



Dengue Virus Infection of Blood–Brain Barrier Cells: Consequences of Severe Disease

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More than 500 million people worldwide are infected each year by any of the four-dengue virus (DENV) serotypes. The clinical spectrum caused during these infections is wide and some patients may develop neurological alterations during or after the infection, which could be explained by the cryptic neurotropic and neurovirulent features of flaviviruses like DENV. Using *in vivo* and *in vitro* models, researchers have demonstrated that DENV can affect the cells from the blood–brain barrier (BBB) in several ways, which could result in brain tissue damage, neuronal loss, glial activation, tissue inflammation and hemorrhages. The latter suggests that BBB may be compromised during infection; however, it is not clear whether the damage is due to the infection *per se* or to the local and/or systemic inflammatory response established or activated by the BBB cells. Similarly, the kinetics and cascade of events that trigger tissue damage, and the cells that initiate it, are unknown. This review presents evidence of the BBB cell infection with DENV and the response established toward it by these cells; it also describes the consequences of this response on the nervous tissue, compares these evidence with the one reported with neurotropic viruses of the *Flaviviridae* family, and shows the complexity and unpredictability of dengue and the neurological alterations induced by it. Clinical evidence and *in vitro* and *in vivo* models suggest that this virus uses the bloodstream to enter nerve tissue where it infects the different cells of the neurovascular unit. Each of the cell populations respond individually and collectively and control infection and inflammation, in other cases this response exacerbates the damage leaving irreversible sequelae or causing death. This information will allow us to understand more about the complex disease known as dengue, and its impact on a specialized and delicate tissue like is the nervous tissue.

Keywords: blood–brain barrier cells, breakdown, severe dengue, neurological manifestations, pathogenesis

INTRODUCTION

Febrile illness caused by the dengue virus (DENV) is the most important arthropod-borne disease in the world. In the last four decades dengue cases have increased by 30-fold due to the adaptive nature and wide-spread localization of the mosquito vector (Chretien et al., 2015). This increase has also been caused by human migration, chaotic, and rapid urbanization, and deforestation in

underdeveloped tropical countries (Arunachalam et al., 2010). Even though dengue is a well-known disease, its clinical manifestations are changing. Nowadays, an increasing number of dengue cases present damage in organs such as brain, liver, heart, or kidneys, an interesting and important feature that has been reported in some cases, but that has been very difficult to relate with the virus given the late onset of these complications. These changes have led the World Health Organization (WHO) to include organ damage in the diagnostic criteria for severe cases of dengue (World Health Organization [WHO], 2009). Neurological manifestations, for example, may occur during or after acute infection, may be transient or irreversible (Bopeththa and Ralapanawa, 2017), and may affect both the central (Ng and Sadarangani, 2017) and peripheral nervous systems (Mota et al., 2017). Questions that still need to be answered include: how the DENV enters into the nervous system, where the susceptible cells are located, what kind of cell/tissue responses occur, and what kind of cell/tissue signaling is responsible for nervous tissue damage. It has been proposed that studies on blood–brain barrier (BBB) cells could greatly add to our understanding of the neurotropism, neuro-susceptibility, and neuropathogenesis associated with the DENV.

DENV BIOLOGY AND PATHOGENESIS

Dengue virus belongs to the *Flavivirus* genus which is part of the Flaviviridae family. There are four different serotypes (DENV1 to DENV4) that infect and produce disease in humans. This virus has an icosahedral capsid, a lipoproteic envelope, and a positive, single stranded RNA genome that codes for three structural proteins (C, capsid; M, membrane; and E, envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Pierson and Diamond, 2013). The envelope (E) glycoprotein is involved in virus entry into target cells, mainly monocytes, macrophages, hepatocytes, and endothelial cells (EC) using membrane receptors (Grove and Marsh, 2011; Cruz-Oliveira et al., 2015). The non-structural proteins promote RNA translation (NS3) and transcription/replication (NS2B/NS3 and NS5), modulate the innate immune response (NS4A and B) (van Cleef et al., 2013), and play a role in the virion assembly and release (Miller et al., 2007; Pierson and Diamond, 2013).

The epidemic cycle is human–mosquito–human, and the main vectors are the *Aedes aegypti* and *Aedes albopictus* females (Bhatt et al., 2013). After a mosquito bite, the inoculated saliva/virus is transported via skin dendritic cells toward the lymphatic nodes where it infects monocytes, replicates, and spreads to the blood and other organs (Pierson and Diamond, 2013). Most infected people do not have symptoms (Endy et al., 2011; Castellanos J. et al., 2016), but others develop subtle symptoms such as fever and malaise. Some patients present muscle and bone pain, high fever, ocular headache, abdominal pain, thrombocytopenia, and mucosal bleeding. This is classified as dengue with warning signs. Around 10% of patients may develop severe signs and symptoms include severe hemorrhages, massive plasma leakage with edema, and severe

organ dysfunction. This is diagnosed as severe dengue which may lead to death (World Health Organization [WHO], 2009).

In addition to clinical signs and symptoms, a case confirmation is necessary and should be determined through serology or molecular tests. However, the sensibility and specificity of tests depends on which day of illness they are undertaken (Guzman and Harris, 2015). Unfortunately, neither clinical following nor laboratory tests allows for the accurate prediction of the outcome in each case.

