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Pathogenesis and clinical aspects of tick-borne encephalitis virus infection

Ph.D. thesis

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Abstract:

This thesis contributes to knowledge about the combined impacts of the pathogenesis of the tick-borne encephalitis virus (TBEV) and the immunopathogenesis of the host on the clinical course of acute tick-borne encephalitis (TBE). The thesis further focuses on the process of TBEV neuroinvasion and the utilization of the host's immune products as potential therapeutic interventions.

Declaration [in Czech]:

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

V Českých Budějovicích, 20. 2. 2020 ……..

RNDr. Jana Elsterová

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List of papers with author's contributions:

The thesis is based on the following papers and manuscripts:

1. Martin Palus, Eva Žampachová, Jana Elsterová and Daniel Růžek. (2014) Serum matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 levels in patients with tick-borne encephalitis. Journal of Infection 68: 165-169. [IF_{2018} = 5,099; $Q1_{2018}$= Infectious Diseases]
   Jana Elsterová participated in data analysis.

2. Martin Palus, Tomáš Bílý, Jana Elsterová, Helena Langhansová, Jiří Salát, Marie Vancová and Daniel Růžek. (2014) Infection and injury of human astrocytes by tick-borne encephalitis virus.
   Journal of General Virology 95: 2411-2426. [IF_{2018} =2,089; $Q2_{2018}$= Virology]
   Jana Elsterová participated in the design and performance of the experiments and contributed to the drafting of the manuscript.

3. Tomáš Bílý, Martin Palus, Luděk Eyer, Jana Elsterová, Marie Vancová and Daniel Růžek. (2015) Electron tomography analysis of tick-borne encephalitis virus infection in human neurons.
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4. Martin Palus, Petra Formanová, Jiří Salát, Eva Žampachová, Jana Elsterová and Daniel Růžek. (2015) Analysis of serum levels of cytokines, chemokines, growth factors, and monoamine neurotransmitters in patients with tick-borne encephalitis: Identifications of novel inflammatory markers with implications for pathogenesis. Journal of Medical Virology 87: 885-892. [IF_{2018} =2,049; $Q2_{2018}$=Infectious Diseases]
   Jana Elsterová participated in data analysis and interpretation.

5. Martin Palus, Marie Vancová, Jana Širmarová, Jana Elsterová, Jan Perner and Daniel Růžek. (2017) Tick-borne encephalitis virus infects human brain microvascular endothelial cells without compromising blood-brain barrier integrity. Virology 507: 110-122. [IF_{2018} =2,657; $Q2_{2018}$= Virology]
   Jana Elsterová participated in the design and performance of the experiments.

6. Jana Elsterová, Martin Palus, Jana Širmarová, Jan Kopecký, Hans Helmut Niller and Daniel Růžek. (2017) Tick-borne encephalitis virus neutralization by high dose intravenous immunoglobulin.
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   Jana Elsterová participated in conceptual design, design of the experiments, data analysis, and drafting of the manuscript.

7. Jana Elsterová, Aleš Chrdle, Dana Teislerová, Václav Hönig, Martin Palus and Daniel Růžek. In prep. The severity of TBE correlates with the age-related physiological changes of blood-brain barrier integrity and immune reaction.
   Jana Elsterová participated in conceptual design, data collection, data analysis and interpretation, and drafting of the manuscript.

Structure of the thesis:

Preface:

Current generations, including mine, have grown up with the belief that everything can be explained with science. But working in science has made me realise that explanations lie not only in the results we obtain from our scientific studies, but also in the journeys we undertake to reach them. These journeys open up many new possibilities that could not have been conceptualised at the outset of a scientific investigation, and are as important as the results themselves. Working on this thesis has been an incredible collection of journeys, ones I could not have embarked on alone. As a result, the thesis does not represent the work of only one individual. Instead, it summarises the results of collaborations within our laboratory and with external colleagues who share enthusiasm for the same topic.

Virology is a very attractive discipline. It is shrouded in many secrets, yet impacts greatly on human life. Early virology was akin to a religion, relying on trust in something that was not visible to the human eye. For today's evidence-based science reliant on modern technology, virology has still not lost its mystery, spurred by the freedom of ideas to push further the boundaries of our knowledge.

In the case of arboviruses, namely the tick-borne encephalitis virus (TBEV), we are trying to understand a difficult and complex natural cycle. It appears that the relationship of the virus, its vector and its natural host is more a relationship of cooperation rather than one of enemy attack. Humans are "dead end" hosts, and it appears that neuroinvasiveness and neurovirulence are more a by-product of the infection than an intentional attack. An individual's immune response and genetic background seem to play a powerful and important role in the development of tick-borne encephalitis. The Laboratory of Arbovirology, together with the Department of Virology with Dr. Daniel Růžek at the helm, is working on understanding all aspects of TBEV from the historical, ecological, molecular and clinical points of view. My aim is to study the clinical pathogenesis of TBEV infection in human patients and to contribute to improving the efficiency of disease therapy.

Introduction:

Tick-borne encephalitis (TBE) is a disease that takes many forms, from an asymptomatic or mild flu-like illness to severe encephalitis, which can lead to paresis and even death. In Europe, TBE represents one of the most serious infections of the central nervous system. South Bohemia is a hyper-endemic region with high TBE prevalence and a relatively low vaccination rate, and as such presents a unique opportunity to study the pathogenesis of the tick-borne encephalitis virus (TBEV), namely the European subtype, as well as a great obligation to help the suffering patient in "real time".

The following section presents an overview of the current knowledge about TBEV. It describes the virus within the system of the *Flaviviridae* family, detailing its reproduction cycle in a host cell and circulation in nature, and examines the impact of the virus on human health, beginning with the molecular mechanism within the infected cells through to the clinical course of the disease.

Classification and evolution of tick-borne encephalitis virus:

The majority of viruses in the genus *Flavivirus* are arboviruses, viruses transmitted by hematophagous vectors, mainly ticks and mosquitoes. However, the genus *Flavivirus* also includes viruses which are restricted only to arthropods or viruses with no known vectors. TBEV is the main medically relevant representative of the tick-borne encephalitis serocomplex, which is further composed of the closely related Langat virus, Louping ill virus, Alkhurma virus, Kyasanur forest disease virus, Omsk hemorrhagic fever virus, Powassan virus and others (Grard et al., 2007), (Dobler, 2010), (Grabowski & Hill, 2017).

Phylogenetic analysis has revealed three main subtypes of TBEV. The subtypes differ in geographic distribution and exhibit a restricted vector range. The European subtype, primarily transmitted by the tick *Ixodes ricinus*, includes the prototype strain Neudorfl. Its geographic distribution covers the area from Western Europe across Central European countries and up to Scandinavia and the European part of Russia. The Far Eastern subtype, where the Sofjin strain is

the concerned prototype, is distributed throughout Ukraine and eastern Russia, China and Japan. The primary vector of this subtype is the tick *Ixodes persulcatus*. The same tick species is the vector of the Siberian subtype, present in central Siberia, with Vasilchenko and Zausaev as the prototype strains (Ecker et al., 1999). However, new risk areas are being discovered every year. Genetic proximity and close antigenic linkages have contributed to the theory that all TBEV subtypes represent variants of a single ancestor virus (Gould et al., 2001). Replication of TBEV is constrained by the long life cycle of a tick and a low total virus turnover (Gritsun, Nuttall & Gould, 2003). Since the absence of recombination of flaviviral genes, the variability of the virus subtypes and the progression of TBEV evolution can be explained in several ways: by slow adaptive evolution and by the existence of a quasispecies mix where some quasispecies are more suitable for vectors and some for vertebrate hosts (Wang et al., 2002), (Růžek et al., 2008), (Asghar et al., 2017). Another driving force in flavivirus variability could be vector switch (Kovalev & Mukhacheva, 2014). Many field observations have provided evidence of various subtypes present in different tick species that have not led to an emergence of new virus variants (Kim et al., 2009), (Jääskeläinen et al., 2011). Moreover, two other potential TBEV subtypes have been discussed in current arbovirology research. A potential new Baikal subtype lineage of TBEV, spread over eastern Siberia, contains 22 strains with an "886-84" strain as a prototype (Demina et al., 2010), (Kozlova et al., 2018). Recently, two novel isolates of TBEV were found in rodent hosts, suggesting a new Himalayan subtype of TBEV (Dai et al., 2018). The determination of different TBEV subtypes has been done on the basis of phylogenetic analysis of E protein (Ecker et al., 1999), (Dai et al., 2018), (Kozlova et al., 2018). The evolution of flaviviruses and their relationship with other viral families has been studied from various perspectives in our laboratory, namely by Dr. Jiří Černý. By combining sequence and structure analyses, Černý has brought a new perspective on the evolution of viral polymerases (Černý et al. 2014), (Černý et al. 2015). Černý's work is summarized in his PhD thesis (Černý, 2015).

Structure of tick-borne encephalitis virus and its replication cycle:

The TBEV genome consists of a single strand RNA of positive polarity, which is covered by a spherical envelope approximately 50 nm in diameter (Figure 1). The viral genome of approximately 11,000 nucleotides is primed by a 5´cap followed by a single open reading frame, which is surrounded by 5´and 3´untranslated regions. The coding region is translated to a single polyprotein, which is co-translationally and post-translationally cleaved by viral and cellular proteases to three structural proteins (protein C, prM and protein E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4B and NS5) (summarized by Pulkkinen, Butcher &Anastasina, 2018).

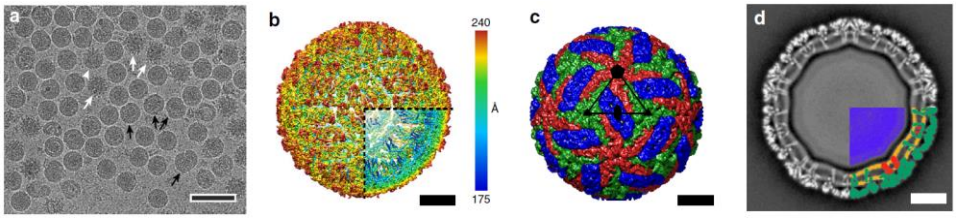

Figure 1: Structure of a TBEV virion (Füzik et al., 2018). a) Structure of mature and immature virions seen with electron microscopy methods. Scale bar 100 nm. b-d) Electron-density map of a TBEV virion showing the envelope icosahedral asymmetric unit consisting of M and E proteins and the central slice of a TBEV electron density map.

The virus replication cycle is presented in Figure 2. Briefly, attachment to a cell during an infection is receptor-mediated. The virion is taken up to the cell mainly via endocytosis and driven to endosomes. The acidic environment in the endosome causes conformational change of the envelope of the viral particle, which leads to a fusion of the viral and endosomal membranes. The viral genome is then released to the cytoplasm and its replication is mediated through anti-sense RNA intermediates, which serve as a template for the synthesis of genomic RNA, which also plays the role of mRNA for the translation of viral proteins. The replication occurs in so-called virus replication factories in close association with the membranes of endoplasmic reticulum. Immature particles then pass through the Golgi apparatus, where prM protein is cleaved and the reorganization of E protein into homodimers results in mature viral

particles, which are transported to the cell membrane with cytoplasmic vesicles and released by exocytosis (Roby et al., 2015), (Pulkkinen, Butcher & Anastasina, 2018).

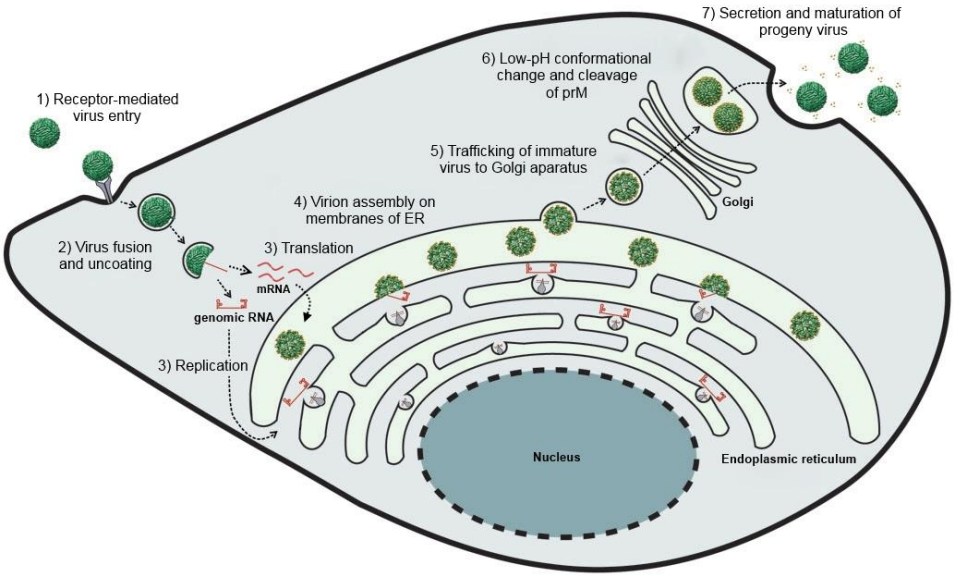

Figure 2: Replication machinery of TBEV in a mammalian cell (adapted from Roby et al., 2015)

Vector-host interactions and virus transmission:

Tick-borne encephalitis virus is an arbovirus that requires a tick as a vector and a virus reservoir. The possible routes of transmission are shown in Figure 3. Ticks were identified as a source of infection, namely of the Louping ill virus, by Sir Stewart Stockman more than 100 years ago (Stockman, 1918), (Jeffries et al., 2014). The tick's multi-stage lifecycle is one of the main reasons why arthropod-borne viruses choose the tick as their vector. Ixodid ticks have three developmental stages: larva, nymph and adult, all requiring blood meal and thus enabling transstadial virus transmission (Nuttall & Labuda, 2003), (Slovák et al., 2014). Transovarial transmission also contributes to the survival of the virus in the vector population, although the transmission rate from one

generation of ticks to another is very low (Řeháček, 1962), (Benda, 1958), (Danielová et al., 2002). Vertebrate hosts serve as a blood source for viremic horizontal transmission of the virus and as an entity allowing localized transmission among co-feeding ticks (without infecting the host) (Labuda et al., 1993). Many rodents and insectivorous species are therefore regarded as maintenance hosts. The tick *Ixodes ricinus* is a great generalist, able to feed on more than 300 species of vertebrate hosts (Anderson, 1991). Different developmental stages prefer different types of host. The abundance and distribution of ticks as vectors of various pathogens has been studied extensively in our laboratory and was the main thesis focus of our colleague Dr. Václav Hönig (Hönig, 2015). In his thesis, Hönig constructed a spatial model to track the distribution of tick-borne pathogens in South Bohemia, Czech Republic, an endemic region of TBEV and other tick-borne pathogens (Hönig et al., 2015), (Hönig et al., 2017), (Hönig et al., 2019).

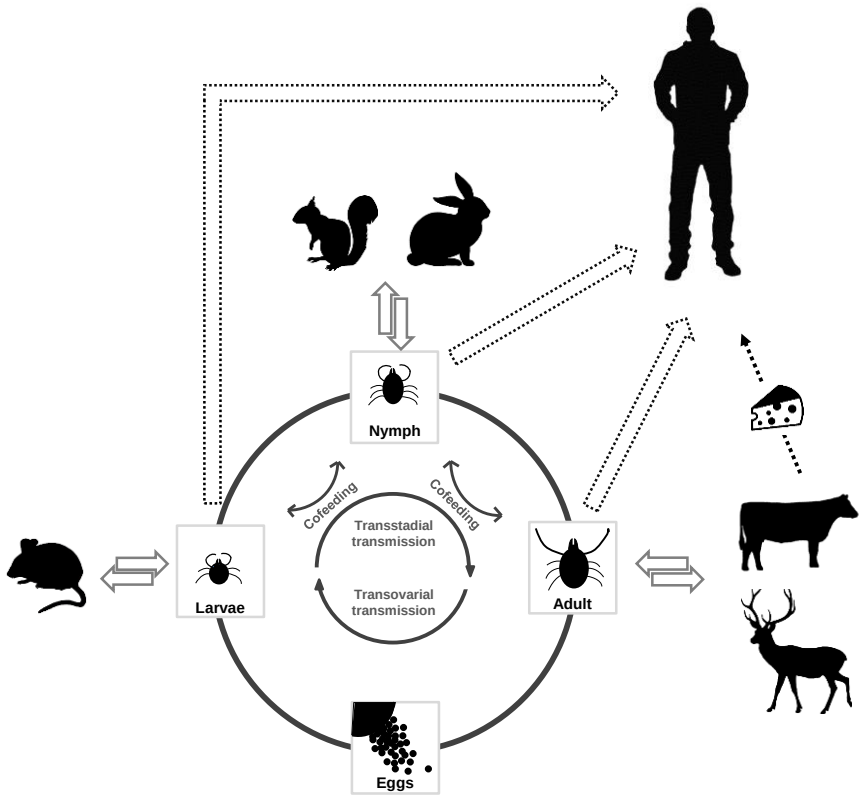

Figure 3: Diagram of the life stages of the Ixodid tick and its hosts with regard to TBEV circulation in nature (bold arrows) and transmission pathways to humans (dashed arrows).

Epidemiology of tick-borne encephalitis virus:

Tick-borne encephalitis is the most important arthropod-borne viral disease found throughout Europe and Russia. Pioneer research of the disease was done mainly in the former USSR in the 1930s (Zilber, 1939), (Chumakov & Zeitlenok, 1940). The first description and isolation of the virus in the former Czechoslovakia followed soon after (Gallia, Rampas & Hollender, 1949), (Rampas & Gallia, 1949), (Krejčí, 1950).

Currently, there are 10–15,000 TBE cases registered annually worldwide (Bogovič & Strle, 2015). The disease is endemic in 27 European and at least four Asian countries (Steffen, 2016). The incidence of TBE correlates with specific ecosystems in the endemic areas and with periods of tick activity (Nuttall et al., 1994). Currently, new risk areas have been identified at higher altitudes (Danielová et al., 2010) and in more northern parts, with rising incidence in Scandinavia (Andreassen et al., 2012), (Soleng et al., 2018), and northern Russia (Tokarevich et al., 2017). The virus has also been recently detected in the United Kingdom, which was previously considered a TBEV-free country (Kreusch et al., 2019), (Holding et al., 2019), (Holding et al., 2020). On the other hand, TBE cases have been on the decline in the Baltic States (Kunze and ISW-TBE, 2012) and in Austria, Germany and Switzerland (Kunze and ISW-TBE, 2013).

In the Czech Republic, there are generally 400 to 800 cases reported each year. The largest number of TBE cases (1,029) was recorded in 2006. The Czech Republic is considered a high-risk area with a high incidence of TBE human cases and a low vaccination rate (Luňáčková et al., 2003), (Kunze and ISW-TBE, 2011), (Kříž et al., 2012), (Amicizia et al., 2013), (Heinz et al., 2013).

Alimentary infections occur via consumption of unpasteurized milk or milk products from grazing domestic ruminants, and frequently result in small local epidemics. First such cases were registered in the former Czechoslovakia in the 1950s (Raška et al., 1954), (Grešíková, 1958). Currently, the most serious (in terms of numbers) outbreaks of alimentary TBEV infection are being reported in Slovakia (Kerlik et al., 2018). For Europe as a whole, there is only limited data for the number of food-borne TBEV infections. Alimentary transmission has been reported for the Czech Republic, Poland, Austria, Hungary, Slovenia, Serbia, Bosnia-Herzegovina and the Baltic countries (Matuszczyk et al., 1997), (Kříž, Beneš & Daniel, 2009), (Holzmann et al., 2009), (Balogh et al., 2010), (Süss, 2011), (Caini et al., 2012), (Hudopisk et al., 2013).

Route of infection:

During a tick bite, TBEV penetrates the skin via the tick saliva. Tick saliva consists of pharmacologically active substances that modulate the host's immunity (Langhansová et al., 2012), (Kotál et al., 2015). Comprehensive research is being done on this by the team led by Professor Jan Kopecký, who has shown that tick salivary molecules compromise antiviral and anti-inflammatory immunity, thus promoting TBEV replication (Lieskovská et al., 2015), (Lieskovská et al., 2018). Immune cells, which infiltrate the skin site during tick feeding, serve as vehicles for the virus (Labuda et al., 1996). Langerhans cells are the main dendritic cell type population in skin epidermis. Previous studies have proposed that these cells are infected by the virus and are used by the virus for transmission to local lymph nodes where the virus further infects macrophages, leading to viremia. Infection of macrophages is crucial for TBEV for further expansion of the infection (Málková & Filip, 1968), (Málková, Mayer & Vrubel, 1969), (Ahantarig et al., 2009). The virus is disseminated to different organs during the short period of primary viremia (Kaiser, 1999). There the virus replicates causing secondary viremia, which can last for several days. During prolonged viremia the virus reaches the central nervous system by crossing the blood-brain barrier (BBB) (McMinn, 1997). The mechanism of BBB disruption and the course of TBEV infection in the central nervous system (CNS) has been studied in depth by my colleague Dr. Martin Palus with my participation (Palus, 2016). The target cells for the infection within CNS are neurons (Hirano et al., 2014), (Bílý et al., 2015). Preferential targets in CNS are large neurons of anterior horns, medulla oblongata, pons, dentate nucleus, Purkinje cells and striatum (Gelpi et al., 2005), although other cell types – like astrocytes, endothelial cells and microglia – are also susceptible to TBEV infection *in vitro* (Růžek et al., 2009), (Palus et al., 2014), (Palus et al., 2017).

Pathogenesis and immune reaction to tick-borne encephalitis:

In terms of innate immunity, it has been shown that type I interferons, which are produced upon viral infection, protect TBEV induced inflammation in CNS and control TBEV replication (Weber et al., 2014), (Lindqvist et al., 2016),

(Selinger et al., 2017). Moreover, NK cell proliferation is activated during the onset of TBE, which leads to the production of proinflammatory cytokines and activation of an adaptive immune response (Blom et al., 2016), (Blom et al., 2018).

The main drivers of pathogenesis during a TBEV infection are still to be confirmed. The pathological consequences of an immune reaction to the virus may play a key role in neuronal damage. Immune mechanisms have been proposed to contribute to the crossing of TBEV through BBB. Cytokines may facilitate this process, since proinflammatory cytokines such as TNF-α (tumor necrosis factor- α) or IL-6 (interleukin-6) have an impact on endothelial cells which, making the tight junction between them, are the key unit of BBB (Vries et al., 1996), (Banks & Erickson, 2010). The proinflammatory cytokines and chemokines were shown to be produced during TBEV infection by astrocytes, which are the key players in the inflammatory response in CNS during neural infections (Palus et al., 2014).

Activation of CD8+ T cell phenotype predominated over CD4+ phenotype in patients diagnosed with TBE (Blom et al., 2015). On the other hand, neither number nor level of activation of T cells infiltrating the brain correlated with the severity of TBE in mouse models (Fujii et al., 2011). Immunopathology is primarily mediated by the CD8+ T cell type, whereas CD4+ T cells confine the development of TBE (Gelpi et al., 2006), (Růžek et al., 2009). T cells and macrophages or microglial cells predominate in inflammatory infiltrates in post mortem brain screening (Gelpi et al., 2005).

TBEV infection also elicits increased levels of proinflammatory cytokines in patient serum together with elevated levels of HGF (hepatocyte growth factor) and VEGF (vascular endothelial growth factor) (Atrasheuskaya, Fredeking & Ignatyev, 2003), (Palus et al., 2015). VEGF has been shown to enhance activity of MMP9 (metalloproteinase 9) (Valable et al., 2005). The levels of MMP9 and its tissue inhibitors, which play an important role in the functioning of BBB, were altered in patients experiencing an acute phase of TBE (Kang et al., 2013), (Palus et al., 2014). In terms of immune responses in patients suffering from TBE, elevated levels of cytokines and chemokines associated with an innate and

proinflammatory adaptive Th1 immune response in CSF correlated with the severity of the disease. Moreover, Th17-type immune response and humoral immunity prevailed in patient serum (Zajkowska et al., 2011), (Grygorczuk et al., 2018), (Bogovič et al., 2019), (Guziejko et al., 2020).

It should be noted that since the recent discovery of lymphatic vasculature in CNS, there is a strong inclination to understand CNS as an immune-regulated site functioning on behalf of an immune-privileged site. Importantly, immune cells, namely T cells, B cells, and dendritic cells, are present in meningeal lymphatic vessels under normal conditions, suggesting that meningeal lymphatics participate in the trafficking of immune cells out of CNS (Aspelund et al., 2015), (Louveau, Harris & Kipnis, 2015), (Louveau et al., 2016). The role of meningeal lymphatics as a novel path for CSF drainage, which represents a conventional path for immune cells to egress CNS, should be studied more deeply in the future, as it could influence TBEV entrance to CNS and potentiate immunopathology in CNS.

Humoral immunity after a natural TBEV infection is known to confer lifelong protection by presence of neutralizing antibodies. The antibody response to TBEV is targeted primarily against the E and NS1 viral proteins and is critically important in controlling and clearing the infection (Heinz, 1986), (Kreil et al., 1998). The antibodies are present in serum and intrathecally produced in CSF (Günther et al., 1997). Passive administration of specific anti-TBEV antibodies can protect mice against an otherwise highly lethal challenge with TBEV (Kreil & Eibl, 1997), (Elsterová et al., 2017). On the other hand, there is a controvening benefit of humoral immunity against flaviviruses, since it has been demonstrated that it also plays a role in immunopathological processes during TBE. Antibody-dependent enhancement (ADE) of infection is a phenomenon occurring when virus-antibody immunocomplexes at sub-neutralizing concentrations interact with cells bearing complement or Fc receptors, which promotes internalization of the virus and an increase in infection (Taylor et al., 2015), (Haslwanter et al., 2017).

Clinical course of tick-borne encephalitis infection:

TBEV infection causes a variable scale of symptoms, from an entirely asymptomatic infection to severe neurological complications that can lead to death. The course of acute TBE and its long-term outcome depend on the subtype of the TBEV infection. Progression to chronic TBEV disease is rather rare in Europe. In Russia, 1-3 % of TBE cases are reported to develop chronic infection, and this is mostly true for the Siberian subtype (Gritsun, Nuttall & Gould, 2003), (Pogodina, 2005). Apart from the Far Eastern subtype, meningoencephalitis is registered less frequently than the meningeal form of TBE; hence the clinical course of the majority of cases is similar to the course of infection caused by the European subtype (Růžek et al., 2019).

After a tick bite, the incubation period to first onset of symptoms can last from 2 to 28 days. A shorter incubation period is typical for alimentary cases of TBEV infection. During the first viremic phase of the disease, flu-like symptoms occur, such as fever, headache, myalgia, arthralgia, fatigue or nausea (Kaiser, 1999). In most patients the infection manifests as abortive and has no further complications, resulting in a monophasic disease. In some patients the virus penetrates to CNS, causing a biphasic disease. The disease results in various focal forms with differing severity, namely in meningitis, meningoencephalitis, meningoencephalomyelitis or encephaloradiculitis. Symptoms vary from fever, headache, photophobia and vertigo in the case of meningitis, to more severe consequences including cognitive or impaired consciousness, behavioral disorders, tremors or neuropsychiatric complaints. Flaccid paresis is characteristic in most severe cases resulting in meningoencephalomyelitis and encephaloradiculitis. Approximately one fourth of patients completely recover within 2 months, while up to 50 % of patients remain with permanent neurological or neuropsychiatric sequelae at long-term follow up. These patients suffer from so called post-encephalitic syndrome, which can last from months to years and includes apathy, sleeping disorders, visual and hearing disorders, gait disturbances and paresis. Fatal outcome occurs in 0.2% to 6.3% of cases (Haglund & Günther, 2003), (Kaiser, 2012), (Bogovič & Strle, 2015), (Růžek et al., 2019).

Clinical and laboratory parameters for diagnosing tick-borne encephalitis:

As the viremic phase, in which the virus is present in blood and can therefore be detected, is relatively short, serological tests for measuring specific IgM and IgG antibodies are preferred. Antibodies are detected in blood or cerebrospinal fluid at the time a patient is admitted to hospital. By this time the patient has usually already suffered from the second phase of TBE with neurological complications. The immune assays used for serological methods for ordinary diagnostics (which are based on the ELISA (enzyme-linked immunosorbent assay) technique) are not specific to TBEV, thus cross-reactivity with other flaviviruses must be taken in account. To increase the specificity of the diagnostic tools, a neutralization assay can be used as a confirmatory method (Kaiser, 2012). Its added value also lies in avidity assessment of IgG antibodies (Gassmann & Bauer, 1997), (Vilibic-Cavlek et al., 2016). Intrathecal antibody production together with moderately raised protein levels and pleocytosis with predominance of neutrophils is the typical scenario in CSF during TBE (Günther et al., 1997), (Kaiser & Holzmann, 2000), (Elsterová et al., in prep.). It has been suggested that using molecular methods to detect the nuclear acid of TBEV in human serum of CSF samples in early stages of TBE is a complementary method for diagnosing TBEV (Saksida et al., 2005), (Schultze et al., 2007), (Saksida et al., 2018), (Veje et al., 2018). Cranial and spinal magnetic resonance imagining (MRI) is the standard neuroimaging method for evaluating various types of encephalitis damage, including TBE. Abnormal findings are rather rare in TBE and restricted to the thalamus, occasionally the cerebellum, nucleus caudatus, basal ganglia and brainstem in severe cases (Kaiser, 1999), (Bender et al., 2005), (Lenhard et al., 2016). New methods for diagnostics and disease prognosis are promising, such as H1-MRI (Proton magnetic resonance spectroscopy), which enables us to detect brain metabolite alterations without any macroscopic changes in the brain regions (Zawadzki et al., 2019). The altered metabolite status in TBE patients could explain post-encephalitic sequelae with the generation and persistency of psychiatric symptoms.

Antiviral therapy:

Despite the fact that reported cases of TBE are increasing (Mansfield et al., 2017) and the geographic ranges of the tick vector species are expanding (Medlock et al., 2013), there is no specific antiviral treatment of TBE. The only option is symptomatic treatment by antipyretics, analgesics, antiemetics, maintenance of water and electrolyte balance, and if necessary, administration of anticonvulsive agents. Corticosteroid management in TBE as an anti-edematous treatment factor is questionable, but it has been used in certain cases (Mickiene et al., 2002).

As an option, specific immunoglobulins are used as post-exposure prophylaxis and treatment in Russia, though this was abandoned in Europe due to complications associated with antibody dependent enhancement (Kluger et al., 1995), (Waldvogel et al., 1996). In addition, it has been shown *in vivo* that administration of recombinant antibodies was more effective than specific antibodies pooled from plasma donors (Baykov et al., 2014), (Matveev et al., 2019). Another possible approach is the administration of pooled high-dose intravenous immunoglobulins, which is approved for clinical use in various diseases including flaviviral encephalitis (Růžek, Dobler & Niller, 2013). Its effect on viral replication of TBEV was studied as part of my doctoral program together with my colleagues Dr. Martin Palus and Dr. Jana Širmarová-Bojčuková, and our collaborating partner Dr. Hans Helmut Niller from the Institute of Medical Microbiology and Hygiene in Regensburg.
An important work on antiviral therapy for TBEV infection was carried out by my colleague Dr. Luděk Eyer (Eyer et al., 2015), (Eyer et al., 2017), (Krol et al., 2019). Various nucleoside analogs were investigated *in vitro* as potential candidates for the treatment of TBEV infections. So far, inhibitors of flaviviral RNA-dependent RNA polymerase, methyltransferase, protease and helicase/NTPase and entry-fusion inhibitors have been described. Together with host nucleoside biosynthesis inhibitors and therapeutics affecting lipid biosynthesis, signalling and metabolism, they represent promising tools for regulating flaviviral infections (Boldescu et al., 2017), (Eyer et al., 2018), (Procházková et al., 2019). The therapeutic approach affects various steps of the flavivirus replication cycle. These are summarized in Figure 4.

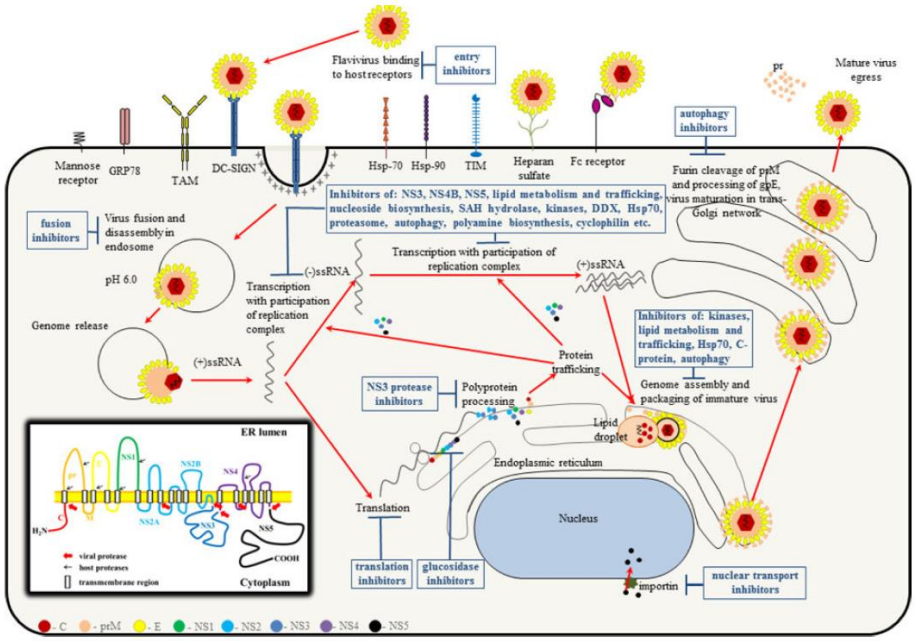

Figure 4: Replication cycle and polyprotein organization of flaviviruses outlined by red arrows. Potential therapeutic approaches are marked in blue boxes (Boldescu et al., 2017).

Prevention of tick-borne encephalitis:

The most important strategy for the reduction of TBE cases is prevention. Besides active immunization, protection against tick bites and early removal of an infesting tick are highly beneficial. Moreover, there are several synthetic and natural repellent substances available to prevent a tick bite (Pages et al., 2014).

There are two vaccines licensed in Europe for the European TBEV subtype based on TBEV strains Neudorfl (FSME-IMMUN, Pfizer) and K23 (Encepur, GSK), and two vaccines licensed in Russia for the Far-Eastern subtype, strain Sofjin (TBE-Moscow, Chumakov FSC R&D IBP RAS) and strain 205 (EnceVir, Microgen). The vaccines have adult and paediatric formulations and are cross protective (Holzmann et al., 1992), (Orlinger et al., 2011), (Chernokhaeva et al., 2018). All are produced in primary chicken embryonic cells with aluminium hydroxide as an adjuvant (Lehrer & Holbrook, 2011). Standard and rapid vaccination

schedules are available. Immunogenicity is not lifelong and revaccination by a booster dose is needed. It is recommended to repeat the booster every 5 years, although it has been declared that even after 10 years 75–90 % of vaccine recipients remain seropositive with neutralizing capacity. Immunogenicity dissipates earlier in older people (Paulke-Korinek et al., 2013), (Konior et al., 2017), (Beran et al., 2019).

Conclusion:

Modern science currently proposes several diverse approaches for dealing with TBEV infection. The key is to understand the virus life cycle and its impact on a host cell. Modern imaging and molecular study methods allow for detailed studies of the structure of the virus, viral entry, replication, release and eventually the pathological effect and adjustment of the host metabolism and immune reaction during infection. This knowledge is a scaffold for developing efficient antiviral therapy, which is still missing. Understanding of the clinical issues surrounding TBEV infection in terms of genetics and the metabolic and immune status of a patient may lead to solving the question of the variable course of TBEV infections.

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Specific aims of the thesis:

The aim of this thesis is to characterize the clinical impact of TBEV infection on a human organism. The thesis is divided into three research topics. The first chapter focuses on the clinical course of TBE and the potential reasons of TBEV neuropathogenesis in relation to the effect of a patient's immune reaction. The second chapter presents a more detailed understanding of the neuroinvassion of the virus and the role of various cell types as compounds of CNS during an infection. The third and last chapter deals with the potential use of human intravenous immunoglobulins in treating severe cases of TBE.

