential for the role of immune cells to be investigated in experimental mouse models, including experimental autoimmune encephalomyelitis (EAE), lipopolysaccharide (LPS), and virus induced CNS vascular permeability model systems, including the lymphocytic choriomeningitis virus (LCMV) and the peptide induced fatal syndrome (PIFS) models. These models may lead to the development of effective therapeutic approaches for these devastating diseases.

2. The Concept of the Blood Brain Barrier (BBB)

Before delving into how the BBB can be altered by immune cells, contributing to a variety of neurological diseases, it is important to understand the basic concept of the BBB and how different molecules gain access to it. The BBB is a highly specialized brain endothelial structure that plays a role in separating components of the circulating blood from neurons (Zlokovic, 2008). It is important to note that diffusion of soluble factors in the blood is controlled by capillaries, which contain specialized tight junctions. Meanwhile, recruitment of immune cells takes place at postcapillary venules (Bechmann et al., 2007). While an intact BBB is essential, it is also important that certain blood derived factors, such as nutrients, be able to cross this barrier. This is achieved through tranacellular transport systems, including carrier-mediated, ion, active efflux, receptor-mediated, and caveolae-mediated transport. Carrier-mediated transport allows the transport of essential nutrients such as glucose to meet the metabolic needs of the brain. Ion transporters such as the sodium pump are important in order to meet energy demands. Active efflux of molecules from the brain endothelium is important for rapid removal of harmful substances such as ingested toxic lipophilic metabolites. Receptor-mediated transport systems have played an important role as targets for drug delivery to the brain. Caveolae, a special type of lipid raft, contributes to
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transcellular permeability by regulating endocytosis and transcytosis (Zlokovic, 2008). Therefore, the BBB has multiple mechanisms by which blood derived agents could enter the CNS. In this review, we will focus on the role of immune-mediated alterations of the neurovascular unit and the subsequent alterations in vascular endothelial cell tight junctions.

3. Organization of the Neurovascular Unit

The vascular permeability observed in ADEM and AHLE has generated a central question of how inflammation promotes alteration of the BBB. The BBB has unique features that enable it to create a solute impermeable barrier (Begley 2004). Based on electron microscopy data, the current model of the BBB is composed of CNS vasculature consisting of interlocking cerebral endothelial cells (CECs). Surrounding CECs are astrocytes, pericytes, axonal endings, and microglia (Begley 2004). Collectively, this group of cells is referred to as the neurovascular unit (NVU). Several recent models of BBB disruption place the NVU as a dynamic interacting multicellular network that ultimately controls permeability of the BBB (Begley, 2004; Minagar & Alexander, 2003; Zlokovic, 2008). Specific properties of each NVU cell type in maintaining BBB integrity are described below and depicted in Figure 1.

3.1 Cerebral Endothelial Cells (CECs)

CECs form tight junctions with one another, creating microvasculature that serves as a seal between the blood and CNS tissue (Fanning et al., 1999; Itoh et al., 1999; Tsukita et al., 1999; Wolburg & Lippoldt 2002). While these tight junctions are considered the actual gate of the BBB, it is thought that CECs alter tight junctions following signaling from other cells of the NVU (Demeuse et al., 2002; Marchi et al., 2009). Numerous in vitro and in vivo studies have demonstrated that CECs only form blood brain barrier properties if they are in the presence of astrocytes or astrocyte-derived factors (Hayashi et al., 1997; Janzer & Raff, 1987; Maxwell et al., 1987; Neuhaus et al., 1991; Sobue et al. 1999).

3.2 Astrocytes

Greater than 90% of the albuminal surface of CECs are in direct contact with astrocytes (Abbott, 2002; Demeuse, et al., 2002). Astrocytes have been shown in vitro to promote the opening and maintenance of CEC tight junctions upon stimulation (Schroeter et al., 2001; Siddharthan et al., 2007; Smith, 2007). For this reason, it is hypothesized that astrocytes play a critical role in maintaining CEC integrity through direct contact and chemical messengers in vivo (Abbott, 2005; Abbott et al., 2006; Haseloff et al., 2005). In addition, astrocytes form complex networks with vasculature, potentially communicating through endfeet and Ca²⁺ waves (Koehler et al., 2006). Their close association with CECs and their role in BBB formation make astrocytes a potential candidate NVU cell type involved in altering vascular permeability.

3.3 Pericytes

Pericytes have been found to promote tighter BBB formation and reduced transendothelial resistance in vitro (Dohgu et al., 2005; Ramsauer, et al., 2002). Pericytes are also an important source of TGF-β in CEC microvessel formation (Dohgu et al., 2005). Transgenic approaches to inhibit migration of pericytes to vasculature through selective disruption of PDGF-β in CECs results in susceptibility to vascular permeability and edema (Hellstrom et al., 2001).
This evidence, while indirect, suggests a potential role for pericytes in maintaining CEC BBB stability in vivo.

3.4 Neurons
Neurons have been implicated in CNS vascular permeability due to the close proximity of axonal endings with astrocyte endfeet and CECs (Hamel, 2006). Neuronal activity could therefore communicate with astrocytes which are in direct contact with CECs. Specific subpopulations of cortical GABAergic interneurons have been demonstrated to promote vasodilation through close association with astrocyte endfeet (Cauli et al., 2004). Furthermore, neurons express high levels of nitric oxide and COX-2, implying a potential mechanism to signal alteration of smooth muscle near vasculature (Vidensky et al., 2003). These observations suggest a potential mechanism for neurons to regulate CEC function in a process dependent or independent of astrocytes.