Disease severity is explained by different factors such as age, nutrition status, genetic background (Guzman et al., 2002; Sierra et al., 2007; Guzman and Harris, 2015), and the infecting virus serotype or genotype (Cologna et al., 2005; Martina et al., 2009). The most prominent factor which leads to severe dengue fever with complications is having a second or third infection with a different DENV serotype (Descloux et al., 2009; Ohainle et al., 2011; Guzman and Harris, 2015). However, a role for NS1 protein has been described, for example activating the complement system or eroding EC glycocalyx (Puerta-Guardo et al., 2016).

The immune response during infection activates monocytes and macrophages (Puerta-Guardo et al., 2013), dendritic cells (Luplertlop et al., 2006), T cells (Lühn et al., 2007), and mastocytes (St John et al., 2013) which release large amounts of cytokines and chemokines to affect, for example, EC (Avirutnan et al., 2006; Trung and Wills, 2010). Synergic immune cell responses lead to cytokine storms, plasma leakage, and organ failure. Therefore, dengue severity depends on the individual response to infection, which has several types of clinical presentations and, frequently, has no correlation with immune markers; this makes accurate diagnosis or prognosis very difficult.

DENGUE WITH NEUROLOGICAL MANIFESTATIONS

As stated above, most DENV infections are asymptomatic or mild, but in less likely cases with severe presentation, signs can begin either early or late and involve different systems and organs such as gastrointestinal, hepatic, renal, cardiovascular, respiratory, musculoskeletal, and neurological (Gulati and Maheshwari, 2007; Puccioni-Sohler et al., 2012). Symptoms and signs in the nervous system have been published since 1976 (Sanguansermisri et al., 1976), however, recently there has been an increase in case reports, experimental *in vivo* and *in vitro* research, meta-analysis, and reviews which show great concerns about these signs and the change in the clinical picture over the last few years.

Neurological alterations occur in both males and females and in every age group in endemic areas. Any of the four DENV serotypes may be involved, although DENV2 and DENV3 are the most frequently identified by RT-PCR or viral isolation (Hapuarachchi et al., 2015). Nevertheless, neurological manifestations as consequence of DENV infection are still reported as infrequent and given the diversity of clinical manifestations, neurological signs can be quite subtle (Wilder-Smith et al., 2019). Nervous system alterations may or may not be reversible and may take several days or months for

complete recovery, as an example, **Table 1** summarizes some of the most interesting clinical reports of patients that presented neurological manifestations after or during DENV (**Table 1**), or other flavivirus (**Supplementary Table S1**) infection.

The nervous alterations could be classified following certain criteria, such as: (i) direct tissue infection (encephalitis, meningitis, myositis, myelitis, rhabdomyolysis); (ii), signs related with systemic or metabolic imbalance (encephalopathy, stroke) and (iii) early or late post-infection sequelae (transverse myelitis, Guillain-Barré syndrome – GBS-, acute disseminated encephalomyelitis) (Murthy, 2010). A detailed description of neurological complications during dengue infection was recently published (Li et al., 2017).

According to this evidence, nervous tissue damage could be caused directly by the virus and/or the immune response, which was originally established to get rid of the virus. However, it is not clear how the virus enters the central nervous system (CNS), which cells are infected and respond, and how equilibrium is reached (or not) after injury. So far, the most probable DENV entry route to the CNS is the hematogenous route (Lanteri and Busch, 2012; Velandia and Castellanos, 2012; Guzman and Harris, 2015), therefore, the BBB plays an important role in favoring the neuroinvasion and neurotropism of the virus. Alteration in its functioning can help to understand the morphological and physiological consequences of the arrival of the virus to the brain. These aspects and the evidence on BBB participation in flavivirus neurological findings have been presented in this document.

BLOOD–BRAIN BARRIER (BBB) CHARACTERISTICS

Brain capillary structures carry oxygen and nutrients to nervous cells (Obermeier et al., 2013). EC lining these vessels have a primary role in permeability regulation and with other cells, form highly organized barriers between the vascular space and nervous tissue parenchyma (Zhao et al., 2015). The neurovascular unit (NVU) consists of the aforementioned capillary EC, pericytes, astrocytes, microglial cells, and neurons joined by close direct and indirect interactions that maintain and regulate brain homeostasis (Abbott et al., 2006; Cardoso et al., 2010). Despite the high selectivity for solutes and molecules, sometimes bacteria, parasites, and viruses can pass through the BBB and invade nervous tissue (Zhang et al., 2015); in these cases, the local or systemic immune response contributes to altering NVU integrity (Hou et al., 2016).

Some viruses can reach the nervous parenchyma in different ways: (i) entering of infected leukocytes; (ii) axonal transport; and (iii) olfactory bulb epithelium infection. Other virus entry involves EC, (iv) inter-endothelial tight junction disruption and (v) EC infection and basolateral release (Miner and Diamond, 2016; Tohidpour et al., 2017). The latter two ways as well as the passing through of infected monocytes may be the most critical routes used by DENV to reach the CNS. For example, Hapuarachchi et al. (2015) reported a fatal case due to DENV4 infection. Importantly, the patient presented an accelerated

neurological damage and although the virus was detected in serum, the viral titer was higher in cerebrospinal fluid (CSF), suggesting a rapid virus entry through BBB (Hapuarachchi et al., 2015). Below, we present the *in vivo* and *in vitro* evidence on the single participation of each of the NVU cells and the whole structure participation in CNS infection by DENV. We also recapitulate evidence of BBB cells infection by other important flaviviruses (**Supplementary Tables S2, S3**).