Since the symptomatic course of TBE varies, a deeper understanding of a patient´s immunity status and the conditions of the blood-brain barrier is crucial for determining the variable levels of TBEV neuropathogenicity (**CHAPTER I**). The aims of the first part of the thesis were to:

1) Determine levels of cytokines and chemokines in patient serums during TBEV infection
2) Determine markers of the blood-brain barrier disruption in patient serums
3) Compare the markers in CSF with respect to the severity of TBE

The disruption of the blood-brain barrier and the pathogenesis of TBEV were studied on primary human tissue cultures. The goal was to contribute to a construction of a functional *in vitro* model of the blood-brain barrier (**CHAPTER II**). The aims of the second part of the thesis were to:

1) Characterize TBEV infection of primary human astrocytes, neurons and microvascular endothelial cells
2) Characterize the penetration of TBEV via the blood-brain barrier in an *in vitro* model

To contribute to a potential therapy for patients suffering from TBE, the third part of the thesis studied the potential impact of pooled human immunoglobulins (IVIG) for therapeutic treatment of TBE as immunomodulators, thereby improving immunopathological consequences (**CHAPTER III**). The aims of the third part of the thesis were to:

1) Characterize pooled human IVIG, which are distributed as intravenous therapy for various diseases
2) Determine the impact of IVIG on the replication of TBEV in human tissue cultures and confirm the results *in vivo*

CHAPTER I:

1. **The severity of TBE correlates with the age-related physiological changes of blood-brain barrier integrity and immune reaction.**
   Jana Elsterová, Aleš Chrdle, Dana Teislerová, Václav Hönig, Martin Palus and Daniel Růžek
   In the form of a manuscript

2. **Analysis of serum levels of cytokines, chemokines, growth factors, and monoamine neurotransmitters in patients with tick-borne encephalitis: Identifications of novel inflammatory markers with implications for pathogenesis.**
   Martin Palus, Petra Formanová, Jiří Salát, Eva Žampachová, Jana Elsterová and Daniel Růžek
   Journal of Medical Virology 2015, 87: 885-892. DOI: 10.1002/jmv.24140

3. **Serum matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 levels in patients with tick-borne encephalitis.**
   Martin Palus, Eva Žampachová, Jana Elsterová and Daniel Růžek
   Journal of Infection 2014, 68: 165-169. DOI: 10.1016/j.jinf.2013.09.08

Current research shows great interest in the variability of TBEV infection severity. Patients infected by TBEV tend to have a wide range of symptoms, from subclinical symptoms to severe neuroinfections. Consequently, we studied the role of the integrity of the blood-brain barrier (BBB) in pathogenesis during TBEV infection. BBB is a critical component of the central nervous system (CNS) in terms of limiting flow from blood or lymphatic vessels to CNS, thus playing a critical role in the neuroinvasiveness of TBEV (McMinn, 1997).

The South Bohemian region of the Czech Republic, from where the studied samples originate, is a TBEV-endemic area with a high annual incidence of TBE infections and a low vaccination rate. It thus presents a great opportunity for studying TBEV infections from a clinical point of view. In collaboration with

České Budějovice Hospital, we obtained a comprehensive dataset and serum samples of patients suffering from acute TBE.

Our research has shown that at the beginning of the second phase of TBE, markers of BBB disruption do not change with the severity of the infection. However, findings showed that age had an impact on the permeability of BBB as measured by the content of protein and albumin in cerebrospinal fluid (CSF). This finding correlates with general experience that older patients suffering from TBE tend to have more severe infections. In addition, the level of intrathecal antibodies and the number of cells that migrate to CSF vary with age in TBE patients (Elsterová et al., in prep.). Since impaired immune cell function and its altered migration occurs in older individuals, the role of age in the pathogenesis of TBE should be taken into account (Shah & Mooradian, 1997), (Erdö, László & Lange, 2017).

In addition, the levels of matrix metalloproteinases (MMPs) and its regulators were evaluated in the TBE patient sera. MMPs are responsible for extracellular matrix protein degradation and play a role in inflammation and BBB disruption. MMP-9 is capable of degrading proteins in the membrane of the cerebral endothelium. Therefore, we measured the association of the levels of MMP-9 and its inhibitor (common tissue inhibitor TIMP-1) with TBE. Increased levels of MMP-9 and TIMP-1 might serve as an indicator of inflammatory damage in brain during TBE (Palus et al., 2014), which is consistent with previous findings in CSF (Kang et al., 2013).

Since the immune system of a host contributes to the pathology and development of disease during a TBEV infection, a set of 30 markers of inflammation was measured in serum samples of patients suffering from TBE (Palus et al., 2015). Analysis included cytokines, chemokines, growth factors and monoamine neurotransmitters, which reflected the global proinflammatory balance with the elevation of cytokines IL-6, IL-8 and IL-12. Furthermore, decreased levels of the neurotransmitters dopamine, serotonin and noradrenalin indicate damage in CNS due to TBEV infection. An enhanced level of the growth factor HGF (hepatopoetin A growth factor) reflects a response to virus spread during the first phase of TBEV infection in terms of tissue regeneration. A higher level of VEGF (vascular endothelial growth factor)

promotes MMP-9 activity, which correlates with the increased levels of MMP-9 in the sera of studied TBE patients. Therefore an alteration of levels of MMP-9, which is related to increased levels of VEGF, has an impact on the permeability of peripheral and CNS vasculature.

The overall status of patients suffering from TBE tends to support the crucial role of BBB disruption and its impact on neuropathology. It is important to note that all the data were sampled at the start of neurological complications in the second phase of TBE. More extensive changes would follow in a prolonged disease in terms of selected factors and markers that influence CNS integrity and immune reaction.

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1. **The severity of TBE correlates with the age-related physiological changes of blood-brain barrier integrity and immune reaction**

Severity of TBE correlates with the age-related physiological changes of blood-brain barrier integrity and immune reaction

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Abstract:

Tick-borne encephalitis (TBE) virus is a neurotropic and neuropathogenic virus transmitted to host via hematophagous vector causing human infections across Europe and northeastern Asia. A retrospective study of total of 56 patients suffering from acute TBE with neurologic symptoms was done to compare the

severity of TBE to age-related physiological status of the patient. Elder patients had more severe course of TBE. Direct proportion of age and fitness of blood-brain barrier (BBB) permeability and immune status measured by markers in cerebrospinal fluid (CSF) was proven. Elderly patients suffering from acute TBE had altered immune status in CSF measured by cell count and levels of intrathecal antibodies and had higher levels of protein, marking the altered BBB integrity. The markers, which are related to physiological status of an individual in terms of immunity and conditions within CNS, should be therefore taken in account in progression and severity of TBE.

Key words: tick-borne encephalitis virus, disease severity, blood-brain barrier permeability, inflammation

2. Analysis of serum levels of cytokines, chemokines, growth factors, and monoamine neurotransmitters in patients with tick-borne encephalitis: Identifications of novel inflammatory markers with implications for pathogenesis

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Analysis of Serum Levels of Cytokines, Chemokines, Growth Factors, and Monoamine Neurotransmitters in Patients With Tick-borne Encephalitis: Identification of Novel Inflammatory Markers With Implications for Pathogenesis

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Tick-borne encephalitis (TBE) is a leading human neuroinfection in Europe and northeastern Asia. However, the pathophysiology of TBE is not understood completely. This study sought to determine the specific serum mediators that are associated with acute TBE. The levels of 30 cytokines, chemokines, and growth factors were measured in serum samples from 87 patients with clinically and serologically confirmed acute TBE and from 32 control subjects using the Cytokine Human Magnetic 30-Plex Panel for the Luminex platform. Serum levels of the monoamine neurotransmitters serotonin, dopamine, and noradrenaline were measured via enzyme-linked immunosorbent assay. TBE virus infection elicited increased levels of the pro-inflammatory cytokines interleukin (IL)-6, IL-8, and IL-12. TBE patients had higher IL-12:IL-4 and IL-12:IL-10 ratios than control patients, reflecting the global pro-inflammatory cytokine balance. Serum levels of the monoamine neurotransmitters serotonin, dopamine, and noradrenaline were significantly lower in TBE patients than in the control group. Most interestingly, increased levels of hepatocyte growth factor and vascular endothelial growth factor were observed in TBE patients; these proteins may be novel and mechanistically important inflammatory biomarkers of TBE. ***J. Med. Virol. 87:885–892, 2015.*** © 2015 Wiley Periodicals, Inc.

KEY WORDS: tick-borne encephalitis virus; neuroinflammation; luminex; neuroinfection

INTRODUCTION

Tick-borne encephalitis (TBE) is one of the most important human viral infections of the central nervous system (CNS) in Eurasia. The etiological agent, TBE virus (TBEV), is a member of the family *Flaviviridae*, genus *Flavivirus*, together with other important human viruses like West Nile virus, Yellow fever virus, Japanese encephalitis virus, Dengue virus, and Omsk hemorrhagic fever virus. Although TBE can be prevented effectively by vaccination, more than 13,000 clinical cases of TBE, including numerous deaths, are reported annually in more than 30 countries in Europe and northeastern Asia [Charrel et al., 2004; Mansfield et al., 2009]. The TBE prevalence is increasing and the virus is spreading to areas that previously were non-endemic. In the years 1974–2003, a continuous increase in TBE morbidity was observed in Europe, and from 2004 to 2006, another considerable increase was seen in the Czech Republic, Germany, Poland, Slovenia, and Switzerland [Suss, 2008].

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The typical clinical features of TBE caused by European TBEV strains are characterized by a biphasic course. The primary phase includes nonspecific symptoms such as fever, muscle pain, headache, and malaise, and the typical secondary phase involves neurological symptoms including meningitis, meningoencephalitis, and meningoencephalomyelitis [Růžek et al., 2010]. There is no specific therapy for TBE other than supportive measures.

The pathophysiology of TBE is unclear [Růžek et al., 2009]. The pathology observed during TBE suggests that some components of the host immune system contribute to disease development and immunopathology [Gelpi et al., 2006; Růžek et al., 2009]. Therefore, the roles of cytokines, chemokines, and growth factors during TBE are among the main key issues that have not been addressed fully to date. Previous reports characterized an acute immune response to TBEV by profiling certain cytokines and chemokines (e.g., tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, CXCL10, CXCL11, CXCL12, CXCL13) in the sera or cerebrospinal fluid of TBE patients [Kondrusik et al., 2001; Atrasheuskaya et al., 2003; Zajkowska et al., 2011]. However, limited sample sizes often restricted the analysis of multiple cytokines of interest via traditional methodologies.

In this study, acute immune response to TBEV was characterized by measuring a panel of 30 cytokines, chemokines, and growth factors, including molecules that have been never or rarely studied in TBE patients; the method employed a fluorescent bead-based technology in a multiplex array system. In addition, the levels of monoamine neurotransmitters were measured in TBE patients and in control subjects via enzyme-linked immunosorbent assay (ELISA), since it was suggested recently that decreased levels of monoamine neurotransmitters such as serotonin may correlate with TBE severity [Sumlivaya et al., 2013]. Here, the serum levels of proinflammatory cytokines IL-6, IL-8, and IL-12 were increased and the levels of monoamine neurotransmitters were decreased in TBE patients compared to control subjects. This investigation also provides the first demonstration that TBE patients harbor increased serum levels of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF), which may represent novel and mechanistically important inflammatory biomarkers of TBE.

MATERIALS AND METHODS

Serum samples were obtained from 87 patients (53 males and 33 females, aged 4–80 years; median, 47 years) with acute TBE that was confirmed via serology (clinically manifest TBE and ELISA-based detection of specific anti-TBEV IgM and IgG antibodies [Test-Line, Brno, Czech Republic]). The control subjects for measurements of serum cytokine/chemokine/growth factors/neurotransmitters were 32 healthy individuals comprising a matched-age group (13 males and 19 females; aged 4–77 years; median, 35.5 years). Samples were collected from patients and control subjects in 2012 in South Bohemia, Czech Republic; aliquots of all serum samples were stored at −80°C. The work described in this study was carried out in accordance with the Declaration of Helsinki. Patients and control subjects (or their parents) provided informed consent before sample collection. Samples were investigated anonymously. The study protocol was approved by the Institutional Ethical Committee (PARU AVCR No. 01/11).

Concentrations of 30 cytokines, chemokines, and growth factors were measured in serum samples using the Human Cytokine Magnetic 30-Plex Panel for the Luminex platform (Life Technologies, Frederick, MD) using a MAGPIX instrument (Luminex, Austin, TX). All procedures were performed according to the manufacturer's instructions. The immunoassay detects IL-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-2 receptor (IL-2R), IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, TNF-α, interferon alpha (IFN-α), interferon gamma (INF-γ), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), eotaxin (CCL11), inducible protein (IP)-10 (CXCL10), macrophage chemotactic protein (MCP)-1, monokine induced by gamma interferon (MIG; CXCL9), macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), regulated upon expression normal T-cell expressed and secreted (RANTES; CCL5), and the growth factors epidermal growth factor, basic fibroblast growth factor, HGF, and VEGF.

Concentrations of serum monoamine neurotransmitters were measured with the Serotonin ELISA Fast Track kit (LDN, Nordhorn, Germany), the Noradrenaline Sensitive ELISA kit (DLD Diagnostika, Hamburg, Germany), and the Dopamine Sensitive ELISA kit (DLD Diagnostika, Hamburg, Germany). Concentrations of all analyzed mediators are reported as pg per ml of serum.

Statistical significance in the between-group differences in the concentration of inflammatory mediators in sera was analyzed using the Mann–Whitney test (P). Identification of outliers was performed with Dixon's Q test. Correction for multiple hypothesis testing used Benjamini and Hochberg's false discovery rate adjustment (q). The Kruskal–Wallis test and Dun's multiple comparison test were used to determine differences among groups in age and sex comparisons. Differences with $P < 0.05$ and $q < 0.05$ were considered significant. Analyses and calculations were performed using GraphPad Prism 5.04 (GraphPad Software, La Jolla, CA).

RESULTS

Soluble factor concentrations were measured in 87 patients during the acute phase of TBE and in

32 uninfected and healthy control subjects who were selected randomly. Relative to controls, TBE patients had significantly elevated levels of cytokines IL-6 ($P < 0.0001$; $q < 0.01$), IL-8 ($P < 0.01$; $q < 0.01$), and IL-12 ($P < 0.01$; $q < 0.05$) (Fig. 1; Supplemental Table 1). Serum samples from TBE patients contained significantly higher concentrations of growth factors HGF ($P < 0.001$; $q < 0.01$) and VEGF ($P < 0.01$; $q < 0.05$) than serum samples from uninfected control subjects (Fig. 1). The concentration of VEGF in TBE patients was at the level of detection, but most control subjects had VEGF levels under the detection limit. Concentrations of IL-5 ($P < 0.05$; $q < 0.05$), GM-CSF ($P < 0.05$; $q < 0.05$), and MCP-1 ($P < 0.0001$; $q < 0.01$) were lower in sera from TBE patients than in sera from control subjects (Fig. 1). TBE patients had noticeably lower serum levels of serotonin ($P < 0.05$; $q < 0.05$), dopamine ($P < 0.0001$; $q < 0.01$), and noradrenaline ($P < 0.0001$; $q < 0.01$) than uninfected control subjects (Fig. 2). In addition, the Mann–Whitney test identified significantly increased concentrations of G-CSF and epidermal growth factor ($P < 0.05$) in the sera of TBE patients, but these differences were insignificant after correction for multiple-hypothesis testing ($q > 0.05$) (Supplemental Table 1).

There were no significant differences ($P > 0.05$) between patients and control subjects in terms of the concentrations of cytokines/chemokines TNF-α, IL-1β, IL-1RA, IL-2, IL-2R IL-4, IL-7, IL-10, IL-13, IL-15, IL-17, IP-10, IFN-α, IFN-γ, MIP-1α, MIP-1β, eotaxin/CCL11, MIG, RANTES, and fibroblast growth factor (Supplemental Table 1). The levels of IL-2, IL-7, IL-17, and TNF-α were at or below the limit of detection. There was no difference in the IL-2:IL-2R and IL-1β:IL-1RA ratios between TBE patients and control subjects ($P > 0.05$) (data not shown).

Pro/anti-inflammatory immune system balance was analyzed by comparing the ratios of pro/anti-inflammatory cytokines. Patients with TBE infection had significantly higher ($P < 0.05$) IL-12:IL-4 and IL-12:IL-10 ratios than control subjects (Fig. 3). No significant difference was detected in other comparisons (IFN-γ:IL-10, IFN-γ:IL-4, IL-2:IL-4, IL-2:IL-10) (data not shown).

When patients and control subjects were divided into two groups according to age (<50 years and ≥50 years), significant differences between groups were observed only in the levels of GM-CSF ($P < 0.05$) and MCP-1 ($P < 0.05$); TBE patients <50 years had significantly ($P < 0.05$) lower levels of GM-CSF and MCP-1 than patients ≥50 years (Fig. 4). No differences were seen between controls younger and older than 50 years of age (Fig. 4).

No significant differences in the levels of any analyzed cytokine/chemokine/growth factor were detected between male and female TBE patients, or between male and female control subjects (data not shown).

DISCUSSION

The role of cytokines, chemokines, growth factors, and other soluble mediators during TBE is a major issue that has not been addressed fully to date. In this study, an acute immune response to TBEV was subjected to a multiplex analysis of 30 cytokines, chemokines, and growth factors in sera from TBE patients and control subjects. In addition, ELISA was used to measure the levels of three monoamine neurotransmitters in TBE patients and control subjects. Due to the unavailability of clinical data for the TBE patients included in this study, the overall immune response during TBE was characterized independent of disease severity and the timing of sample collection.

TBE patients had significantly elevated levels of proinflammatory cytokines IL-6, IL-8, and IL-12 (Fig. 1). In addition to its proinflammatory role, the anti-inflammatory properties of IL-6 have been described. Moreover, IL-6 plays important roles in regulatory and inflammatory processes within the CNS [März et al., 1999; Atrasheuskaya et al., 2003]. In most cases, IL-6 production is associated with a protective immune response, but unregulated overproduction can be harmful to the host. A previous study reported high concentrations of serum IL-6 in TBE patients during the first week of hospitalization, but these levels decreased in parallel with increased levels of their soluble receptors [Atrasheuskaya et al., 2003]. This decrease in cytokine levels was accompanied by an increase in the levels of IL-10, an immunoregulatory cytokine with anti-inflammatory properties [Atrasheuskaya et al., 2003]. Interestingly, pediatric TBE patients displayed the highest levels of serum IL-6 at admission, but did not manifest symptoms of meningitis or meningoencephalitis, as the adults did [Atrasheuskaya et al., 2003]. Another study demonstrated that only some TBE patients harbored elevated serum levels of IL-6; these levels correlated with age, but not with gender or disease outcome [Toporkova et al., 2008]. No correlation between serum IL-6 levels and age was detected in the present study.

IL-8 plays a crucial role in the recruitment of neutrophils and T cells into the CNS and is associated with blood-brain barrier dysfunction. IL-8 is also known to induce the expression of matrix metalloproteinase (MMP)-9, which was shown to be increased in serum [Palus et al., 2014] and cerebrospinal fluid [Kang et al., 2013] in patients with TBE. Higher concentrations of IL-8 in sera from TBE patients indicate this molecule's possible role in immunopathology in the CNS.

IL-12 makes important contributions to the primary activation of cell-mediated specific immunity, a critical part of the immune response to viral infections. IL-12 activates natural killer cells and T lymphocytes and mediates enhancement of the cytotoxic activity of natural killer cells and $CD8^+$ T cells

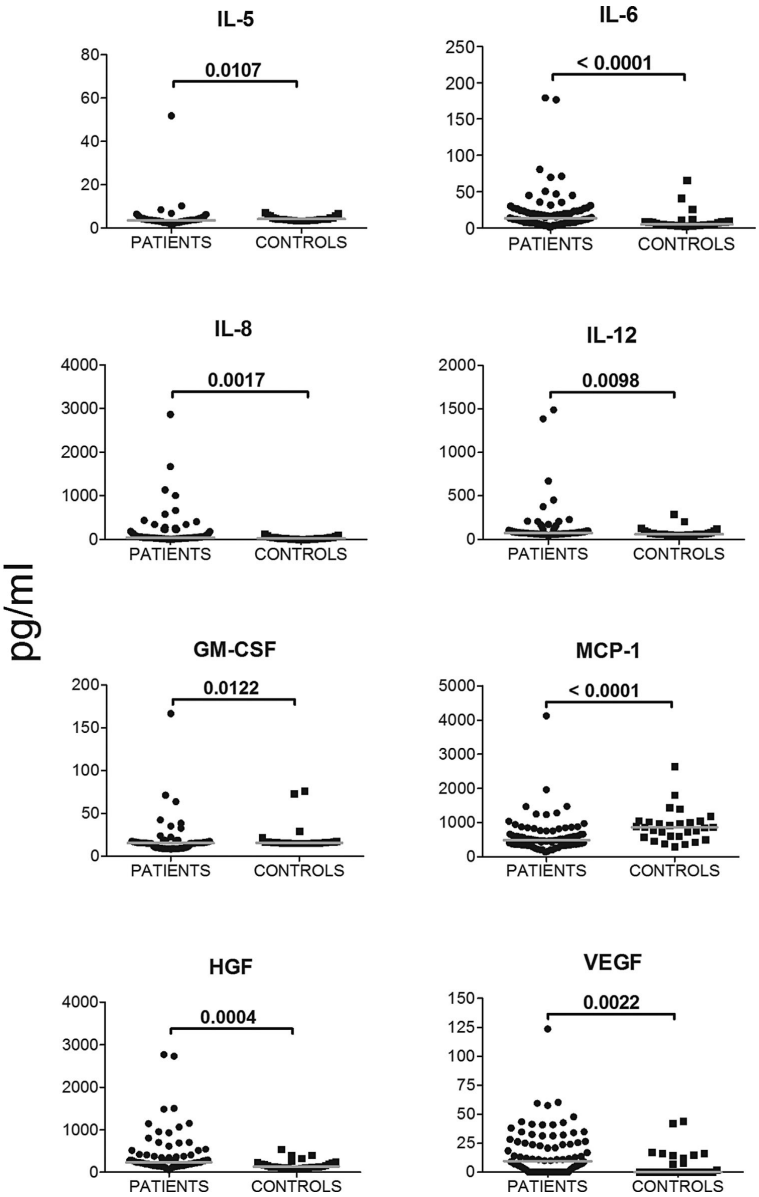

Fig. 1. Serum concentrations of IL-5, IL-6, IL-8, IL-12, GM-CSF, MCP-1, HGF, and VEGF in TBE patients and control subjects evaluated by the multiplex system described in Materials and Methods. Horizontal lines indicate median values.

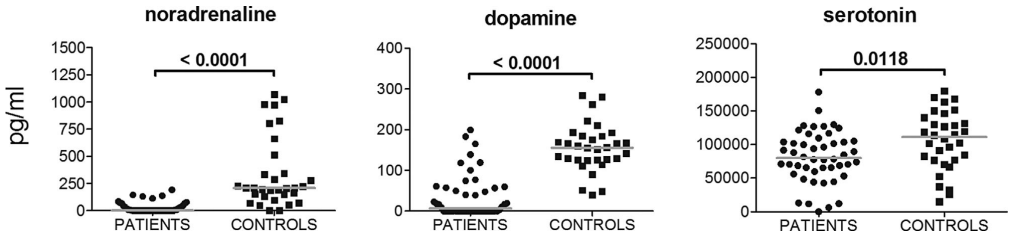

Fig. 2. Serum concentrations of serotonin, dopamine, and noradrenaline in TBE patients and control subjects. Horizontal lines indicate median values.

[Trinchieri, 2003]. Here, IL-12 levels were higher in TBE patients than in control subjects. Moreover, significantly higher IL-12:IL-4 and IL-12:IL-10 ratios were detected in sera from TBE patients, reflecting the global proinflammatory cytokine balance in infected human hosts.

The levels of IL-5, GM-CSF, and MCP-1 were lower in sera from TBE patients than in sera from control subjects. IL-5 is a typical cytokine produced by Th2 cells, playing a central role in differentiation and in the activation of eosinophils. Further, IL-5 triggers B-cell differentiation into antibody-producing plasma cells [Kouro and Takatsu, 2009]. During the acute phase, suppression of IL-5 production below control levels indicates the existence of Th1 (cell-mediated) proinflammatory polarization of the host immune response during TBE neuroinfection.

GM-CSF and macrophage colony-stimulating factor are principal microglial growth factors; their expression (along with that of their cognate receptors) is upregulated in the injured or diseased CNS [Giulian and Ingeman, 1988; Raivich et al., 1991; Lee et al., 1994; Cosenza-Nashat et al., 2007]. Here, GM-CSF levels were slightly lower in sera from TBE patients than in that from control subjects, which is puzzling and requires further study.

MCP-1 plays an essential role in several peripheral and CNS inflammatory disorders characterized by mononuclear cell infiltrates. However, MCP-1 may also perform several vital functions in addition to its participation in cell recruitment, and may have either a protective or detrimental role depending on the inflammatory stimulus, cell type, or disease state [Thompson et al., 2008]. Interestingly, significantly lower levels of MCP-1 in sera from TBE patients than in sera from control subjects were detected in the present investigation. A previous study reported a clear correlation between MCP-1 mRNA production in TBEV-infected mice and disease severity [Palus et al., 2013]. The observed lower serum levels of MCP-1 in TBE patients require further confirmation in a future study.

There were no significant differences between TBE patients and control subjects in terms of the serum concentrations of cytokines/chemokines TNF-α, IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-7, IL-10, IL-13, IL-15, IL-17, IP-10, IFN-α, IFN-γ, MIP-1α, MIP-1β, G-CSF, eotaxin/CCL11, MIG, RANTES, epidermal growth factor, or fibroblast growth factor (Supplemental Table 1); the levels of IL-2, IL-7, IL-17, and TNF-α were at or below the limit of detection. Decreased concentrations could be caused by analyte instability during storage of the serum samples. Consistent with previous studies, the concentrations of circulating IL-1RA were higher than the corresponding levels of IL-1β [Dinarello, 1998; Atrasheuskaya et al., 2003]. The ratio of IL-1β to IL-1RA is considered to be more important for the outcome of the infection than the

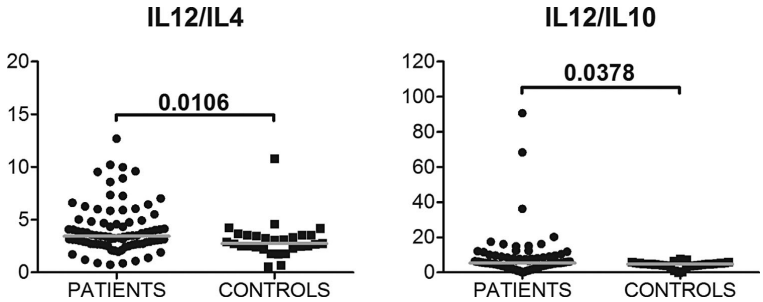

Fig. 3. Ratios of serum levels of IL-12:IL-4 and IL-12:IL-10 in TBE patients and control subjects. Horizontal lines indicate median values.

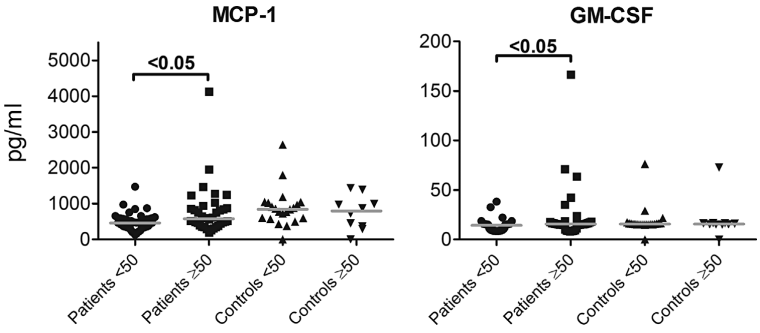

Fig. 4. Serum concentrations of MCP-1 and GM-CSF in TBE patients and in control subjects aged <50 years and ≥50 years. Horizontal lines indicate mean values.

concentration of IL-1β itself; this ratio can serve as a good prognostic marker [Atrasheuskaya et al., 2003]. However, in the present study, no significant difference was observed between the IL-1β:IL-1RA ratios in TBE patients and control subjects. This lack suggests differences in IL-1β:IL-1RA ratios between European TBE cases that are usually mild and Russian TBE, which is frequently severe. Previous reports of increased serum levels of IP-10 and TNF-α in TBE patients [Kondrusik et al., 2001; Zajkowska et al., 2011] were not confirmed in this study, probably due to high variation in the data caused by inconsistent timing of sample collection.

Generally, TBE patients older than 50 years have significantly more sequelae and a higher fatality rate [Haglund et al., 1996]. In this study, patients <50 years had significantly lower levels of GM-CSF and MCP-1 than patients ≥50 years, suggesting stronger down-regulation of the innate immune response in the case of these markers in younger patients. However, there were no differences in the other analytes between TBE patients <50 years and ≥50 years of age.

The serum levels of the monoamine neurotransmitters serotonin, dopamine, and noradrenaline during acute TBE infection were also determined (Fig. 2). Sumlivaya et al. [2013] demonstrated that during the acute peak period of TBE, serum serotonin levels were significantly lower in TBE patients than in healthy controls. In the paralytic form of TBE, the levels of serum serotonin were noticeably lower than in the nonparalytic form [Sumlivaya et al., 2013]. Based on these data, the authors concluded that serum serotonin levels can serve as a marker of CNS damage during TBE and that they predict the development of severe forms of TBE [Sumlivaya et al., 2013]. The present study confirmed the lower levels of serotonin in TBE patients versus levels in control subjects, but the serum levels of catecholamines (dopamine and noradrenaline) were decreased in TBE patients as well. Catecholamines are known to be decreased in the cerebrospinal fluid of Japanese encephalitis patients [Misra et al., 2005]. The lower levels of monoamine neurotransmitters detected in sera from TBE patients may be association with neuropsychological complications observed frequently during or after TBE [Juchnowicz et al., 2002; Schmolck et al., 2005].

Among the prominent findings of this study was the novel observation that serum concentrations of HGF and VEGF were increased in TBE patients in comparison to control subjects (Fig. 1). HGF, also known as hepatopoietin A or scatter factor, serves as a mitogen (stimulating growth and enhancing the cell motility of various epithelial cells) and as a morphogen [Matsumoto and Nakamura, 1991; Ozden et al., 2004]. Increased amounts of HGF have been observed in sera from patients after acute injuries, especially to the liver, kidneys, and lungs, where HGF serves as a regeneration factor. HGF is produced by mesenchymal cells in response to injuries to a distant organ [Kono et al., 1992]. Increased levels of HGF were reported in the cerebrospinal fluid of patients with various diseases affecting brain tissue [Yamada et al., 1994; Fenton et al., 1998; Tsuboi et al., 2002; Ozden et al., 2004]. In TBE, increased serum HGF levels may reflect a response to virus multiplication in peripheral tissues and organs during the first phase of the infection or a response to virus-mediated brain tissue damage; increased HGF levels may be responsible for tissue regeneration. The specific role of HGF production during TBE remains to be defined.

Here, the serum levels of VEGF were mostly under the detection limit in control subjects, but were detectable in TBE patients. VEGF is a signal protein that stimulates vasculogenesis and angiogenesis. VEGF overexpression can contribute to the development of encephalitis because VEGF increases the permeability of peripheral and CNS vasculature by permeating endothelial cells in venules and capillaries [Roberts and Palade, 1995; Mayhan, 1999; Ay et al.,

2008]. VEGF has been shown to enhance MMP-9 activity [Valable et al., 2005]. A previous study demonstrated that serum MMP-9 levels and the ratio of MMP-9 to tissue inhibitor of metalloproteinase-1 were significantly higher in TBE patients than in control subjects [Palus et al., 2014]. Another study showed that elevated CSF MMP-9 levels in TBE patients were associated with brain inflammatory reactions, disruption of the blood-brain barrier, and disease severity [Kang et al., 2013]. Measuring the levels of VEGF in TBE patients yielded further evidence that injury to the blood-brain barrier is critical during the development of TBE.

In summary, the present study is the first report of increased serum levels of various cytokines and growth factors, as well as decreased serum levels of important monoamine neurotransmitters, in patients with TBE. The growth factors HGF and VEGF represent new inflammatory biomarkers of TBE with potentially important biological functions, findings worthy of further investigation. Increased knowledge of inflammatory mediators activated during TBE may provide a basis for the design and development of new pharmacological approaches to treat this important neuroinfection.

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SUPPORTING INFORMATION

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3. Serum matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 levels in patients with tick-borne encephalitis

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Serum matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 levels in patients with tick-borne encephalitis

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Summary *Objectives:* Matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) play important roles in the function of the blood—brain barrier (BBB). To investigate the function of the BBB during tick-borne encephalitis (TBE), the levels of MMP-9 and its common tissue inhibitor, TIMP-1, were measured in serum from patients with acute phase of TBE.
Methods: Serum MMP-9 and TIMP-1 levels were measured in 147 patients with TBE and 153 controls by ELISA.
Results: Serum MMP-9 levels and MMP-9/TIMP-1 ratios of TBE patients were significantly higher than controls ($p < 0.0001$ and $p < 0.005$, respectively). There were no significant differences in serum TIMP-1 levels between TBE patients and controls. Serum MMP-9 and TIMP-1 levels and MMP-9/TIMP-1 ratios were not associated with age of the patients. However, TBE-positive males with TBE had higher levels of MMP-9 than TBE-positive females ($p < 0.05$).
Conclusions: Our results suggest that the increased serum level of MMP-9 and MMP-9/TIMP-1 ratio is associated with the pathogenesis of TBE. Serum MMP-9 can serve as an indicator of breakdown of the BBB and inflammatory brain damage during TBE.

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Introduction

Tick-borne encephalitis (TBE) is considered an important health problem in Europe and Russia because of protracted course and in some cases severe illness resulting in long-lasting cognitive dysfunction and even persisting pareses of spinal nerves. Although mortality in Europe is relatively low (<1%), rates as high as 20% have been observed in Asia.[1] TBE is endemic in regions of 27 European countries, and new risk areas are discovered every year. In the 30 years between 1974 and 2003, a continuous increase in TBE morbidity reaching up to 400%, was observed in Europe.[2] From 2004 to 2006, another considerable increase was seen in some TBE countries, notably the Czech Republic, Germany, Poland, Slovenia, and Switzerland.[3] The etiologic agent, tick-borne encephalitis virus (TBEV), a member of the family *Flaviviridae*, genus Flavivirus, is an arbovirus that is transmitted to humans by infected ixodid tick vectors.

The pathophysiology of the development of encephalitis is unclear.[4] In particular, very little is known about the role of the blood—brain barrier (BBB) in the neuropathogenesis of TBE.[5] In this study, the function of the BBB during TBE was investigated by measurements the levels of matrix metalloproteinase-9 (MMP-9) and its common tissue inhibitor (TIMP-1) in serum from patients with acute phase of TBE. Various experimental studies demonstrated that MMP-9 might be a potential mediator for the disruption of BBB.[6–8] Matrix metalloproteinases (MMPs) represent a family of enzymes that are responsible for the degradation of extracellular matrix proteins. MMPs play important roles in normal and pathological processes, including inflammation.[9] MMP-9 is capable of degrading collagen IV, a major component of the basement membrane of the cerebral endothelium, and promotes the migration of cells through tissue or across the BBB.[9,10] The activity of MMPs is controlled by specific tissue inhibitors of metalloproteinases (TIMPs). TIMP-1 has a high affinity for MMP-9.[9,11] The levels of MMP-9, TIMP-1 and the ratio between these two proteins were extensively studied in various diseases of the central nervous system, including subacute sclerosing panecephalitis,[9,10] fungal or tuberculous meningoencephalitis,[12] HIV-associated neurological diseases,[13] herpes simplex virus encephalitis,[14] and influenza-associated encephalopathy.[15]

Here, we demonstrate that the development of TBE is associated with increase of MMP-9 levels and MMP-9/TIMP-1 ratio in serum. Our results suggest that the increased serum level of MMP-9 and MMP-9/TIMP-1 ratio is associated with the pathogenesis of TBE, and serum MMP-9 can serve as an indicator of inflammatory damage to the brain during TBE.

Patients and methods

Serum samples were obtained from 147 patients (78 males and 69 females, aged from 5 to 91 years; median, 46 years) with serologically confirmed acute TBE (detection of specific anti-TBEV IgM and IgG antibodies by ELISA). The samples were collected in years 2011 and 2012. Patients (or their parents) signed an informed consent before sample collection. Then the samples were investigated anonymously. The study was approved by Institutional Ethical Committee (PARU ASCR No. 01/11).

The control subjects for the serum levels of MMP-9 were 239 and TIMP-1 were 153 healthy individuals of a corresponding age group (115 males and 124 females; aged from 4 to 83 years; median, 40 years).

The serum concentrations of MMP-9 and TIMP-1 were determined by sandwich-type ELISA kits (Human TIMP-1 and Human MMP-9, Invitrogen Corporation CA, USA). Assays were performed following the instructions of the manufacturer. The detection limits were 0.0235 ng/ml for MMP-9, and 1.56 ng/ml for TIMP-1.

The statistical differences in MIP-9 and TIMP-1 concentration in sera between groups were analyzed using the Mann—Whitney test. Differences with $p < 0.05$ were considered significant. Correlations were analyzed using Pearson's coefficient correlation. Analyses and calculations were performed using GraphPad Prism 5.04 (GraphPad Software, Inc., USA).