Fig. 1. A model depicting the organization of the neurovascular unit (NVU). The NVU is composed of cerebral endothelial cells (CECs) surrounded by astrocytes, pericytes, axonal endings, and microglia. The NVU may ultimately control permeability of the BBB through tight junctions between CECs of the vasculature.
3.5 Microglia
Microglia have been implicated as mediators of CNS vascular permeability due to their expression of proinflammatory cytokines that promote BBB disruption in vitro, the most notable being TNF-α (Zhao et al., 2007). Microglia are potent antigen-presenting cells of the CNS that express both class I and class II molecules. Ex vivo isolated microglia have also been demonstrated to activate T cells in vitro (Mack et al., 2003). Therefore, microglia have the capacity to both interact with T cells and express proinflammatory factors that could alter the NVU resulting in BBB disruption.

4. Cerebral endothelial cell tight junctions and CNS vascular permeability
The NVU has been implicated in controlling entry of blood derived products across the BBB through tight junctions between CECs of the vasculature. Tight junctions are composed of both cytoplasmic and transmembrane proteins. These proteins are linked to an actin-based cytoskeleton allowing for a tight seal among cerebral endothelial cells of CNS vasculature (Petty & Lo, 2002). Cytoplasmic proteins collectively referred to as the Membrane-Associated Guanylate Kinase-Like homolog family provide structural support and play an organizational role for CECs (Hawkins & Davis, 2005). The current model of how the transmembrane proteins occludin, claudin and junctional adhesion molecules (JAMs) present in tight junctions interact is shown in Figure 2. Among these proteins, occludin and claudin appear to form the primary seal of the tight junction, whereas JAMs are proposed to

![Diagram of tight junctions between cerebral endothelial cells (CECs). Occludins, claudins and junctional adhesion molecules (JAMs) regulate adhesion of CECs. Occludins and claudins adhere to the zona occludin complex which consists of an interaction of ZO-1, ZO-2, and ZO-3 proteins. In contrast JAMs adhere only to zona occludin-1 (ZO-1). The zona occludin proteins in turn adhere to actin cytoskeleton proteins. Opening of tight junctions during inflammation appears to be an orchestrated event of degradation and expression of occludins and claudins, respectively and contraction of actin cytoskeleton. Model is modified from (Minagar & Alexander, 2003).](image-url)
be involved in monocyte and leukocyte adhesion and transmigration through the BBB (Ballabh et al., 2004; Brooks et al., 2005). The zona-occludin proteins, ZO-1, ZO-2 and ZO-3, have been shown to connect the transmembrane proteins to actin, providing stability for tight junction formation (Lai, 2005). Expression of occludin is much higher in neural endothelial cells when compared to peripheral endothelial cells whereas claudins are found in both (Ballabh et al., 2004). Occludin could therefore contribute to structural differences in BBB tight junctions as compared to tight junctions found in peripheral tissue.

Following insult to the CNS, inflammatory immune cells home to the CNS tissue. Despite the ability of immune cells to enter the CNS, the BBB remains relatively impermeable to other blood derived products. This is potentially due to immune cells entering the CNS without opening tight junctions between CECs via post capillary venules (Bechmann et al., 2007; Burns et al., 2000; Wolburg et al., 2005). However, tight junctions do not remain closed in all cases of CNS inflammation. Some instances of CNS inflammation promote opening of tight junctions and increased permeability of blood derived products through the BBB. Why tight junctions and the BBB open under some inflammatory circumstances but not others remains unknown. Current models suggest inflammatory immune cells promote opening of tight junctions between CECs through the activation of cells that line the BBB (Minagar & Alexander, 2003; Zlokovic, 2008). Chemokine and cytokine emission from immune cells have been implicated in this process with some examples being VEGF, IFN-γ, TNF-α, IL-1β and IL-6 (Argaw et al., 2009; Blamire et al., 2000; Didier et al., 2003; Ferrari et al., 2004; Krizanac-Bengez, et al., 2003; Laflamme, 1999; Paul et al., 2003; Stoll et al., 2000; Suidan et al., 2010; Wong et al., 2004). Chemokines enable adhesion of immune cells by mediating the activation of integrins on these cells. Additional signals lead to cytoskeletal reorganization, permitting transmigration of immune cells across the BBB, which is directed by matrix metalloproteinases (MMPs). Current therapies focus on blocking the integrins, which have resulted in reductions of BBB breakdown in patients with active multiple sclerosis. Targeting MMPs may also serve to stabilize BBB dysfunction (Zlokovic, 2008).

5. Importance of vascular endothelial growth factor (VEGF) in promoting CNS vascular permeability

Clinical observations and current experimental model systems used to address the contribution of inflammatory cells in BBB disruption have put forth a potential role for VEGF, a cytokine that has an instrumental role in vascular development and angiogenesis (Deininger et al., 2003; Karamysheva, 2008; Olsson et al., 2006; Su et al., 2006). However, VEGF was originally defined as ‘vascular permeability factor’ which was found to be secreted by tumor cells (Senger et al., 1983). VEGF is over 50,000 fold more potent than histamine in promoting vascular permeability in in vitro assays, demonstrating the potency of this cytokine to promote deregulation of the vasculature (Shulman et al., 1996). VEGF mediated permeability occurs through modification of endothelial cells to develop (a) vesiculo-vacuolar organelles, (b) increased caveolae formation, (c) fenestrations in membranes, and (d) alteration of tight junctions (Antonetti et al., 1999; Esser et al., 1998; Feng et al., 1999; Roberts & Palade, 1995). These modifications in endothelial cells could result in different levels of vascular permeability that range from the entry of small particles up to whole erythrocytes (Weis & Cheresh, 2005). The prevailing hypothesis is that VEGF’s effects on endothelial cells of vasculature result in local edema, hemorrhage and tissue damage (Chi et al., 2005). However, the specific interactions between inflammatory cells that result in VEGF-mediated vascular permeability remain a developing area of research.
The role of VEGF in the CNS is further complicated by the dual role of this cytokine in both angiogenesis and development, as well as in mediating vascular permeability. VEGF-deficient mice are an embryonic lethal phenotype which fail to develop normal vasculature (Carmeliet et al., 1996; Ferrara et al., 1996). Adding to the complexity of VEGF in the CNS is the rather ubiquitous nature of VEGF expression. During the onset of neuropathology, several CNS cell types including astrocytes, neurons and microglia have been reported to upregulate VEGF expression (Hayashi et al., 1997; Issa et al., 1999; Marti & Risau, 1998; Plate et al., 1999). Meanwhile, the CNS cell types that express the VEGF receptors, Flt-1 and Flk-1, include endothelial cells, smooth muscles and neurons (Carmeliet & Storkebaum, 2002; Ishida et al., 2001). VEGF is expressed in the CNS within 6 hours following a stroke and this cytokine has also been localized to MS lesions (Marti et al., 2000; Proescholdt et al., 2002). In experimental rodent models of hypoxia, VEGF expression in the CNS increases with the incidence of vascular permeability. Blocking VEGF in this model resulted in reduced vascular permeability (Schoch et al., 2002). Therefore, there is significant evidence in the literature that VEGF may be a viable target to treat neurological diseases characterized by vascular permeability and hemorrhage.