ENDOTHELIAL CELLS

Plasma leakage is the central phenomenon in severe dengue (SD) cases. It is caused either by EC homeostasis alteration due to direct DENV infection or because EC are a target of inflammatory mediators secreted by either them or by other infected cells (Avirutnan et al., 1998). The first report of EC infection due to DENV used serotype 2 and primary cultures of rabbit cava vein and human umbilical vein EC (Andrews et al., 1978). Despite the existence of many studies, until recently the molecular events and specific receptors involved in DENV binding and entry to EC were unclear (Yang et al., 2016).

Dengue virus entry can occur in a receptor-mediated binding with or without clathrin participation manner, but in immune cells it also uses virion-IgG complex to bind Fc-gamma receptors to infect these cells (Wei et al., 2003; van der Schaar et al., 2008). This could also be a mechanism used in EC (Cruz-Oliveira et al., 2015).

Regarding the latter, using a virus overlay protein binding assay (VOPBA) in ECV304 cells, it was possible to identify three new cell membrane proteins of 29, 34, and 43 kDa, which binds to a recombinant E protein, suggesting that these proteins may be important for DENV entry and internalization in these cells (Wei et al., 2003).

Later, integrin $\beta 3$ was also identified as an EC receptor to DENV, since protein knockdown reduced viral entry by approximately 90% (Zhang et al., 2007). Similarly, HMEC-1 cells upregulate protein disulfide isomerase (PDI) during infection. Silencing its expression reduces the number of infected cells and the production of new virions (Wan et al., 2012). Recently, it was shown that the Rod domain of the intermediate filament vimentin, which is exposed at the EC surface, may interact with domain III of the DENV envelope protein. This indicates a role in the binding of the virus to the endothelium (Yang et al., 2016).

Additional evidence shows that DENV binding to EC induces Rho-associated coiled-coil-containing kinase (ROCK) activation and vimentin reorganization, which induces endoplasmic reticulum redistribution for more efficient virus replication and assembly (Lei et al., 2013). Membrane sugars and proteoglycans, such as heparan sulfate proteoglycan, act as virus-binding molecules, given that treatment with heparin or heparan-, chondroitin-, and dermatan-sulfate inhibited the infection up to 60% (Dalrymple and Mackow, 2011). Finally, evidence exists of the following molecules as EC receptors to DENV: DC-SIGN, ICAM-3, CD14, mannose receptor (CD206), heat shock proteins (HSP70 and HSP90), glucose-regulated protein (GRP78), and

TABLE 1 | Clinical reports of dengue cases with neurological involvement.

| Patients/ age (years) | Viral symptoms | Neurologic symptoms | DENV | | Molecular and other tests | Dx | Outcome (number of cases) | References |
|---|---|--|-------------------|-------------------|--|----------------|---------------------------------|--|
| | | | Lab tests | | | | | |
| | | | Serum | CSF | | | | |
| 55 CH (0.1–12); F (14); M (9–67) | Nausea, headache, vomiting, rigors, fever, rash, myalgia. | Confusion, seizures, unusual behavior, focal neurological signs, fluctuating consciousness, somnolence. | IgM NS1 IgG | IgM | CT: Edema and ischemic infarcts. MRI: Restricted diffusion. EEG: Encephalitis PNRT and IMH: DENV (+). BTB: Cerebral edema. | ENC ENP | L (12) RWS (2) CR (8) | Lum et al., 1996; Angibaud et al., 2001; Nogueira et al., 2002; Chotmongkol and Sawanyawisuth, 2004; Méndez and González, 2006; Reo et al., 2013; Mathew et al., 2014; Fong et al., 2017 |
| M (17; 25) F (27) | Fever, myalgia, arthralgia, vomiting, headache. | Seizures, coma, stiffness, amnesia, memory impairment | IgM IgG | IgM IgG | BTB: Infection of N, A, M and EC. MRI: Subdural hematoma | HVS MI | L (1) CR (1) | Ramos et al., 1998; Yeo et al., 2005; Mittal and Jain, 2011 |
| F (17; 28) M (42) | Headache, fever, vomiting, chills, diarrhea. | Confusion, K/B test (+), seizures, (↓) consciousness, fixed pupils, no motor response. | IgM NS1 IgG | IgM IgG | RT-PCR: DENV (+) | AP DF SD | RWS (1) CR (2) L (1) | Goswami et al., 2012; Hapuarachchi et al., 2013 |
| M (41), F (45) | Headache, myalgia, arthralgia, fever, chills | Loss of balance | IgM IgG NS1 | IgM IgG NS1 | TEM: VLP in brain parenchyma. NE: Lack of coordination, ataxic gait | HVS AC | L (1) CR (1) | Limonta et al., 2012; Withana et al., 2014 |
| M (18) | Fever, myalgia, headache, lethargy | Loss of balance, speech difficulty, weakness. | IgM IgG NS1 | IgM IgG NS1 | NE: Slight tremors, tongue in right protrusion | PMN/CA/BP | RWS | Azmin et al., 2013 |
| F (13, 24) | Fever, arthralgia, myalgia, headache | Seizures, rigidity in extremities. | IgM IgG IgG | IgM IgG | MRI: Hemorrhages CT: Brain stem herniation | HENC SD | RWS (1) L (1) | Nadarajah et al., 2015 |
| F (12; 39) | Fever, myalgia, nausea, diarrhea, chills | Weakness, flaccid quadriplegia, autonomic instability, involuntary muscle tightening and twitching | IgM IgG | IgM IgG | MRI: Epidural hematoma. TMBS: Motor potentials absent. | TM Mild NMC | RWS (1). CR (1) | Fong et al., 2016; Doi et al., 2017 |