Results

The geometric means of serum MMP-9 and TIMP-1 levels, and MMP-9/TIMP-1 ratios of the controls were 251.8 ng/ml (range, 58.8—342.5 ng/ml), 742.8 ng/ml (range, 153.4—3635.7 ng/ml), and 0.5400 (range, 0.0004—2.1804), respectively. The geometric means of serum MMP-9 and TIMP-1 levels, and MMP-9/TIMP-1 ratios of the TBE patients were 403.1 ng/ml (range, 93.9—599.3 ng/ml), 749.3 ng/ml (range, 188.1—3384.1 ng/ml), and 0.6801 (range, 0.0302—2.0236), respectively (Fig. 1). Serum MMP-9 levels and MMP-9/TIMP-1 ratios of TBE patients were significantly higher than the controls ($p < 0.0001$ and $p < 0.005$, respectively). There were no significant differences in serum TIMP-1 levels between TBE patients and controls ($p = 0.7964$).

When patients and control subjects divided into two groups according to their age (<50 and ≥50 years), there were no statistical differences between the groups (Fig. 2). The geometric means of serum MMP-9, TIMP-1 levels, and MMP-9/TIMP-1 ratios of the TBE patients <50 years-old were 350.8 ng/ml (range, 65.62—599.26 ng/ml), 758.90 ng/ml (range, 202.00—3384.13 ng/ml), and 0.6766 (range, 0.0302—1.8104), respectively. The geometric means of serum MMP-9, TIMP-1 levels, and MMP-9/TIMP-1 ratios of the TBE patients ≥50 years-old were 359.40 ng/ml (range, 74.88—588.28 ng/ml), 736.00 ng/ml (range, 188.11—1902.71 ng/ml), and 0.6848 (range, 0.0532—2.0237), respectively. There was no correlation of serum MMP-9 and TIMP-1 levels and MMP-9/TIMP-1 ratios with age of the TBE patients as well as controls (Fig. 3).

The geometric means of serum MMP-9, TIMP-1 levels, and MMP-9/TIMP-1 ratios of the male TBE patients were 372.80 ng/ml (range, 74.88—599.26 ng/ml), 763.9 ng/ml (range, 188.1—3384.1 ng/ml), and 0.709 (range, 0.532—2.024), respectively. The geometric means of serum MMP-9, TIMP-1 levels, and MMP-9/TIMP-1 ratios of the female TBE patients were 333.8 ng/ml (range, 65.6—567.3 ng/ml), 732.70 ng/ml (range, 199.67—2751.42 ng/ml), and 0.65 (range, 0.03—1.81), respectively. In case of controls, no difference in serum MMP-9 and TIMP-1 levels, and MMP-9/TIMP-1 ratios was seen between males and females. Males

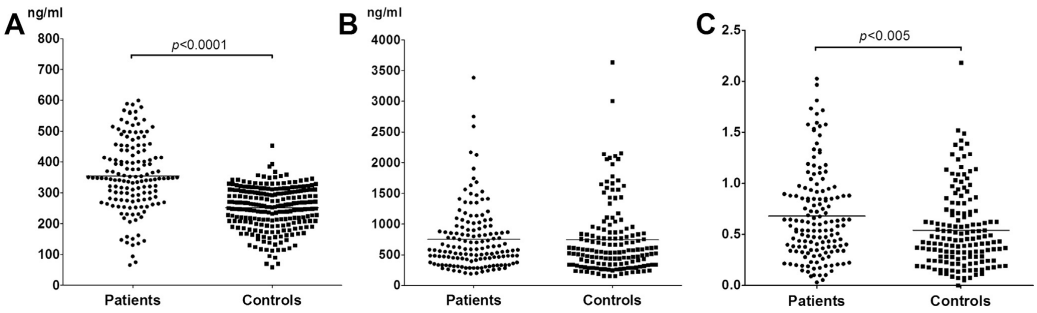

Figure 1 Serum concentrations of MMP-9 (A) and TIMP-1 (B) and MMP-9/TIMP-1 (C) ratio in TBE patients and in controls. The horizontal lines indicate mean values.

with TBE had significantly higher levels of MMP-9 than females with TBE ($p < 0.05$). There were no significant differences in serum TIMP-1 levels and MMP-9/TIMP-1 ratios between male and female patients with TBE ($p = 0.5181$) (Fig. 4).

Discussion

The BBB is a critical component of the CNS in that it limits the flow of material between the general circulation, either lymphatic or circulatory, and the CNS.[16] In human patients with severe forms of TBE, BBB breakdown has been suggested based on the increased level of neurospecific proteins, such as α-1 brain globulin (α1BG) or neuron-specific enolase (NSE), in serum.[17] The kinetic serum profiles of α1BG and NSE in severe TBE followed the general time courses of specific clinical manifestations.[17] Similarly, increased sICAM-1 concentrations in CSF of TBE patients suggested BBB disturbances occurring during early phase of the disease.[18] In TBEV infected mice, breakdown of the BBB was demonstrated at later times post-infection. This breakdown was, however, not necessary for virus entry in the CNS, was independent on migration of $CD8^+$ T-cells into brain parenchyma, and more likely represented a bystander effect of virus-induced cytokine/chemokine overproduction in the brain.[5] Here we present further evidence that the BBB injury occurs during TBE by measurement of serum MMP-9 and TIMP-1 levels, and MMP-9/TIMP-1 ratio. It is well documented that elevated levels of MMP-9 in the serum/plasma and brain are associated with BBB disruption, leading to an exacerbation of neurodegenerative diseases.[19] MMP-2, MMP-3 and MMP-9 increase the permeability of the BBB and inhibitors of MMPs can reduce damage to the BBB.[20] Whenever the BBB is affected, MMP-9 is a key factor in the injury process. MMP-9 is produced in the cells constituting the BBB, including brain microvascular endothelial cells[21] and astrocytes[22] under pathological conditions. The changed levels of MMP-9, its inhibitor TIMP-1, and the ratio between these two proteins were shown to play important role in pathological processes associated with BBB breakdown in various diseases of the central nervous system, including subacute sclerosing panecephalitis,[9,10] fungal or tuberculous meningoencephalitis,[12] HIV-associated neurological diseases,[13] herpes simplex virus encephalitis,[14] and influenza-associated encephalopathy.[15]

The present study is the first to demonstrate that serum MMP-9 levels and MMP-9/TIMP-1 ratios are markedly higher

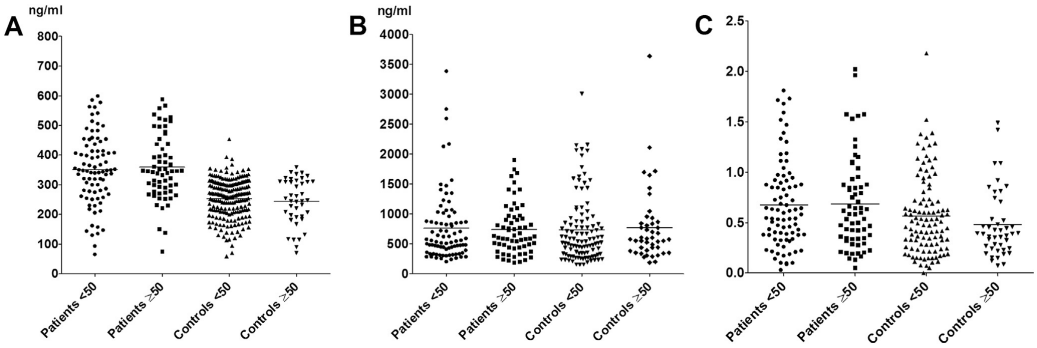

Figure 2 Serum concentrations of MMP-9 (A) and TIMP-1 (B) and MMP-9/TIMP-1 (C) ratio in TBE patients and in controls aged <50 and ≥50 years. The horizontal lines indicate mean values.

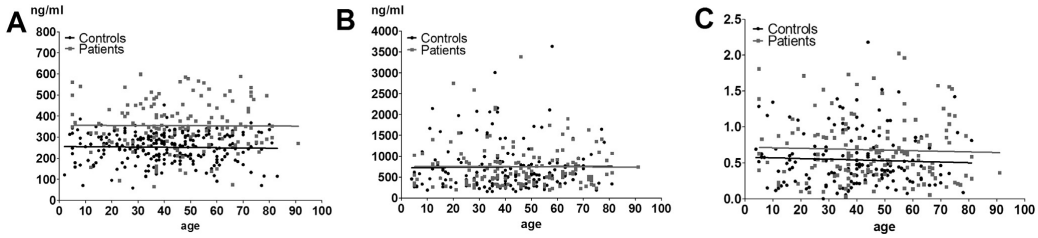

Figure 3 Correlation of the serum concentrations of MMP-9 (A) and TIMP-1 (B) and MMP-9/TIMP-1 (C) ratio in TBE patients and in controls with age of the individuals.

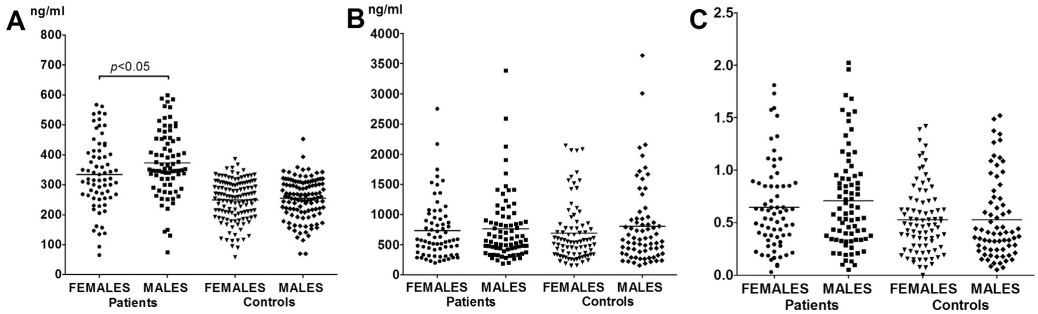

Figure 4 Serum concentrations of MMP-9 (A) and TIMP-1 (B) and MMP-9/TIMP-1 (C) ratio in male and female TBE patients and in controls. The horizontal lines indicate mean values.

in TBE patients compared to controls (Fig. 1). There is no specific therapy for TBE, but it was demonstrated that corticoids (5—10 mg/kg/day intravenously) have favourable effect during the acute stage of this disease.[23] Since corticosteroid is known to improve capillary functions by reducing activity of MMP-9 and increasing levels of TIMPs,[24] it can be speculated that the effect of corticoids in TBE may be explained at least in part by the down-regulation of MMP-9 activity, similarly as it was suggested for fungal and tuberculous meningoencephalitis.[12] In West Nile virus infection, it was demonstrated that MMP-9 facilitates virus entry into the brain by enhancing BBB permeability.[25] However, the role of MMP-9 in TBEV entry into the CNS and subsequent neuropathogenesis remains unknown and requires further study.

The incidence and severity of TBE are highest in people aged $\geq$50 years. Generally, patients above the age of 50 years experience significantly more sequelae and a higher case fatality rate.[26] It was also demonstrated that serum levels of interleukin 6 (IL-6) correlate positively with age of TBE patients.[27,28] IL-6 and also IL-8 act as stimulators of MMPs, which comes along with our results. However, in our study we did not find any differences in serum levels of MMP-9, TIMP-1 and MMP-9/TIMP-1 ratios in patients below and above the age of 50 years (Fig. 2) and there was also no correlation of the serum levels of MMP-9, TIMP-1 and MMP-9/TIMP-1 ratios with increasing age (Fig. 3). This indicates that the MMP-9 activity during TBE is not dependent on the age of the patient. The fact that there is no correlation between the MMP-9 levels and age, while IL-6 titers correlate with age,[27] suggests a more complicated mechanism.

Higher serum levels of MMP-9 were detected in male than female TBE patients. Sex-specific incidence of TBE is higher in men than in women in the Czech Republic (men to women ratio 1.5:1),[29] but there are no data if there are differences in sequelae and case fatality rates between males and females.

In conclusion, we have shown that serum MMP-9 levels and MMP-9/TIMP-1 ratios were higher in TBE patients than in controls. The increased levels of MMP-9 and MMP-9/TIMP-1 ratios in serum were not dependent on age of the patients, but TBE-positive males had higher levels of serum MMP-9 than TBE-positive females. Our data in the present study indicate that MMP-9 may play an important role in the BBB breakdown and development of brain damage during TBE.

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CHAPTER II:

1. **Infection and injury of human astrocytes by tick-borne encephalitis virus**
   Martin Palus, Tomáš Bílý, Jana Elsterová, Helena Langhansová, Jiří Salát, Marie Vancová and Daniel Růžek
   Journal of General Virology 2014, 95: 2411-2426. DOI: 10.1099/vir.0.068411-0

2. **Tick-borne encephalitis virus infects human brain microvascular endothelial cells without compromising blood-brain barrier integrity**
   Martin Palus, Marie Vancová, Jana Širmarová, Jana Elsterová, Jan Perner and Daniel Růžek
   Virology 2017, 507: 110-122. DOI: 10.1016/j.virol.2017.04.012

3. **Electron tomography analysis of tick-borne encephalitis virus infection in human neurons**
   Tomáš Bílý, Martin Palus, Luděk Eyer, Jana Elsterová, Marie Vancová and Daniel Růžek
   Scientific Reports 2015, 5: 10745. DOI: 10.1038/srep10745

The process of the breakdown of BBB during tick-borne encephalitis is not yet fully understood, nor is the entry of the virus into CNS. The blood-brain barrier is a highly selective semipermeable membrane barrier formed by endothelial cells that are interconnected by tight junctions. The integrity of BBB is supported by various cell types, such as microglia, astrocytes and pericytes, which together form a so-called neurovascular unit.

There are several hypotheses about how TBEV reaches CNS and causes neuropathology in humans. The virus might enter CNS directly via the olfactory nerve, by a hematogenous mode of neuroinvasion during BBB breakdown, with the infected immune cells that enter CNS during inflammation, or by infecting BBB cells (McMinn, 1997), (Koyuncu, Hogue & Enquist, 2013).

Based on the results of previous studies that showed that BBB permeability increased in the later stages of TBEV infection, when there was already a high viral load in mice brains before actual BBB disruption (Růžek et al., 2011), we focused on the mechanisms that facilitate the crossing of the virus through the intact BBB. An *in vitro* model of BBB was constructed according to previous studies (Verma, Lo, et al., 2010), (Verma, Kumar, et al., 2010).

Neuropathogenesis followed by neuroinflammation in TBEV infection was studied directly on human neurons as the main targets of a virus infection (Bílý et al., 2015). It was observed that in the early stages of TBEV infection, the virus spreads throughout the whole neuron, including the dendrites. This is consistent with the findings of our colleagues from Hokkaido University, Japan (Hirano et al., 2014). In later phases of the infection, the viral antigen concentrates in aggregates that are localized within the rough endoplasmic reticulum. The structural changes taking place during neuronal injury were visualized by advanced high-pressure freezing and freeze-substitution techniques followed by high-resolution 3D electron tomography. These images were done by our colleagues from the laboratory of electron microscopy, Dr. Marie Vancová and Dr. Tomáš Bílý. The ultrastructural changes revealed alteration in the rough endoplasmic reticulum, where tubule-like structures were formed due to the viral infection. Moreover, signs of autophagy were observed in neuronal dendrites enhancing TBEV replication.

The role of astrocytes in TBEV infection was also studied, as the key factor in an immune response arising from viral neuroinfections (Palus, Bílý, et al., 2014). Astrocytes are considered as providers of homeostasis in the brain, where they modulate extracellular levels of neurotransmitters and gliotransmitters and maintain BBB (Abbott, Rönnbäck & Hansson, 2006). TBEV is able to produce a persistent infection in primary human astrocytes and to activate them, resulting in the production of proinflammatory cytokines and chemokines. Astrocytes contribute to the immunopathological processes in CNS, since they produce elevated levels of matrix metalloproteinase 9, an indicator of BBB degradation (Paul et al., 1998), (Roe et al., 2012), (Palus, Žampachová, et al., 2014). Ultrastructural changes during a TBEV infection confirmed the existence of tubule-like structures associated with RER cisternae of the cell.

Finally, human microvascular endothelial cells, the crucial composite of the blood-brain barrier, were characterized in the context of TBEV infection (Palus et al., 2017). Viral negotiation of BBB is a critical event in the development and progression of CNS infection. The integrity of BBB is compromised in later stages of TBEV infection. However, BBB breakdown is not necessary in order for the virus to penetrate into the brain (Růžek et al., 2011). The mechanism of TBEV crossing the BBB was investigated in an *in vitro* model. Human microvascular endothelial cells are permissive for TBEV replication, though in very limited numbers. A transcellular pathway for TBEV to enter the brain is supported by this fact. Nevertheless, the infected endothelial cells remained intact, with no disruption of the tight junction proteins.

Taken together, there are possibly several effects of neuroinvasiveness and neuropathogenesis of TBEV. The role of the neurovascular unit seems to be a crucial one. Our observations imply that neuroinflammation as a consequence of TBEV infection is one of the major hallmarks of TBE neuropathogenesis, a finding that is supported by previous studies (Palus et al., 2013), (Růžek et al., 2009), (Růžek et al., 2011).

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1. **Infection and injury of human astrocytes by tick-borne encephalitis virus**

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Infection and injury of human astrocytes by tick-borne encephalitis virus

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Tick-borne encephalitis (TBE), a disease caused by tick-borne encephalitis virus (TBEV), represents the most important flaviviral neural infection in Europe and north-eastern Asia. In the central nervous system (CNS), neurons are the primary target for TBEV infection; however, infection of non-neuronal CNS cells, such as astrocytes, is not well understood. In this study, we investigated the interaction between TBEV and primary human astrocytes. We report for the first time, to the best of our knowledge, that primary human astrocytes are sensitive to TBEV infection, although the infection did not affect their viability. The infection induced a marked increase in the expression of glial fibrillary acidic protein, a marker of astrocyte activation. In addition, expression of matrix metalloproteinase 9 and several key pro-inflammatory cytokines/chemokines (e.g. tumour necrosis factor α, interferon α, interleukin (IL)-1β, IL-6, IL-8, interferon γ-induced protein 10, macrophage inflammatory protein, but not monocyte chemotactic protein 1) was upregulated. Moreover, we present a detailed description of morphological changes in TBEV-infected cells, as investigated using three-dimensional electron tomography. Several novel ultrastructural changes were observed, including the formation of unique tubule-like structures of 17.9 ± 0.15 nm diameter with associated viral particles and/or virus-induced vesicles and located in the rough endoplasmic reticulum of the TBEV-infected cells. This is the first demonstration that TBEV infection activates primary human astrocytes. The infected astrocytes might be a potential source of pro-inflammatory cytokines in the TBEV-infected brain, and might contribute to the TBEV-induced neurotoxicity and blood–brain barrier breakdown that occurs during TBE. The neuropathological significance of our observations is also discussed.

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INTRODUCTION

Tick-borne encephalitis (TBE) is a serious viral infection of the central nervous system (CNS) caused by tick-borne encephalitis virus (TBEV). TBEV is a single-stranded positive-sense RNA virus belonging to the genus *Flavivirus*, family *Flaviviridae* (Mansfield *et al.*, 2009). More than 13 000 clinical cases of TBE, including numerous deaths, are reported annually in Europe and north-eastern Asia (Mansfield *et al.*, 2009). Despite the medical importance of this disease, some crucial steps in the development of encephalitis remain poorly understood. In humans, TBEV may produce a variety of clinical symptoms. The clinical spectrum of acute TBE ranges from symptoms of undifferentiated febrile illness or mild meningitis to severe meningoencephalitis with or without myelitis (Haglund & Günther, 2003; Růžek *et al.*, 2010). Chronic TBE occurs less frequently and has been reported only in some regions of Russia, mainly in Siberia and the Far East, where this form comprises 1–3 % of all TBE cases (Gritsun *et al.*, 2003).

Major hallmarks of TBEV neuropathogenesis are neuro-inflammation followed by neuronal death and disruption of the blood–brain barrier (BBB) (Růžek *et al.*, 2009a, 2011; Palus *et al.*, 2013, 2014). The response of TBEV infection in the brain is characterized by massive inflammatory events, including the production of cytokines (e.g. IFN-γ, TNF-α, and IL-1β, IL-6 and IL-10) and chemokines [e.g. monocyte chemoattractant protein (MCP)-1/CCL2, IFN-γ-induced protein 10 (IP-10)/CXCL10, macrophage inflammatory

†These authors contributed equally to this study.

A supplementary movie is available with the online version of this paper.

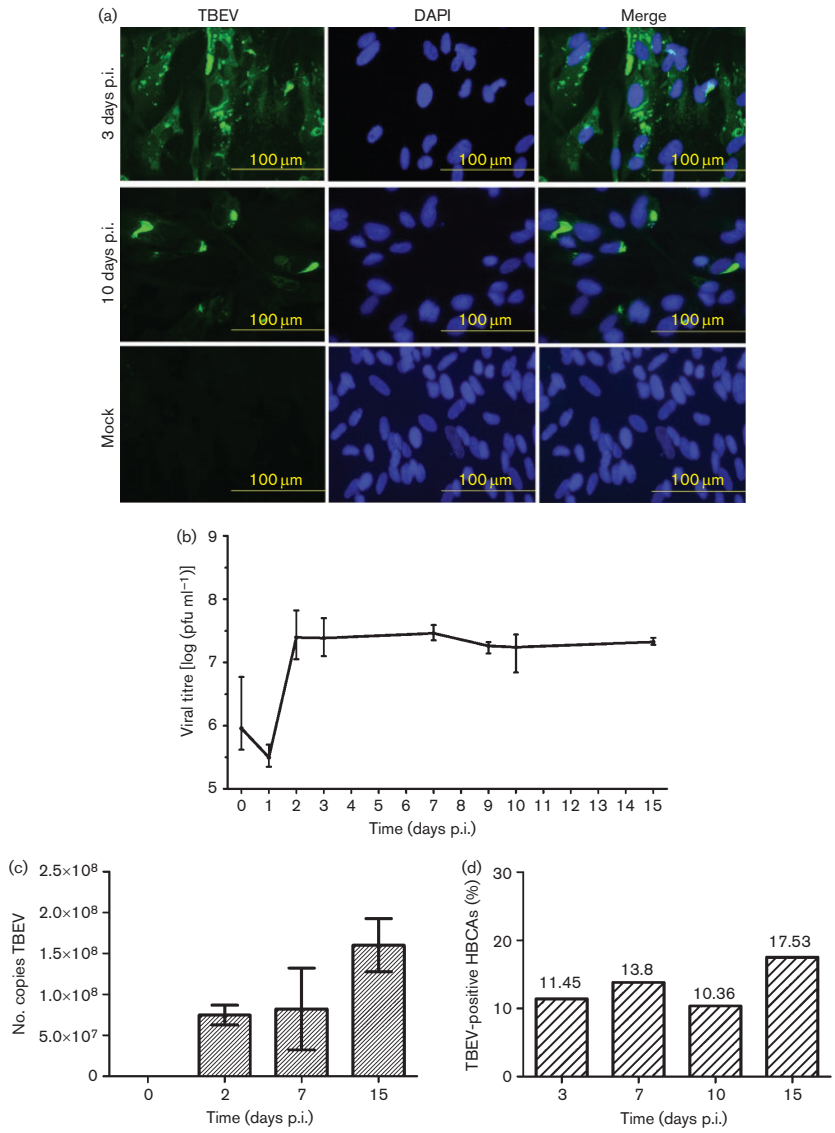

Fig. 1. TBEV can infect human primary astrocytes. (a) HBCAs grown and fixed on slides at days 3 and 10 post-infection (p.i.) were stained with anti-flavivirus envelope antibody (green) and counterstained with DAPI (blue). TBEV-infected HBCAs immunostained with flavivirus-specific antibody demonstrated virus replication in the cytoplasm, with antigen aggregates forming at day 3 p.i. At later time points (10 days p.i.), only the brightly stained aggregates of viral antigen were observed. Mock-infected HBCAs stained with primary and secondary antibodies were used as a negative control, and did not exhibit any TBEV antigen staining. (b) TBEV titres in culture supernatant from HBCAs collected at 0, 1, 2, 3, 7, 9 and 15 days p.i. were determined by plaque assay using porcine kidney stable cells. Viral titres are expressed as p.f.u. ml^{-1}. Data represent means ± SEM. (c) Total RNA extracted from HBCA cell lysates at 0, 2, 7, and 15 days p.i. was used to determine the number of

intracellular TBEV RNA copies by quantitative RT-PCR. Values represent means ± SEM. (d) The percentage of HBCAs that were positive for TBEV antigen in culture at 3, 7, 10 and 15 days p.i. was determined. Data were obtained based on a total of 23 000 cells counted in at least seven independent fields.

protein (MIP)-1α and RANTES] (Palus *et al.*, 2013). Microglia and astrocytes are classically believed to serve as the predominant source of these cytokines and chemokines in the CNS, and therefore may act as important processors of neuroinflammation and neurodegeneration (Ramesh *et al.*, 2013). The pro-inflammatory chemokines attract immunocompetent cells to the CNS (Réaux-Le Goazigo *et al.*, 2013), including $CD8^+$ T-cells, which may mediate immunopathology during TBE (Růžek *et al.*, 2009a). Moreover, the pro-inflammatory molecules can further activate downstream apoptotic signalling pathways in neurons, resulting in neuronal death (Kumar *et al.*, 2010) or inducing breakdown of the BBB (Růžek *et al.*, 2011; Erickson *et al.*, 2012; Palus *et al.*, 2014). We used a rodent model to demonstrate that TBEV infection is associated with the dramatic BBB breakdown that occurs during the later stages of infection. The BBB breakdown most likely represents a bystander effect of virus-induced cytokine/chemokine overproduction in the brain (Růžek *et al.*, 2011). However, the specific cell types that express these cytokines and chemokines have not been characterized.

Although neurons are primary targets after TBEV enters the CNS (Hirano *et al.*, 2014), other brain cells may also be infected (Potokar *et al.*, 2014). Infection of non-neuronal CNS cells including astrocytes has, albeit infrequently, been reported in cases of flavivirus encephalitis (Desai *et al.*, 1995; Nogueira *et al.*, 2002; German *et al.*, 2006; Balsitis *et al.*, 2009; de Araújo *et al.*, 2009; Sips *et al.*, 2012). It was shown recently that TBEV infects cultured primary rat astrocytes without affecting their viability. Therefore, it was suggested that astrocytes might represent an important reservoir of TBEV in brain during the infection (Potokar *et al.*, 2014). Astrocytes are the most abundant glial cell population in the human brain (Nedergaard *et al.*, 2003) and have various leading roles in the brain, including integrating neuronal functions, neuronal support and regulation of the BBB. Thus, astrocytes serve as a structural and functional bridge between endothelial cells of the BBB and neurons; together, they form the 'neurovascular unit' (Stanimirovic & Friedman, 2012), which regulates blood flow, the integrity of the BBB and neuronal activity in response to physiological and pathophysiological changes (Hussmann *et al.*, 2013).

Astrocytes are key players in the inflammatory response during neural infections caused by flaviviruses, namely Japanese encephalitis (Bhowmick *et al.*, 2007; Yang *et al.*, 2012) and West Nile encephalitis (Diniz *et al.*, 2006; Verma *et al.*, 2011; Hussmann *et al.*, 2013); however, their role in the development of TBE remains largely unstudied. Here, we aimed to investigate the sensitivity of primary human astrocytes to TBEV infection, virus growth, virus-induced astrocyte activation, and cytokine and chemokine production. We demonstrate here for the first time, to the best of our knowledge, that TBEV is capable of productive, persistent infection in primary human astrocytes, and that this infection is associated with astrocyte activation and the production of various pro-inflammatory cytokines and chemokines.

On the ultrastructural level, the infection causes massive morphological changes that include the proliferation and rearrangement of the rough endoplasmic reticulum (RER) and lead to the formation of new compartments with an optimal microenvironment that provides functional sites for protein synthesis, processing and RNA replication, whilst providing protection against the host immune system (Welsch *et al.*, 2009; Gillespie *et al.*, 2010; Offerdahl *et al.*, 2012; Miorin *et al.*, 2013). These newly transformed compartments are represented by vesicles or vesicle packets that contain a pore opening to the cytosol (Offerdahl *et al.*, 2012; Miorin *et al.*, 2013) and convoluted membranes with a putative polyprotein processing function (Welsch *et al.*, 2009). A number of other functions have been ascribed to the proliferation of this membrane network, including the concentration of virus replication machinery, the provision of a solid-state platform for viral protein synthesis and replication, and the sequestration of viral dsRNA (the replicative form) from innate immune sensors (Overby *et al.*, 2010; Offerdahl *et al.*, 2012).

We also used electron tomography to provide important insights into the three-dimensional (3D) morphology of the infected cells, and, to the best of our knowledge, this is the first description of the 3D architecture of the tubule-like structures found in the RER of TBEV-infected human astrocytes. Taken together, our findings suggest that astrocytes can significantly contribute to the development of inflammation in the CNS during TBE. This information may facilitate novel strategies for treating this important neural infection.

RESULTS

TBEV can infect and replicate in human astrocytes

We employed a plaque assay and immunofluorescence staining for viral antigen to determine TBEV infection and replication kinetics in primary human brain cortex astrocytes (HBCAs) (Fig. 1). Viral antigen was not detected in mock-infected HBCAs (Fig. 1a) or in cells stained with secondary antibody alone. Based on a total of 23 000 cells counted in at least seven independent fields, approximately 11 % of HBCAs were infected with TBEV at day 3, 14 % at day 7, 10 % at day 10 and 18 % at day 15 post-infection (p.i.) (Fig. 1d).

TBEV replication was quantified using a plaque assay in TBEV-infected cell supernatants collected daily from days 0

to 3 and then at 7, 9, 10 and 15 days p.i. Productive TBEV replication in the form of release of virions was first detected at day 2 after infection, and day 2 also represented the limit of virus production (Fig. 1b). Intracellular TBEV replication assessed by quantitative real-time reverse transcription (RT)-PCR also confirmed virus replication for the first 15 days p.i., and the number of TBEV RNA copies increased in a time-dependent manner (Fig. 1c). We also used phase-contrast microscopy to examine TBEV-infected astrocytes for cytopathogenic effect (CPE) and cell death; neither was observed at any time point (data not shown).

Immunofluorescence staining revealed that the TBEV antigen was distributed mostly diffusely throughout the entire body of the astrocyte at early time points after infection (Fig. 1a). However, at later time points (as early as day 3 after infection), we observed brightly staining aggregates of viral antigen. A co-localization study with protein disulfide isomerase family A, member 3 (PDIA3) antigen (also known as Erp57, Er-60 and GRP58) suggested that the antigen was localized primarily in extremely hypertrophied and rearranged endoplasmic reticulum of the cells as early as day 3 p.i. (Fig. 2).

TBEV induces the expression of multiple pro-inflammatory cytokines/chemokines in human astrocytes

Pro-inflammatory cytokines, such as IL-1β and TNF-α, play an important role in mediating neuronal death and neuroinflammation in various diseases. Therefore, we investigated the effect of TBEV infection on the mRNA expression of key pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, IFN-α and TNF-α. We also measured the expression of MCP-1/CCL2, MIP-1β/CCL4 and IP-10/CXCL10 mRNAs in infected and control astrocytes (Figs 3 and 4). On day 1 p.i., we observed no significant increase in the mRNA expression of any cytokine/chemokine.

The expression of CCL2/MCP-1 mRNA did not change significantly at any time point (Fig. 3f). Robust upregulation of TNF-α mRNA was detected at 3 and 4 days p.i.; however, the expression was decreased at 15 days p.i. (Fig. 3a). IFN-α mRNA expression was slightly upregulated only at day 4 p.i. (Fig. 3b). Although the expression of IL-1β, IL-6 and IL-8 mRNAs increased from day 3 to 4, no upregulation was observed at day 15 p.i. (Fig. 3c–e, respectively). We observed strong increases of CCL4/MIP-1β and CXCL10/IP-10 mRNA expression from 3 to 4 days p.i.; however, at day 15, the expression level for these molecules was similar in infected cells and control cells (Fig. 4a, c).

We used ELISA to investigate the release of secreted MIP-1β/CCL4 and IP-10/CXCL10 cytokines/chemokines in the culture medium of TBEV-infected and control cells. The amount of soluble MIP-1β/CCL4 did not increase until day 4 p.i. At day 5 p.i., the amount of soluble MIP-1β/CCL4 was significantly increased (Fig. 4d). Basal levels of IP-10/CXCL10 in culture media were very low. Starting on day 2

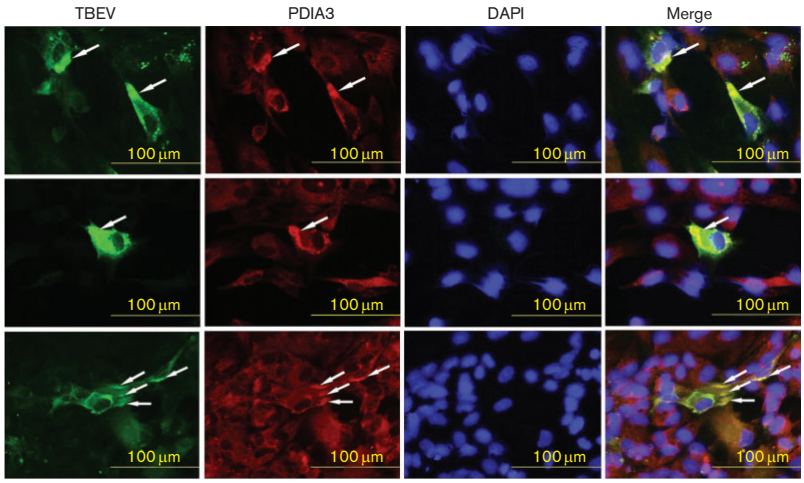

Fig. 2. TBEV antigen is co-localized with PDIA3 antigen in infected HBCAs at later times p.i. HBCAs grown and fixed at day 3 p.i. were stained with anti-flavivirus envelope antibody (green) and anti-PDIA3 antibody (red), and counterstained with DAPI (blue). Co-localization of TBEV and PDIA3 antigens was observed at all investigated time points p.i. Three representative examples are shown. Mock-infected HBCAs stained with primary anti-flavivirus and secondary antibodies (or cells stained with secondary antibodies only) were used as negative controls and did not exhibit any TBEV or PDIA3 antigen staining (not shown).

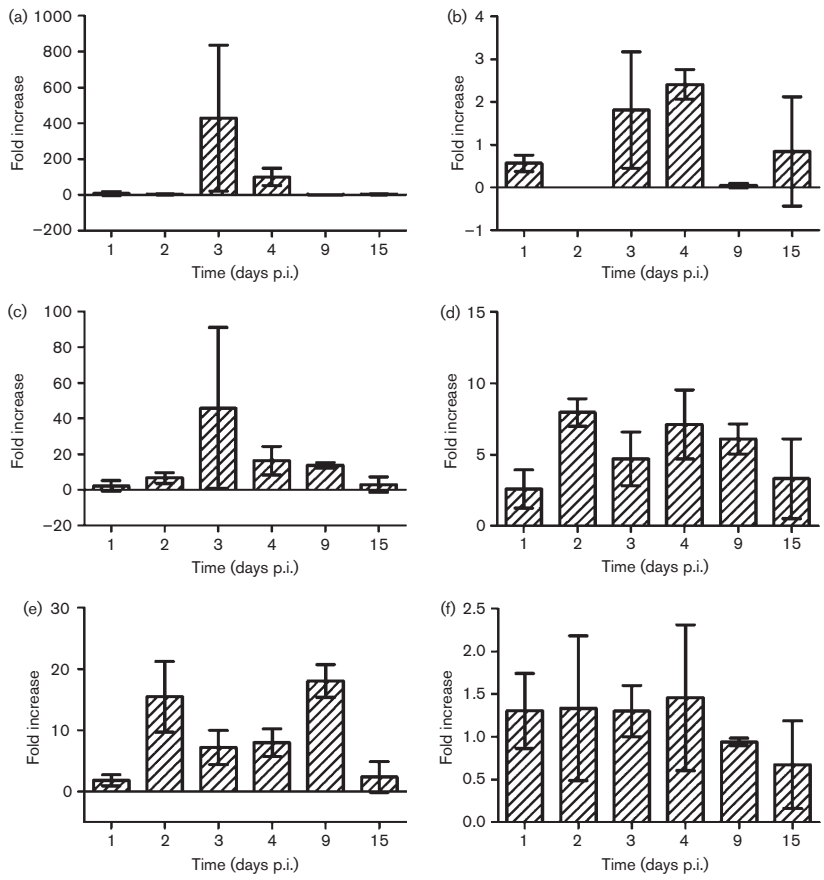

Fig. 3. TBEV differentially modulates the expression of pro-inflammatory cytokines and chemokines in HBCAs. Total RNA from mock-infected and TBEV-infected HBCAs at days 1–4, 9 and 15 p.i. were used to determine the fold change of TNF-α (a), IFN-α (b), IL-1β (c), IL-6 (d), IL-8 (e) and MCP-1/CCL2 (f) mRNAs with quantitative RT-PCR. Changes in cytokine and chemokine levels were first normalized to the expression of housekeeping genes (human β-actin and glyceraldehyde 3-phosphate dehydrogenase) and the fold change in the infected cells was calculated compared with the corresponding controls. Data are expressed as means ± SEM.

p.i., the amount of soluble IP-10/CXCL10 increased substantially ($P<0.001$; Fig. 4b).