6. Tight junction alterations in the Experimental Autoimmune Encephalomyelitis (EAE) model

Experimental autoimmune encephalomyelitis (EAE) is an animal model used to study brain inflammation, and its clinical and pathological characteristics closely resemble those of ADEM. Induction of EAE produces neurological deficits, inflammatory CNS lesions, and disruption of the BBB (Morgan, Shah et al. 2007). This demyelinating disease of the CNS is induced by immunizing mice with well characterized myelin peptides, such as PLP, MAG, or myelin oligodendrocyte glycoprotein (MOG)35-55 peptide in complete Freund’s adjuvant (CFA), which causes inflammatory responses to the protein/peptide. It is also common to co-inject the mice with pertussis toxin to break down the blood brain barrier (BBB), allowing immune cells to gain access to the CNS tissue (Chen & Brosnan, 2006; Gurfein et al., 2009). The use of this mouse model has revealed the role that VEGF, CD4 T cells, astrocytes, tight junction proteins, and certain cytokines, such as IL-17, IL-22, and IFN-γ, have in the breakdown of the BBB.

Although IFN-γ was originally believed to play a pathogenic role in EAE, evidence has emerged that this cytokine may actually play a suppressive role. Since EAE in general is mediated by CD4 T cells, the proliferation and apoptosis of these cells in IFN-γ-deficient mice was investigated in order to define the role of IFN-γ in EAE. It was discovered that in the absence of IFN-γ, activated CD4 T cells exhibited a more extensive proliferation as well as decreased apoptosis when compared to wild-type mice. Therefore, it appears that IFN-γ is essential to limit the extent of EAE by serving to suppress the expansion of activated CD4 T cells (Chen & Brosnan, 2006).

Argaw et al (2009) have employed the MOG peptide induced EAE model to investigate the potential links between the reactivity of astrocytes and BBB disruption. Through the use of confocal imaging, they have shown that astrocyte-derived VEGF-A causes down-regulation and disruption of the tight junction proteins claudin-5 and occludin, promoting breakdown of the BBB, as shown in Figure 3 (Argaw et al., 2009).

Morgan et al took a closer look at occludin and the signaling events that occur during EAE through analysis of spinal cord tissue of naïve and EAE animals. Neurological impairment
Fig. 3. (A) VEGF down-regulates claudin-5 and occludin in human BMVECs. Confocal imaging of human BMVEC treated with 100 ng/mL VEGF-A for 24 hours and immunostained for claudin-5 and occludin. In controls, both proteins localize to the plasma membrane in areas of cell-cell contact. Note that claudin-5 and occludin are both down-regulated by VEGF-A. Data are representative of at least 3 separate experiments on 3 distinct cultures. (B) Sections of cerebral cortex from 12-week adult C57BL/6 mice 24 h following stereotactic microinjection of murine VEGF_{165} (60 ng in 3 μl PBS/BSA) or vehicle control. Sections are immunostained for claudin-5 (red channel) plus fibrinogen (green channel). Note that BBB permeability is observed around vessels in VEGF-injected areas. (C) Immunoreactivity for claudin-5 and occludin appears patchy and discontinuous in VEGF-injected areas. (Scale bars: A, 20 μm; B, 75 μm; C, 20 μm.) Data shown in B and C are representative of findings from at least 4 mice per time point per condition. (This figure was reproduced from Argaw, A. T., B. T. Gurfein, et al. (2009). VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown. Proceedings of the National Academy of Sciences of the United States of America 106(6): 1977-1982. Permission was granted by PNAS).

was observed in the animals undergoing EAE, which demonstrated a progressive development of tail and hind limb weakness and eventually became paraplegic. Immunohistochemical analysis of tissue revealed an increase in the number of nuclei through Hoechst staining and the presence of infiltrated macrophages and activated microglia through ED1 labeling in animals treated with EAE, thus confirming the
inflammatory nature of the disease. Additionally, an increase in the abundance of the plasma proteins albumin and transferrin was observed in EAE tissue, which is also consistent with inflammation leading to BBB breakdown. Immunoprecipitation revealed increased electrophoretic mobility of occludin in EAE animals which was found to be a result of occludin dephosphorylation. This was confirmed through the use of phosphatase treatment, which caused a further increase in electrophoretic mobility, while phosphatase inhibitors blocked this effect. These changes in occludin phosphorylation may signal changes in permeability of tight junctions. Furthermore, localization of occludin was analyzed via immunohistochemistry and was observed at intercellular junctions in naïve tissue, which is consistent with previous findings. However, localization of occludin to intercellular junctions in EAE tissue was often not apparent. Junctionally-localized claudin-5 was also disrupted in EAE tissue, indicating that the structure of tight junctions is disrupted in EAE. These findings reveal a potential role for occludin in BBB disruption (Morgan et al., 2007). Therefore, further investigation of this tight junction protein, along with its phosphorylation changes and signaling pathways, may open the door to new potential therapeutics for diseases involving breakdown of the BBB.