Summary of clinical reports of patients with neurological consequences during or after DENV infection. AC, acute cerebellitis; AP, axonal polyneuropathy; BP, brachial plexopathy; BTB, brain tissue biopsy; CA, cerebellar ataxia; CH, children; CR, complete recovery; CT, computed tomography; DF, dengue fever; Dx, diagnosis; EEG, electroencephalogram; ENC, encephalitis; ENP, encephalopathy; F, female; HVS, hypovolemic shock; HENC, hemorrhagic encephalitis; IMH, immunohistochemical analysis; K/B, Kernig and Brudzinski test; L, lethargy; M, male; MI, memory impairment; MRI, magnetic resonance imaging; NE, neurological exam; NMC, neuromuscular complications; PMN, Parkinsonism with multiple cranial neuropathies; PRNT, plaque reduction neutralization test; RWS, recovered with sequelae; RT-PCR, polymerase chain reaction with retro-transcription; SD, severe dengue; TEM, transmission electron microscopy; TM, transverse myelitis; TMBS, transcranial magnetic brain stimulation; VLPs, viral like-particles; (+), positive; (–), decrease.

laminin receptor (Dalrymple and Mackow, 2014). However, their direct participation remains to be investigated.

Although, *in vitro* it is possible to evaluate and quantify DENV infection in EC, infection and replication is often not detected *in vivo*. In some cases, antigens or viral RNA have been detected in EC from brain biopsies of dengue fatal cases -particularly EC's from the inferior olive nucleus, medulla (Calvert et al., 2015) and cerebellar granular layer (Ramos et al., 1998), other reports, had shown viral RNA or proteins in EC from liver and lung (Jessie et al., 2004; Póvoa et al., 2014), suggesting viral replication in these cells (Basu and Chaturvedi, 2008).

There is no consensus about the effect of DENV on EC. Some reports have found no morphological changes in EC after infection (Srikiatkachorn and Kelley, 2014). However, apoptotic EC were detected in the lungs, guts, and brains of Cuban patients who died due to DENV infection (Limonta et al., 2007). In another study, ECV340 cells infected with DENV2 had a 7–40% increase in the number of apoptotic cells at 48 h post infection (p.i.) (Avirutnan et al., 1998). A similar result was reported after using an HMEC-1 line after infection with a DENV2 clinical isolate. Apoptosis was detected and activated by the cleavage of caspase 3 and poly (ADP-ribose) polymerase 1 (PARP-1) by the viral protease complex NS2B/NS3 (Vásquez et al., 2009).

Rodents are not naturally susceptible to DENV infection, delaying the implementation of suitable animal models to study infection and pathogenesis (Yauch and Sheresta, 2008). Viremia and clinical signs in non-human primates are mild (Zompi and Harris, 2012). This leads to the use of immunodeficient, humanized, or modified mice (Wu-Hsieh et al., 2009; Plummer and Shresta, 2014), or the production of adapted virus to specific hosts, with the purpose of implement models to study the disease, antiviral, or vaccine development (Puccioni-Sohler and Rosadas, 2015). These infection models have advantages and flaws but are frequently used to better understand dengue disease (Srikiatkachorn and Kelley, 2014).

Using mouse models, the nervous tissue infection has been evaluated. A previous work reported BBB damage in Balb/c mice inoculated intracerebrally (i.c.) with DENV2. Researchers found Evans blue leakage and radiolabeled erythrocytes in brain parenchyma, a phenomenon explained by the increase of cytokines (Chaturvedi et al., 1991). Recently, we established a DENV neuroinfection model inoculating the neuroadapted D4MB-6 strain intraperitoneally (i.p.) in 2- and 7-day-old Balb/c mice. This adapted strain (DENV4 mouse brain passaged six times) reaches the CNS and induces severe brain cytoarchitectural changes, leukocyte infiltration, and BBB dysfunction associated with EC activation (Velandia-Romero et al., 2012). A similar finding in adult mice was reported recently, and viral replication occurred in EC from the cortex, hippocampus, and cerebellum detected by the NS3 expression, concomitant with BBB disruption (Solomão et al., 2018). In this case, the DENV2 was isolated from a patient with confirmed encephalitis, suggesting that natural DENV strains are neurotropic and neurovirulent, able to evade the immune system, and invade the brain efficiently through BBB ECs, leading to replication in the brain parenchyma which induces nervous injury.

Dengue virus-infected EC are low in most *in vitro* experiments using either primary cultures or cell lines (Calvert et al., 2015). The proportion of DENV-4-infected mouse brain microvascular EC (MBEC) cultured in our laboratory were 7 and 12% at 24 and 48 h p.i. respectively, but the neuroadapted D4MB-6 strain infected half of the cells at 48 h p.i. (Velandia-Romero et al., 2016). These differences between models may be explained by the presence of heparin in some media cultures for EC which may inhibit the infection. Infection percentages also depend on DENV strain or the concentration of NS1 in the inoculation media, since this protein forms complexes with HSPG competitively, inhibiting the EC infection (Calvert et al., 2015). In fatal cases, the likelihood of finding viral proteins or RNA depends on the time of tissue collection, because in later periods the virus may be cleared making detection impossible (Srikiatkachorn and Kelley, 2014).