TBEV infection induces the production of matrix metalloproteinase 9 by astrocytes

The expression of matrix metalloproteinases (MMPs), especially MMP-9, correlates with BBB disruption during many neuroinflammatory diseases. Therefore, we investigated the effect of TBEV infection on the production of MMP-9 by astrocytes. The release of soluble MMP-9 into the culture medium of mock- and TBEV-infected astrocytes was detected using ELISA. Starting at day 2 after infection, the amounts of soluble MMP-9 increased continuously until the end of the experiment, with a dramatic increase at day 7 (Fig. 5).

TBEV infection is associated with the activation of infected astrocytes, as demonstrated by increased glial fibrillary acidic protein (GFAP) expression

To provide additional evidence that TBEV infection per se causes astrocyte activation, we measured the production of

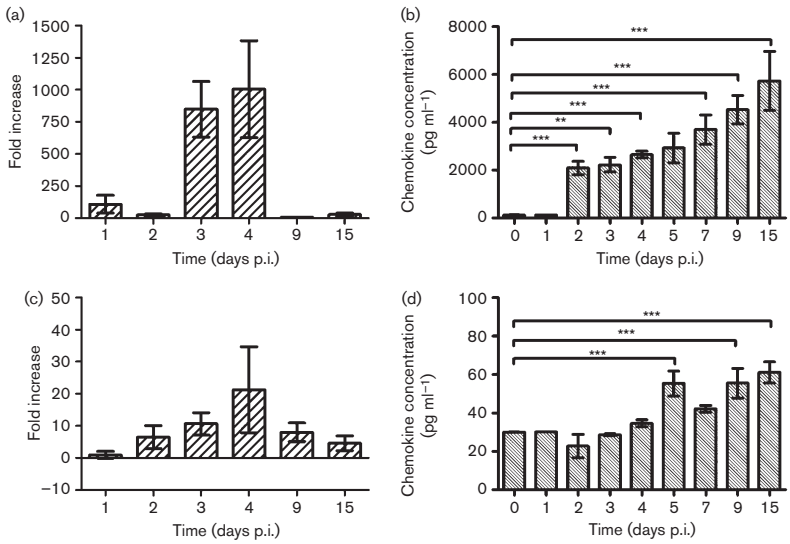

Fig. 4. TBEV increases the production of IP-10/CXCL10 and MIP-1β/CCL4 in infected HBCAs. (a, c) Total RNA from mock-infected and TBEV-infected HBCAs at 1–4, 9 and 15 days p.i. was used to determine the fold change of IP-10/CXCL10 (a) and MIP-1β/CCL4 (c) mRNAs with quantitative RT-PCR. Changes in the cytokine and chemokine mRNA levels were first normalized to the expression of housekeeping genes (human β-actin and glyceraldehyde 3-phosphate dehydrogenase) and the fold change in the infected cells was calculated compared with corresponding controls. Data are expressed as means ± SEM. (b, d) Levels of IP-10/CXCL10 (b) and MIP-1β/CCL-4 (d) in culture supernatants were determined using ELISA at 0–7, 9 and 15 days p.i. Data represent mean concentrations ± SEM. $^{**}P<0.01$; $^{***}P<0.001$.

GFAP, a marker of astrocyte activation, in mock-infected and TBEV-infected astrocytes, as well as in cells treated with lipopolysaccharide (LPS) at various times p.i. Flow cytometry indicated that the intensity of GFAP production increased significantly in TBEV-infected HBCAs at 3, 7 and 15 days p.i. compared with mock-infected and LPS-treated cells, clearly demonstrating astrocyte activation (Fig. 6a, b). The intensity of GFAP production in the TBEV- and mock-infected cells was also visualized with fluorescence microscopy using a specific anti-GFAP antibody (Fig. 6c).

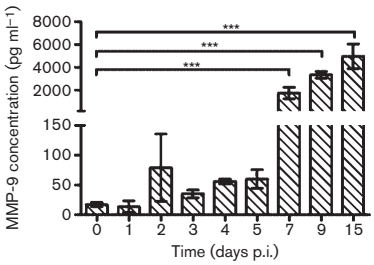

Fig. 5. TBEV-infected HBCAs release large quantities of MMP-9. Levels of MMP-9 in culture supernatants were determined using ELISA at 0–7, 9 and 15 days p.i. Data represent mean concentrations ± SEM. $^{***}P<0.001$.

TBEV causes dramatic ultrastructural morphological changes in infected astrocytes

We used transmission electron microscopy and electron tomography to investigate ultrastructural changes in mock- and TBEV-infected HBCAs at 3 and 9 days p.i. At 3 days p.i., we observed rearranged cisterns of the RER with typical virus-induced vesicles and viral particles, as described elsewhere (Fig. 7a) (Růžek *et al.*, 2009b). Next, we observed that many enveloped TBEVs were crowded into the Golgi complex (Figs 7b and 8b). In contrast, at 9 days p.i., we observed a lower number of viral particles in the cisterns of the RER (Fig. 7c) and in vacuoles close to the Golgi complex (Fig. 7d). At 9 days p.i., we observed intra-mitochondrial electron-dense granules that formed annular structures in the mitochondrial matrix (Fig. 7c). Moreover, these cells contained both swollen mitochondria with mitochondrial cristae located in the periphery, and mitochondria that apparently lacked any alteration in morphology (Fig. 7e). Furthermore, some infected cells displayed other

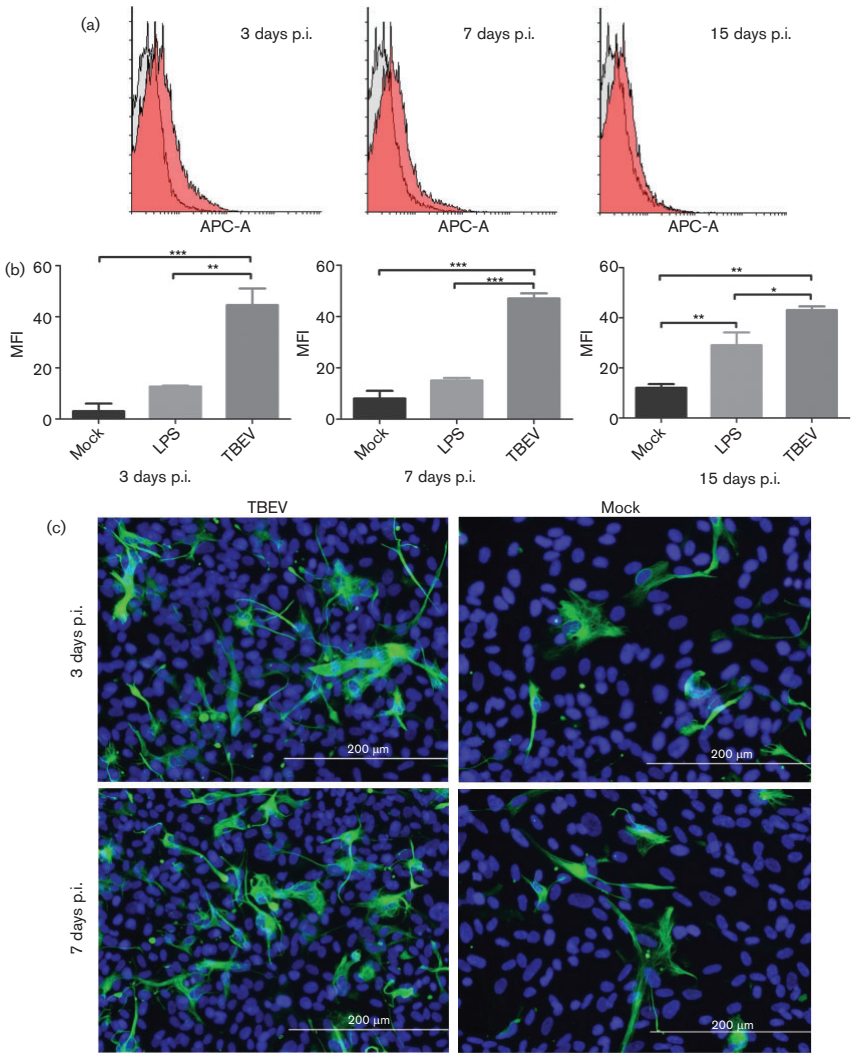

Fig. 6. TBEV infection activates HBCAs, as demonstrated by increased GFAP production. (a) Flow cytometry analysis of GFAP production in mock-infected and TBEV-infected HBCAs at 3, 7 and 15 days p.i. is shown as overlapping histograms of relative fluorescence intensity of the analysed cells. (b) The mean fluorescence intensity (MFI) of GFAP-positive cells in culture after mock infection or TBEV infection and LPS treatment at 3, 7 and 15 days p.i. was determined by flow cytometry; it was significantly increased in TBEV-infected HBCAs at all time points investigated. (c) HBCAs grown and fixed at days 3 and 7 p.i. were stained with anti-GFAP antibody (green) and counterstained with DAPI (blue). HBCAs stained with secondary antibody alone were used as a negative control and did not exhibit any GFAP antigen staining (not shown). *$P<0.05$; **$P<0.01$; ***$P<0.001$.

morphological features of cells undergoing necrotic cell death, such as chromatin aggregates in the nuclear periphery, irregularly shaped cells and surface blebs, and enlarged cisternal space in the endoplasmic reticulum (Fig. 7c–e). In contrast, neither virus replication nor ultrastructural alterations were evident in several other cells from the same

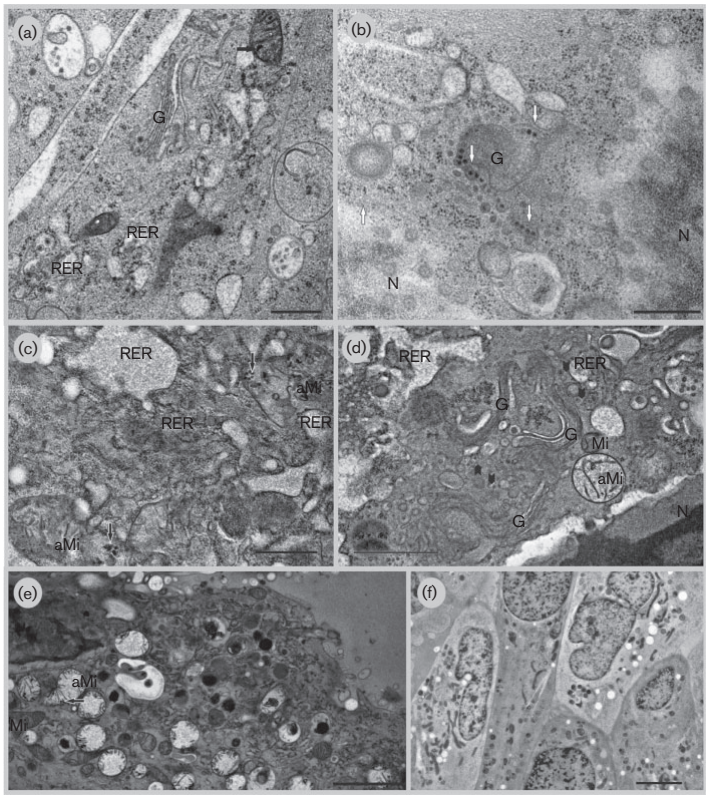

Fig. 7. Morphological changes in TBEV-infected HBCAs at 3 (a, b) and 9 (c–f) days p.i. (a) TBEV particles located inside the remodelled RER and the Golgi complex (G). In particular, note the mitochondrion with the electron-dense granules (black arrow). (b) Viral particles (white arrows) accumulated in the periphery of the Golgi stack (G). N, nucleus. (c–e) Ultrastructural alterations were observed in infected cells at 9 days p.i.: enlarged cisternae of the RER, rearranged RER membranes, swollen mitochondria (aMi) with annularly arranged granules (black arrows) and mitochondria without structural changes (Mi), as well as viral particles inside vacuoles (arrowheads). (f) Transmission electron microscopy did not reveal any ultrastructural abnormalities in several HBCAs at 9 days p.i. Bars: 500 nm (a, b), 100 nm (c); 1 μm (d); 2 μm (e); 5 μm (f).

culture and the same time interval (Fig. 7f), which is in accordance with immunofluorescence staining for viral antigen. The ultrastructure of these cells was similar to that of the mock-infected HBCAs (Fig. 8a). Morphological changes induced by TBEV infection involved almost all cell compartments (RER, Golgi complex, mitochondria and phagosomes) as visible on the 3D model of the infected cells (Fig. 8b).

TBEV induces the formation of tubule-like structures in the endoplasmic reticulum of some infected astrocytes

We observed tubule-like structures, which were located inside the RER cisternal space of only a very few of the infected astrocytes (Fig. 9 and Movie S1, available in the online Supplementary Material). The tubule-like structures were laid out in many parallel groupings of bundle-like fascicles. The electron density of these tubule-like structures was consistent throughout their shape (Fig. 10a–c), and they were 17.9 nm ($\pm$0.2 nm; n=101) in diameter. In contrast, when viewed using the electron microscope, cellular microtubules outside the ER appeared to be less electron-dense on the inside and were bordered on the outside by two dense lines; the diameter of these microtubules was 20.3 nm (microtubules in Fig. 10d–f). All enveloped viral particles were observed in the lumen of the RER, and most were directly connected to the tubule-like structures (Fig. 11a–d). The diameter of the enveloped

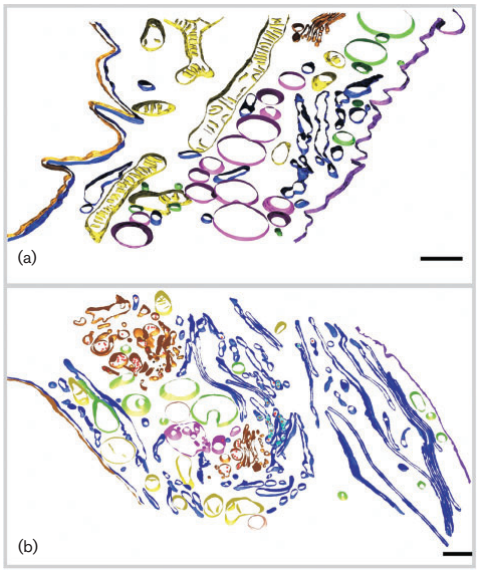

Fig. 8. 3D models of mock-infected (a) and TBEV-infected HBCAs (b) at 3 days p.i. TBEV infection causes extensive morphological changes, including membrane reorganization of the RER; differences are evident in the Golgi complex, mitochondria and phagosomes. 3D reconstructions of single axis tomograms are shown. Tilt series images were collected with either ±60° tilt range in 0.65° increments (a) or ±65° tilt range in 1° increments (b). The final 3D reconstructed thicknesses are 52 nm (a) and 56 nm (b). Pixel resolution: 1.66 nm (a), 2.16 nm (b). Orange, the Golgi complex; red, TBEV particles; light blue, TBEV-induced structures; dark blue, RER; yellow, mitochondria; green, vacuole; pink, phagosome/endosome; violet, cell membrane; brown, nuclear membrane. Bars, 500 nm.

viral particles was 42.5 nm (±0.7 nm; n=12), and the diameter of the nucleocapsid was 26.1 nm (±0.7 nm; n=12). Several TBEV-induced vesicles that were nearly spherical and ranged from 60 to 90 nm in diameter were observed in close proximity to viral enveloped particles. Some TBEV-induced vesicles were connected in a manner identical to that described regarding the tubule-like structures (Fig. 11e–h). We also noted the presence of a subviral particle enclosed in a vesicle in close proximity to the tubule-like structures. The presence of subviral particles (Fig. 11i, j) indicated defective virus assembly.

DISCUSSION

Although TBEV is a significant cause of encephalitis in humans, relatively little attention has been given to TBEV infection of cells in the human CNS. Neurons are primary targets for TBEV (Hirano *et al.*, 2014) in the CNS, and other brain cells may also be infected (Potokar *et al.*, 2014). Although TBEV antigen was not detected in astrocytes in a study investigating brains from fatal human TBE cases (Gelpi *et al.*, 2005), data from other studies indicate that non-neuronal CNS cells including astrocytes are also, albeit infrequently, infected in cases of flavivirus encephalitis (Desai *et al.*, 1995; Nogueira *et al.*, 2002; German *et al.*, 2006; Balsitis *et al.*, 2009; de Araújo *et al.*, 2009; Sips *et al.*, 2012). Infection of non-neuronal cells might play some role in the entry of the virus into the CNS, development of neuroinflammation and viral persistence in the CNS during chronic infection. A recent report demonstrated that primary rat astrocytes are sensitive to TBEV infection, although the infection did not affect cell viability (Potokar *et al.*, 2014). Therefore, it was suggested that astrocytes might represent an important reservoir of dormant TBEV during chronic brain infection (Potokar *et al.*, 2014), for example in cases of chronic TBEV infections reported in humans in Siberia and the Far East (Gritsun *et al.*, 2003). Moreover, an increasing number of studies have demonstrated the important role of astrocytes during encephalitis caused by other flaviviruses, such as West Nile virus and Japanese encephalitis virus (Chen *et al.*, 2000, 2004; Diniz *et al.*, 2006; Kumar *et al.*, 2010; Verma *et al.*, 2011; Yang *et al.*, 2012; Hussmann *et al.*, 2013; Hussmann & Fredericksen, 2014). However, cultured astrocytes were not sensitive to infection with dengue virus (Imbert *et al.*, 1994). In this study, we showed that primary human astrocytes could be infected with TBEV and produce relatively high virus titres (Fig. 1). The viability of the infected cells was not altered during the monitored time interval after infection (15 days), which is in accordance with the findings of a study on primary rat astrocytes (Potokar *et al.*, 2014). In agreement with other authors (Potokar *et al.*, 2014), we can conclude that primary human astrocytes are much more resilient to TBEV infection than other cell types, such as human neuroblastoma, glioblastoma and medulloblastoma cells (Růžek *et al.*, 2009b). However, the number of infected cells in the culture did not exceed 20 % during the entire investigated period, suggesting that only a fraction of the cells is sensitive to infection, whilst the rest remain resistant. It remains unknown exactly what renders some cells sensitive to the infection and others resistant.

TBEV infection in the brain is associated with the induction of several cytokines and chemokines. Accumulation of cytokines and chemokines in the CNS may accentuate the progression of encephalitis instead of restricting virus replication (Ramesh *et al.*, 2013). Although viral infection is not generally as robust in human glial cells as in neurons, they secrete much higher levels of immune mediators, such as cytokines and chemokines (Verma *et al.*, 2011). Therefore, during the inflammatory response, astrocytes and other glial cells may influence the balance between host protection and neurotoxicity. We reported previously in a mouse study

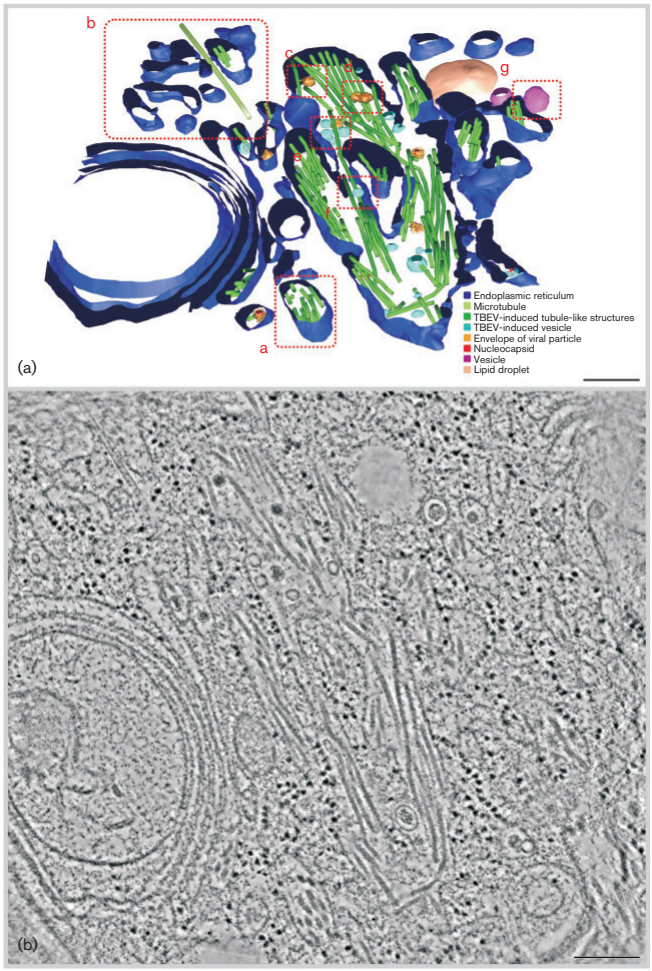

Fig. 9. Tubule-like structures observed in TBEV-infected HBCAs at 3 days p.i. This is a 3D reconstruction of a dual axis tomogram (a) and a slice of the tomogram (b). Tilt series images were collected in the range ±65° in 0.65° increments. The final reconstructed section thickness was approximately 60 nm, which was divided into 75 slices. Pixel resolution: 0.81 nm. Bars, 200 nm. This tomogram is shown in Movie S1.

that high expression of various cytokines/chemokines during TBE is able to mediate immunopathology, and might be associated with a more severe course of infection and increased fatality (Palus *et al.*, 2013). In the present study, we observed that TBEV infection of astrocytes is associated with the dramatically increased production of various pro-inflammatory cytokines and chemokines. In particular, quantitative real-time RT-PCR and ELISA indicated that the expression/production of IL-1β, IL-6, IL-8, IFN-α, TNF-α, IP-10/CXCL10 and MIP-1β/CCL4 was significantly elevated in TBEV-infected astrocytes (Figs 3 and 4), which is consistent with other studies describing cytokine/chemokine production by flavivirus-infected astrocytes (Verma *et al.*, 2011; Yang *et al.*, 2012; Hussmann & Fredericksen, 2014). The greatest increase in cytokine/chemokine production was observed between days 2 and 3 p.i. (Figs 3 and 4). This finding was consistent with the time of peak virus production in HBCAs (Fig. 1b).

Cytokines such as TNF-α and IL-1β have been reported as potent inducers of neuronal injury (Brabers & Nottet, 2006; Ghoshal *et al.*, 2007; McColl *et al.*, 2008; Kumar *et al.*, 2010; Verma *et al.*, 2011). IL-1β, IL-6 and IL-8 are endogenous pyrogens that exert multiple downstream inflammatory signalling pathways (Verma *et al.*, 2011). These cytokines are elevated during various CNS infections, including TBE (Palus *et al.*, 2013). Increased concentrations of pro-inflammatory cytokines, including TNF-α and IL-6, were detected in sera from TBE patients, and their elevated levels corresponded with the acute phase

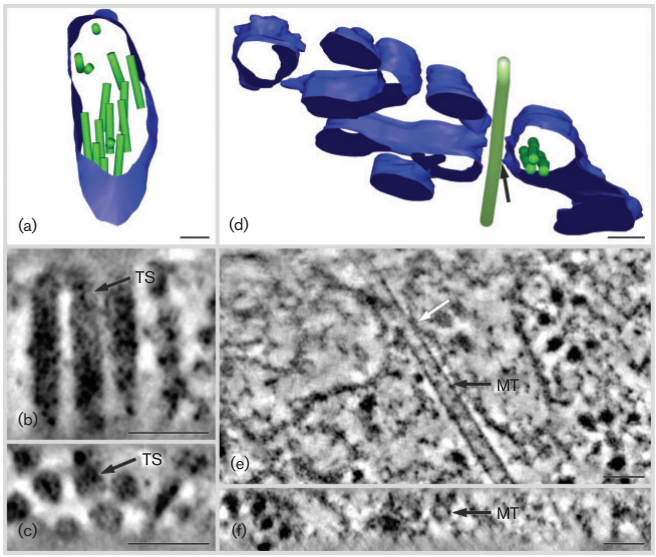

Fig. 10. Comparison of the ultrastructure of tubule-like structures (a–c) and a microtubule (d, e) in TBEV-infected HBCAs. (a–c) Detail of tubule-like structures from Fig. 9(a), area *a*. The model is shown in (a), a slice of a dual axis tomogram in (b) and the side view in (c). (d–f) Detail of microtubule from Fig. 9(a), area *b*. The model is shown in (d), a slice of a dual axis tomogram in (e) and the side view in (f). The white arrow in (e) indicates a connection between the microtubule and the membrane of the RER. Tilt series images were collected in the range ±65° in 0.65° increments. The final reconstructed section thickness was approximately 60 nm, which was divided into 75 slices. Pixel resolution: 0.81 nm. Bars, 50 nm.

of the disease (Atrasheuskaya *et al.*, 2003). The chemokine IP-10 has the ability to attract activated T-cells in the CNS (Klein *et al.*, 2005). Excessively high levels of IP-10 in the CNS can be very harmful to the host (Sasseville *et al.*, 1996; Westmoreland *et al.*, 1998; Sui *et al.*, 2006), possibly by activating a calcium-dependent apoptotic pathway (Sui *et al.*, 2004). In human TBE patients, higher levels of IP-10 can be detected in serum, as well as in cerebrospinal fluid (Lepej *et al.*, 2007; Zajkowska *et al.*, 2011). The attraction of $CD8^+$ T-cells to the CNS by IP-10 can have important consequences for viral clearance, as well as for immunopathological reactions observed during TBE (Růžek *et al.*, 2009a). Similar to our study, astrocytes have been described as a predominant source of IP-10 in Japanese encephalitis (Bhowmick *et al.*, 2007). The expression of MCP-1/CCL2, a compound that is able to disrupt the integrity of the BBB and modulate the progression of neuroinflammation (Yao & Tsirka, 2014), is highly upregulated in TBEV-infected brain tissue (Palus *et al.*, 2013). However, its expression was not increased in astrocytes after TBEV infection in the present study, suggesting that astrocytes are not responsible for the production of this cytokine in the CNS during TBE. Together, TBEV-infected astrocytes produce a variety of cytokines that can mediate a diverse range of neurodegenerative functions, including disruption of the BBB, chemoattraction of peripheral immune cells into the CNS and neuronal damage.

We reported previously that TBE is associated with the disruption of BBB integrity, which is most likely caused by cytokine/chemokine overproduction in the brain (Růžek *et al.*, 2011). In human TBE patients, higher levels of MMP-9 (a compound with multiple functions, including disruption of the BBB) have been observed in serum (Palus *et al.*, 2014) and cerebrospinal fluid (Kang *et al.*, 2013). However, it was not clear which cells were involved in MMP-9 production and BBB disruption. In the present study, we observed that TBEV-infected astrocytes produced large quantities of MMP-9 (Fig. 5), and therefore might represent the main cell population responsible for the increase of BBB permeability during TBE. The entry of TBEV into the CNS precedes the breakdown of the BBB (Růžek *et al.*, 2011). The invasion of TBEV into the CNS brings the virus into close proximity with the second component of the BBB, astrocytes (Hussmann *et al.*, 2013). TBEV-activated astrocytes then produce MMP-9, which might cause the BBB breakdown. Moreover, MMP-9 is capable of causing neuronal apoptosis (del Zoppo, 2010).

Additional evidence of astrocyte activation by TBEV was demonstrated by the increased production of GFAP (Fig. 6). GFAP is involved in many important CNS processes, including cell communication and BBB function. Increased GFAP expression/production as a marker of astrocyte activation has been documented in many studies (Brodie *et al.*, 1997; Zhou *et al.*, 2004; Pozner *et al.*, 2008; Watanabe *et al.*, 2008; Kumar *et al.*, 2010; Fan *et al.*, 2011; Ojeda *et al.*, 2014), represents one of the earliest responses to CNS injury and is a distinguishing feature of reactive astrogliosis (Montgomery, 1994). The activation of glial cells including astrocytes represents one of the major histopathological features of TBE (Környey, 1978; Gelpi *et al.*, 2006). The astrocyte activation leads to a downstream cascade of inflammatory cytokine production that results in the death of neurons (Kumar *et al.*, 2010; Pekny *et al.*, 2014). In our

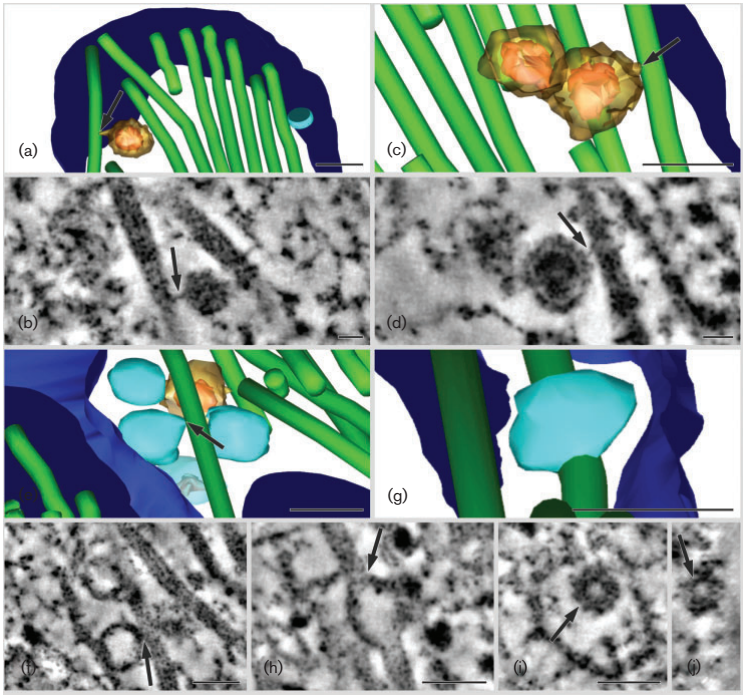

Fig. 11. 3D architecture of tubule-like structures observed in TBEV-infected HBCAs. The connection (arrow) between tubule-like structures and enveloped virions in the model (a, c, e, g) and in a slice of a dual axis tomogram (b, d, f, h). Enlargement of Fig. 9(a), areas *c* (a, b), *d* (c, d), *e* (e, f) and *f* (g, h) is shown. Supposed subviral particle enclosed in the vesicle outside the RER, detail from Fig. 9(a), area *g*, is shown in the tomogram top view (i) and side view (j). Tilt series images were collected in the range ±65° in 0.65° increments. The final reconstructed section thickness was approximately 60 nm, which was divided into 75 slices. Pixel resolution, 0.81 nm. Bars, 50 nm.

study, GFAP production was higher in TBEV-infected astrocytes than in mock-infected or LPS-stimulated astrocytes. Treatment with LPS had no effect on GFAP production at 3 and 7 days after treatment, which is in accordance with a previous study that demonstrated that LPS downregulates the expression of GFAP mRNA (Letournel-Boulland *et al.*, 1994). However, on day 15 post-treatment, LPS treatment increased GFAP production in astrocytes compared with controls, although at a lower level than in TBEV-infected cells (Fig. 6a, b).

We demonstrated previously that TBEV infection of human neural cells (neuroblastoma, medulloblastoma and glioblastoma cells) is associated with a number of morphological changes. The infection of medulloblastoma and glioblastoma cells led to proliferation of the RER and extensive rearrangement of cytoskeletal structures (Růžek *et al.*, 2009b). With the exception of typical remodelling of the RER (Figs 7 and 8), here we have described the morphology and 3D organization of TBEV-induced structures, called tubule-like structures, located in the RER of infected HBCAs (Figs 9–11). Tubular structures (also called elongated vesicles or rod-like particles) of sizes ranging from 50 to 100 nm in diameter and 100 nm to 3.5 µm in length have been reported previously inside the RER of other cells infected with either tick-borne flaviviruses (Lorenz *et al.*, 2003; Offerdahl *et al.*, 2012) or mosquito-borne flaviviruses (Welsch *et al.*, 2009). Tubules with closed ends and without pores or connections to other tubules or other structures have been observed in the RER of ISE-6 tick cells persistently infected with Langat virus (Offerdahl *et al.*, 2012). Tubular structures measuring 50 nm in diameter have been found in mammalian COS-1 cells expressing proteins prM and E of TBEV (Lorenz *et al.*, 2003). We supposed that enveloped virus particles were also connected to tubule-like structures in TBEV-infected neuroblastoma cells (Růžek *et al.*, 2009b; Fig. 5). However, the tubular structures that we observed in TBEV-infected astrocytes differed from those reported previously with

respect to diameter and the appearance of the inner part of the structure. The presence of these tubule-like structures solely in the RER and their small diameter dispute their microtubular origin. Although the function and origin of the tubule-like structures remain unexplained, these structures appear to be a feature of persistent infection (Lorenz *et al.*, 2003; Offerdahl *et al.*, 2012). We propose that viral activity leads to the production of tubule-like structures, either directly as a result of defective virus assembly, or due to disruption of the host's cellular metabolism.

In TBEV-infected HBCAs at 9 days p.i., we observed the presence of swollen mitochondria next to mitochondria without any ultrastructural alterations. This finding might indicate irreversible injury of some astrocytic mitochondria that is connected with the permeability of mitochondrial membranes and the uptake of water from the cytosol. The remaining mitochondria inside astrocytes may help maintain the energy balance, supporting cell survival and thus contributing (as the glial cells) to neuronal protection. The absence of TBEV particles and lack of ultrastructural alteration in other HBCAs in the culture is in accordance with immunofluorescence staining and the observation of Potokar *et al.* (2014) regarding the resistance of astrocytes to TBEV-mediated cell death. In contrast to previous observations of TBEV infections of neural cells, we did not observe any signs of apoptosis (Růžek *et al.*, 2009b) or the formation of structures described recently as laminal membrane structures in neurons (Hirano *et al.*, 2014).

In summary, these results demonstrate for the first time, to the best of our knowledge, that cultured human primary astrocytes are sensitive to TBEV infection and are a potential source of pro-inflammatory cytokines in TBEV-infected brain cells, which might contribute to TBEV-induced neurotoxicity and/or BBB breakdown during TBE.

METHODS

Virus and cells. The TBEV strain Neudoerfl, a prototype strain of the European subtype, kindly provided by Professor F. X. Heinz from the Medical University of Vienna, was used in all experiments. The virus was originally isolated from the tick *Ixodes ricinus* in Austria in 1971. The virus has been characterized extensively, including its genome sequence (GenBank accession no. U17495) and the 3D structure of its envelope protein, E (Rey *et al.*, 1995). The virus was passaged four times by infecting suckling mice intracranially before its use in the present study.

HBCAs were purchased from ScienCell at passage 1 and propagated in CSC Complete Medium with 10 % serum (ACBR) at 37 °C and 5 % CO_2. In all experiments, low-passage-number cells were used. Porcine kidney stable (PS) cells (Kožuch & Mayer, 1975) were grown at 37 °C in L-15 medium supplemented with 3 % newborn calf serum and a 1 % antibiotic mixture of penicillin and streptomycin (Sigma).

Viral growth in HBCAs. Monolayer HBCA cultures grown in 96-well plates were inoculated with virus diluted in the culture medium to an m.o.i. of 5. Virus-mediated CPE was investigated using light microscopy. At 0, 1, 2, 3, 5, 7, 9 and 15 days p.i., supernatant medium from appropriate wells was collected and frozen at −70 °C. Titres were determined by plaque assay.

Plaque assay. Virus titres were assayed on PS cell monolayers, as described previously (De Madrid & Porterfield, 1969). Briefly, 10-fold dilutions of the virus sample were placed in 24-well tissue culture plates and PS cells were added in suspension (0.6×10^5–1.5×10^5 cells per well). After incubation for 4 h, the suspension was overlaid with carboxymethylcellulose (1.5 % in L-15 medium). After incubation for 5 days at 37 °C, the plates were washed with PBS, and the cell monolayers were stained with naphthalene black. Infectivity was expressed as p.f.u. ml^{-1}.

Immunofluorescence staining. Infected and non-infected cells on slides were subjected to cold acetone : methanol (1 : 1) fixation for 10 min, rinsed in PBS and blocked with 10 % FBS. Cells were labelled with flavivirus-specific mAb (1 : 250; Sigma-Aldrich) or with anti-GFAP antibody conjugated with Alexa Fluor 488 (1 : 200, eBioscience) for 1 h at 37 °C. Flavivirus-specific, mouse mAb and anti-PDIA3 rabbit antibody (1 : 250; Sigma-Aldrich) were used for double labelling. After washing with Tween 20 (0.05 %, v/v) in PBS, the cells were labelled with anti-mouse, goat secondary antibody conjugated with FITC (1 : 500; Sigma-Aldrich) or anti-rabbit, goat secondary antibody conjugated with Atto 550 NHS (1 : 500, Sigma-Aldrich) for 1 h at 37 °C. The cells were counterstained with DAPI (1 μg ml^{-1}; Sigma) for 30 min at 37 °C, mounted in 2.5 % 1,4-diazabicyclo(2.2.2)octane (Sigma) and examined with an Olympus BX-51 fluorescence microscope equipped with an Olympus DP-70 CCD camera.