In addition to claudin-5 and occludin, the tight junction adaptor protein ZO-1 has also been analyzed using the EAE model. CNS tissue from naïve and EAE animals was analyzed for expression of ZO-1. Immunohistochemistry and confocal microscopy revealed disrupted expression of ZO-1 in lesion areas of EAE animals when compared to naïve animals. In order to correlate this disruption with clinical symptoms, time-course studies of ZO-1 distribution were performed. It was found that disruption of ZO-1 preceded clinical disease in EAE in lesion areas and correlated with symptomatic clinical signs in non-lesion areas. Additionally, vascular permeability was analyzed through the use of Evans blue dye, and an increase in permeability was found at the peak of EAE. Taken together, it is evident that a complete understanding of the regulation of tight junction architecture is essential for the development of therapies (Bennett et al., 2010).

Kebir et al employed the EAE model to examine the effects of Th17 lymphocytes, which are known to have encephalitogenic potential, on BBB disruption, and found that significantly more Th17 lymphocytes migrated across the BBB in vitro than did either Th1 or ex vivo CD4+ lymphocytes. To confirm the ability of these lymphocytes to cross the BBB in vitro, cells were stained for IL-17 and IL-22, a cytokine product of Th17 cells, before and after migration. There was a significant increase in IL-17+ and IL-22+ lymphocytes after migration. Furthermore, it was found that both IL-17 and IL-22-producing lymphocytes expressed the cytolytic molecule granzyme B, enabling these cells to kill human fetal neuron-enriched cultures. Similar to the previously mentioned studies, the effect on tight junctions was also investigated. It was discovered that treating monolayers of human BBB-ECs with IL-17 produced an increase in the diffusion of fluorescently-labeled BSA, which coincided with a decrease in occludin and ZO-1 expression in EAE mice. Additionally, it was found that Th17 lymphocytes contribute to inflammation through the recruitment of CD4+ lymphocytes (Kebir et al., 2007).

The mechanisms of IL-17-induced BBB disruption were further investigated using the EAE model by Huppert et al. It was discovered that IL-17 treatment disrupted tight junction molecules by decreasing occludin levels and causing a reorganization of ZO-1, thus impairing BBB integrity. It was also found that IL-17 induced an increase in reactive oxygen species (ROS) formation, a mechanism known to be involved in BBB disruption. To link this observation to the effect on tight junctions, the involvement of ROS signaling was
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Investigated, and DPI, an NAD(P)H oxidase inhibitor, was found to prevent the effect of IL-17 on occludin. Additionally, it has been demonstrated that phosphorylation of myosin light chain (MLC) in BECs is induced by IL-17, leading to BBB permeability. When EAE mice were treated with ML-7, a MLCK inhibitor, they developed significantly milder clinical signs and had a reduced number of infiltrating lymphocytes and macrophages when compared with vehicle-treated mice. This treatment also reduced the levels of IL-17 and IL-22, which are pathogenic cytokines associated with EAE. Furthermore, IL-17-deficient mice portrayed lower levels of oxidative stress and were protected from BBB disruption induced by EAE (Huppert et al., 2010). Taken together, these two studies portray a role for proinflammatory cytokines, particularly IL-17, on the integrity of the BBB. Targeting the mechanisms of these cytokines could bring about novel therapeutic strategies for inflammatory diseases such as ADEM and AHLE. The results from these studies can be incorporated into a model, which is shown in Figure 4.

**Fig. 4. Mechanisms of CD4 T cell initiated BBB disruption.** (A) Th17 lymphocytes contribute to inflammation through IL-17, which induces NAD(P)H oxidase-dependent ROS formation, leading to phosphorylation of MLC, and subsequent BBB permeability. (B) Additionally, CD4 T cells induce astrocytes to release VEGF, causing down-regulation of the tight junction proteins claudin-5 and occludin, which promotes breakdown of the BBB.

### 7. Lipopolysaccharide (LPS) model

Injection of small amounts of lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, can induce shock and systemic inflammation. The signaling pathway begins by LPS binding to LPS binding protein (LBP), with subsequent transfer of
LPS to CD14. In order for the proper targeting of TLR-4 to the surface and its ability to recognize LPS, MD-2 binds to TLR-4. This complex then interacts with CD14:LPS, sending a signal that activates the transcription factor NFkB, which activates genes that encode proteins that play a role in defense against infection.

Qin et al sought to understand the effects of systemic LPS on brain inflammation. Upon peripheral injection of LPS, there was a rapid increase in the cytotoxic factor TNF-α, which remained elevated in the brain even 10 months after treatment. Injection of LPS also promoted the activation of brain microglia through TNFα receptors. Brain sections were immunostained with Iba1, an antibody specific for microglia. Increased cell size, irregular shape, and intensified Iba1 staining are apparent in LPS-treated mice when compared with controls. This activated microglia may lead to the production of more inflammatory factors, which could possibly lead to neuronal death. This reactive microgliosis may be the cause of progressive and chronic neuroinflammation (Crews et al., 2007).