The apparent low susceptibility of EC to DENV infection observed in post-mortem samples contrasts with the profound effect that the infection has on these cells, in particular on the BBB. This suggests that other factors, such as inflammatory molecules, amplify the effects of DENV and alter the endothelial tissue and other cells of the NVU (Calvert et al., 2015).

Endothelial cell activation due to infection was reported based on the expression or secretion of sVCAM-1 and sICAM-1 (Cardier et al., 2006), which has also been detected in the membrane of HMEC-1 and LSEC (Peyrefitte et al., 2006). The D4MB-6 neuroadapted strain after the infection of MBEC also induced VCAM-1 and ICAM-1 expression, as well as PECAM-1 and E-selectin (Velandia-Romero et al., 2016). Interestingly, the immune mediators secreted by infected EC depends on the serotype used, i.e., DENV1 induced interleukin (IL) -6, TNF- α , chemokine (C-X-C motif), ligand 1 (CXCL1), CCL2, CCL5, and CCL20 (Soe et al., 2017). All of these molecules are associated with endothelial hyperpermeability and change the coagulatory balance related to hemorrhages and disseminated intravascular coagulation seen during severe dengue (Srikiatkachorn and Kelley, 2014). However, DENV2 infection induced monocyte chemoattractant protein 1 (MCP-1), matrix metalloproteinases (MMP), and macrophage migration inhibitory factor – MIF – (Lima-Juniora et al., 2013).

PERICYTES

These cells surround the EC and are immersed in the basal membrane of microvessels and capillaries. Pericytes have a crucial role in the maturation and function of the BBB since they interact with both EC and astrocytes inside the NVU (Yamakazi and Mukouyama, 2018). To our knowledge, there have been no reports on DENV infection or susceptibility to it of pericytes, although Japanese encephalitis virus (JEV), another *Flavivirus*, is known for infecting them and affecting the BBB function.

Japanese encephalitis virus is responsible for most encephalitis cases in Asia, with around 68,000 cases reported each year, 30% of which result in fatal outcomes (Thongtan et al., 2012). This virus was isolated for first time in 1935 (Solomon et al., 2003; Mishra et al., 2008) and is transmitted via *Culex* spp. mosquitoes. Fever, headaches, meningeal irritation, and an altered state of

consciousness are the primary signs and symptoms (Thongtan et al., 2010). During infection, 30–50% of patients may present paralysis, loss of speech, and behavioral changes (Chen et al., 2000; Thongtan et al., 2012).

Several molecules have been proposed as cell receptors to JEV, including HSPG, HSP70, and HSP90B, vimentin, CD4, laminin receptor, and $\alpha 5\beta 3$ integrin. However, the pericyte molecule involved in the infection is not yet known (Nain et al., 2016).

Even though EC obtained from brain microvascular vessels are susceptible to JEV, the *in vivo* BBB function and integrity is not affected during infection. However, EC cultures exposed to JEV-infected pericyte supernatants experienced an increase in permeability and loss of *trans*-endothelial electrical resistance (TEER). These changes in function were related to the presence of IL-6, MMP-2 and -9, and the proteasomal degradation of the zonula occludens protein – ZO-1 – (Chen et al., 2014), which is an important scaffold protein, whose interaction with others tight junctions proteins (TJP) and the actin filaments of the EC are crucial for the maintenance of endothelial barrier function and the regulation of vascular permeability (Garcia-Ponce et al., 2015). Together, these findings suggest that the immune response of the pericytes play a central role in endothelial dysfunction during JEV infection (Chang et al., 2017).

Recently, it was reported that the *Flavivirus* ZIKV infects retinal pericytes and retinal EC in addition to pigmented epithelium cells (Roach and Alcendor, 2017). Interestingly, pericytes replicate ZIKV more efficiently than EC, but they do not die during infection. Both cell types, after 4 days p.i., secreted high levels of RANTES, a chemokine that favors the retina inflammation and ocular injury that occurs in ZIKV infection (Roach and Alcendor, 2017). In this work, it is suggested that the virus enters in a hematogenous way, through EC from retinal arteries and capillaries. This is because both pericytes and EC are susceptible to infection and able to replicate the virus, allowing it to spread to other eye cells. These findings suggest that pericytes may play a role in BBB, permitting the infection and secreting the immune mediators that modify the barrier physiology.

ASTROCYTES

These cells are the main components in the architecture and function of the CNS. In addition, to providing metabolic support to neurons, astrocytes can also regulate neuronal activation and function (Abbott et al., 2006), as they store information and regulate the synaptic transmission participating in the tripartite synapsis with pre- and post-synaptic neurons (Perea et al., 2009). Astrocytes modify the EC and BBB function (McConnell et al., 2017) due to their permanent contribution to nervous system defense and recovery after an infection, or physical or metabolic damage (Zorec et al., 2018). For example, astrocytes express pathogen-associated molecular patterns (PAMP) and Toll receptors (Krasowska-Zoladek et al., 2007; Suh et al., 2009) that recognize bacteria and viruses (Kigerl et al., 2014) in addition to MHC-I and II (Dong and Benveniste, 2001) to recognize and present antigens. These cells also secrete at least 200 different molecules immunomodulators, hormones, and growth factors

(Verkhatsky et al., 2016). This means that after a stimulus they respond with cell activation (astrogliosis or astrocytosis) and promote neuroinflammation. There are few studies on DENV infection effect on astrocytes, and those that are available provide contradictory evidence. For example, Balb/c astrocytes (Type I and II) were exposed to three different strains of DENV2 and one of DENV4 and no infection was reported after 15 days p.i. (Imbert et al., 1994). Similarly, GFAP positive astrocytes were not infected after the i.p. inoculation of the neuroadapted D4MB-6 dengue strain (Velandia-Romero et al., 2012), and neither viral protein nor RNA were detected in astrocyte cultures at 48 h p.i. (Velandia-Romero et al., 2016). Despite this, *in vitro* astrogliosis post viral inoculation was confirmed evaluating the cells morphological changes.