Flow cytometry. HBCAs were cultured in a 96-well plate at a concentration of 5×10^4 cells ml^{-1} and infected with the TBEV strain Neudoerfl (m.o.i. of 5) 1 day after seeding. Mock-infected or LPS-treated (at a final concentration of 100 ng ml^{-1}) cells were used as controls. Cells were harvested at 3, 7 and 15 days p.i. Astrocytes were fixed with a Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Briefly, the cultured cells were washed twice with Dulbecco's PBS (Sigma-Aldrich), trypsinized, inactivated with FCS and centrifuged at 160 ***g*** for 5 min at 4 °C. Harvested cells were fixed and permeabilized for 45 min according to the manufacturer's protocol. An anti-flavivirus group antigen antibody (1 : 500; Merck-Millipore) and a secondary FITC-conjugated anti-mouse polyvalent antibody (1 : 500; Sigma-Aldrich) were used to stain the TBEV-positive cells. An anti-GFAP antibody (1 : 100, Santa Cruz) conjugated to Alexa Fluor 488, was used to stain GFAP-positive cells. The cells were washed with 1 × permeabilization buffer after each staining and centrifuged at 300 ***g*** for 5 min at room temperature. Finally, the cells were resuspended in 1 % FCS and used for flow cytometry analysis on a BD FACS Canto II with BDFACS Diva software. Obtained data were analysed using Flowing Software 2, version 2.5.1 (Perttu Terho, University of Turku, Finland).

Quantitative real-time RT-PCR. Total RNA was extracted from TBEV-infected HBCAs and control cells and cDNA was synthesized by reverse transcription using an Ambion Cells-to-CT kit (Applied Biosystems) according to the manufacturer's instructions. The synthesized cDNAs were used as templates for quantitative real-time PCR. The PCR was performed using pre-developed TaqMan Assay Reagents [Assay IDs: IL-6 (Hs00985639_m1), IL-1β (Hs01555410_m1), IL-8 (Hs00174103_m1), IFN-α (Hs00819693_sH), TNF-α (Hs00174128_m1), MIP-1β/CCL4 (Hs99999148_m1), MCP-1/CCL2 (Hs00234140_m1) and IP-10 (Hs01124251_g1)] and TaqMan Gene Expression Master Mix (Applied Biosystems) on a Rotor Gene-3000 (Corbett Research). Human β-actin (Hs99999903_m1) and glyceraldehyde 3-phosphate dehydrogenase (Hs03929097_g1) were used as housekeeping genes. The amplification conditions were as follows: 2 min at 50 °C (to allow UNG to destroy any contaminating templates); 10 min at 95 °C (to denature UNG and activate the

enzymes); 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min.

To calculate the fold change in gene expression, the cycle threshold (C_t) of the housekeeping genes was subtracted from the C_t of the target gene to yield ΔC_t. Change in expression of the normalized target gene was expressed as $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_{tsample} - \Delta C_{tcontrol}$, as described previously (Livak & Schmittgen, 2001).

Viral RNA was quantified in cells grown in 96-well plates at 0, 2, 7 and 15 days p.i. with TBEV strain Neudoerfl (m.o.i. of 5). The cells were lysed using an Ambion Cells-to-CT kit and subsequently subjected to RNA purification with a QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions. Viral RNA was quantified using a TBEV Real-time RT-PCR kit (Liferiver) on a Rotor Gene-3000 (Corbett Research) following the manufacturer's instructions.

ELISA. We used human ELISA kits (Invitrogen) to measure the effect of TBEV exposure on cytokine/chemokine and MMP-9 expression in HBCAs. Mock-infected and TBEV-infected HBCAs were plated in 96-well plates at a density of 2×10^4 cells per well and infected 24 h later. The cells were infected with TBEV (m.o.i. of 5) and mock infected with the same dilution of brain suspension without virus. At 0, 1, 2, 3, 4, 5, 9 and 15 days p.i., supernatant medium from appropriate wells was collected and frozen at −70 °C. Cell supernatants were then assayed for CXCL10/IP10 (Human ELISA kit, KAC2361; Invitrogen), MIP-1β (Human ELISA kit, KAC2291; Invitrogen) and MMP-9 (Human ELISA kit, KHC3061; Invitrogen) according to the manufacturer's instructions.

Transmission electron microscopy and electron tomography. TBEV-infected and control HBCAs that had been grown on sapphire discs were high-pressure frozen at either 3 or 9 days p.i. in the presence of 20 % BSA diluted in growing medium using a Leica EM PACT2 high-pressure freezer. Freeze substitution (Leica EM AFS2) was carried out in 2 % osmium tetroxide diluted in 100 % acetone at −90 °C for 16 h, and then warmed up at a rate of 5 °C h^{-1} to remain at −20 °C for 14 h, and finally warmed up again at the same rate to a final temperature of 4 °C. Samples were rinsed three times in anhydrous acetone at room temperature and infiltrated stepwise in acetone mixed with SPI-pon resin (SPI) (acetone : SPI ratios of 2 : 1, 1 : 1 and 1 : 2, for 1 h at each step). The samples, now in pure resin, were polymerized at 60 °C for 48 h.

Sections were prepared using a Leica Ultracut UCT microtome (Leica Microsystems) and collected on 300 mesh copper grids. Staining was performed using alcoholic uranic acetate for 30 min and in lead citrate for 20 min. Images were obtained using a JEOL 2100F or JEOL 1010 transmission electron microscope. For electron tomography, protein A-conjugated 10 nm gold nanoparticles (Aurion) were added to both sides of each section as fiducial markers.

Tilt series images were collected in the range of ±65° with 0.65° increments using a 200 kV JEOL 2100F transmission electron microscope equipped with a high-tilt stage and Gatan camera (Orius SC 1000) and controlled by SerialEM automated acquisition software (Mastronarde, 2005). Images were aligned using the fiducial markers. Electron tomograms were reconstructed using the IMOD software package. Manual masking of the area of interest was employed to generate a 3D surface model (Kremer *et al.*, 1996).

Statistical analysis. Data were analysed using version 5.04 of the GraphPad Prism5 software program (GraphPad Software). ELISA measurements of increased chemokine and cytokine production were analysed using one-way ANOVA (Tukey's multiple comparison test). All other data were analysed using one-way ANOVA (Newman–Keuls multiple comparison test). Differences of $P<0.05$ were considered statistically significant.

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2. Tick-borne encephalitis virus infects human brain microvascular endothelial cells without compromising blood-brain barrier integrity

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Tick-borne encephalitis virus infects human brain microvascular endothelial cells without compromising blood-brain barrier integrity

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ABSTRACT

Alteration of the blood-brain barrier (BBB) is a hallmark of tick-borne encephalitis (TBE), a life-threating human viral neuroinfection. However, the mechanism of BBB breakdown during TBE, as well as TBE virus (TBEV) entry into the brain is unclear. Here, primary human microvascular endothelial cells (HBMECs) were infected with TBEV to study interactions with the BBB. Although the number of infected cells was relatively low in culture (< 5%), the infection was persistent with high TBEV yields (> 10^6 pfu/ml). Infection did not induce any significant changes in the expression of key tight junction proteins or upregulate the expression of cell adhesion molecules, and did not alter the highly organized intercellular junctions between HBMECs. In an *in vitro* BBB model, the virus crossed the BBB via a transcellular pathway without compromising the integrity of the cell monolayer. The results indicate that HBMECs may support TBEV entry into the brain without altering BBB integrity.

1. Introduction

Tick-borne encephalitis (TBE) is a severe infectious disease affecting the central nervous system (CNS) of humans. TBE is caused by TBE virus (TBEV), a member of family *Flaviviridae*, genus *Flavivirus* (Lindquist, 2014; Mansfield et al., 2009). Over the past few decades, TBE has become a growing public health concern in Eurasia, with more than 10,000 clinical cases of TBE, including numerous deaths, reported every year (Bogovic and Strle, 2015). Clinical presentation of TBE ranges from fever or meningitis to more severe meningoencephalitis or encephalomyelitis. Long-term neurological sequelae are common after TBE and usually involve paresis, ataxia, or gait disturbance (Bogovic et al., 2010; Růžek et al., 2010).

Major hallmarks of TBEV pathogenesis are neuroinflammation, followed by neuronal death (Gelpi et al., 2005, 2006; Bílý et al., 2015) and disruption of the blood-brain barrier (BBB) (Růžek et al., 2011; Palus et al., 2014b; Chekhonin et al., 2002; Moniuszko et al., 2012; Kang et al., 2013). The BBB is a critical component of the CNS formed by epithelial-like high-resistance tight junctions (TJs) within the endothelium comprising brain microvascular endothelial cells in capillaries perfusing the brain. The BBB represents a physical barrier between the blood stream and brain parenchyma. TJ proteins include claudins and occludin, which are joined to the cytoskeleton by cytoplasmic proteins, such as zonula occludens (ZO) (Persidsky et al., 2006). The other components of the BBB include glial cells/astrocytes, neurons, and perivascular pericytes, which form a neurovascular unit with the brain microvascular endothelial cells (Cardoso et al., 2010).

TBEV negotiation of the BBB is a critical event in the development and progression of CNS infection. Using a rodent model, we previously demonstrated that BBB integrity is compromised in TBEV-infected mice. The permeability of the BBB increases at later stages of TBE infection when high virus load is already present in the brain (Růžek et al., 2011). Thus, BBB breakdown is not necessary for TBEV entry into the brain; it represents a consequence, rather than a prerequisite, of TBEV infection in the brain. Increased permeability of the BBB can also be observed in mice deficient in CD8+ T-cells, indicating that these cells, which normally respond to the inflammatory signals released from the CNS by migrating from the periphery into the brain, are not involved in breaking down the BBB. The BBB is most likely compromised by the activity of pro-inflammatory mediators, such as cytokines, chemokines, and matrix metalloproteinases (MMPs), mainly MMP-9, expressed in the infected brain tissue (Růžek et al., 2011). The role of MMP-9 as a mediator causing disruption of the BBB was confirmed experimentally (Paul et al., 1998; Roe et al., 2012; Verma et al., 2010).

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Our previous *in vitro* study demonstrated that TBEV-infected or activated human astrocytes are strong MMP-9 producers (Palus et al., 2014a). In human patients, higher levels of MMP-9 are found in both serum (Palus et al., 2014b) and cerebrospinal fluid (Kang et al., 2013). Although these studies collectively demonstrate that BBB disruption is a common pathological feature during TBE and influenced by cytokines, chemokines, and MMP-9, the means by which TBEV evades the BBB, resulting in virus entry into the brain, remain largely unknown.

Viruses use a number of different mechanisms to gain access to the CNS. One of these mechanisms is direct infection of the endothelial cells forming the BBB (Verma et al., 2009; Cosby and Brankin, 1995; Lathey et al., 1990; Li et al., 2015). In this study, we investigated the interaction of TBEV with primary human brain microvascular endothelial cells (HBMECs), the main component of the human BBB, and examined the expression of key TJ proteins and cell adhesion molecules. Moreover, virus migration through the BBB and its effect on BBB permeability were studied using an *in vitro* BBB model. The results indicate that HBMECs may support TBEV entry into the brain via a transcellular pathway, but without altering the expression of key TJ proteins and cell adhesion molecules or compromising BBB integrity.

2. Material and methods

2.1. Viruses and cells

Two well-characterized, low-passage representatives of the European TBEV subtype, strains Neudoerfl (kindly provided by Professor F. X. Heinz, Medical University in Vienna; passaged four times in brains of suckling mice before use in this study) and Hypr (passaged six times in brains of suckling mice before use in this study), were used in the study. Hypr strain is considered to be highly neuroinvasive in laboratory mice, while Neudoerfl strain exhibits medium level of neuroinvasiveness.

Low-passage primary HBMECs (ACBRI 376) were purchased from CellSystems (Kirkland, WA, USA). The cells were initiated by elutriation of dispase-dissociated normal human brain cortex tissue and propagated in ECM medium (ScienCell, Carlsbad, CA, USA) in rat collagen I (Cultrex) coated culture flasks. All experiments were performed with cells at passage < 12 and initiated 3 days post-seeding in fibronectin-coated wells (~0.32 cm^2) at a seeding density of 5×10^4 cells per well. The cells were infected with TBEV at a multiplicity of infection (MOI) of 5 and virus adsorption allowed for 2 h before the inoculum was removed by two washing steps and replaced with fresh medium. MOI=5 was used because it provided the most robust infection rates. In parallel, uninfected cells were used as controls.

Porcine stable kidney (PS) cells were used for plaque assay (see below). The cells were cultured at 37 °C in Leibovitz (L-15) medium supplemented with 3% newborn calf serum and a 1% mixture of antibiotics/antimycotics and glutamine (Biosera) (Kozuch and Mayer, 1975).

2.2. Viral growth in HBMECs

Monolayer HBMEC cultures grown in fibronectin-coated 96-well plates were inoculated with virus diluted in the culture medium to an MOI of 5. Virus-mediated CPE was investigated using light microscopy. At 2, 6, 24, 48, and 96 h p.i., supernatant media from appropriate wells was collected and stored at −70 °C. Titres were determined by plaque assay.

2.3. Plaque assay

The production of infectious virus by HBMECs was determined from cell-free supernatants using plaque assays as described previously (De Madrid and Porterfield, 1969). Briefly, 10-fold dilutions of the virus sample were placed in 24-well tissue culture plates and PS cells added in suspension (1.3×10^5 cells per well). After 4 h of incubation at 37 °C and 0.5% CO_2, a carboxymethylcellulose (1.5% in L-15 medium) overlay was added to each well. Following 5-day incubation at 37 °C and 0.5% CO_2, the cell monolayers were visualized with naphthalene black. Infectivity was expressed as plaque forming units per millilitre (pfu/ml).

2.4. Immunofluorescent staining for detection of TBEV antigen and TJ proteins

Infected and non-infected HBMECs on fibronectin-coated slides were subjected to 4% formaldehyde fixation for 15–30 min, washed with Tween 20 (0.05%, v/v) in PBS, permeabilized by 0.2% Triton X-100 in PBS with Tween 20 for 15 min at room temperature, and blocked with 5% normal goat serum. Cells were labelled with flavivirus-specific mAb (1:250; Millipore), anti-occludin (1:14, Invitrogen), or anti-ZO-1 (1:100, Invitrogen) for 1 h at 37 °C. Flavivirus-specific mouse mAbs were used in combination with anti-occludin, anti-ZO-1, or anti-protein disulfide isomerase family A member 3 (PDIA3) rabbit antibody (1:250; Sigma-Aldrich) for double labelling. After washing with Tween 20 (0.05%, v/v) in PBS, the cells were labelled with anti-mouse, goat secondary antibody conjugated with FITC (1:500; Sigma-Aldrich) or anti-rabbit, goat secondary antibody conjugated with Atto 550 NHS (1:500, Sigma-Aldrich) for 1 h at 37 °C. The cells were counterstained with DAPI (1 mg/ml 21; Sigma-Aldrich) for 10 min at 37 °C, mounted in 2.5% 1,4-diazabicyclo(2.2.2)octane (Sigma-Aldrich), and examined using an Olympus BX-51 fluorescence microscope equipped with an Olympus DP-70 CCD camera.

2.5. Scanning electron microscopy and correlative immunofluorescence

After immunofluorescent staining, the slides were washed in 0.1 M HEPES several times (final volume 100 ml) and left at 4 °C overnight. Cells were fixed in 2.5% glutaraldehyde in 0.1 M HEPES for 20 min at room temperature, washed three times in buffer, and post-fixed in 2% OsO_4 for 15 min. After washing, the samples were dehydrated though a graded acetone series (each step 5 min) and dried using the critical point drying method. Glass slides were mounted on aluminium stubs, gold coated, and observed in an FE-SEM JEOL 7401 F at 4 kV using the conventional Everhart-Thornley detector.

Using the Adobe Photoshop software, fluorescence images were merged with SEM micrographs and the level of the transparency changed to see both image layers.

2.6. Real-time quantitative RT-PCR

Total RNA was extracted and cDNA synthesized from mock- and TBEV-infected HBMECs 12, 24, 48, and 72 h p.i. (peak period of virus production in the culture) using Ambion® Cells-to-CT™ Kit (Applied Biosystems) according to the manufacturer's instructions. The synthesized cDNAs were used as templates for real-time PCR. Real-time quantitative PCR was performed using pre-developed TaqMan® Assay Reagents (Assay IDs: ICAM1 (Hs00174037_m1), TJP1 (Hs01551876_m1), OCLN (Hs00170162_m1), VCAM1 (Hs01003372_m1), SELE (Hs00174057_m1), and TaqMan® Gene Expression Master Mix (Applied Biosystems) on a Rotor Gene-3000 (Corbett Research). Human beta actin (Hs01060665_g1) and glyceraldehyde-3-phosphate dehydrogenase (Hs02758991_g1) were used as housekeeping genes. The amplification conditions and calculation of fold change were described previously (Palus et al., 2014a).

Viral RNA was quantified 12, 24, 48, and 72 h p.i. in cells grown in 96-well plates with TBEV at MOI 5. The cells were lysed using Ambion® Cells-to-CT™ Kit (Applied Biosystems) and subsequently subjected to

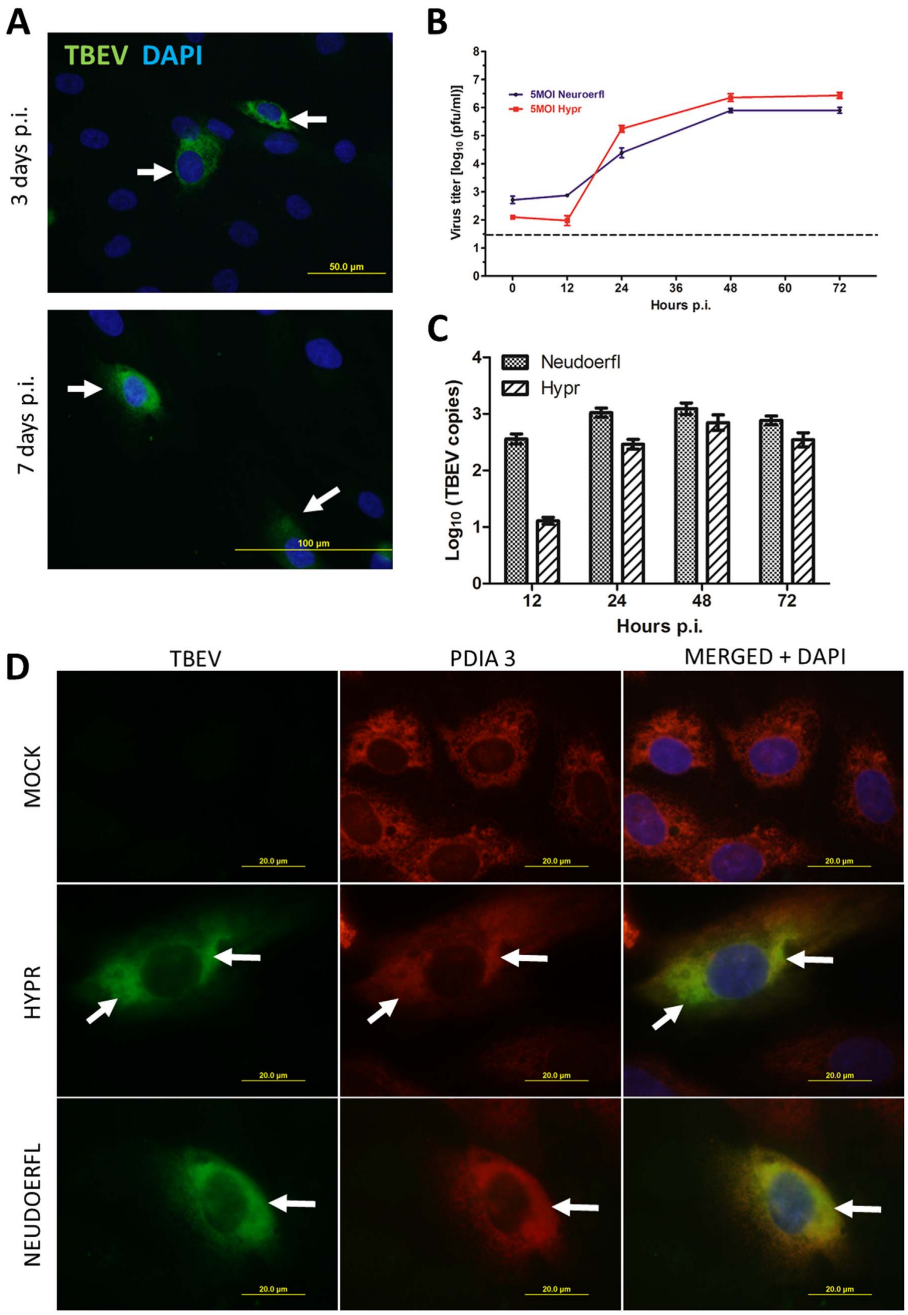

Fig. 1. TBEV can infect primary HBMECs. (A) HBMECs grown and fixed on slides 3 and 7 days post-infection (p.i.) were stained with anti-flavivirus envelope antibody (green) and counterstained with DAPI (blue). (B) TBEV titres in culture supernatant from HBMECs collected 0, 12, 24, 36, 48, 60, and 72 h p.i. were determined by plaque assay using porcine stable kidney cells. Viral titres are expressed as pfu/ml. Data are presented as mean ± SEM based on four independent experiments conducted in at least duplicate. (C) Total RNA extracted from HBMEC lysates 12, 24, 48, and 72 h p.i. was used to determine the number of intracellular TBEV RNA copies by quantitative RT-PCR. Values represent mean ± SEM. (D) TBEV antigen co-localized with PDIA3 antigen in the infected HBMECs 7 days p.i. (white arrows). HBMECs grown and fixed on slides 7 days after infection were stained with anti-flavivirus envelope antibody (green) and anti-PDIA3 antibody (red), and counterstained with DAPI (blue).

RNA purification using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Viral RNA was quantified using the Genesig Real-time PCR Detection Kit for Tick-borne Encephalitis Virus (PrimerDesign Ltd, UK) using the Oasig lyophilized OneStep qRT-PCR (PrimerDesign Ltd, UK) on a Rotor Gene-3000 (Corbett Research) following the manufacturer's instructions.

2.7. Western blot

Total cellular protein was extracted from mock- and TBEV-infected (MOI =5) cells 12, 24, 48, and 72 h p.i. using Cell Lysis Buffer (Cell Signaling). A total of 10 μg of cellular protein extract was fractioned on a gradient (4–15%) Criterion™ TGX Stain-Free™ Precast gel (BioRad) and transferred to a PVDF membrane (BioRad). Blocking was performed with 3% skim milk in TBST for 1 h at room temperature and the membranes incubated overnight at 4 °C with monoclonal anti-β-actin (Ambion), monoclonal anti-VCAM-1 (Invitrogen), monoclonal anti-ICAM-1 (Invitrogen), or monoclonal anti-E-selectin (Invitrogen) antibodies. After four washes with TBST, the membranes were incubated with secondary alkaline-phosphatase conjugated antibody solution (Novex) for 1 h at room temperature and developed using Novex® AP Chemiluminescent Substrate. The blots were scanned and quantified using ChemiDoc™ MP imager (BioRad).

2.8. In vitro BBB model, measurement of BBB permeability, and TBEV transmigration

An *in vitro* BBB model was constructed according to Verma et al. (2010) using BioCoat® Cell Culture Inserts with human fibronectin-coated polyethylene terephthalate (PET) membrane (3.0-μm pore and 0.33 cm^2) in a 24-well plate (BD Bioscience, Bedford, MA). Briefly, 5×10^4 HBMECs were seeded on the upper part of the insert after membrane rehydration, and the inserts with cells were incubated at 37 °C in a 5% CO_2 atmosphere and 100% humidity. The integrity of the *in vitro* BBB model was determined by measuring the TEER using EVOMX (World Precision Instruments, USA) according to the manufacturer's instructions. TEER values are presented as fold variations from the average control reading after deducting the empty insert values. When HBMECs developed a monolayer with high TEER, the cells were infected with TBEV at MOI =5. Virus adsorption was allowed for 1 h, and then the inoculum was removed and replaced with fresh medium. Virus titres were determined in both media from both compartments by plaque assay, and the integrity of the barrier was evaluated by TEER and FITC-dextran permeability assays. Three independent experiments were conducted in duplicate to obtain the mean ± standard deviation (SD).

The FITC-dextran permeability assay was performed as described previously (Verma et al., 2009). FITC-dextran (4 kDa, Sigma; 200 μl of 100 μg/ml medium) has low BBB penetration and is widely used as a barrier integrity marker in *in vitro* models. FITC-dextran was added to the upper chamber (TBEV, mock-control, and no-cell inserts) and, after 2 h incubation, 100 μl of medium was removed from the lower chamber. The fluorescence of transmigrated FITC-dextran was measured on an Infinite 200 PRO multimode reader (TECAN). The amount of FITC-dextran that crossed the insert membranes was normalized to the mean of the mock-controls. Before the barrier permeability measurement, 100 μl of the media from both the upper (luminal) and lower (abluminal) chambers was collected for the transmigration assay. The virus titre was analysed by plaque assay and the kinetics of TBEV transmigration across the BBB determined. The BBB permeability was assessed from days 1–11 after seeding. At 3 days, the TEER plateaued and the cells were infected with the virus. TEER and FITC-dextran transmigration were measured in inserts with and without HBMECs. TBEV was quantified in the culture media from both chambers 2, 6, 24, and 48 h p.i. using plaque assay.

2.9. Statistical analysis

Data are expressed as means ± SD, and the significance of differences between groups was evaluated by using Mann-Whitney *U* test with GraphPad Prism 5 (GraphPad Software, Inc., USA), version 5.04. Differences with $p < 0.05$ were considered significant.

3. Results

3.1. TBEV can infect and replicate in HBMECs

The ability of TBEV to infect and replicate in HBMECs, the main component of the human BBB, is unknown. To determine infection and the replication kinetics of TBEV strains with different virulence in primary HBMECs, we performed plaque assays, qRT-PCR, and immunostaining (Fig. 1). Productive TBEV replication in the form of the release of infectious virus particles was first detected 1 day after infection, and day 2 represented the limit of virus production, with mean titres reaching 6.49 ± 0.31 $\log_{10}$ pfu/ml for Hypr and 5.96 ± 0.23 $\log_{10}$ pfu/ml for Neudoerfl (Fig. 1B). Intracellular TBEV replication assessed by qRT-PCR confirmed active virus replication as early as 12 h post-infection (p.i.) (Fig. 1C). The number of TBEV RNA copies reached a maximum 2 days p.i.; the mean virus titre reached 2.84 ± 0.27 $\log_{10}$ genome copies/μl for Hypr and 3.09 ± 0.20 $\log_{10}$ genome copies/μl for Neudoerfl (Fig. 1C). Phase contrast microscopy was used to examine TBEV-infected endothelial cells for a cytopathic effect (CPE) and cell death; neither was observed at any time point with any of the TBEV strains used (data not shown). Moreover, no eminent CPE was evident 19 days p.i., and infected primary HBMEC culture exhibited better conditions then control cells. No significant differences in HBMEC infectivity and virus replication were observed following infection with any of the TBEV strains used.

3.2. TBEV envelope protein distribution in infected HBMECs

Immunofluorescent staining revealed that the TBEV antigen (envelope (E) protein) was distributed diffusely throughout the entire body of the endothelial cell (Fig. 1). However, in some infected cells we observed brightly stained aggregates of viral antigen (Fig. 1A). The number of infected cells in the culture did not exceed 5% during the investigated period (up to 19 days p.i.; data not shown). Viral antigen was not detected in mock-infected HBMECs or cells stained with secondary antibody alone. A co-localization study with protein disulfide isomerase family A, member 3 (PDIA3) antigen (also known as Erp57, Er-60, and GRP58) suggested that the antigen localized primarily in rearranged endoplasmic reticulum (Fig. 1D). No significant difference was observed in antigen distribution between Hypr- and Neudoerfl-infected HBMECs.

3.3. TBEV infection does not alter TJ proteins in HBMECs

TBEV-infected HBMEC monolayers were investigated for alterations of the distribution of key TJ membrane proteins by immunofluorescent staining of occludin, and zonula occludens-1 (ZO-1). The infected cells were identified by staining TBEV antigen (E protein) as an intracellular marker of the infection. The cells were fixed and stained 3, 11, and 19 days p.i. Immunofluorescence revealed no significant TJ disruption, fragmentation, or absence of immunoreactivity for occludin and ZO-1 between infected and uninfected HBMECs at any time point investigated (Figs. 4A, 5A). HBMECs grown and fixed on slides 3 (data not shown) and 5 days p.i. (Figs. 2 and 3) were used for scanning electron microscopy (SEM). This analysis provided further evidence that HBMECs in the culture were attached by TJ connections, and no differences were seen between mock- and TBEV-infected HBMECs (Figs. 2, 3, 4A, 5A). Connections between cells composed from a net of delicate intertwined long processes are shown in Fig. 2,

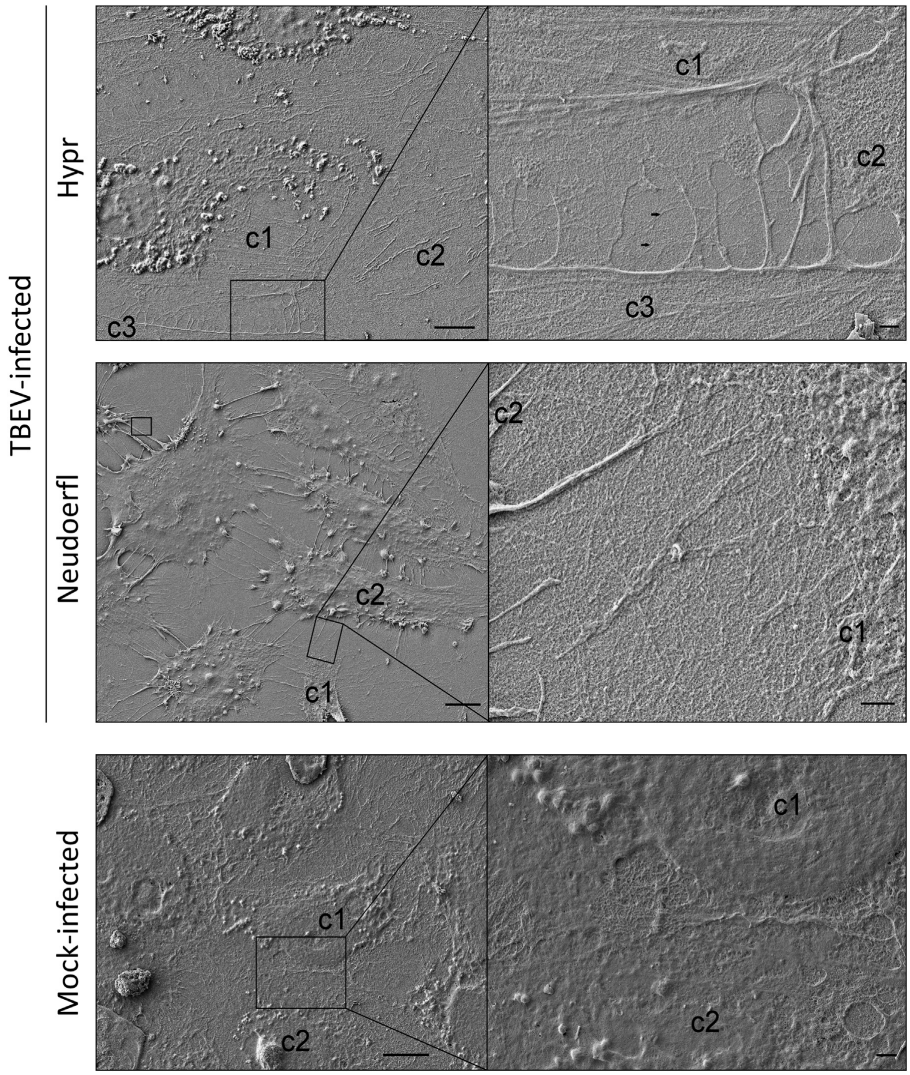

Fig. 2. Scanning electron micrographs of TBEV-infected HBMECs grown on cover slips 5 days p.i. Overview images (left panel, scale bars =10 μm) show positions of individual cells (c) and the areas from which the details were imaged (right panel, scare bars =1 μm). At high magnification, a dense junctional net connecting individual cells is clearly seen. The images were obtained using a JEOL 7401 F scanning electron microscope at 4 kV in secondary electron imaging mode.

with more obvious detail on the right. TBEV-infected HBMECs did not exhibit any alteration in cell connections compared to mock-infected controls. Moreover, no differences in TJ organization were observed by SEM after infection with either of the two TBEV strains (Figs. 2 and 3). Correlative immunofluorescent staining and SEM were used to visualize TJ cell-cell interactions after TBEV infection in high resolution (Fig. 3). In mock-infected cells or cells infected with either of the two TBEV strains, TJ molecules ZO-1 and occludin were localized in the intercellular space and no differences observed in the labelling intensity between mock- and TBEV-infected cells (Fig. 3). The intercellular space was formed by a network of fine and highly organized and frequently overlapping filaments. Both TJ proteins were localized predominantly in the thickened areas of the cell boundaries identified as TJs (Fig. 3). These observations confirmed that TBEV infection has no effect on occludin and ZO-1 distribution between HBMECs in culture.

BBB integrity is influenced by both the cellular distribution and expression of TJ proteins in the HBMEC TJs. Therefore, the mRNA expression of key TJ proteins, such as ZO-1 and occludin, were analysed by qRT-PCR in mock- and TBEV-infected HBMECs at the peak interval of virus production (i.e., from 12 h to 3 days p.i.). The expression of mRNA for the key TJ proteins was normalized to the expression of two housekeeping genes. TBEV infection did not significantly affect the expression of ZO-1 or occludin at the mRNA level

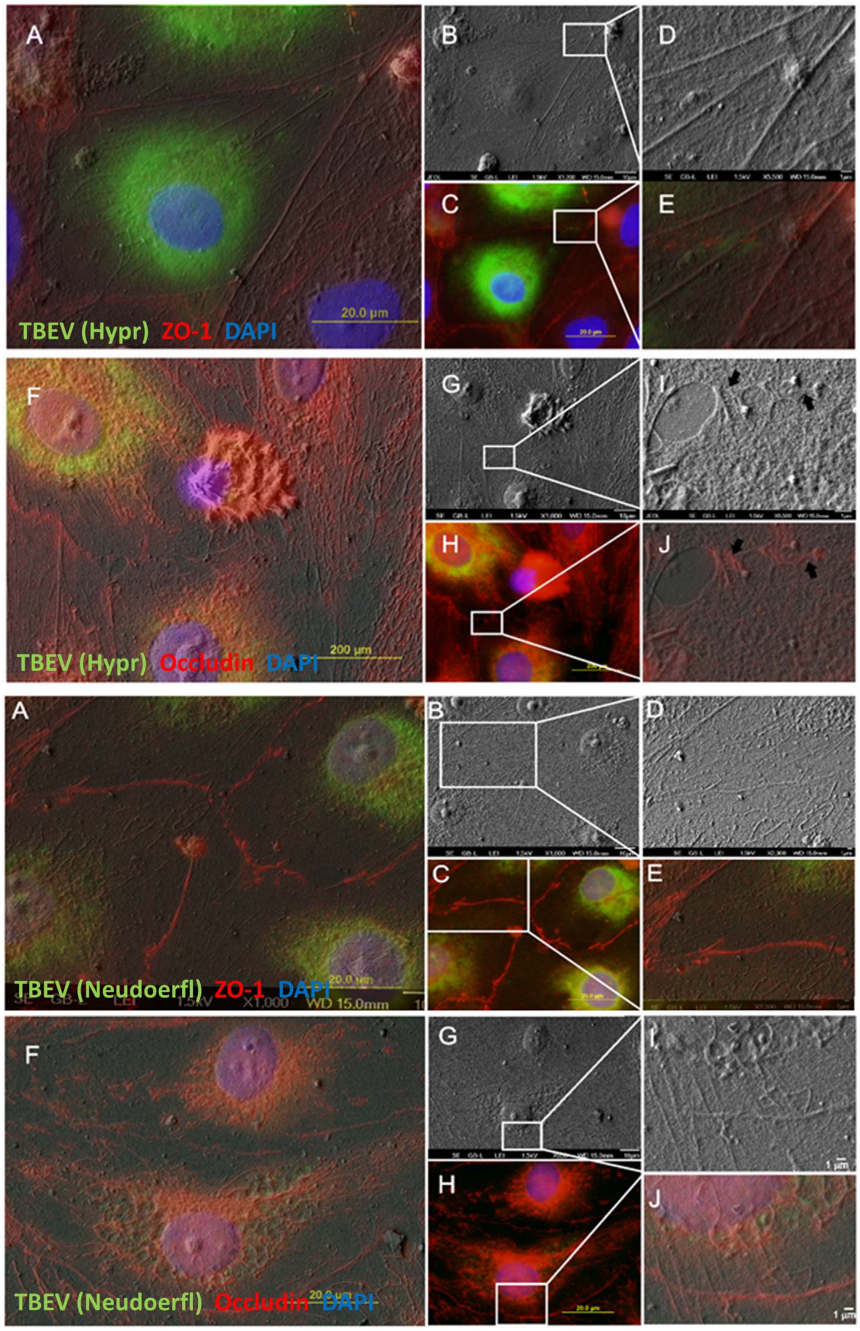

Fig. 3. Co-localization of TBEV antigen and TJ proteins in TBEV-infected HBMECs (3 days p.i.) using correlative immunofluorescence and scanning electron microscopy (SEM). Cell monolayers were labelled with anti-flavivirus envelope antibody (green) and either anti-occludin or anti-ZO1 antibody (red). Nuclei were counterstained with DAPI (blue). (A, C, E) Overlays of SEM images and merged fluorescence images. (G, I, B, D) SEM images were taken using the JEOL 7401 F scanning electron microscope at 4 kV in secondary electron imaging mode. (H, C) Fluorescence images were obtained with a BX-51 fluorescence microscope equipped with an Olympus DP-70 CCD camera.