Using a similar model, Brooks et al employed Complete Freund’s adjuvant (CFA), which has also been known to stimulate the production of TNF. It was discovered that changes in certain transmembrane tight junction proteins alter the function of the BBB. For example, occludin was significantly decreased in CFA-injected rats, while claudin-3 and claudin-5 were both significantly increased when compared to saline-treated controls. The alteration of these tight junction protein levels coincided with BBB disruption (Brooks et al., 2005). Matrix metalloproteinases (MMPs) mediate BBB disruption and trafficking of immune cells into the CNS and can be induced by LPS. It has previously been shown that expression of stromelysin-1 (MMP-3) occurs after intracerebral injection of LPS. To investigate the effect of MMP-3 on BBB disruption, MMP-3 knockout mice have been used. LPS injection increased CNS vascular permeability in wild-type mice but not in mice lacking MMP-3. Additionally, quantitative stereology showed that MMP-3 KO mice exhibited a significant reduction in neutrophil infiltration. Furthermore, when analyzing alterations in tight junction proteins, it was discovered that both claudin-5 and occludin were decreased after LPS injection in both WT and MMP-3 KO mice. However, there was less tight junction damage in the mice lacking MMP-3. Therefore, further investigating the mechanisms of MMP-3 activity may aid in the development of MMP inhibitors to be used in the treatment of neuroinflammatory diseases (Gurney et al., 2006). Importantly, this model system illustrates the potential of peripheral inflammation to induce microglia activation and subsequent BBB disruption in the CNS, the tentative model of which is put forward in Figure 5.

8. LCMV model of Vascular Permeability: Lymphocytic Choriomeningitis Virus (LCMV)

Lymphocytic choriomeningitis virus (LCMV) is a well-studied fatal model of CNS vascular permeability. CD8+ T cells are important mediators in the response to several viral infections. These immune cells are activated in secondary lymphoid organs, particularly the spleen, and then migrate to sites of infection, where they kill virus-infected cells and secrete pro-inflammatory cytokines such as IFN-γ and TNF-α. It has previously been shown that the immunopathology resulting from CNS infection with LCMV correlates with the cytotoxic activity of virus-specific CD8+ T cells and this process is perforin-dependent (Kagi et al., 1994). Storm et al sought to re-evaluate the role of perforin in this process. They discovered that perforin-deficient mice infected with LCMV still die from inflammation mediated by CD8+ T cells, although this death is delayed 2-5 days when compared to wild-
type mice infected with LCMV. Nevertheless, death occurred despite the absence of perforin, impaired pro-inflammatory cytokine production, and a deficit in the cytotoxic ability of CD8+ T cells. However, it was also shown that CD8+ T cells were required for the lethality, because those that received CD8-depleting antibodies survived. The delay in fatality in the absence of perforin may be attributed to virus-specific T cells being prevented from entering the CNS. Additionally, these T cells may not be effectively recruited to the CNS within the first 6-7 days after infection. However, once they are able to enter the CNS, they have the ability to induce fatal CNS pathology (Storm et al., 2006).

Similarly, Kim et al, using the LCMV model, employed two-photon microscopy to visualize the dynamics of immune cells in the meninges. They observed motile CTL which expressed chemoattractants that recruited myelomonocytic cells, such as neutrophils. Therefore, it was inferred that a disorder that depends on CD8+ T cells may rely solely on CTL recruited myelomonocytic cells. Using mice with single mutations in all major CTL effector pathways, including perforin and TNF-α, it was found that mice still experienced convulsive seizures after LCMV infection. Therefore, no single deficiency had the ability to prevent disease. This group then questioned the potential role for monocytes and/or neutrophils in the seizure-induced death on day 6. To investigate this, they depleted both monocytes and neutrophils.
by administering high-dose anti-GR-1 to CCR2 knockout mice. This treatment extended survival by 3 days and preserved vascular integrity on day 6, as evidenced by leakage of Evans blue dye into the brain. Therefore, they concluded that CD8+ T cells may primarily serve to attract other effector populations. Potential therapies could involve reduction of myelomonocytic activation or targeting the CTL chemotactic mechanisms that recruit myelomonocytic cells (Kim et al., 2009).

Another study addressing LCMV-mediated death once again showed the importance of CD8+ T cells in generating the lethality of the disease. This study showed that CD8-deficient mice survived after i.c. injection of LCMV. Additionally, they showed that RAG knockout mice, which lack T and B cells, do not exhibit morbidity or mortality, thus providing evidence attributing these effects to immunopathology instead of viral damage. This group also addressed a possible connection between BBB disruption and LCMV pathogenesis through the use of Evans blue staining. While leakage of Evans blue dye was observed in C57BL/6 mice on day 5, before the onset of seizures, RAG knockout mice did not show signs of BBB disruption, indicating that BBB damage is induced by adaptive immunity. The role of perforin was also addressed in this study, with perforin knockout mice showing an intermediate phenotype after LCMV challenge and no clinical signs of the disease. However, surviving perforin knockout mice showed an increase in BBB disruption similar to C57BL/6 mice. Therefore, although BBB disruption occurs after LCMV infection, it may not be associated with mortality. This group proposed that other neuroanatomical changes, such as brain herniation, may be the most likely cause of mortality. Understanding the basis of LCMV neuropathogenesis may be helpful for designing therapies for viral hemorrhagic fevers in addition to ADEM and AHLE (Matullo et al., 2010).

9. Inducible model of severe vascular permeability: Peptide-Induced Fatal Syndrome (PIFS)

The Daniel’s strain of Theiler’s murine encephalomyelitis virus (TMEV) is a murine picornavirus that causes a transient early meningoencephalitis in all mouse strains, and persistent infection of the white matter with demyelination in susceptible strains (Njenga et al., 1997; Rodriguez & David, 1985; Rodriguez, 1986). During acute TMEV infection, mice of H-2b haplotype mount an antiviral CD8 T cell response that is highly focused on an immunodominant TMEV peptide, VP2

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121-130, presented in the context of the Db class I molecule (Johnson et al., 1999; Johnson et al., 2001). It was demonstrated that intravenous injection of this VP2

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121-130 peptide prior to TMEV infection inhibited VP2 peptide specific CD8 T cell expansion in the CNS and prevented IFN-γ receptor-/- mice from developing paralytic disease. However, injection of VP2 peptide during acute TMEV infection, after the expansion of Db:VP2

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121-130 epitope specific CD8 T cells in the CNS, resulted in death in 24 to 48 hours. It was determined that this fatal syndrome was antigen specific, because mice were asymptomatic when given the mock control Db binding E7 peptide. In subsequent studies, our laboratory determined that CNS vascular permeability and hemorrhage are a major component of this condition. This peptide induced fatal syndrome (PIFS) is a readily reproducible means to investigate CD8 T cell-mediated CNS vascular permeability (Figure 6). The major advantage of this model system is that BBB disruption is inducible through administration of the antigenic VP2 peptide, enabling a kinetic analysis of early and late cellular and gene expression events. While BBB disruption is a common feature of numerous neurological conditions, the PIFS model with its high level of CNS vascular permeability,
edema, rapid demyelination, and onset of multiple focal hemorrhages, most closely resembles AHLE (Figure 7).