Astrocytes infection with DENV has been reported in fatal human biopsy cases (Bhoopat et al., 1996), like the one reported in Mexico (Ramos et al., 1998). However, in every case, regardless of whether the astrocytes were infected or not, researchers reported the early and robust response to infection as astrogliosis, which was maintained even after nervous tissue viral clearance. Therefore, astrogliosis could be involved in long-term complications presented in some patients (Lee et al., 2016).

During the activation process, astrocytes change function, morphology, and biochemistry (Middeldorp and Hol, 2011), induced by local signals that depend on the localization and duration of the harmful stimulus or aggression (White and Jakeman, 2008). Astrocytes in injured tissue secrete pro-inflammatory molecules such as IL-6, TNF- α , and interferon β (Boonnak et al., 2011; Burkert et al., 2012).

Significant astrogliosis and progressive cell hypertrophy with longer and thicker astrocytic processes may be induced by strong or continuous stimuli (Eng et al., 2000). These morphological changes are associated with the overexpression of cytoskeleton proteins such as GFAP, vimentin, and nestin (Buffo et al., 2010), which are essential for cell proliferation process and glial scar formation (Sofroniew, 2009).

Rhesus macaques inoculated with intravenous or subcutaneous injections of DENV2 experienced a reduction in the number of astrocytes in the frontal lobe compared to non-infected control monkeys. However, cytoplasmic enlargement and an increase in the number and length of astrocytic end-feet in white matter were found (Lee et al., 2016). Generalized astrogliosis was seen in 2-, 7-, and 14-day-old Balb/c mice infected with the neuroadapted strain D4MB-6, finding large numbers of GFAP+ cells that were larger in size, more branched, and with greater fluorescence intensity. The latter results were related with the evidence of paralysis, which is a sign of neural impairment along with the histopathological evidence of neuron infection and efficient viral replication in these cells. Interestingly, the older mice (14 days old) were refractory to infection and showed no neurological symptoms. Nevertheless, they had astrocytic activation with the same changes, demonstrating that the immune and physiological development of these individuals determines their susceptibility to DENV infection, favoring brain virus clearance (Velandia-Romero et al., 2012).

On the other hand, it has been shown that astrocytes are susceptible to the West Nile Virus (WNV) and that

they can become viral reservoirs because WNV persists for up to 114 days after infection in astrocytes without inducing cell death (Diniz et al., 2006). This virus shares some characteristics with DENV and JEV and currently is a concern in many countries experiencing permanent outbreaks (Solomon and Mallewa, 2001). In addition to infection in Langerhan's cells and keratinocytes, after the initial replication, WNV infects neurons, astrocytes, microglial cells, and EC, leading to a massive recruitment of inflammatory cells toward the brain parenchyma (Hussmann and Fredericksen, 2014; Winkelmann et al., 2016). Clinically, the infection is characterized by meningitis, encephalitis, and acute flaccid paralysis. In severe cases it can cause the death of the patient (Hussmann and Fredericksen, 2014).

Another factor to consider during infection and glial activation is the brain lymphatic system (BLS) (Louveau et al., 2015; Sandrone et al., 2019), represented by the classic lymphatic vessels in the dura mater, and the canals formed by the walls of the veins and astrocytic endfeet (called the glymphatic system) (Iliff et al., 2012; Yang et al., 2013; Louveau et al., 2015). Importantly, the function of this structure is to drain debris and interstitial macromolecules from the brain (Aspelund et al., 2015). Additionally, BLS allows the circulation of T lymphocytes, which in response to infection can be protective or pathogenic (Louveau et al., 2015).

In fact, the drainage of viral antigens from the parenchyma to the CSF (made by the glymphatic system), and from there to the lymphatic vessels, can contribute to suppression or control of the T cells response in the periphery (McGavern and Kang, 2011; Louveau et al., 2015). This would explain the tolerance and little activity of the extra neural immune system during some nervous system infections with virus like HIV (Tan et al., 2012), MCMV (Torti et al., 2011) and parasites like *Toxoplasma gondii* (Landrith et al., 2015). Interestingly, although it is known that the lymphatic system is supremely important during the dispersion of DENV in the periphery (Yam-Puc et al., 2016), its participation in the dispersion of the virus within the nervous system by recirculating the virus that might be present in the CSF is not known or evaluated. Similarly, its role in the control of infection and its contribution to the evasion of the response within nervous tissue is unknown.

MICROGLIAL CELLS

According to their mesodermal origin, microglial cells are immune cells of the CNS (Ginhoux and Prinz, 2015) which express macrophage markers such as F4/80, Fc receptors, and CD11b. During brain injury, microglial cells become reactive and secrete inflammatory mediators similar to those produced by macrophages (Lannes et al., 2017). Microglia establishes bidirectional communication with neurons, astrocytes and pericytes, which is a determining factor during development and brain function in adults. In pathological conditions, an early reaction of microglia inducing and regulating the astrocytes function occurs (Jha et al., 2018). These cells are susceptible to DENV, possibly due to the expression of previously reported viral

receptors such as, HSPG, CD14, DC-SIGN, GRP78, HSP70 and HSP90, β 3 integrin, mannose receptor, and C-type lectin domain containing 5A (CLEC5a), and others (Thongtan et al., 2012), just like it has been described in the murine microglial cell line BV2, previously reported as susceptible to all four DENV serotypes (Bhatt et al., 2015).