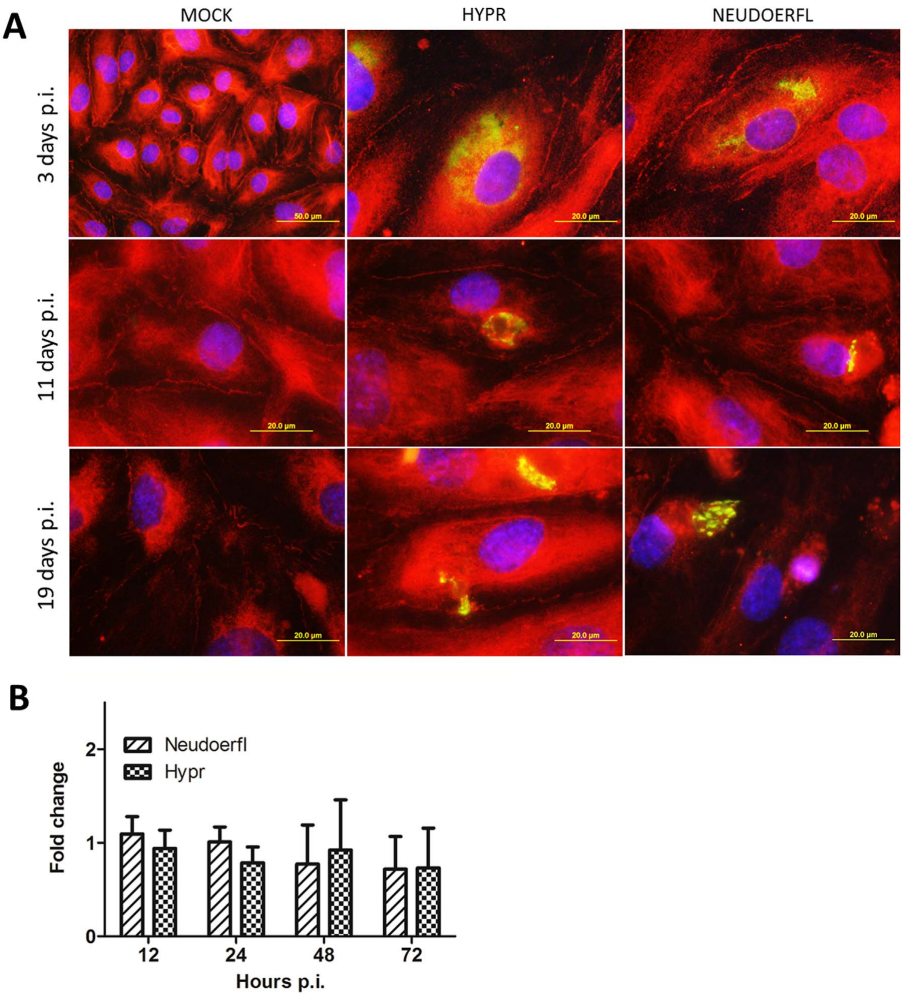

Fig. 4. TBEV infection has no effect on occludin expression and distribution in HBMECs. (A) HBMECs grown and fixed on slides 3, 11, and 19 days p.i. were stained with anti-flavivirus envelope (green) and anti-occludin (red) antibodies and counterstained with DAPI (blue). (B) Total RNA from mock- and TBEV-infected HBMECs 12, 24, 48, and 72 h p.i. were used to determine the fold-change in occludin mRNA expression by qRT-PCR. Changes in the levels of occludin mRNA were normalized to the expression of housekeeping genes human beta actin and glyceraldehyde-3-phosphate dehydrogenase, and then the fold change in the infected cells determined relative to corresponding controls. Data represent three independent experiments conducted in duplicate. Data are expressed as mean ± SD.

in infected HBMECs at any time point investigated (Figs. 4B, 5B). For both TBEV strains, similar mRNA expression patterns were observed for each measured TJ protein.

3.4. TBEV infection does not upregulate the expression of cell-matrix adhesion molecules in HBMECs

To investigate whether TBEV infection of HBMECs would potentially affect the expression of cell-matrix adhesion molecules (CAMs), proteins located on the cell surface involved in adhesive interactions with leukocytes. Transcript levels of important CAMs, such as ICAM-1, VCAM-1, and E-selectin, were quantified in TBEV-infected HBMECs 12, 24, 48, and 72 h p.i. using qRT-PCR and compared to mRNA levels in mock-infected controls. The expression was normalized to the mRNA levels of two housekeeping genes. At 48 and 72 h p.i., the mRNA levels for ICAM-1, VCAM-1, and E-selectin were lower in TBEV-infected HBMECs compared to mock-infected controls (Fig. 6A). However, quantification of ICAM-1, VCAM-1, and E-selectin in mock-infected and TBEV-infected HBMECs by western blotting did not demonstrate any downregulation of the expression of these CAMs (Figs. 6B, 6C). These results indicate that TBEV infection does not upregulate the expression of important CAMs in HBMECs.

3.5. TBEV crosses the BBB without altering barrier permeability

We used an *in vitro* BBB model to investigate the ability of cell-free TBEV to migrate across the HBMEC monolayer. TBEV titres released by the infected HBMECs into the cell culture medium in the luminal (UCS)

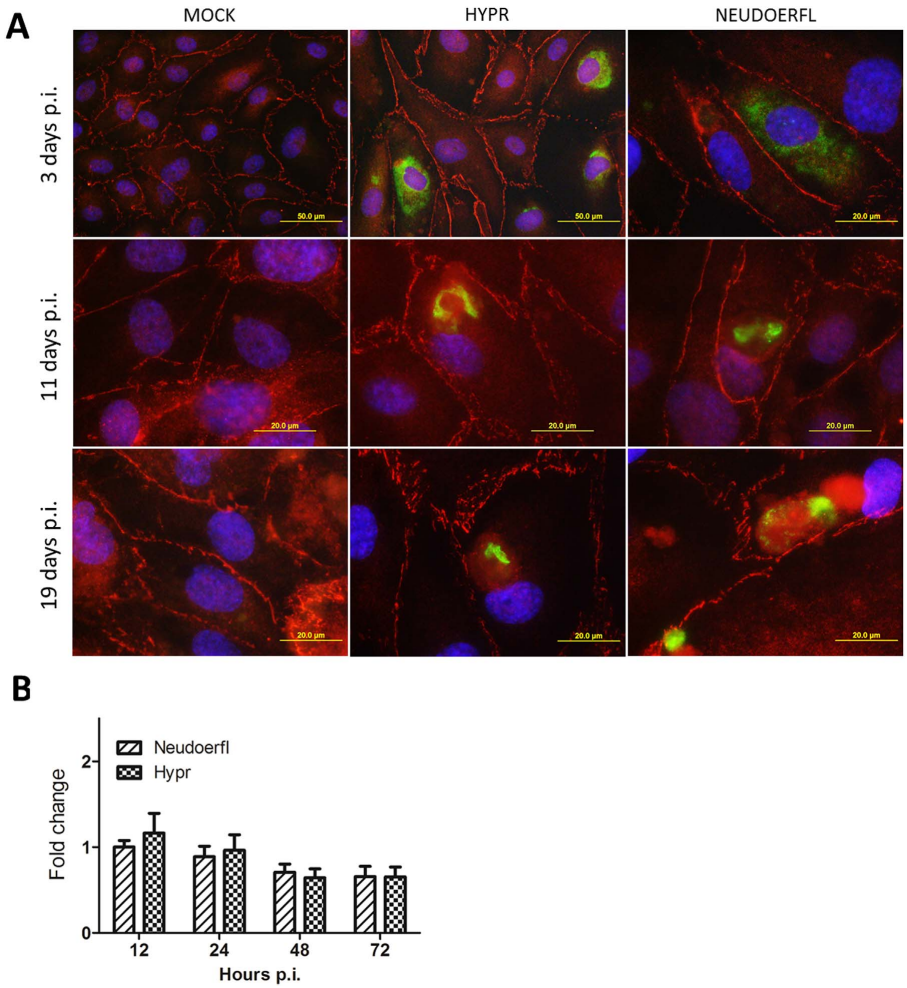

Fig. 5. TBEV infection has no effect on ZO-1 expression and distribution in HBMECs. (A) HBMECs grown and fixed on slides 3, 11, and 19 p.i. were stained with anti-flavivirus envelope (green) and anti-ZO-1 (red) antibodies and counterstained with DAPI (blue). (B) Total RNA from mock- and TBEV-infected HBMECs 12, 24, 48, and 72 h p.i. were used to determine the fold-change in ZO-1 mRNA expression by qRT-PCR. Changes in the levels of ZO-1 mRNA were normalized to the expression of housekeeping genes human beta actin and glyceraldehyde-3-phosphate dehydrogenase, and then the fold change in the infected cells determined relative to corresponding controls. Data represent three independent experiments conducted in duplicate. Data are expressed as mean ± SD.

and abluminal (LCS) compartments of the model at various time points (2, 6, 24, and 48 h p.i.) were quantified by plaque assay. At early infection time points (i.e., at 2 and 6 h p.i.), TBEV was detected in the UCS only, with titres reaching approximately 2.5 $\log_{10}$ pfu/ml (Hypr) and 3.3 $\log_{10}$ pfu/ml (Neudoerfl). The virus titre in the LCS 2 and 6 h p.i. was below or at the detection limit of the plaque assay (1.44 $\log_{10}$ pfu/ml). Twenty-four hours p.i., a shift increase in TBEV titre was observed in both the UCS and LCS of the BBB model (Fig. 7A). At this time point, Hypr reached 5.36 ± 0.47 $\log_{10}$ pfu/ml in the UCS and 4.17 ± 0.67 $\log_{10}$ of pfu/ml in the LCS, and Neudoerfl reached 4.72 ± 0.65 $\log_{10}$ pfu/ml in the UCS and 3.75 ± 1.66 $\log_{10}$ pfu/ml in the LCS. Forty-eight hours p.i., the virus titres slightly increased and reached a plateau in both the UCS and LCS (Fig. 7A). Therefore, the TBEV replication kinetics in the *in vitro* BBB model were analogous the kinetics in HBMEC culture (Fig. 1B).

The integrity of the HBMEC monolayer in the *in vitro* BBB model was measured by transendothelial electrical resistance (TEER) and FITC-dextran transmigration assay. Both of these methods are widely accepted quantitative techniques for measuring the integrity of TJ dynamics in cell culture models of endothelial and epithelial monolayers (Srinivasan et al., 2015; Verma et al., 2009). Three days after seeding the cells on the culture insert, TEER measured between the UCS and LCS separated by the membrane with a cell monolayer reached its highest values, and at this time the cells were infected with TBEV. TEER and FITC-dextran transmigration were measured in no-cell controls and in mock- and TBEV-infected HBMEC monolayers (Fig. 7B, C). TEER values are presented as the fold variation from the average control reading after deducting the empty insert values.

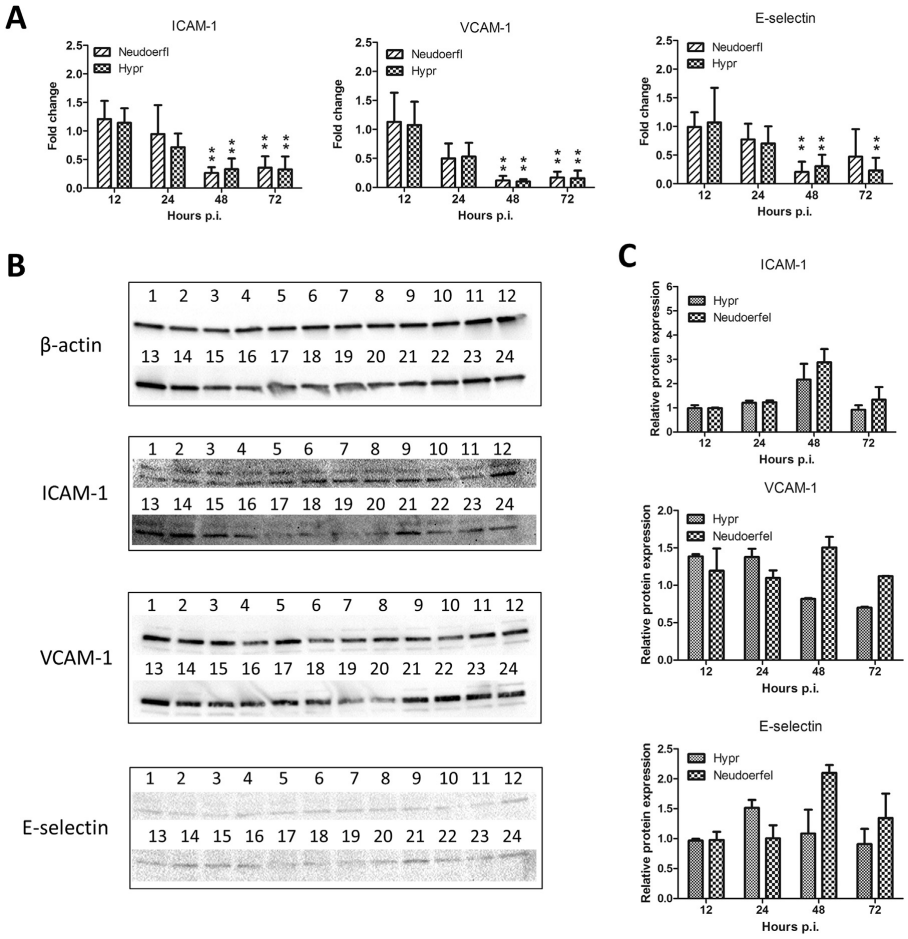

Fig. 6. Effect of TBEV infection on the expression of key adhesion molecules in HBMECs. (A) Changes in mRNA expression of adhesion molecules after TBEV infection in HBMECs. Total RNA from mock- and TBEV-infected HBMECs 12, 24, 48, and 72 h p.i. was used to determine the fold-change in ICAM-1, VCAM-1, and E-selectin by qRT-PCR. Changes in the levels of intercellular adhesion molecules were normalized to the expression of housekeeping genes human beta actin and glyceraldehyde-3-phosphate dehydrogenase, and then the fold change in infected cells determined relative to corresponding controls. Data represent three independent experiments conducted in duplicate. Data are expressed as mean ± SD. ΔCT of infected culture were compared with control ΔCT using Mann-Whitney *U* test. *, $p < 0.05$; **, $p < 0.01$. (B) Changes in protein expression of adhesion molecules after TBEV infection in HBMECs. Total protein from mock- and TBEV-infected HBMECs 12, 24, 48, and 72 h p.i. was used to determine the fold-change in ICAM-1, VCAM-1, and E-selectin by Western blot. Data represent one experiment conducted in duplicate: 1–2, Hypr-infected HBMECs 12 h p.i.; 3–4, Neudoerfl-infected HBMECs 12 h p.i.; 5–6, mock-infected HBMECs 12 h p.i.; 7–8, Hypr-infected HBMECs 24 h p.i.; 9–10, Neudoerfl-infected HBMECs 24 h p.i.; 11–12, mock-infected HBMECs 24 h p.i.; 13–14, Hypr-infected HBMECs 48 h p.i.; 15–16, Neudoerfl-infected HBMECs 48 h p.i.; 17–18, mock-infected HBMECs 48 h p.i.; 19–20, Hypr-infected HBMECs 72 h p.i.; 21–22, Neudoerfl-infected HBMECs 72 h p.i.; 23–24, mock-infected HBMECs 72 h p.i. (C) The results from panel B were normalized to the expression of housekeeping protein human β-actin and densimetrically quantified as the fold change over that of mock-infected controls. Data are expressed as mean ± SD.

The TEER values were in the range of all BBB models before infection and were not significantly altered 2 or 4 days p.i. in mock- or TBEV-infected BBB models; the TEER value relative to control was virtually the same for both time points and both TBEV strains used (Fig. 7B). In addition, similar to the TEER data, the amount of FITC-dextran that crossed the BBB models was virtually the same for both time points and both TBEV strains used, whereas approximately 20-times higher FITC-dextran transmigration was observed if culture inserts with no cells were used (Fig. 7C). These data collectively demonstrate that transmigration of cell-free TBEV does not alter the integrity of the HBMEC monolayer in the *in vitro* BBB model.

4. Discussion

The BBB is a highly selective semipermeable membrane barrier that separates the circulating blood from the CNS. The barrier is formed by brain endothelial cells, which are interconnected by TJs. The BBB maintains brain homeostasis by regulating the entry of nutrients, xenobiotics, cytokines/chemokines, and immune cells, but also acts effectively to protect the CNS from most pathogens. Neurotropic viruses have developed several mechanisms by which they can cross the BBB and reach the CNS, such as transcellularly, paracellularly, and/or inside circulating immune cells (the so-called Trojan-horse mechanism) (Spindler and Hsu, 2012). Some viruses require disrup-

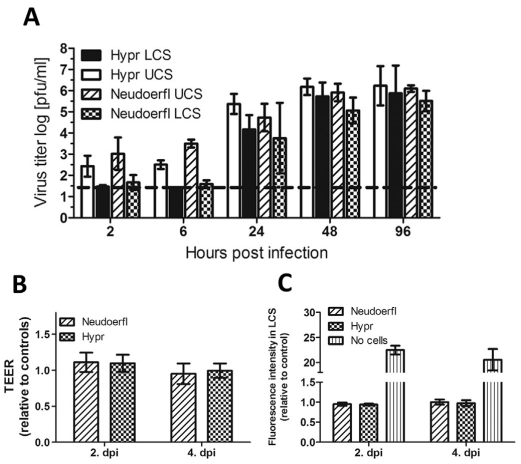

Fig. 7. TBEV infection does not compromise BBB integrity in an *in vitro* model. (A) An *in vitro* BBB model based on HBMEC culture on microporous filter membrane in a Transwell system was used to measure changes in BBB permeability during TBEV infection. HBMECs in the BBB model were infected with TBEV at a MOI of 5. Viral titres in the upper (UCS) and lower (LCS) compartments were determined by plaque assay 2, 6, 24, 48, and 96 h p.i. TBEV titres are expressed as pfu/ml of the supernatant and represent mean ± SD of three independent experiments conducted in duplicate. The dashed line represents the detection limit of the method. (B) The integrity of the *in vitro* BBB model was determined by measuring the TEER 2 and 4 days after infection. TEER values are presented as fold variations from the average control reading after deducting the empty insert values. The data were obtained from three independent experiments conducted in duplicate (mean ± SD). (C) FITC-dextran transmigration assay presented as fluorescence intensity in LCS measured 2 and 4 days after infection. The values are presented as the fold variation from its corresponding average control reading (culture inserts with uninfected HBMECs). The data are based on three independent experiments conducted in duplicate (mean ± SD).

tion of the BBB to enter the CNS, whereas others can enter the CNS through the intact BBB. In the case of TBEV, we demonstrated previously that the permeability of the BBB increases at later stages of infection when high virus loads are present in the brain (Růžek et al., 2011). Therefore, TBEV entry into the brain precedes BBB disruption, but how the virus crosses the BBB remains unknown (Růžek et al., 2011). Studies on other flaviviruses, such as West Nile virus (WNV) or Japanese encephalitis virus (JEV), have provided evidence that these viruses can target HBMECs without compromising the integrity of the barrier (Verma et al., 2009; Li et al., 2015). The infection of these cells is a possible mechanism by which some encephalitic flaviviruses gain entry into the CNS via the transcellular pathway (Verma et al., 2009). Other flaviviruses, such as Murray Valley encephalitis virus or St. Louis encephalitis virus, are speculated to enter the CNS via olfactory nerves (Monath et al., 1983). In this study, HBMECs were used as an *in vitro* model of the BBB and the interaction of TBEV with these cells and effect of the infection on BBB permeability investigated. We show that TBEV, similar to WNV and JEV, establishes productive infection in HBMECs, which can lead to the transfer of the virus from the blood to the CNS via an intracellular pathway. Intracellular TBEV replication in HBMECs was demonstrated by qRT-PCR, TBEV antigen was detected in HBMEC by immunostaining, and released infectious TBEV was quantified by plaque assay. The results suggest a short TBEV replication cycle in HBMECs; released virions were first detected 1 day p.i. with peak viral RNA production between 12 and 24 h p.i. TBEV infection of HBMECs was persistent; viral antigen production was observed in the infected cells for the whole investigation period (to 19 days p.i.). However, the infection was accompanied by no apparent CPE. Persistent TBEV infection was demonstrated previously in primary neural cells, including human neurons (Bílý et al., 2015) and human or rat astrocytes (Lindqvist et al., 2016; Potokar et al., 2014; Palus et al., 2014a), whereas infection of immortalized human neural cells, such as neuroblastoma, medulloblastoma, and glioblastoma cells, is accompanied by gross CPE (Růzek et al., 2009). Though primary HBMECs are sensitive to infection and produce high virus titres, TBEV infection of immortalized HBMEC line hCMEC/D3 (Weksler et al., 2013) is not productive (unpublished results). The number of infected primary HBMECs in the culture was low (< 5%), but we detected relatively high virus titres in culture supernatants (> 10^6 pfu/ml), indicating that a single infected HBMEC is able to produce large quantities of novel virus particles. The main limitation of our study is the use of single cell type in the BBB model. Interaction of HBMEC with infected monocytes, infected/activated astrocytes or other cell types may have dramatic effects on HBMEC infection rates and BBB integrity (Roe et al., 2014; Verma et al., 2010).

The low TBEV infection rate in HBMEC corresponds to the few reports demonstrating infection of BBB endothelial cells by flaviviruses *in vivo* (King et al., 2011; Velandia-Romero et al., 2012). It should be also noted that the sensitivity of HBMEC to TBEV infection *in vivo* can be influenced by pro-inflammatory cytokines expressed in the periphery and released into the circulation during TBE. However, taking into account the high sensitivity of neural tissue to TBEV infection, the infection of very few HBMECs may be sufficient for efficient virus transmission through the BBB and the initiation of infection in the brain. Our data demonstrating the release of infectious TBEV on the abluminal side of the *in vitro* BBB model indicates the ability of a cell-free virus to migrate across the BBB. The release of infectious particles to the abluminal side of the *in vitro* BBB model was independent of the virulence of the particular TBEV strain used. However, the infection of HBMECs in the *in vitro* BBB model was not associated with any changes in barrier permeability, as demonstrated by TEER and FITC-dextran permeability. These results are in accordance with data published for WNV (Verma et al., 2009) and JEV (Li et al., 2015). We previously found in a mouse model that TBEV entry into the brain directly follows the viremia peak (Růžek et al., 2011). A dose as low as 10 pfu of TBEV injected intracerebrally could cause 100% mortality in a mouse model (Hayasaka, 2011), implying that even a very low virus titre entering the brain is sufficient to establish generalized CNS infection. The low infection rate of HBMECs, which is followed by high virus production, supports the idea that infection of HBMECs represents a possible route for TBEV entry into the brain (Haglund and Günther, 2003; Chambers and Diamond, 2003). This corresponds with the observation of a widespread distribution of TBEV antigens in the CNS already in early disease stages, supporting a haematogenous mode of neuroinvasion by TBEV (Gelpi et al., 2005). The low sensitivity of HBMECs to TBEV could be a factor in why only some individuals infected with TBEV develop CNS infection and experience neurological symptoms (Haglund and Günther, 2003).

Several studies of HBMEC infection with neurotropic viruses, such as HTLV-1 (Afonso et al., 2007), HIV-1 (Kanmogne et al., 2005), SIV (Luabeya et al., 2000), and HCMV (Bentz et al., 2006), have shown that infection is associated with a decrease and/or redistribution of TJ proteins. Conversely, an increase in TJ protein expression was observed in HBMECs infected with WNV (Verma et al., 2009). In this study, the expression of key TJ proteins in TBEV-infected HBMECs was investigated at the mRNA level using qRT-PCR. No significant changes were found for occludin and ZO-1 during peak virus production. We also employed immunofluorescent staining of TJ proteins and viral antigen to visualize the integrity of the barrier between non-infected and infected cells; no changes in the distribution of TJ proteins were found. This was confirmed by SEM. A correlative SEM-immunofluorescent staining of the viral antigen clearly demonstrated presence of both the viral antigen and TJ proteins expressed by the infected cells. Therefore, TBEV infection of HBMECs has no effect on TJ protein expression and distribution.

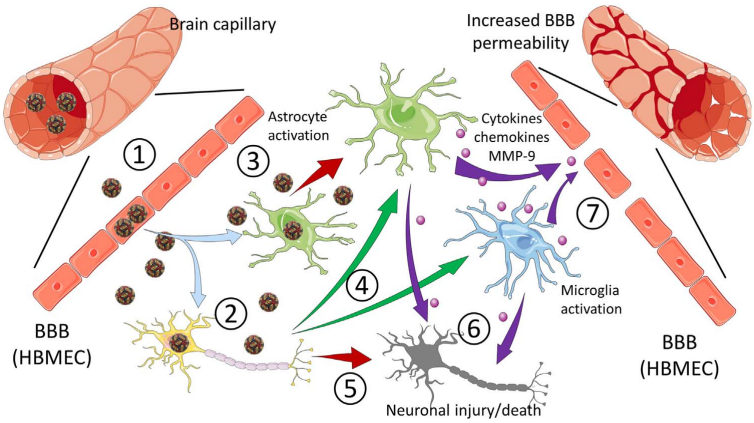

Fig. 8. Schematic presentation of possible mechanisms of TBEV entry into the brain, development of neuroinflammation, and BBB breakdown. (1) During viremia, TBEV can infect HBMECs, which multiply the virus and release novel virus particles into the CNS. However, this occurs without compromising BBB integrity. TBEV infection of HBMECs represents one possible mechanism of virus entry into the brain, but other mechanisms can be involved. (2) In the CNS, neurons represent the main target of virus infection. (3) In addition, other neural cells, such as astrocytes, may support virus replication, leading to their activation. (4) Infected neurons release cytokines that activate astrocytes and microglia. (5) TBEV infection of neurons leads to their dysfunction or death. (6) Activated astrocytes and microglia release large quantities of cytokines/chemokines/MMP-9; some of them may have neurotoxic effects and (7) induce BBB breakdown. Figure created using Servier Medical Art available on www.servier.com.

Infection of HBMECs by WNV was associated with increased expression of CAMs, such as VCAM-1 and E-selectin, at the peak of viral replication (Verma et al., 2009; Roe et al., 2014). A similar effect has been observed in many viral neuroinfections (Shen et al., 1997; Seigneur et al., 1997; Cosby and Brankin, 1995; Velandia-Romero et al., 2016; Roe et al., 2014). The induction of CAMs has an impact on the migration of immunocompetent cells through the BBB. In WNV, the permeability of the *in vitro* BBB model increased dramatically following the transmigration of monocytes and lymphocytes across the models infected with the virus, which was reversed in the presence of a cocktail of blocking antibodies against ICAM-1, VCAM-1, and E-selectin (Roe et al., 2014). Increased ICAM-1 mRNA expression was reported previously in the brains of TBEV-infected mice (Růžek et al., 2011). Conversely, no significant changes in CAM expression were seen in TBEV infection of HBMECs during the peak virus replication. Slight down-regulation of the expression of ICAM-1, VCAM-1, and E-selectin mRNA in infected HBMECs was observed, but protein levels did not differ between infected and uninfected HBMECs.

We previously speculated that the BBB is most likely compromised by the activity of pro-inflammatory mediators, such as cytokines, chemokines, and MMPs, mainly MMP-9, expressed in the TBEV-infected brain tissue (Růžek et al., 2011). Increased expression of cytokines/chemokines positively correlated with an increase in BBB permeability in TBEV-infected mice. Large amounts of cytokines and chemokines were produced in the CNS in response to TBEV infection, namely TNF-α, IL-6, IFN-γ, RANTES (CCL5), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), and IP-10 (CXCL10) (Růžek et al., 2011; Palus et al., 2013; Hayasaka et al., 2009). Increased expression of several of these cytokines/chemokines, NF-κB, MMP-9, and other pro-inflammatory soluble factors is also observed in TBE patients (Grygorczuk et al., 2006, 2015, 2016; Moniuszko-Malinowska et al., 2017; Palus et al., 2014b, 2015; Zajkowska et al., 2011). Brain extracts from JEV-infected mice containing high levels of cytokines, chemokines, and other soluble factors, but not virus alone, increased the permeability of an *in vitro* BBB model, which strongly suggests that cytokines and/or chemokines contribute to BBB disruption *in vivo* (Li et al., 2015). Pro-inflammatory cytokines and chemokines, such as IL-6, IFN-γ, CXCL10, and CCL2-5, can compromise BBB integrity by downregulated TJ proteins claudin-5, occludin, and ZO-1 (Roe et al., 2012). In addition, MMPs are thought to play a major role in promoting BBB disruption via the degradation of TJ proteins, oedema formation, and disintegration of the neurovascular unit (Jian Liu and Rosenberg, 2005; Rosenberg, 2002; Roe et al., 2012). We previously reported that astrocytes, which are also adjacent BBB component cells, represent a target for TBEV infection (Palus et al., 2014a). Infected or activated astrocytes induce robust production of MMP-9 and a broad array of cytokines/chemokines, including IL-1β, IL-6, IL-8, IFN-α, TNF-α, CXCL10, and CCL4, with a well-documented contribution to BBB disruption (Palus et al., 2014a). This all strongly indicates that inflammatory cytokines, chemokines, and MMPs, but not the virus itself, mediate BBB breakdown during TBE.

Based on results from this study and data from the literature, we speculate that TBEV invades the CNS by infecting HBMECs without compromising the integrity of the BBB (Fig. 8). Other pathways for TBEV to cross the BBB, including paracellular transfer and/or virus transport inside circulating immune cells, cannot be ruled out. In the CNS, neurons represent the main target for virus infection (Bílý et al., 2015). Infected neurons produce cytokine signals, which induce the activation of astrocytes and other glial cells. In vitro studies suggest that astrocytes can also be infected. Fast induction of type I IFN production by astrocytes plays an important role in their self-protection (Lindqvist et al., 2016). Infected or activated astrocytes and other activated glial cells produce large quantities of cytokines/chemokines and MMPs (Palus et al., 2014a), which may induce BBB breakdown by reducing the expression of TJ proteins (Li et al., 2015). Chemokines and their receptors are involved in coordinating complex leukocyte trafficking patterns into the CNS (Bardina and Lim, 2012). Infection of neurons leads to their dysfunction/damage/death (Růzek et al., 2009). The cytokines and chemokines produced by activated astrocytes and other glial cells play a role not only in virus control (Tun et al., 2014), but also neuronal injury and death (Kumar et al., 2010; Chen et al., 2010; Das et al., 2008; Ghoshal et al., 2007).

We report here that HBMECs are sensitive to TBEV infection, produce high virus titres, and support TBEV entry into the brain without altering BBB integrity. However, also other ways of TBEV entry into the brain can be involved. Further research is needed to elucidate the precise mechanisms of TBEV entry into the brain and molecular mechanisms of BBB breakdown during TBE. Understanding these mechanisms can promote the development of novel strategies for treating this important human neuroinfection.

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3. Electron tomography analysis of tick-borne encephalitis virus infection in human neurons

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Electron Tomography Analysis of Tick-Borne Encephalitis Virus Infection in Human Neurons

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Tick-borne encephalitis virus (TBEV) causes serious, potentially fatal neurological infections that affect humans in endemic regions of Europe and Asia. Neurons are the primary target for TBEV infection in the central nervous system. However, knowledge about this viral infection and virus-induced neuronal injury is fragmental. Here, we directly examined the pathology that occurs after TBEV infection in human primary neurons. We exploited the advantages of advanced high-pressure freezing and freeze-substitution techniques to achieve optimal preservation of infected cell architecture. Electron tomographic (ET) reconstructions elucidated high-resolution 3D images of the proliferating endoplasmic reticulum, and individual tubule-like structures of different diameters in the endoplasmic reticulum cisternae of single cells. ET revealed direct connections between the tubule-like structures and viral particles in the endoplasmic reticulum. Furthermore, ET showed connections between cellular microtubules and vacuoles that harbored the TBEV virions in neuronal extensions. This study was the first to characterize the 3D topographical organization of membranous whorls and autophagic vacuoles in TBEV-infected human neurons. The functional importance of autophagy during TBEV replication was studied in human neuroblastoma cells; stimulation of autophagy resulted in significantly increased dose-dependent TBEV production, whereas the inhibition of autophagy showed a profound, dose-dependent decrease of the yield of infectious virus.

Tick-borne encephalitis virus (TBEV), a member of the *Flaviviridae* family, genus *Flavivirus*, causes tick-borne encephalitis (TBE) in humans, a neuroinfection prevalent in large areas of Europe and North-eastern Asia. Humans develop a febrile illness, and a subset of cases progress to neurological manifestations ranging from mild meningitis to severe encephalomyelitis[1,2]. Despite the medical importance of TBE, some crucial steps in the development of encephalitis remain poorly understood. TBEV is mainly transmitted to the host when infected ticks feed. Virus replication is first detected in draining lymph nodes; this is followed by development of viremia; during the secondary viremic phase, the virus crosses the blood-brain barrier (BBB) and enters the brain[3]. Major hallmarks of TBEV neuropathogenesis are neuroinflammation, followed by neuronal death[4,5], and disruption of the BBB[3,6]. Neuronal injury may be directly caused by viral infection, but destruction has also been attributed to infiltrating immunocompetent cells (mainly $CD8^+$ T-cells), inflammatory cytokines, and activated microglial cells[2,7,8]. Tissue culture models of TBEV infections in primary neurons can distinguish between injuries caused by the virus and those caused by the immune response[9,10]. Various cellular models, including neuronal cell lines, primary cultures of embryonic or neonatal mouse and rat neuronal cells, and neurons derived from embryonic stem cells, have been used to explore infections with various neurotropic viruses (e.g., polio, herpes simplex type 1, varicella-zoster, West Nile, Japanese encephalitis, and rabies)[10–13]. We previously examined

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TBEV infections in human neural (neuroblastoma, glioblastoma, and medulloblastoma) cell lines[14]. On the ultrastructural level, the infection caused massive morphological changes in cells, including the proliferation and rearrangement of rough endoplasmic reticulum (RER) and signs of apoptosis or necrosis[14].

Recently, replication features of neurotropic flaviviruses, West Nile virus, Japanese encephalitis virus, and TBEV were compared in primary mouse neuronal cultures. Viral antigen accumulation in neuronal dendrites was induced to a greater extent in a TBEV infection than in infections with the other flaviviruses[15]. TBEV replication induced characteristic ultrastructural membrane alterations in neurites, known as laminal membrane structures (LMSs)[15]. However, conventional chemical fixation for sample visualization presents obstacles in obtaining sufficient morphological detail[16]. Therefore, in the present study, we exploited the advantages of high-pressure freezing and freeze-substitution techniques to improve the preservation of virally modified structures in TBEV-infected neurons.

Here, we visualized TBEV infections in primary human neurons in three-dimensional (3D) space at ultrastructural resolution with electron tomography. To the best of our knowledge, this study was the first to visualize the architecture of cellular components involved in TBEV replication and transport in neurons and the major ultrastructural changes that occur in response to TBEV infections. These novel data revealed the neuronal injury caused by TBEV infections, independent of the immune system response. Our results may facilitate the development of novel strategies for treating this serious human neuroinfection.

Results

Replication of TBEV and distribution of virus antigen in human neurons. We employed a plaque assay to determine TBEV infection and replication kinetics in human neurons (HNs; Fig. 1A). The HNs were infected with TBEV, and at 0, 3, 5, 7, and 12 days post infection (p.i.), cell supernatants were collected. Productive TBEV replication was detected in the form of released virions on day 3 p.i. The virus titer in the culture supernatant remained the same until the end of the experiment (Fig. 1A).