Fig. 6. Mouse model of CNS vascular permeability. CD8+ T cell-mediated vascular permeability is induced in C57BL/6 mice. Mice are intracranially infected with TMEV at day 0. During the peak of CD8 T cell expansion, mice are intravenously administered VP2\textsubscript{121-130} peptide to induce permeability or E7 control peptide. The CNS is harvested on the following day.

In addition to being mediated by antigen-specific CD8+ T cells, PIFS is influenced by perforin expression and genetic background. C57BL/6 perforin-deficient mice are resistant to this fatal syndrome. Another key determinant in the development of PIFS is the genetic background of the animal. While C57BL/6 mice are highly susceptible to PIFS, both FVB and 129 SvIm strains are significantly more resistant. Interestingly, this holds true despite these strains having a substantial population of antigen-specific CD8+ T cells (Johnson et al., 2005).

Another important aspect of the PIFS model is the observation that mice lacking major histocompatibility complex (MHC) class II, and thus CD4 T cells, IFN-γR, TNF-α, TNFR1, TNFR2, and TNFR1/TNFR2 still succumbed to the fatal syndrome. Inhibiting interleukin-1 and lymphotoxin-β did not serve to protect the mice from PIFS (Johnson et al., 2005). Therefore, it appears that the cytokines and CD4 T cell subsets heavily studied in other model systems of immune-mediated CNS vascular permeability do not play a role in the development of fatal blood brain barrier disruption in the PIFS model. However, removal of antigen-specific CD8+ T cells conferred protection, preventing mice from becoming moribund (Johnson et al., 2005). Suidan et al took this into consideration and investigated the contribution of the effector functions of CD8+ T cells, notably perforin and FasL, on disruption of the BBB and PIFS. It was first observed that the expansion of CNS infiltrating antigen-specific CD8+ T cells was not significantly different between C57BL/6 mice and C57BL/6 mice deficient in perforin or FasL. MRI analysis revealed that while C57BL/6 and C57BL/6 FasL deficient mice both exhibited extensive vascular permeability, mice lacking
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Fig. 7. In vivo MRI images of 8 day TMEV infected C57BL/6 mice, 24 hours after VP2\(^{121-130}\) peptide injection (right panel) or irrelevant E7 peptide injection (left panel). Top row: axial images extracted from the gadolinium enhanced T1 weighted dataset demonstrate extensive contrast enhancement of confluent areas of the brain in the VP2 injected mouse, and very faint enhancement in the parahippocampal area in the E7 injected animal. Middle row: T2 weighted images demonstrate T2 hyperintensities, corresponding with areas of edema, inflammatory infiltrates, demyelination and tissue damage in the VP2 injected mouse; minimal hyperintense changes are also demonstrated in the parahippocampal areas of the E7 injected animal. Bottom row: T2* weighted images demonstrate punctuate T2 hypointensities, corresponding with areas of microhemorrhages in the VP2 injected mouse. (Reproduced with permission from Pirko, I., G. L. Suidan, et al. (2008). Acute hemorrhagic demyelination in a murine model of multiple sclerosis. Journal of neuroinflammation 5: 31).

perforin were protected. These same results were obtained when employing the FITC permeability assay, in which FITC-albumin is intravenously given to VP2 or E7 treated mice. Since albumin does not readily cross the BBB under normal conditions, this technique enables analysis of CNS vascular permeability with both microscopy and analysis of brain homogenate on an immunofluorescent plate reader. The combination of these two techniques, in addition to T1 gadolinium enhanced, T2 and T2* MRI, revealed that perforin, but not FasL, is essential for VP2 peptide induced CNS vascular permeability, edema and microhemorrhage formation, respectively. It was also found that astrocyte activation as measured by GFAP expression is dependent on perforin expression (Figure 8). High-resolution microscopy revealed the expression of GFAP co-localized with FITC-albumin leakage. This is important because astrocytes have been shown to be involved in regulation of BBB integrity (Suidan et al., 2008).
Time course experiments have been conducted in order to determine the sequence of events that occur in the development of PIFS. It was discovered that astrocyte activation and degradation of the tight junction protein occludin occur prior to peak levels of CNS vascular permeability and motor deficits. In accordance with the results from the LPS model employed by Brooks et al using the same microvessel isolation technique, it was found that expression of claudin-5 was significantly increased, while expression of occludin was significantly decreased. In both the LPS and the PIFS model, these alterations of tight junction protein levels coincide with BBB disruption. Furthermore, in the PIFS model, concurrent with a lack of vascular permeability, tight junction protein alterations were not observed in perforin-deficient mice. Additionally, to investigate the possibility that apoptosis is the cause of CNS vascular permeability, active caspase-3 protein levels were assessed. These levels did not increase until after peak levels of CNS vascular permeability, indicating that apoptosis does not initiate this permeability. In addition, the increase in claudin-5 levels serves as evidence that CECs were viable during peak permeability and not undergoing apoptosis (Suidan et al., 2008).