After binding to a receptor, clathrin-mediated endocytosis is used to internalize the DENV (Jhan et al., 2017) and start replication, an event that triggers cell activation, leading the morphological change from round cells to multipolar cells, and the secretion of inflammatory molecules. These changes can be observed *in vitro* in BV2 cells (Bhatt et al., 2015), and in the brain of young mice infected with neuroadapted D4MB-6 strain, where infected microglial cells were also observed frequently in the analyzed tissue (Puccioni-Sohler and Rosadas, 2015). Interestingly, each DENV serotype infection induces different responses in BV2 microglial cells. DENV1 induces a cytokine profile related with changes in vascular permeability, while DENV2 alters the oxidative stress-mediated apoptotic response, and DENV3 establishes an anti-inflammatory and antiviral mediators profile. On the other hand, mediators induced by DENV4 could have a significant role in the alteration of BBB function, mainly by enhancing the secretion of MMP (Bhatt et al., 2015). Infection with DENV2 and DENV4 caused the activation of rat microglial cells (HAPI), but serotype 4 induced a higher ROS production and greater cell death compared to DENV2 or even JEV (Suwanprinya et al., 2017), suggesting a noxious role in brain inflammation and neurotoxicity.

The response of microglia may be essential to protect DENV-infected mice, as the animals treated for macrophage/microglia depletion with clodronate liposomes showed higher viral RNA and protein expression as well as more severe neurological signs. The reduction of microglial cells in the brain was also related with lower IFN- γ and IL-12 secretion and less infiltrated TL CD8 cells (Tsai et al., 2016). Taken together, these results support that, despite microglial cells susceptibility to DENV infection, their response contributes to delay nervous tissue damage, and like it happens during infections with JEV, microglial cells may play the role of Trojan horse, transporting the virus and serving as viral reservoirs for up to 16 weeks (Nain et al., 2016).

GLIAL ACTIVATION

Microglial and astrocytic cells are the essential pillars of the immune response during a viral infection in the CNS (Ramesh et al., 2013; Bhatt et al., 2015). Overall, after the pathogen is recognized, astrocytes and microglial cells trigger two types of events: (i) an activation of the innate immune response in neighbor cells, amplifying the signal, and (ii) a favoring of the changes in BBB permeability, contributing to blood leukocyte migration to the brain parenchyma, and helping in the adaptive immune response (Jhan et al., 2017). As part of the response, mediators such as IL-6, IFN- γ , GM-CSF, IL-1, and TNF- α are secreted. These induce changes in BBB permeability and together are the necessary stimuli to differentiate and induce proliferation of monocytes, astrocytes, microglial cells, and EC

(van Kralingen et al., 2013). The production of chemokines and the induction of adhesion molecule expression facilitate T cells entering and surviving, and the proliferation of B cells in the tissue (van Kralingen et al., 2013). As part of a general response, during infection, astrocytes and microglial cells secrete anti-inflammatory and neuroprotector molecules that induce suppression and neural protection. The expression of co-stimulatory molecules such as B7 and B40 of the adaptive immune response (Farina et al., 2007) is a key mediator to diminish the damage in nervous tissue (Miljkovic et al., 2007).

NEURONS

In addition to their role in information processing, neurons contribute to regulating the other nervous system cell functions. These cells secrete VEGF and establish physical contact with NVU cells during development to regulate their activity and stimulate BBB growth and branching (Ruhrberg and Bautch, 2013). Once the vascular network is established, the cytoplasmic processes of neurons and astrocytes control the vessels lumen diameter, releasing vasoactive substances such as nitric oxide (Gotoh et al., 2001), acetylcholine (Scremin et al., 1973), neuropeptide Y (Abounader et al., 1995), and somatostatin (Cauli et al., 2004; McConnell et al., 2017), demonstrating the principal role of neurons in BBB homeostasis.

Peripheral DENV infection and replication induces a “cytokine storm” which affects many tissues and even modifies brain homeostasis and neurological signs without direct infection. Neuronal infection has been detected in tissues obtained from fatal cases (Ramos et al., 1998; Nogueira et al., 2002; Guzman and Harris, 2015), however, there have been documented cases of infections and neurological alterations such as loss of consciousness, sensory or motor dysfunction and analgesia in the early post-infection times (Shweta et al., 2014).

Animal models of dengue infection have shown neuronal infection and nervous tissue injury (Hotta et al., 1981). One of the first works describing viral antigens in neurons after intracerebral inoculation also detected proteins of the virus in neuronal axons. Ultrastructural descriptions showed neuronal necrosis and nuclear clefts (Sriurairatna et al., 1973).