Immunofluorescence staining was used to assess viral antigen distribution in HNs. Viral antigen was not detected in mock-infected HNs. Immunofluorescence staining revealed that TBEV antigen was mostly distributed diffusely throughout the entire neuron bodies at early time points after infection (Fig. 1B). However, at later time points, we observed brightly stained aggregates of viral antigen in some cells (Fig. 1B). A co-localization study with protein disulphide isomerase family a, member 3 (PDIA3) antigen (also known as Erp57, Er-60, and GRP58) suggested that the viral antigen was localized primarily in the hypertrophied, rearranged RER. Occasionally, we observed viral antigen accumulation in the dendrites of TBEV-infected HNs (Figs. 1B,2A,B, yellow arrows). Viral antigen also accumulated in association with RER alterations, such as large whorl formations (Fig. 2B, white arrows), or in places that exhibited a local loss of network structure (Fig 2C). In the latter locations, RER alterations were accompanied by the presence of numerous longitudinal fibers (white arrows) that were positively immunolabeled with anti-viral protein E (Figs. 1B,2C).

TBEV-induced RER alterations and formation of tubule-like structures in HNs. Mock-infected and TBEV-infected HNs were examined with transmission electron microscopy at two time points (3 and 12 days p.i.) to delineate TBEV-induced morphological structures that were associated with early and late phases of the infection. Compared to mock-infected HNs, TBEV-infected cells exhibited a broad range (a complex set) of virus-induced subcellular structural changes at both time points.

At 3 days p.i., HN RER cisternae contained several resident virions (approximately 45 nm in diameter), virus-induced vesicles, and tubule-like structures (Fig. 3). Virus-induced vesicles were often arranged in two tightly apposed cisterns of the RER. Some of these vesicles contained electron-dense material, which represented newly-formed nucleocapsids (Fig. 3C,D). In other parts of the RER compartment, the cisterns accommodated tubule-like structures that ran either in parallel lines or in different directions (Fig. 3C,D, movie S1). The tubule-like structures measured 22 ± 1.3 nm ($N = 51$) in diameter.

The tubule-like structures were frequently observed at both investigated time points in TBEV-infected HNs. In several cases, tubule-like structures with different diameters (e.g., 22 ± 1.3 nm and 43.8 ± 4.3 nm, $N = 7$) were observed separately in the RER cisternae of single cells (Fig. 4A–D, movie S2); typically, virus-induced vesicles and virions were observed in neighboring RER spaces (Fig. 4B–D; Fig. 3C,D).

A prominent morphological change in TBEV-infected HNs was proliferation of the RER membranes that harbored sites of TBEV replication (Fig. 5A,B, movie S3). The proliferative parts of the RER were devoid of ribosomes (Fig. 5A, inset; movie S3, white arrows). At the later time point (12 days p.i.), proliferated RER had rearranged into large whorls (Fig. 5C,D, movie S4). The 3D reconstruction showed complex, lamellar whorls that enclosed a central space containing the cytoplasm of the same structure, and an electron-density as outside, limited by four membranes (Fig. 5D, light blue, movie S5). Numerous flattened ER cisternae were located on the periphery (Fig. 5D, blue). Electron tomography confirmed a continuation/connection of this peripheral part with the RER space, which accommodated TBEV-induced structures (vesicles or tubule-like structures) (Fig. 5D). These observations suggested that the membranous whorls were formed from the peripheral parts of the ER due to extensive ER stress. The large whorls did not contain virions or virus-induced structures. The central part of the whorl had features of an autophagosome, but it did not show signs of degraded content. Finally, we observed a tiny

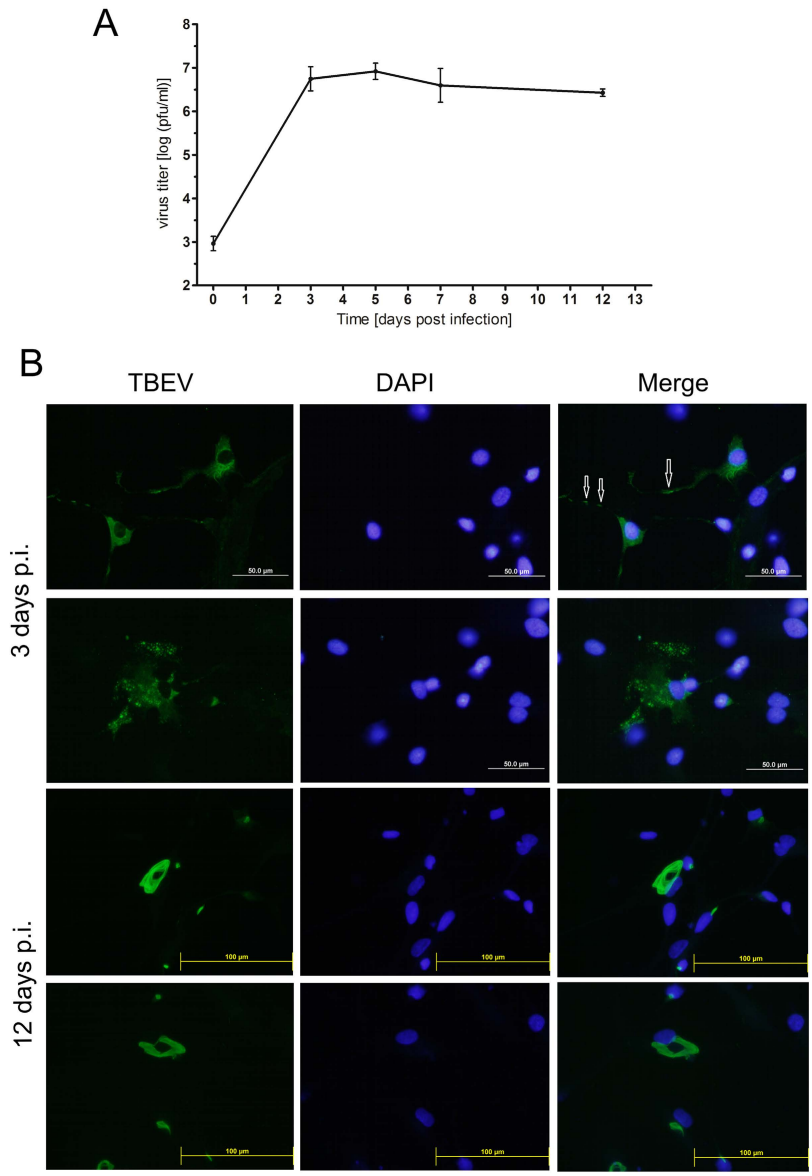


Figure 1. TBEV can infect human neurons. (**A**) TBEV titers in culture supernatants from HNs collected at 0, 3, 5, 7, and 12 days post-infection (p.i.) were determined in plaque assays with porcine kidney stable cells. Viral titers are expressed as pfu/ml. Data represent means ± SEM. (**B**) HNs grown and fixed on slides at days 3 and 12 p.i. were stained with anti-flavivirus envelope antibody (green) and counterstained with DAPI (blue). TBEV-infected HNs immunostained with flavivirus-specific antibody demonstrated virus replication in the cytoplasm at an early time point (3 days p.i.); antigen accumulated into aggregates at later a time point (12 days p.i.). Mock-infected HNs stained with primary and secondary antibodies were used as a negative control, and did not exhibit any TBEV antigen staining (not shown). The arrows indicate accumulation of viral antigen in dendrites of the infected HNs.

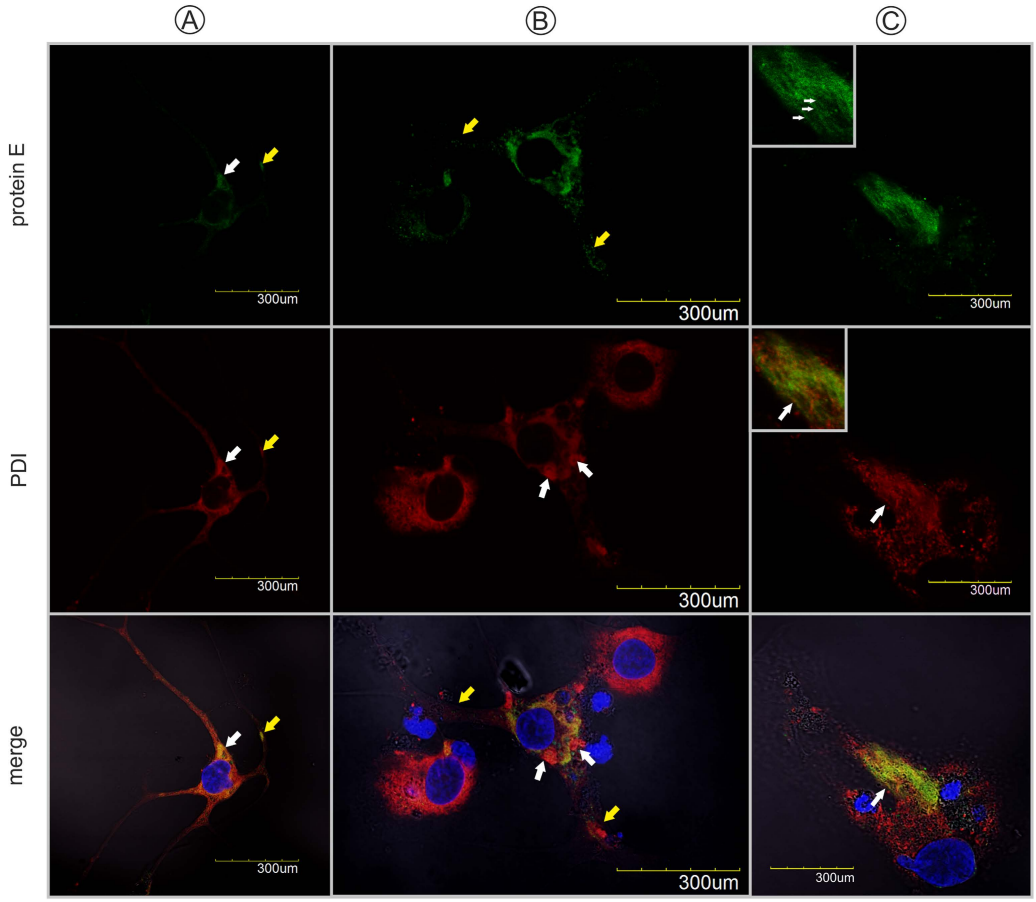


Figure 2. Confocal images of human neurons infected with TBEV. Neurons were fixed at (**A**,**B**) 3 days p.i. and (**C**) 12 days p.i. and double immunolabeled with antibodies against TBEV protein E (green) (*top panels*), PDIA3 (red) (*middle panels*), and (*merge panels*) counterstained with DAPI. (**A**) Replication complexes were observed in the perikaryon (white arrow) and dendrites (yellow arrow). (**B**) Infected cells with intact endoplasmic reticulum networks were observed next to infected cells with large whorls (white arrows). (**C**) Localization of viral protein E in the numerous longitudinal fibers (white arrows) associated with the ER.

connection between two primary virus-induced structures, as shown in detail in a slice of the tomogram (Fig. 5E) and in the 3D model (Fig. 5F; movie S5).

Signs of autophagy observed in TBEV-infected HNs. In several cases, particularly in HN extensions, which represented dendrites, we observed that the TBEV replication space was enclosed by flattened ER cisternae. This suggested the formation of autophagosomes that sequestered cell structures damaged by TBEV. Electron tomography was performed to view the complex architecture of these structures. In most cases, the sequestration was incomplete, as shown in Fig. 6A–E (movie S6 and movie S7), even when the projection images suggested that the structure might be interpreted as an autophagic vacuole (Fig. 6B). The TBEV particles and virus-induced vesicles resided in the RER (Fig. 6A,C,D, black arrows), but the enclosing ER cisterns lacked ribosomes (Fig. 6D, white arrows). Lipid droplets were also found associated with these structures (Fig. 6D,E). No morphologically similar structures were found in the extensions of mock-infected HNs (Fig. 6F). However, in the perikaryon (Fig. 6G), several autophagosomes or autophagolysosomes (with signs of degraded material) were observed. In some TBEV-infected

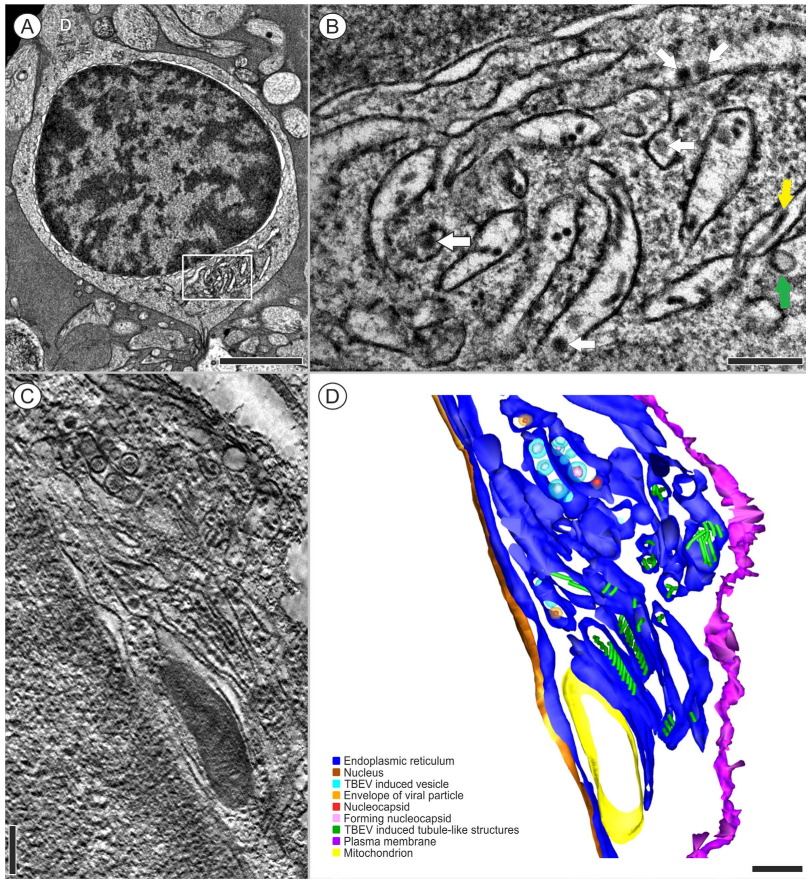


Figure 3. HNs infected with TBEV for 3 days. (**A**) A neuron with two cytoplasmic extensions on opposite sides of the cell body. D- Dendrite. (**B**) Detail of the boxed region in (**A**) shows the RER, which contains viral particles (44.75 nm, n = 4, white arrows), virus-induced vesicles (green arrow), and tubule-like structures (yellow arrow). (**C**,**D**) The presence of tubule-like structures (22 ± 1.3 nm, N = 51) inside the RER. (**C**) A slice of the tomogram was rendered as (**D**) a 3D reconstruction of a single axis of the tomogram. A series of images were collected in a ±65° tilt range with 0.65° increments. Pixel resolution: 1.1 nm. This single-axis tomogram is shown in movie S1. Bars: (**A**) 2 mm, (B-D) 200 nm. The transmission electron microscope images were acquired with (**A**,**B**) a JEOL 1010 80 kV and (**C**,**D**) a JEOL F2100 200 kV.

HN extensions, we observed autophagosomes with all the typical morphological features (Fig. 7 and movie S8). These autophagosomes were limited by numerous membranes that did not have ribosomes, but they resembled rearranged, small, membranous ER whorls. In the 3D reconstruction, we have identified numerous membranes that sequestered intact mitochondria, lipid droplets, and vacuoles within the inner space with TBEV particles (Fig. 7B).

Autophagy induction enhances TBEV replication. To study whether autophagy plays a role in TBEV replication, we treated human neuroblastoma cells with rapamycin, an autophagy inducer. The cells were subsequently infected with TBEV at m.o.i. 0.1 pfu per cell. Culture supernatants were harvested after 24, 48, and 72 hours, and viral titers were determined by plaque assay. The viral yields were increased in cells treated with a range of concentrations rapamycin on day 2 and 3 p.i. (Fig. 7C). The induction levels were quite high reaching 1.5 $\log_{10}$ pfu/ml higher titers in cells treated either with 0.05 or 0.1 μM of rapamycin in comparison with the untreated control on day 3 p.i. (Fig. 7C).

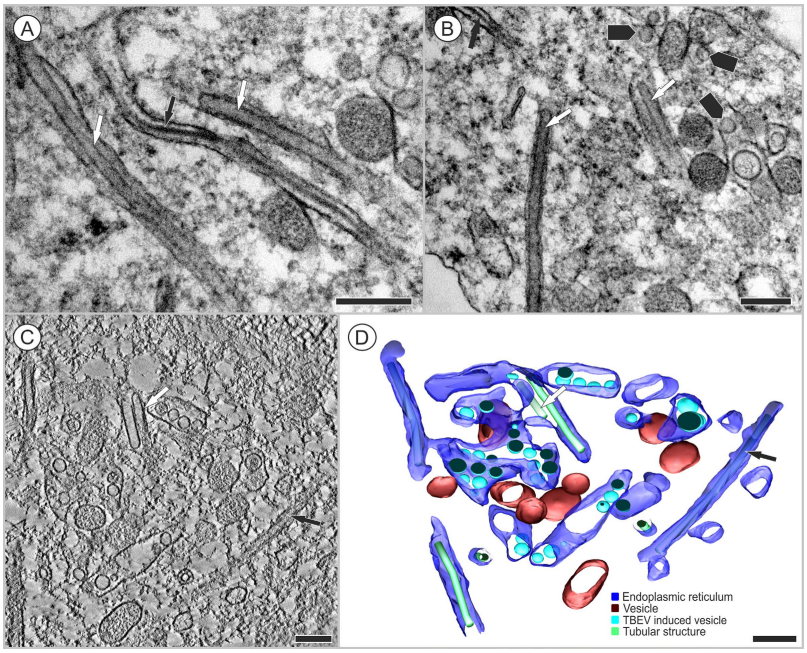


Figure 4. Tubule-like structures of different diameters were localized inside a single neuron infected with TBEV. Transmission electron microscope images were acquired at (**A**) 3 days p.i. or (**B**) 12 days p.i. (**A**) Inside the ER, tubules of 43.8 ± 4.3 nm (N = 7) in diameter (white arrows) were observed; other cisternae contained tubules of 22 ± 1.3 nm in diameter (black arrow). (**B**) The TBEV infection induced ER rearrangements (black arrowheads). (**C**) A slice of the tomogram and (**D**) a 3D reconstruction of a single axis tomogram. Tilt series images were collected in the ±65° tilt range with 1° increments. Pixel resolution: 0.8 nm. This single-axis tomogram is shown in movie S2. Bars: 200 nm. (**A–D**) The transmission electron microscope images were acquired with a JEOL F2100 200 kV.

Suppression of autophagy reduces TBEV replication. To investigate the effect of spautin-1, a compound known to be an effective autophagy inhibitor, on viral replication in the neuronal cells, we tested its effects on TBEV growth in human neuroblastoma cells. The cells pretreated with a range of concentrations of spautin-1 for 30 min, were infected with TBEV at m.o.i. 0.1 pfu per cell, after which the incubation was continued in the presence of the drug. Culture supernatants were harvested after 24, 48, and 72 hours, and viral titers were determined by plaque assay. Treatment with a range of concentrations of spautin-1 showed a profound, dose-dependent inhibition of the yield of infectious virus, with statistically significant drops in TBEV titer of more than two orders of magnitude at the highest spautin-1 concentration (10 μM) on day 3 p.i. (Fig. 7D).

Transport of the TBEV in the neuron. One projection image of a 40-nm thick ultrathin section showed that, in the extensions of TBEV-infected HNs, two virions (48.2 nm and 48.9 nm diameters, with nucleocapsids of 30.3 and 30.9 nm diameters, respectively) were located separately inside vacuoles that were directly connected to cellular microtubules (Fig. 8A). The presence of that connection was confirmed by examining a double-axis electron tomography (±60° tilt range with 0.6° increments, pixel resolution: 0.55 nm), and then creating a 3D reconstruction (Fig. 8B, movie S9). Both connections are shown in detail on slices of the tomogram (Fig. 8C,D, arrows). Extensions of mock-infected HNs contained vesicles with electron-dense cores with diameters that ranged from 70 to 90 nm; those vesicles were observed in direct contact with the cytoplasm (Fig. 9A,B). Electron-dense granules (similar to the vacuoles that contained virions in Fig. 8A) were found in close proximity to bunches of microtubules and mitochondria, as seen in the transverse section (Fig. 9A). Other vesicles, 43.8 nm in diameter, but different in structure from virions (for comparison see Fig 8A), were observed in the control cells (Fig. 9C).

We used nocodazole to assess the effect of microtubule destabilizing agent on virus growth in human neuronal cells. Nocodazole is known to induce depolymerization of actin filaments and microtubules. Human neuroblastoma cells pretreated with a range of non-cytototoxic concentrations of nocodazole

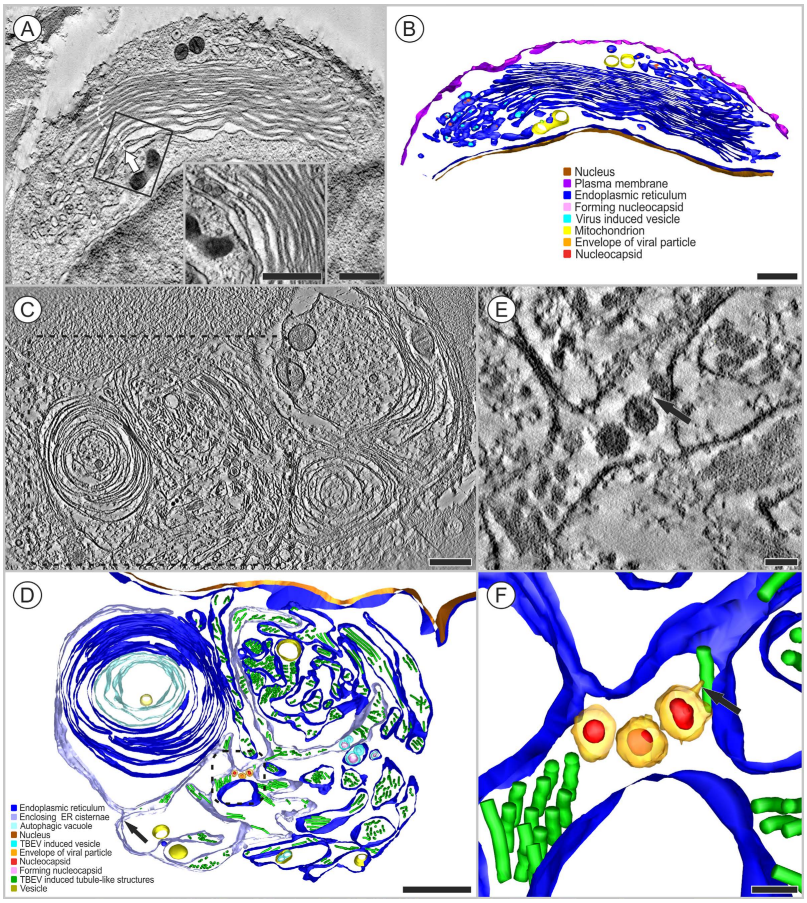


Figure 5. Proliferation of the endoplasmic reticulum observed in HNs infected with TBEV. Transmission electron microscope images were acquired at (**A**,**B**) 3 days p.i. and (**C**–**E**) 12 days p.i. (**A**) TBEV particles and TBEV-induced vesicles are located inside the proliferated and reorganized cisterns of the rough endoplasmic reticulum; the boxed region is enlarged in the inset. (**B**) The image in (**A**) was rendered as a colored 3D model. (**C**) Large whorls are clearly observed in abnormal endoplasmic reticulum. (**D**) The boxed region in (**C**) is rendered in a 3D model to clarify the different components. (**D**) Lamellar whorls are surrounded by cisternae (light purple) arising (arrow) from the rough endoplasmic reticulum (blue), which accommodates tubule-like structures (green). The central part of the whorls comprises concentric circles of flattened ER cisternae (light blue). We observed several lipid droplets (yellow) in proximity of the whorls. (**E**) Detailed image of the boxed region in (**D**) shows the connection between a TBEV particle and a tubule-like structure (arrow) inside the rough endoplasmic reticulum. (**F**) The image in (**E**) was rendered as a colored 3D model. (**A**) A single axis tomogram; ±65° tilt range with 0.65° increments, pixel resolution: 2.2 nm; (**C**) a single axis tomogram, 2 × 2 montage, ±65° tilt range with 1° increments, pixel resolution: 0.8 nm. Bars: (**A**–**D**) 500 nm, (**E**,**F**) 50 nm. (**A**–**F**) The transmission electron microscope images were acquired with JEOL F2100 200 kV.

for 30 min were infected with TBEV at m.o.i. 0.1 pfu per cell, after which the incubation was continued in the presence of the drug. Culture supernatants were harvested after 24, 48, and 72 hours, and viral titers were determined by plaque assay. Treatment with a range of concentrations of nocodazole showed a profound inhibition of the yield of infectious virus, with statistically significant drops in TBEV titer of more than two orders of magnitude at the all concentrations of nocodazole on days 2 and 3 p.i. (Fig. 8E).

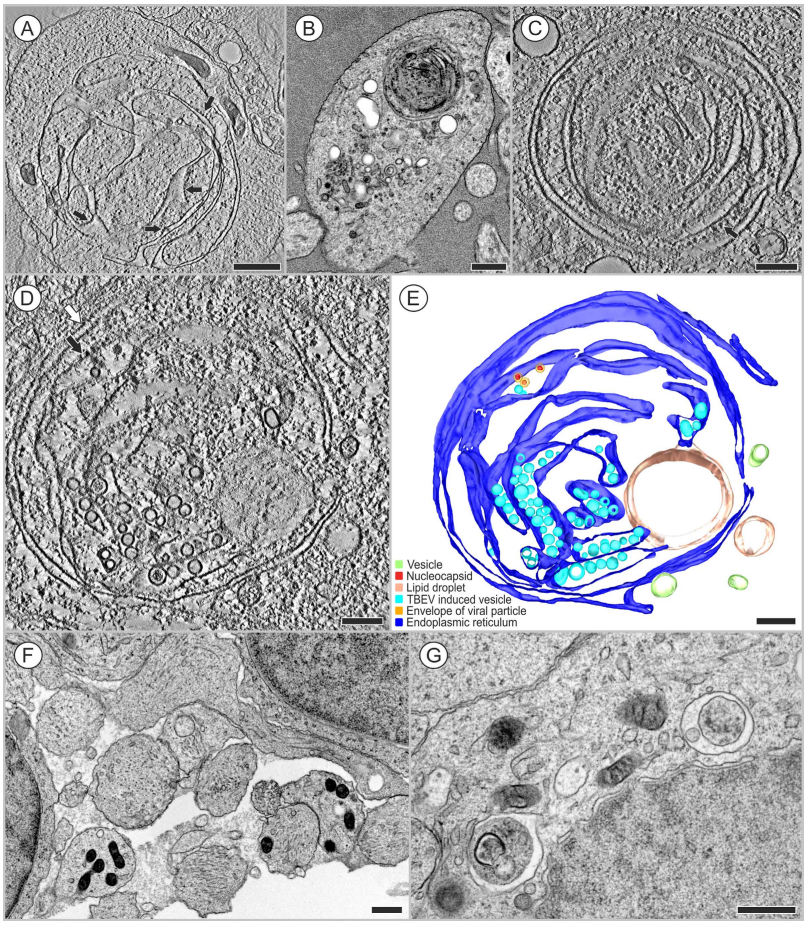


Figure 6. Formation of autophagic vacuoles in HNs infected with TBEV. Transmission electron microscope images were acquired at (**A**–**C**) 3 days p.i., (**D**,**E**) 12 days p.i., and in (**F**,**G**) mock-infected cells. (**A**,**B**) The RER (ribosomes are indicated with black arrows), which contain TBEV particles and virus-induced structures, were nearly completely sequestered by peripheral cisterns (see electron tomography in supplement) in neuronal extensions. (**C**) Detail of (**B**) shows the coiled RER with TBEV-induced structures. (**D**) The cisterns of the RER (ribosomes indicated with black arrow) with replicating TBEV particles and virus-induced vesicles are surrounded by one flattened ER cistern that nearly encloses this space, and a lipid droplet. Enclosing cisternae of the ER were devoid of ribosomes (white arrow). (**E**) 3D model of (**D**). (**F**) Similar vacuoles/autophagosomes were not observed in control neuronal extensions. (**G**) Several vacuoles that sequestered cell parts (debris, fragments of degraded membranes) were found in the cell body of control neurons. Bars: (**A**,**B**,**F**,**G**) 500 nm, (**C**–**E**) 200 nm. (**A**,**C**,**D**) Slices of a single axis tomogram; ±65° tilt range with 1° increments, pixel resolution: (**A**) 1.1 nm, (**C**) 0.7 nm; (**D**) 0.8 nm. Transmission electron microscope images were acquired with (**A**,**C**–**E**) JEOL f2100 200 kV and (**B**,**F**,**G**) JEOL 1010 80 kV.

Discussion

We demonstrated that neuronal TBEV infections produced cell-free, infectious virus by using the supernatant from TBEV-infected HNs to infect PS cells and measuring the viral titers with a plaque assay. TBEV replication was detected in the form of released virions on day 3 p.i., and the culture supernatant maintained a virus titer of approximately 10^7 pfu/ml until the end of the experiment (Fig. 1A). This finding suggested that the TBEV-infected HNs had apparently transitioned from an acute to a persistent infection. In a previous study, we compared TBEV growth in human neuroblastoma, glioblastoma,

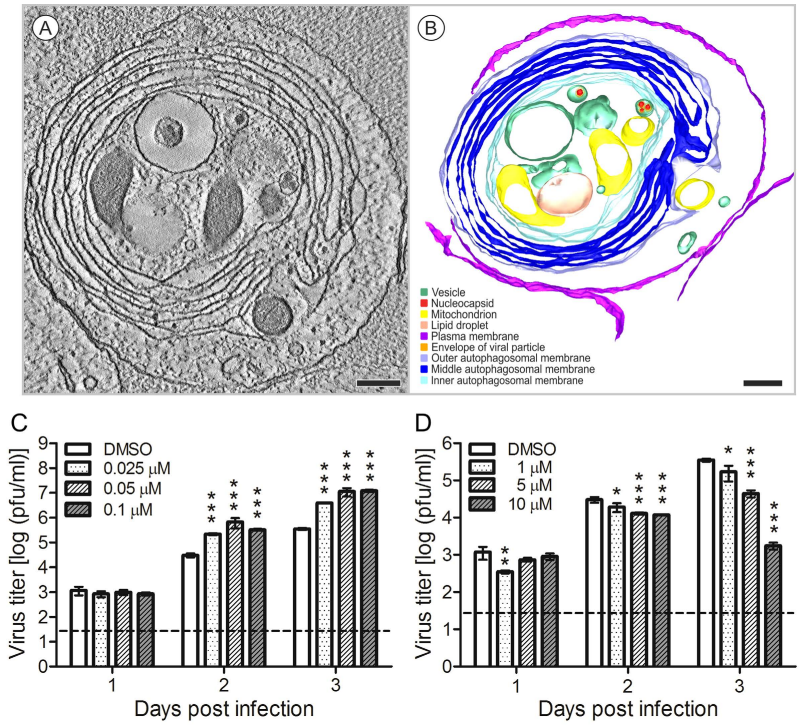
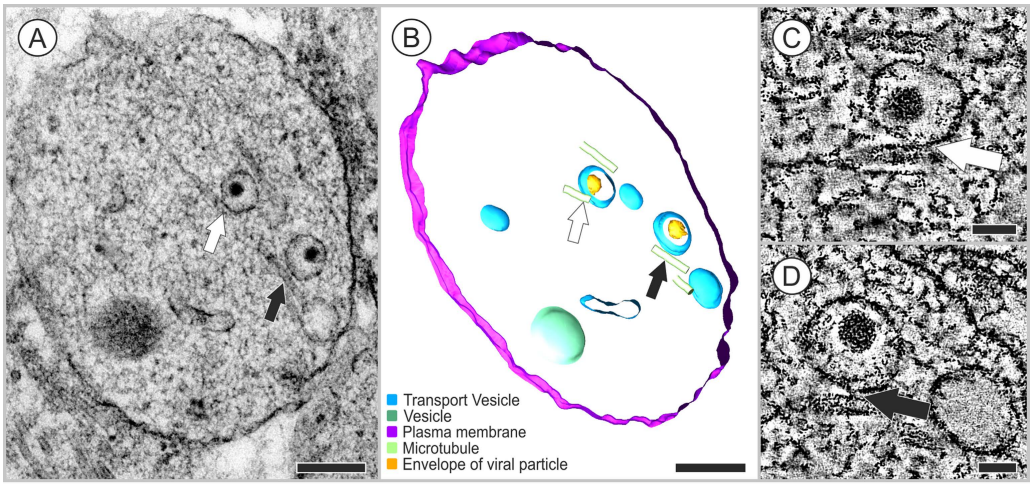


Figure 7. An autophagosome in a neural extension observed 3 days after TBEV infection. The cytoplasm contains mitochondria and TBEV particles in vacuoles, encircled by several double membranous structures, apparently of RER origin (but devoid of ribosomes). (**A**) A slice of a single axis tomogram; ±65° tilt range with 0.6° increments, pixel resolution: 0.8 nm. (**B**) A 3D model of (**A**). Bars: 200 nm. (**A**,**B**) Transmission electron microscope images were acquired with JEOL F2100 200 kV. (**C**,**D**) Autophagy enhanced TBEV production in human neuroblastoma cells. The cells were pretreated with the solvent control (DMSO), rapamycin (0.025, 0.05, or 0.1 μM) (**C**), or spautin-1 (1, 5, or 10 μM) (**D**) then infected with TBEV at m.o.i. of 0.1 pfu per cell for 24, 48, or 72 hours. The culture supernatants were collected for plaque assay on PS cells. The virus titers (pfu/ml) are shown as the means ± SEM. The horizontal dashed line indicates the minimum detectable threshold. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

medulloblastoma, and PS cells. That study showed that virus replication in neural cell lines was more than 100 times more efficient than in the PS cells[14]. In the present study, we showed that primary HNs were also highly sensitive to TBEV infection, and they also produced high virus titers.

A previous study in primary mouse neurons showed that TBEV antigen accumulated in the dendrites of infected neurons[15]. Viral proteins were synthesized principally in the neuronal cell bodies in the early stages of infection, but they were distributed to dendrites later[15]. In the present study, TBEV-infected HNs also showed antigen accumulation in dendrites (Fig. 1B, arrows), but this was not a frequent event. At early time points after infection, virus antigen was present in practically the whole body of the neuron (Figs. 1B,2); at later time points, antigen accumulation appeared mainly in highly reorganized, proliferated RER (demonstrated in the co-localization experiments with the PDIA3 antigen) (Fig. 2), and only occasionally in dendrites. Viral protein accumulation in dendrites may affect neural function[15] and TBEV infections can arrest neurite outgrowth[17]. It was hypothesized that viral protein accumulation in dendrites might affect the distribution and function of host proteins, which in turn, might cause neural dysfunction and cellular degeneration[15].

Our results suggested that vesicles containing TBEV particles were transported in infected neurons, based on observations that TBEV-containing vesicles were associated with microtubules in HNs (Fig. 8A–D). In human neuroblastoma cells, non-cytotoxic concentrations of nocodazole, a compound which disrupts microtubules by binding to β-tubulin and preventing formation of one of the two interchain disulfide linkages and thus inhibiting microtubules dynamics, resulted in significant reduction



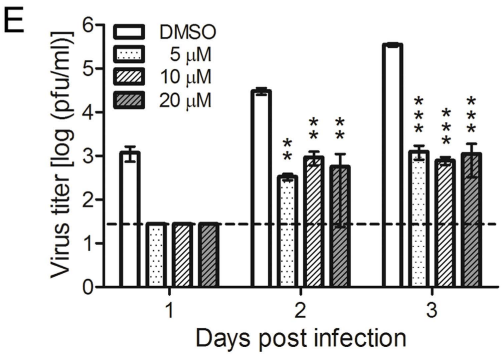


Figure 8. Two vacuoles that accommodated TBEV particles in a neuronal extension at 12 days after infection. (**A**–**D**) Arrows indicate connections between vacuoles and microtubules. (**A**) The projection image and (**B**) the 3D model. (**C**,**D**) Images show slices of a double-axis tomogram acquired with a ± 60° tilt range in 0.6° increments; pixel resolution: 0.55 nm Bars: (**A**,**B**) 200 nm; (**C**,**D**) 50 nm. Transmission electron microscope images were acquired with (**A**) JEOL 1010 80 kV and (**C**,**D**) JEOL F2100 200 kV. (**E**) Nocodazole treatment inhibits TBEV replication in human neuroblastoma cells. The cells were pretreated with the solvent control (DMSO), or nocodazole (5, 10, or 20 μM) then infected with TBEV at m.o.i. of 0.1 pfu per cell for 24, 48, or 72 hours. The culture supernatants were collected for plaque assay on PS cells. The virus titers (pfu/ml) are shown as the means ± SEM. The horizontal dashed line indicates the minimum detectable threshold. $^{**}p < 0.01$; $^{***}p < 0.001$.

in virus infectivity (Fig. 8E). Viral spread in neurons is generally mediated by fast axonal transport, a microtubule-associated, anterograde and retrograde transport system. In West Nile virus (WNV) infections, transneuronal viral spread required axonal release of viral particles. WNV underwent bidirectional spread in neurons, and axonal transport promoted viral entry into the CNS[18].