A putative mechanism of CD8+ T cell-mediated CNS vascular permeability is induction of vascular endothelial growth factor (VEGF) in the CNS. This cytokine has a highly vascular-permeating effect, and, along with its receptors, undergoes a change in expression following...
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insult to the CNS (Deininger et al., 2003; Ferrara et al., 2003; Krum & Khaibullina, 2003; Krum et al., 2008; Lafuente et al., 2006; Proescholdt et al., 1999; Proescholdt, et al., 2002; Senger et al., 1983; Su et al., 2006; Srikitakhorn et al., 2007; Zhang et al., 2000). Because signal transduction through binding of VEGF to its receptor, fetal liver kinase-1 (flk-1), leads to vascular permeability, there may be an important role for this cytokine in disruption of the BBB during neuroinflammatory conditions (Ferrara et al., 2003). Through analysis of VEGF protein levels and FITC albumin-leakage into the brain, it was found that both VEGF protein and phosphorylation of flk-1 were significantly increased following administration of VP2 peptide. Coinciding with these increases was an increase in leakage of FITC-albumin, and thus CNS vascular permeability. During this permeability, in situ hybridization revealed that the major source of VEGF expression was neurons. Confocal microscopy further confirmed this by showing that immunostaining of the neuronal marker NeuN colocalizes VEGF cytokine (Suidan et al., 2010). However, since VEGF is not exclusively expressed by neurons clinically and in other model systems, alternative cell types, such as GR-1+ neutrophils, may also contribute to BBB disruption through a VEGF dependant mechanism.

Since it has been demonstrated that neuronal expression of VEGF occurred simultaneously with CD8 T cell-mediated CNS vascular permeability, VEGF had the potential to be a viable therapeutic target in neurological diseases such as AHLE. To investigate this possibility, Suidan et al administered the neuropilin-1 inhibitor, ATWLPPR, to 7-day TMEV-infected C57BL/6 mice given VP2_{121-130} peptide or mock E7 peptide. Neuropilin-1 is a coreceptor for VEGF and has been shown to enhance processes mediated by the VEGF receptor flk-1 (Soker et al., 1998). Through analysis of FITC-albumin leakage into the brain, it was found that inhibition of neuropilin-1 with high doses of ATWLPPR VEGF inhibitor resulted in decreased CNS vascular permeability when compared to mice treated with PBS or scrambled RAPTLWP peptide. Furthermore, there was no significant difference between mice treated with VEGF inhibitor and mice treated with mock E7 peptide (Suidan et al., 2010). As previously reported using the PIFS model, degradation of the tight junction protein occludin occurs prior to CNS vascular permeability (Suidan et al., 2008). However, when mice were treated with ATWLPPR neuropilin-1 inhibitor, occludin protein levels were preserved, likely contributing to the maintenance of an impermeable BBB. Therefore, inhibition of VEGF-mediated pathways may serve as a strong therapeutic strategy for the treatment of neurological diseases characterized by BBB disruption.

The observation that high neuronal VEGF expression coincided with BBB disruption prompts the question of to what extent CNS-infiltrating CD8 T cells could actively engage Théllier’s virus-infected neurons. This area of research has been controversial since neurons express little or no detectable levels of MHC class I (Corriveau et al., 1998; Horwitz et al., 1999; Joly et al., 1991; Neumann, et al., 1995; Rall et al., 1995). To address this controversy, GFP+ CD8+ cells were adoptively transferred to C57BL/6 mice, and it was found that these cells were highly specific for the Db:VP2_{121-130} epitope. The next major question is which cell types are potential targets for these cells. Confocal microscopy revealed that TMEV protein translation occurred in the hippocampus, striatum, hypothalamus, and cortex. Positive staining with the neuronal marker NeuN revealed that neurons were the main cell type translating virus protein. Furthermore, adoptively transferred GFP+ CD8+ cells were found in close proximity to TMEV infected neurons and their processes. (McDole et al., 2010). The finding that CD8 T cells engage neurons, as shown in Figure 9, also reveals a possible
mechanism by which axonal and neuronal damage could take place in neuroinflammatory diseases such as AHLE and ADEM.

Fig. 9. CD8 T cells form immune synapses with target neurons. Both CD8 protein (A and E) and T cell receptor (B and F) polarized toward neurons (C and G) are highly indicative of an immune synapse. Merged images are shown (D and H). For A–H, results are representative of an analysis of eight mice. All microscopy was performed on hippocampus. Scale bars: 10 μm (This figure was reproduced from McDole, J.R., S.C. Danzer, et al. (2010). Rapid formation of extended processes and engagement of thielor’s virus-infected neurons by CNS-infiltrating CD8 T cells. The American Journal of Pathology 177(4): 1823-1833. Permission was granted by Elsevier).

It is apparent that CD8 T cells and expression of VEGF contribute to BBB disruption in the PIFS model. However, it is possible that the NVU or other immune cell types, such as GR-1+ neutrophils, may also contribute to disruption of the BBB in accordance with previously published work with the LCMV models of BBB disruption highlighted above. These potential mechanisms of virus induced vascular permeability are illustrated in Figure 10. Traditional methods of neutrophil depletion, such as those used in the BBB studies of LCMV, employ the anti-GR-1 monoclonal antibody RB6-8C5. These studies conclude that neutrophils are the critical blood-derived cell type promoting BBB disruption (Kim et al., 2009). However, RB6-8C5 has been shown to bind to both Ly-6G on neutrophils and Ly-6C on lymphocytes and monocytes. Therefore, it is possible that GR-1-specific Ab depletion could also remove large numbers of activated CD8 T cells in addition to neutrophils. To address this controversy and determine whether CD8 T cells cause BBB disruption without the contribution of neutrophils, we have recently employed the TMEV model and used both the RB6-8C5 mAb and the Ly-6G-specific mAb 1A8 to deplete neutrophils in vivo. Ablation of epitope-specific CD8 T cells was seen in 7-day TMEV infected mice treated with RB6-8C5 but not those treated with 1A8 or normal rat serum. Therefore, the Ly-6G-specific mAb 1A8 is more selective for neutrophils in accordance with published results (Daley et al., 2008; Dunay et al., 2010). Additionally, anti-GR-1 depletion was shown to preserve motor...
function, as shown through assessment of the mice on a Rotamex Rotarod, and to reduce CNS vascular permeability as demonstrated by assaying FITC-albumin leakage into the CNS (data not shown). However, because 1A8 treatment somewhat reduced the levels of FITC-albumin leakage into the CNS, there may be a potential role for neutrophils in CD8 T cell initiated CNS vascular permeability, and this needs to be investigated further. Nevertheless, anti-GR-1 depletion strategies may hold promise as a potential therapeutic strategy for neurologic disorders characterized by altered permeability of the BBB.