Approximately 40% of primary cultured Balb/c neurons were infected using a MOI 5 (Salazar et al., 2013) and experiments in the mouse cell line Neuro-2a showed viral internalization after 2 h post inoculation. The virus was detected using a plaque assay after 24 h p.i. (Ho et al., 2017), reinforcing the evidence for some DENV neurotropism, although the neuronal molecules responsible for the infection promotion remain unknown. Using VOPBA experiments there has been identified a 65 kDa protein which binds specifically to DENV (Ramos-Castañeda et al., 1997). Interestingly, *in vivo* and *in vitro* dopamine receptor-blocking induced a reduction of neurological symptoms in mice and diminished the number of infected neurons (Simanjuntak et al., 2014). A similar finding was observed in Neuro-2a cells (expressing D2R) and mouse hippocampal neurons (expressing D1, D2, and D5 receptors) since treatment with metoclopramide reduces the infection, suggesting the

participation of dopaminergic receptors in DENV infection (Ho et al., 2017). This work also demonstrated that Neuro-2a cells treated with an inhibitor of clathrin-mediated endocytosis (chlorpromazine) or an inhibitor of clathrin-independent endocytosis (Pitstop-2) induces a significant infection reduction, replication, and virus release, indicating the participation of both endocytosis systems (Hou et al., 2016), such as those previously reported by van der Schaar et al. (2008).

Despite many authors proposed that DENV should not be considered as a neurotropic or neurovirulent flavivirus, there is evidence of CNS entry by hematogenous and axonal transport routes (Chaturvedi et al., 1991; Mc Minn, 1997). However, the DENV neurotropism and neuroinvasion determinants are still little known (Velandia-Romero et al., 2012). A monocyte-released free virus could infect EC in brain capillaries and reach the parenchyma by passive diffusion or transcytosis (Mc Minn, 1997; Liou and Hsu, 1998; Chantick et al., 2016) and infect neurons and microglial cells, like occurs with other *Flaviviruses* (Ludlow et al., 2016). However, axonal transport used by many neurotropic viruses, such as herpes, rabies, JEV, and WNV, may travel inside the axons as nude capsids or in vesicles until they reach the neuronal soma in the brain and spread to other areas using the axonal transport (Hunsperger and Roehrig, 2009; Salinas et al., 2010; Ludlow et al., 2016).

Immunodeficient neonatal mice inoculated with DENV2 showed viral particles in both myelinic and amyelinic fibers of pre- and post-synaptic neurons by electronic microscopy at 5 days p.i., suggesting an axonal spreading of DENV in the brain and spinal cord (An et al., 2003). The neuroadapted D4MB-6 strain used in our laboratory inoculated intraperitoneally or by footpad injection in 3-day-old Balb/c mice produced encephalitis and paralysis.

Once the virus reaches the brain, DENV induces paralysis, myelitis, and transverse myelitis in addition to unconsciousness and behavioral changes, indicating the neuronal involvement during infection. Neuronal apoptosis appears to be responsible for neurological manifestations, at least in infected mice, as hippocampal and cortical neurons die during infection, many of them undergoing apoptotic changes (Deprès et al., 1996). In this regard, the neuroadapted D4MB-6 strain induced a high proportion of TUNEL positive cells in the brain parenchyma (Velandia-Romero et al., 2012), similar to the findings in adult C57/BL6 mice which were intracerebrally inoculated with DENV3. They suffered behavioral changes associated with hippocampal neuron apoptosis mediated by caspase-3 (Silva de Miranda et al., 2012), presumably related to high levels of TNF- α secretion by neurons or glia (Jhan et al., 2018). It is not clear if at least part of the death seen in neurons is due to a bystander effect rather than direct infection.

Neuronal death and apoptosis have been frequently reported in cultures, showing a pattern of chromatin condensation by electronic microscopy and DNA fragmentation (TUNEL positive) (Ludlow et al., 2016). This finding also occurred in N18 mouse neuroblastoma cells, which showed additional arrest in the G1 cell cycle phase (Su et al., 2001). Recently Ho et al. (2017), described cell death, proliferation arrest, and morphological changes in Neuro-2a cells after DENV2 infection

using a high MOI (Ho et al., 2017). Although these results are obtained in mouse neuroblastoma cells, we recently reported that DENV2 infection in SH-SY5Y human neuroblastoma cells induces cell death, expression, and secretion of TNF- α as well as DNA fragmentation and Annexin V translocation, suggesting similar responses among species and validating these models (Castellanos J. E. et al., 2016).

CONCLUSION

Dengue pathogenesis is complex and sometimes may appear as nervous system damage, with neurological signs and symptoms. Clinical evidence, as well as evidence in cells and animals, suggests that DENV may enter the CNS via the hematogenous route and infect both neurons and microglial cells, before eventually infecting the EC of BBB. These infected cells, when activated, prevent virus spread and help to control their deleterious effects. This response appears to be adequate in most human cases and controls infection and inflammation with minor or no sequelae; however, some cases result in severe disease or death. The studies in cells and mice show apoptotic neuronal death associated with direct infection due to inflammatory mediators or excitotoxicity. Glial cell and EC responses during infection indicate that soluble molecules, cytokines, and chemokines such as RANTES, TNF- α , IL-6, IL-10, and MCP-1 among others, favor leukocyte infiltration, worsening brain inflammation. This response is standard for a neurotropic *Flavivirus* such as JEV and WNV and indicates a putative neurotropic feature of dengue. Immune responses in the nervous system may also be responsible for the loss of barrier function, allowing a massive infiltration of infected/activated monocytes that enhance viral spread and tissue injury, establishing a

vicious circle of high permeability, greater infiltration, greater cell infection, and greater inflammation.

AUTHOR CONTRIBUTIONS

M-AC-P formulated the review idea, and wrote, translated, and edited the review. MV-R formulated the review idea, and wrote and edited the review. LB-L, EB, and SC-O wrote and edited the review. JC wrote, translated, and edited the review.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.01435/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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