Fig. 10. BBB disruption in the PIFS model is induced through intravenous injection of VP2_{121-130} peptide at 7 days post TMEV infection. At 7 days post TMEV infection, the CNS infiltrating Db:VP2_{121-130} specific CD8 T cell response peaks. Administration of VP2_{121-130} peptide results in heightened activation of expanded CNS infiltrating Db:VP2_{121-130} specific CD8 T cells interacting with Db class I expressing cells, which could include neurons, astrocytes, cerebral endothelial cells, pericytes, and microglia. Our central hypothesis (1.) is that CD8 T cells engage neurons to promote upregulation of VEGF. Our alternative hypothesis (2.) is that CD8 T cells engage a different CNS cell type that results in BBB disruption and vascular permeability that is dependent or independent of neuronal VEGF.
10. Summary of the experimental model systems to illustrate the contribution of immune cells in BBB disruption

The EAE, LPS, and virus induced CNS vascular permeability model systems have enabled an understanding of how immune cells affect and alter CNS cell types of the neurovascular unit and ensuing BBB disruption. A defined role for CD4 T cells has been put forth through the EAE model, which also demonstrates that astrocyte-derived VEGF and IL-17 cause decreases in the tight junction proteins claudin-5 and occludin. The LPS model has portrayed a role for TNFα, activated microglia, and MMPs in BBB disruption. Similar to the EAE model, occludin has also been shown to be decreased in this model. However, claudin-3 and claudin-5 were found to be increased, and this coincided with BBB disruption. The virus induced models of vascular permeability have demonstrated a role for neutrophils, CD8 T cells, and VEGF. The LCMV model demonstrates an essential role for CD8 T cells in lethality, but puts emphasis on the fact that these cells may primarily serve to attract other effector populations, such as GR-1+ neutrophils. The PIFS model, which has the advantage of being inducible through the administration of an antigen peptide, also validates a role for CD8 T cells in contributing to CNS vascular permeability. Similar to the LPS model, the PIFS model also reveals a decrease in occludin and an increase in claudin-5, both of which are dependent on perforin expression. In accordance with the EAE model, VEGF is also shown to play a major role in BBB disruption. Furthermore, time course experiments using this model enabled the discovery of the order of events involved in BBB disruption, showing that astrocyte activation and degradation of occludin occur prior to CNS vascular permeability and motor deficits. Taken together, these models have put forward a defined role for neutrophils, CD4 and CD8 T cells, and VEGF in BBB disruption. This knowledge provides important information on the extent inflammatory immune cells contribute to CNS vascular permeability in diseases such as ADEM and AHLE. Therefore, continued efforts to control inflammation and dampen VEGF-mediated vascular permeability are important therapeutic approaches for treatment of these conditions.

11. Conclusion

Knowledge of the extent inflammatory immune cells contribute to disruption of the BBB is essential in order to develop treatments for ADEM and AHLE, two diseases that currently have no standard therapies and whose etiology is still not well understood. These potential therapies could also extend beyond ADEM and AHLE to other diseases characterized by increased BBB disruption, such as multiple sclerosis, viral hemorrhagic fevers, and cerebral malaria. The current experimental model systems available to address the contribution of inflammatory cells in BBB disruption open the door to several potential therapies. For example, it is apparent from both the EAE model and the PIFS model that targeting VEGF and inhibiting VEGF-mediated pathways may help ameliorate BBB disruption. In support of this therapeutic approach, we have determined that inhibition of neuropilin-1, a co-receptor for VEGF, decreased CNS vascular permeability (Suidan 2010). It is also evident through the EAE model that targeting the signaling pathways of occludin, the mechanisms of proinflammatory cytokines such as IL-17, and the tight junction architecture may provide avenues for potential therapeutic approaches. Results obtained from the LPS model demonstrate that directing research at MMP inhibitors and targeting microglia to potentially stop the production of inflammatory factors may also be valuable tools. The LCMV model
portrays a need to investigate reducing myelomonocytic activation or targeting CTL chemotactic mechanisms that recruit myelomonocytic cells as a potential means of treatment. Finally, the PIFS model demonstrates that strategies that target CD8 T cells and VEGF cytokine may hold promise as potential therapies to ameliorate severe BBB disruption. Future research in these areas as well as in additional mechanisms by which immune cells cause BBB disruption will aid in the development of more specific therapies to address these devastating immune-mediated neurological disorders.

12. References


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Pathogenesis of Encephalitis
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Many infectious agents, such as viruses, bacteria, and parasites, can cause inflammation of the central nervous system (CNS). Encephalitis is an inflammation of the brain parenchyma, which may result in a more advanced and serious disease meningoencephalitis. To establish accurate diagnosis and develop effective vaccines and drugs to overcome this disease, it is important to understand and elucidate the mechanism of its pathogenesis. This book, which is divided into four sections, provides comprehensive commentaries on encephalitis. The first section (6 chapters) covers diagnosis and clinical symptoms of encephalitis with some neurological disorders. The second section (5 chapters) reviews some virus infections with the outlines of inflammatory and chemokine responses. The third section (7 chapters) deals with the non-viral causative agents of encephalitis. The last section (4 chapters) discusses the experimental model of encephalitis. The different chapters of this book provide valuable and important information not only to the researchers, but also to the physician and health care workers.

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