Here, we also described the morphology and 3D organization of TBEV-induced, tubule-like structures located in the RER of infected HNs (Figs. 3B,4A–D). Similar structures were previously demonstrated in the RER of other vertebrate or arthropod cells infected with TBEV[19], Langat virus[20], and mosquito-borne flaviviruses[16,21,22]. In a previous study, we observed virus-induced vesicles and viral particles directly attached to tubule-like structures in the RER of TBEV-infected human primary astrocytes[19]. The tubule-like structures were only occasionally seen in acutely infected cells, but the number of tubules increased dramatically in persistently infected cells[20]. Unlike previous studies, we frequently observed the presence of tubule-like structures in TBEV-infected neurons. To the best of our knowledge, this study

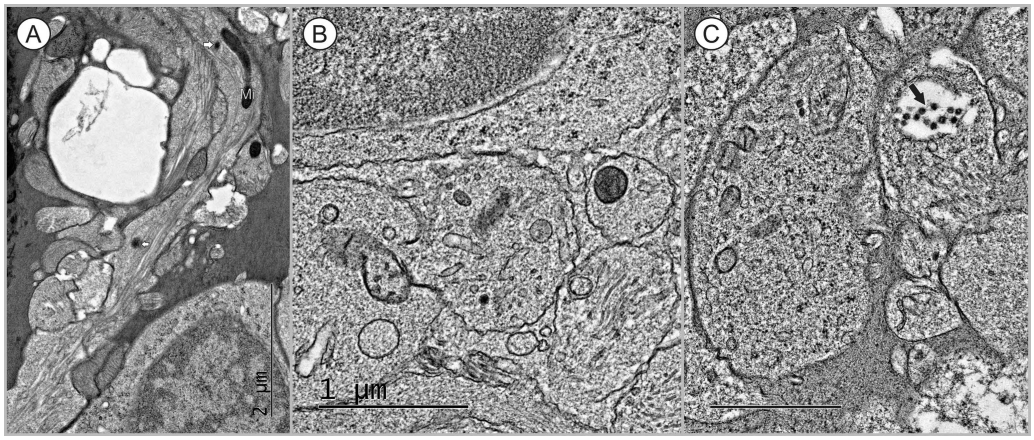


Figure 9. Mock infected HNs examined at 3 days p.i. (**A**,**B**) HN extensions contained a few probable secretion granules with electron-dense cores, with diameters of (**A**) about 70 nm and (**B**) 90 nm, that were in direct contact with the cytoplasm. (**C**) Different vesicles of 43.8 nm (n = 5) in diameter were located outside the cells (black arrow). Bar 1 µm. (**A**–**C**) Transmission electron microscope images were acquired with JEOL 1010 80 kV.

was the first to demonstrate the simultaneous presence of two sizes of tubule-like structures in the RER of single infected cells (Fig. 4A–D). Inside the RER, we observed tubules that were 43.8 ± 4.3 nm (N = 7) in diameter, and in other cisternae, we observed tubules that were 22 ± 1.3 nm in diameter (Fig. 4A–D). Previous studies reported tubule-like structures that ranged from 50 to 100 nm in diameter and from 100 nm to 3.5 µm in length[20–23]. In TBEV-infected human astrocytes, we previously observed tubule-like structures of 17.9 nm (±0.2 nm) in diameter[19]. The function of tubule-like structures is not clear. These structures may represent features of replication, aberrant structures, or features of a cellular process that aims to restrict the infection[20]. With immunofluorescence and confocal microscopy, we observed fibrillary structures composed of viral E protein in cisterns of the ER; these structures suggested that E protein was present in the tubule-like structures (Figs. 1B,2). The functional contribution of these tubule-like structures to TBEV replication should be addressed in future studies.

Our detailed 3D ultrastructural analysis clearly demonstrated that the TBEV infection triggered a remarkable alteration in the ER membrane structures of HNs (Fig. 5B,D). Previous studies have shown alterations in ER membranes by flaviviruses, which included formations of vesicle pockets and convoluted structures that represented a platform for viral RNA replication and virion assembly[15,24,25]. These replication compartments also shielded double-stranded RNA from host cell-intrinsic surveillance mechanisms[24–26].

In TBEV-infected mice, Hirano *et al.*[15] described characteristic ultrastructural changes in neurite membranes, called LMSs. They hypothesized that LMSs were formed by membrane reconstitutions triggered by the viral replication, and that the LMS might serve as a platform for dendritic viral replication and virion assembly[15]. In the present study, we observed TBEV replication sites in dendrites, but also in perinuclear regions of TBEV-infected HNs. However, our 3D tomography data strongly suggested that, in several cases, the TBEV replication sites were enclosed inside newly formed autophagosomes. Moreover, we observed virions inside autophagosomes that were surrounded by numerous membranes (Figs. 5,6,7) with many typical morphological features. Autophagosome-limiting membranes did not have ribosomes, and they always had two or more limiting membranes[27]. Additionally, we observed interactions with lipid droplets (LDs)[28] in both newly forming autophagosomes and fully-formed autophagosomes of TBEV-infected HNs. A recent report noted the existence of a physical connection between the endoplasmic reticulum and newly forming autophagosomes[29,30]. We confirmed this observation (Fig. 5C,D and Fig. 6).

Upon autophagy induction, the initial event is the formation of a membranous cistern called the phagophore, or isolation membrane. Autophagy begins with the isolation of double-membrane structures in the cytoplasm. Later, these membrane structures elongate and mature. The elongated double-membranes form autophagosomes (large vesicles with diameters of 500–1500 nm in mammalian cells), which become mature with acidification; then, they fuse with lysosomes to become autolysosomes. The sequestered content is then degraded by lysosomal hydrolases[31].

There are many proposals for the role of autophagy during virus infection in neurons. In post-mitotic, long-lived cell types, such as neurons, basal and stress-induced autophagy may be particularly important for maintaining cellular health. In addition, it represents an important neuronal antiviral defense mechanism for the sequestration and elimination of viral proteins, viral particles, and viral replication complexes[32]. Thus, autophagy represents an adaptive, pro-survival process, rather than a maladaptive, pro-death response, during CNS viral infection[32]. Autophagy has been shown to restrict the replication of several viruses in neurons, including Rift Valley fever virus[33], Sindbis virus[34], and herpes simplex virus type 1[35]. However, the growth of WNV has been shown to be independent of WNV-induced autophagy in neuronal cultures[36]. Although autophagy is a cellular homeostatic mechanism involved in the antiviral response, it can be also subverted to support viral growth. Virus-induced autophagy promoted the replication of dengue virus[37,38], enterovirus 71[31], poliovirus, and coxsackievirus B4[39]. Japanese encephalitis virus growth is dependent on autophagy activation during the early stages of infection[40]. Our study was the first to demonstrate that autophagy occurred during TBEV replication in HNs, and is also the first report that autophagy enhances TBEV replication. We treated TBEV-infected neuroblastoma cells with rapamycin (inducer of autophagy, as inhibition of mTOR mimics cellular starvation by blocking signals required for cell growth and proliferation), and spautin-1 (highly specific and potent autophagy inhibitor in mammalian cells, which promotes the degradation of Vps34 PI3 kinase complexes by inhibiting two ubiquitin specific peptidases, USP10 and USP13 that target the Beclin1 subunit of Vps34 complexes) and investigated the effect of the treatment of TBEV growth. The induction of autophagy in human neuroblastoma cells by rapamycin increased TBEV replication (Fig. 7C), whereas the inhibition of autophagy by spautin-1 reduced significantly viral titers (Fig. 7D), indicating that autophagy positively regulates TBEV replication.

As mentioned above, we observed direct interactions between autophagosomes and LDs in TBEV-infected HNs (Fig. 6D,E, Fig. 7). A previous report showed that autophagosomes could target cellular lipid stores (LDs) to generate energy for the cell[41]. For example, dengue viral-infection-induced autophagy stimulated the delivery of lipids to lysosomal compartments, which resulted in the release of free fatty acids. These fatty acids underwent β-oxidation in the mitochondria to generate ATP, which produced a metabolically favorable environment for viral replication[28]. Thus, aside from the many roles of autophagy in regulating cellular homeostasis, its regulation of lipid metabolism can represent a major contributor to robust viral replication[41]. The role of autophagy as a provider of free fatty acids as an energy source for RNA replication during viral infection will be a subject of our further studies.

In conclusion, we directly investigated the morphological changes induced by TBEV infections in HNs with advanced high-pressure freezing, freeze-substitution, and electron tomography techniques, with the purpose of achieving optimal preservation of ultrastructure for electron tomography visualization of cellular architecture. These methods enabled clear visualization of connections between microtubules and vacuoles that harbored TBEV virions in neuronal extensions, connections between tubule-like structures and virions, 3D organizations of proliferating endoplasmic reticulum membranes, membranous whorls, and the formation of autophagic vacuoles. These data provided insight into the process of TBEV-induced injury in HNs, and our findings will promote future studies that aim to understand the molecular mechanism of TBEV infection in the human CNS.

Methods

Virus and cells. The virus used in this study was the TBEV strain, Neudoerfl, kindly provided by Professor F. X. Heinz from the Medical University in Vienna. The strain was originally isolated from the tick *Ixodes ricinus* in Austria in 1971. The virus represents a prototype strain of the European subtype of TBEV and was characterized extensively, including its genome sequence and the 3D structure of its envelope protein E[42]. The virus underwent four passages in the brains of suckling mice before use in this study.

Human neurons (HNs) were isolated from the human brain and characterized by immunofluorescence with antibodies specific to neurofilament, microtubule associated protein 2 (MAP2), and β-tubulin III (purchased at passage zero from ScienCell Research Laboratories, Carlsbad, CA). HNs were propagated in Neuronal Medium (ScienCell) with 1% neuronal growth supplement and 1% penicillin/streptomycin (ScienCell) at 37 °C and 5% CO_2. In all experiments, cells were used at passage zero.

Human neuroblastoma cells UKF-NB-4[14] were cultured at 37 °C and 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (Sigma) and 1% mixture of penicillin and streptomycin (Sigma).

Porcine kidney stable (PS) cells[43] were cultured in L-15 medium supplemented with 3% newborn calf serum and 1% penicillin/streptomycin (Sigma-Aldrich) at 37 °C.

Viral growth in HNs. HNs were seeded onto gelatin (0.2%) coated wells of 96-well plates at 20,000–25,000 cells/cm^2. After establishing the culture, cells were inoculated with the virus diluted in culture medium to 5 multiplicity of infection units (MOI). Virus-mediated cytopathic effect (CPE) was investigated with light microscopy. At 0, 3, 5, 7, and 12 days p.i., supernatant medium from appropriate wells was collected and frozen at −70 °C. Viral titers were determined by plaque assay.

Plaque assay. PS cells were used to determine virus titers according to a protocol described previously, with minor modifications[44]. Tenfold dilutions of the virus samples were placed in 24-well tissue culture plates, and suspended PS cells were added (5×10^4 cells per well). After incubating for 4 h, the suspension was overlaid with carboxymethylcellulose (1.5% in L-15 medium). After incubating for 5 days at 37 °C, the plates were washed with PBS, and the cells were stained with naphthalene black (Sigma Aldrich). Virus titer was expressed in units of pfu/ml.

Immunofluorescence staining. TBEV-infected and control HNs were grown on slides. Then, cells were fixed in 4% formaldehyde for 1 h at room temperature, rinsed three times in 0.1 M phosphate buffer (PB) with 0.02 M glycine, permeabilized with 0.1% Triton X-100 with 1% normal goat serum in 0.1 M PB for 30 min, and blocked with 5% normal goat serum. Cells were labeled with flavivirus-specific mouse mAb (1:250; Millipore) for 1.5 h at 37 °C. Flavivirus-specific mAb and rabbit anti-PDIA3 antibody (1:250, Sigma-Aldrich) were used for double labeling. After washing with ten–fold diluted blocking solution, the cells were labeled with goat anti-mouse secondary antibody conjugated with FITC (1:500, Sigma-Aldrich) or goat anti-rabbit secondary antibody conjugated with Atto 550 NHS (1:500, Sigma-Aldrich) for 1.5 h at 37 °C. The cells were counterstained with DAPI (1 μg/ml, Sigma-Aldrich) for 10 min at 37 °C, mounted in 2.5% 1,4-diazabicyclo(2.2.2)octane (Sigma-Aldrich), and examined with an Olympus BX-51 fluorescence microscope equipped with an Olympus DP-70 CCD camera. Confocal microscopy was performed with an Olympus FV-1000; serial Z-series images were acquired in blue, red, and green channels.

Transmission electron microscopy and electron tomography. TBEV-infected and control HNs were grown on sapphire discs. At either 3 or 12 days p.i., cells were high-pressure frozen in the presence of 20% BSA diluted in Neuronal Medium with a Leica EM PACT2 high-pressure freezer. Freeze substitution was carried out in 2% osmium tetroxide diluted in 100% acetone, as described previously[19], with a Leica EM AFS2 at −90 °C for 16 h. The samples were than warmed at a rate of 5 °C/h, incubated at −20 °C for 14 h, and finally warmed again at the same rate to a final temperature of 4 °C. The samples were rinsed three times in anhydrous acetone at room temperature and infiltrated stepwise in acetone mixed with SPI-pon resin (SPI) (acetone:SPI ratios of 2:1, 1:1, 1:2, for 1 h at each step). The samples in pure resin were polymerized at 60 °C for 48 h.

Sections were prepared with a Leica Ultracut UCT microtome and collected on 300 mesh cooper grids. Staining was performed with alcoholic uranic acetate for 30 min, and then, lead citrate for 20 min. Images were obtained with a JEOL 2100F or JEOL 1010 transmission electron microscope. For electron tomography, protein A-conjugated 10 nm gold nanoparticles (Aurion) were added to both sides of each section as fiducial markers.

Tilt series images were collected in the range of ±60° to 65°, with 0.6° to 1° increments. Images were acquired with a 200 kV JEOL 2100F transmission electron microscope equipped with a high-tilt stage and a Gatan camera (Orius SC 1000) and controlled with SerialEM automated acquisition software[45]. Images were aligned based on the fiducial markers. Electron tomograms were reconstructed with the IMOD software package. Manual masking of the area of interest was employed to generate 3D surface models[46].

Autophagy stimulation and inhibition, and microtubule disruption. Stocks of rapamycin (Sigma-Aldrich) as an autophagy stimulator, spautin-1 (Sigma-Aldrich), an autophagy inhibitor, and nocodazole (Sigma-Aldrich) as a microtubule disruptor, were prepared in DMSO. Monolayers of human neuroblastoma cells in 96-well plates were pretreated with different concentrations of either drug (or DMSO as a negative control) for 30 min at 37 °C and infected with TBEV at a multiplicity of infection of 0.1 pfu per cell. Infection was always performed in triplicate. Supernatants were harvested at 24, 48, and 72 hours p.i. and titers were determined by plaque assay as described above.

Statistical analysis. Statistical analyses were performed using version 5.04 of the GraphPad Prism5 (GraphPad Software, Inc., USA). Data were transformed by use of the $X' = \log(X)$ formula and analyzed using one-way ANOVA (Dunnett's Multiple Comparison Test). p-values < 0.05 were considered significant.

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Author Contributions

M.V. and D.R. conceived and designed the experiments and wrote the manuscript. T.B., M.P., L.E., and J.E. performed the experiments and analyzed the data. All authors reviewed the manuscript.

Additional Information

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CHAPTER III:

1. **Tick-borne encephalitis virus neutralization by high dose intravenous immunoglobulin**
   Jana Elsterová, Martin Palus, Jana Širmarová, Jan Kopecký, Hans Helmut Niller and Daniel Růžek
   Ticks and Tick-borne Diseases 2017, 8: 253-258. DOI: 10.1016/j.ttbdis.2016.11.007

As there are no approved specific therapeutics to treat TBE, we tested the potential use of human-pooled high dose intravenous immunoglobulin (IVIG) (Elsterová et al., 2017). Since a host's immune reaction to TBEV infection has serious pathological consequences, an immune modulation was suggested as being potentially beneficial. Pooled intravenous immunoglobulins are used to treat several acute hyper-inflammatory autoimmune conditions, such as Kawasaki vasculitis, Guillain-Barré syndrome, myasthenia gravis and immune thrombocytopenia, and may also be used as adjunctive therapy for sepsis. The beneficial effect of IVIGs during treatment of these diseases is based on their immunomodulatory functions, namely the fact that they block Fc receptors of macrophages, therefore occluding the clearance of antibody coated cells, and block complement activation by abrogating the ability of aggregated antibodies to lead to complement activation. Furthermore, IVIG contains a spectrum of neutralizing antibodies for various pathogens (Stangel & Pul, 2006). A series of case reports of successful IVIG interventions in severe viral encephalitis of arboviral origin resulted in great interest in taking advantage of IVIG in TBE (Růžek, Dobler & Niller, 2013). The inflammation dampening therapeutic effects of IVIG might override the antibody dependent enhancement, which was the reason for cancelling the use of anti-TBEV specific immunotherapy for treating TBE in Europe.

In this study we tested two lots of IVIG of the same substance from Octapharma, Manchester, UK, which is distributed for clinical purpose as Octagam 10%. They varied in the presence or absence of anti-TBEV specific antibodies, which further determined their effect on TBEV replication and pathogenesis. Pooled intravenous immunoglobulins with no detectable specific

anti-TBEV antibodies had no or very low effect on viral replication in human cell lines of neural origin. Moreover, no therapeutic efficacy was achieved by a challenge of a lethal dose of TBEV to mice after treatment by IVIG, with no detectable anti-TBEV specific antibodies. On the other hand, a strong inhibitory effect of IVIG with high titers of anti-TBEV antibodies on virus growth was detected in the *in vitro* model. Similar results were observed in mice infected by a lethal dose of TBEV and treated by IVIG containing high levels of TBEV-neutralizing antibodies.

As had been proposed in earlier studies (Růžek, Dobler & Niller, 2013), we examined the effect of IVIG with a low efficacy of immunomodulative properties, but a high neutralizing pattern with consequences on viral replication and pathogenesis with antibody specific immunotherapy. No antibody dependent enhancement concerning the TBEV-infected mice after treatment by IVIG was observed, regardless of the amount of TBEV-specific antibodies present in the preparation.

Based on our results, we can conclude that IVIG with high titers of specific anti-TBEV antibodies may serve as a useful post-exposure prophylaxis for patients suffering from TBE, particularly for patients with primary immunodeficiency or failed anti-TBEV vaccination who are living in an endemic area of TBEV occurrence. A more detailed study of specific anti-TBEV antibodies is needed, as it would provide a great tool for examining the cross-reactivity of flaviviruses, including ADE syndrome, with the potential of a high therapeutic outcome.

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Elsterová, J., Palus, M., Širmarová, J., Kopecý, J., Niller, H.H. & Růžek, D. 2017. Ticks and Tick-borne Diseases Tick-borne encephalitis virus neutralization by high dose intravenous immunoglobulin. *Ticks and Tick-borne Diseases*. 8(2):253–258. DOI: 10.1016/j.ttbdis.2016.11.007.

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1. **Tick-borne encephalitis virus neutralization by high dose intravenous immunoglobulin**

Ticks and Tick-borne Diseases 8 (2017) 253–258

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Original article

Tick-borne encephalitis virus neutralization by high dose intravenous immunoglobulin

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ABSTRACT

Tick-borne encephalitis (TBE) is a potentially lethal neuroinfection in humans, caused by TBE virus (TBEV). Currently, there are no approved therapeutic agents to treat TBE. Previously, it was suggested that application of high dose intravenous immunoglobulin (IVIG) may pose potentially successful treatment for severe cases of TBE. In this study, we determined the titers of TBEV-neutralizing antibodies in two IVIG lots originating from the same manufacturer, and tested their ability to treat a lethal TBEV-infection in a mouse model. Using an *in vitro* assay, more than 100-fold difference in TBEV-neutralizing capacity was demonstrated between the two individual IVIG lots. High TBEV-neutralizing activity of IVIG containing TBEV-specific antibody was confirmed in two different human neural cell lines, but IVIG without TBEV-specific antibodies had no or little effect on virus titers in the culture. In TBEV-infected mice, 90% of protection was achieved when the mice were treated with IVIG containing higher titers of TBEV-specific antibodies, whereas no immunotherapeutic effect was seen when mice were treated with IVIG without TBEV-specific antibodies. No antibody-dependent enhancement of TBEV infectivity induced by cross-reactive antibodies or by virus-specific antibodies at neutralizing or sub-neutralizing levels was observed either in cell culture or in TBEV-infected mice treated with any of the IVIG preparations. The results indicate that IVIG lots with high TBEV antibody titers might represent a post-exposure prophylaxis or first-line effective therapy of patients with a severe form of TBE.

1. Introduction

Tick-borne encephalitis virus (TBEV) (family *Flaviviridae*, genus *Flavivirus*) is a causative agent of tick-borne encephalitis (TBE), a severe human neuroinfection manifesting as meningitis, meningoencephalitis, meningoencephalomyelitis or meningoencephalomyeloradiculitis. The infection is fatal in approximately 1% of cases, but in up to 46% of cases it may result in long-lasting or permanent neurological damage, known as post-encephalitic syndrome. This include neuropsychiatric problems, balance disorders, headache, dysphasia, hearing defects, spinal paralysis, tremor, etc. (Haglund and Günther, 2003). The most severe cases are reported in elder (Jelenik et al., 2010) or immunocompromised patients (Chmelík et al., 2016). However, severe courses of TBE with accompanying post-encephalitis syndrome do also occur in children (von Stülpnagel et al., 2016) Although an effective vaccine against TBEV is available, a vaccination coverage remains still low in several endemic countries (Süss et al., 2010). During the last 30 years, there has been a continuous increase in the numbers of human cases of TBE in Europe. Currently, between 10,000 and 15,000 TBE cases per year are reported in Europe and Asia (Suss, 2008; Bogovic and Strle, 2015). There are no approved therapeutic agents to treat TBE. The use of specific anti-TBEV immunoglobulins for TBE therapy was discontinued in Europe due to the concerns of antibody-dependent enhancement (ADE) of the infectivity after post-exposure prophylaxis in children (Kluger et al., 1995; Waldvogel et al., 1996). However, ADE was not confirmed in TBEV-infected mice after passive pre- or post-exposure prophylactic administration of the specific anti-TBEV antibodies (Kreil and Eibl, 1997). Moreover, spe-

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cific anti-TBEV immunoglobulins are still in use in Russia, where a single post-exposure administration an anti-TBEV immunoglobulin in a dose of 0.05 ml/kg body weight ensures protection on average in 79% of TBE cases (Pen'evskaia and Rudakov, 2010). Based on an increasing number of case reports on the successful treatment of other arboviral infections, including Japanese encephalitis, Eastern equine encephalitis, West Nile fever, or chikungunya, with high dose intravenous immunoglobulins (IVIG) (Ben-Nathan et al., 2003, 2009; Srivastava et al., 2015; Planitzer et al., 2007; Caramello et al., 2006; Rajapakse, 2009; Golomb et al., 2001; Chusri et al., 2011; Hamdan et al., 2002; Shimoni et al., 2001), we suggested using IVIG also for the treatment of severe cases of TBE (Růžek et al., 2013). IVIG is a commercial preparation of purified human IgG manufactured from pooled plasma from thousands of healthy donors and is approved for clinical use (Rhoades et al., 2000). IVIG has a broad repertoire of antibodies neutralizing various pathogens and neutralization is commonly assumed to be the main mechanism of action. Moreover, IVIG has important immunomodulatory effects, which include activation/blockade of Fc receptors, attenuation of complement-mediated damage, induction of anti-inflammatory cytokines, anti-inflammatory effect by cytokine-specific, or CD4 and MHC class I-specific autoantibodies, autoantibodies against the Fas receptor, etc. (Boros et al., 2005).

In this study, the potency of IVIG for TBEV neutralization was tested *in vitro* in two human neural cell lines, and *in vivo* using a lethal mouse model mimicking severe cases of TBE in humans. Our results demonstrate that individual IVIG lots even from the same manufacturer can differ significantly in the titers of TBEV-neutralizing antibodies. The passive administration of IVIG containing sub-neutralizing levels of TBEV-specific antibodies has no effect on the survival of TBEV-infected mice, but IVIG containing anti-TBEV antibodies can effectively prevent or ameliorate the development of the disease.

2. Material and methods

2.1. Human serum and IVIG preparations

Pooled human serum was obtained from Sigma-Aldrich (St. Louis, MO, USA). Two lots of OCTAGAM® 10% [100 mg/ml] (Octapharma, Manchester, UK) were used; i.e., lot No. A125C8534 (further referred as IVIG1), and lot No. C343A8541 (further referred as IVIG2). The IVIG1 and IVIG2 preparations represented a pooled material from more than 3500 donors, and contained human normal IgG > 95%, with a broad spectrum of antibodies against various infectious agents. IgA content was ≤ 0.2 mg/ml (http://www.octapharma.com.au/fileadmin/user_upload/octapharma.au/product_docs/octagam_10_PI.pdf).

2.2. Cell cultures

Human neuroblastoma cells and human glioblastoma cells (kindly provided by Professor T. Eckschlager, 2nd Faculty of Medicine, Charles University in Prague) were grown at 37 °C/5% CO_2 in Iscoveís modified Dulbeccoís medium (IMDM) supplemented with 10% fetal calf serum (FCS) and 1% mixture of antibiotics/antimycotics (penicillin, streptomycin; Biosera). Porcine kidney stable (PS) cells (Kozuch and Mayer, 1975) were grown at 37 °C in Leibovitz's L-15 medium supplemented with 3% newborn calf serum and 1% mixture of antibiotics/antimycotics (penicillin, streptomycin; Biosera).

2.3. ELISA and plaque reduction neutralization test

Specific anti-TBEV antibodies in the pooled human serum, IVIG1 and IVIG2 were determined by sandwich-type ELISA (IMMUNOZYM FSME (TBE) IgG; PROGEN Biotechnik, Germany). The assay was performed according to the instruction of the manufacturer. The IgG antibody titer was expressed in Vienna Units per ml (VIEU/ml).

Presence of specific neutralizing anti-TBEV antibodies in IVIG preparations and pooled human serum was determined by plaque reduction neutralization test (PRNT) as described previously (Bárdos et al., 1983), with slight modifications. Ten mg/ml of protein content of the IVIG1, IVIG2, and pooled human serum were used for PRNT. Inactivation of complement was done by incubation at 56 °C for 30 min. Two fold serial dilutions of the samples were incubated with 1×10^3 pfu of TBEV strain Hypr for 90 min at 37 °C. After that, 5×10^4 of PS cells was added to the wells of 96-well plate and the suspension was overlaid with carboxymethylcellulose. After incubation for 5 days, the cells were fixed and stained as described previously (De Madrid and Porterfield, 1969). The last dilution of the sample that caused 80–100% reduction of cytolysis was regarded as the endpoint titer.

2.4. Virus stocks, infection of cell cultures, and virus titrations

Low-passage TBEV strains Neudoerfl (kindly provided by Professor F. X. Heinz, Medical University in Vienna; passaged four times in brains of suckling mice) and Hypr (passaged six times in brains of suckling mice) were used in the study. TBEV strain Neudoerfl was isolated in 1971 from an *Ixodes ricinus* tick in Burgenland, Austria; Hypr strain was isolated in 1953 from the blood of a deceased child in Moravia, Czech Republic.

Human neuroblastoma or human glioblastoma cells were seeded in 96-well plates at concentration of 1×10^4 cells/well and cultured overnight. The other day, the cells were infected with TBEV strain Neudoerfl at a multiplicity of infection (m.o.i.) = 5. The cells were treated with either IVIG1 or IVIG2 under three regimens. One group was pretreated with 10 mg/ml of either IVIG1 or IVIG2 for 4 h before the infection. The second group was treated with the same concentrations of the preparations after the infection throughout the whole experiment. The third group represented a combination of both; i.e., combined pretreatment as well as treatment post-infection. A control group represented TBEV-infected cells without any treatment. Cell supernatants were collected daily from day 1 to day 4 (neuroblastoma), and 1 to day 4 and on day 6 (glioblastoma) post-inoculation, and subjected to a plaque assay.

The titer of the virus was assayed on PS cell monolayers based on a modified protocol by De Madrid and Porterfield (1969). Briefly, tenfold dilutions of the virus suspension were placed in 96-well plates and 1×10^4 of PS cells per well was added. After 4 h incubation at 37 °C and 0.5% CO_2, a carboxymethylcellulose (1.5% in L-15 medium) overlay was added to each well. Following 5 day incubation at 37 °C and 0.5% CO_2, the cells were stained with naphtalene black. Infectivity was expressed as pfu/ml.

Viral RNA was isolated from cell culture supernatants by QIAamp Viral RNA Mini Kit (Qiagen), and viral genome copies were quantified using RT-qPCR for TBEV (Path-TBEV-EASY; Primerdesign) on a Rotor Gene-3000 (Corbett Research).

Cell cytotoxicity of both IVIG preparations was tested by Annexin V Apoptosis Detection Kit FITC (eBioscience, CA, USA). Briefly, human neuroblastoma or glioblastoma cells were seeded in 24-well plates in concentration of 3×10^5 of cells/ml. The cells were subsequently incubated in the presence of IVIG preparations or the pooled human serum at concentrations 0 mg/ml, 0.1 mg/ml, 1 mg/ml, and 10 mg/ml for 24 h. Cell viability was tested 72 h after the IVIG/pooled human serum addition according to the instructions in the kit manual. The analysis was performed using BD FACS Canto II flow cytometer with BD FACS Diva Software.

2.5. Mice and virus inoculation

Female BALB/c mice of 5 to 6 weeks of age were obtained from Harlan Laboratories, Inc. (Indiana, USA). The mice were housed in plastic cages with sterilized wood-chip bedding in a specific-pathogen free room under constant temperature of 22 °C and relative humidity of 65%. Sterilized pellet diet and water was provided *ad libitum*. Ten mice per group were used in the experiments.

The mice were inoculated subcutaneously with 10^3 pfu of TBEV Hypr strain. Hypr strain was selected because of its high virulence for laboratory mice. The recipient group of mice was administered with 0.5 ml either human sera or IVIG1 or IVIG2 (50 mg/ml) intraperitoneally daily from 1 to 5 days post-infection (p.i.). Control mice received PBS at the same time intervals. The mice were monitored daily for 28 days p.i.

The research complied with all relevant European Union guidelines for work with animals and was in accordance with the Czech national law guidelines on the use of experimental animals and protection of animals against cruelty (animal Welfare Act No. 246/1992 Coll.). The protocol was approved by the Committee on the Ethics of Animal Experimentation of the Institute of Parasitology and of the Departmental Expert Committee for the Approval of Projects of Experiments on Animals of the Czech Academy of Sciences (permit No. 165/2010).

2.6. Statistical analysis

Kruskal-Wallis test and Dunn's Multiple comparison test was used for comparisons of the concentrations of TBEV-neutralizing antibodies in the individual preparations by PRNT, as well for comparisons of viral titers in cell culture. Results from ELISA were analyzed by two-way ANOVA-Bonferroni multiple comparison. Cell viability after treatment with individual preparations was analyzed by Bartlett's test for equal variances and Dunn's Multiple comparison test. Survival rates were analyzed by log-rank Mantel-Cox test. All tests were performed using GraphPad Prism 5.00 (GraphPad Software, Inc., USA). *P*-values < 0.05 were considered to be statistically significant.

3. Results

3.1. Detection of anti-TBEV antibodies in the investigated preparations

To investigate the titer of antibodies against TBEV in the pooled human serum and both IVIG preparations, the samples were analyzed by ELISA and PRNT. No anti-TBEV antibodies were detected in the pooled human serum. IVIG1 contained detectable titers of anti-TBEV antibodies (approx. 250 VIEU/ml) only when the lowest dilution (i.e., 10 mg/ml) was tested. On the other hand, IVIG2 contained high titers of anti-TBEV antibodies within high range of dilutions from 10 mg/ml (approx. 1250 VIEU/ml) to 0.1 mg/ml (approx. 120 VIEU/ml) (Fig. 1A).

Pooled human serum did not contain any TBEV-neutralizing antibodies as determined by PRNT. The titers of TBEV-neutralizing antibodies in IVIG1 were at the borderline level. IVIG2 contained high levels of TBEV-neutralizing antibodies reaching titers 1:125, which is approximately 100-fold higher titer when compared to IVIG1 (Fig. 1B).

3.2. Reduction of TBEV-growth in human neural cell lines

IVIG1 and IVIG2 were tested for their ability to inhibit TBEV growth *in vitro* in two human neural cell types; i.e., human neuroblastoma, and human glioblastoma cells. Previously, both these cell types were shown to be highly sensitive to TBEV and to produce high virus titers (Růzek et al., 2009). To exclude any false-positive effect caused by potential cytotoxicity of the preparations tested, the toxicity of both IVIG preparations was first examined in both cell systems by detecting apoptotic cells based on the detection of phosphatidylserine on the cell membrane, and necrotic cells by labeling nuclear proteins. No toxicity was observed when the cells were incubated in the presence of preparations at the concentrations ranging from 0.1 to 10 mg/ml (data not shown).

The cells were infected with TBEV strain Neudoerfl at a multiplicity of infection (m.o.i.) = 5. The cells were treated with either IVIG1 or IVIG2 under three regimens as described in Material and Methods. Cell supernatants were collected and subjected to a plaque assay.

TBEV titers reached approximately 10^7 pfu/ml, and 10^6 pfu/ml in untreated neuroblastoma and glioblastoma cells, respectively. Pretreatment or treatment with IVIG1, or combination of both, had no effect on TBEV titer in neuroblastoma cells at any time point investigated with the exception of day 1 p.i. (Fig. 2A). In human glioblastoma cells, a slight decrease in virus titer was seen only in cells treated with IVIG1 at day 4 p.i. (Fig. 2C). On the other hand, IVIG2 had a strong inhibitory effect on virus growth in cell culture (Fig. 2B, D). In human neuroblastoma cells, pretreatment with IVIG2 had no significant effect on virus growth, but when the pretreatment was combined with post-infection treatment, virus titers were significantly reduced up to 10^4-fold compared to the untreated TBEV-infected cells. Treatment with IVIG2 post-infection reduced virus titers in the culture approximately 10^2 fold compared to the untreated cells (Fig. 2B). Similar effect was seen in case of TBEV-infected glioblastoma cells, where the treatment post-infection or combination of the pretreatment and posttreatment resulted in significant reduction of virus titer compared to untreated TBEV-infected cells, reaching a maximum of 10^3-to-10^4-fold reduction on days 2 and 3 (Fig. 2D). Combined pre- and posttreatment of glioblastoma and neuroblastoma cells with IVIG2 reduced also the number of TBEV RNA copies in cell culture supernatant, as measured by RT-qPCR. There was approximately 1.5-2 $\log_{10}$ difference in the number of viral genomic copies in the supernatant of the treated and control cultures on day 3 p.i. (data not shown).

3.3. Protection of mice from a lethal TBEV infection by IVIG

Infection of mice is a well-established model to investigate the mechanism and the efficacy of treatment interventions or immune protection against TBEV infection. In our experiments, mice were infected with 10^3 pfu of TBEV Hypr strain, which is a dose causing 100% mortality. The infection in mice was associated with classical signs of neuroinfection; i.e., ruffled fur, hunched posture, paresis, etc. Due to ethical reasons, mice in a terminal stage of the neuroinfection were euthanized. Groups of 10 TBEV-infected mice were given daily intraperitoneal injections of 50 mg of either IVIG1 or IVIG2 from day 1 to day 5 p.i. Control groups contained TBEV-infected mice that were treated intraperitoneally at the same intervals with 0.8 ml PBS or pooled human serum.

The results summarized in Fig. 3, show that passively transferred IVIG2 conferred 90% survival of this otherwise lethal challenge, whereas no therapeutic efficacy was achieved using IVIG1. Similarly, no mice treated with pooled human serum or PBS survived. All mice treated with IVIG1/pooled human serum/PBS died within 12 days after virus inoculation and no differences were seen in the mean survival time between these groups ($p > 0.05$). The results clearly show that IVIG2 containing high levels of TBEV-neutralizing antibodies had high antiviral effect in TBEV-infected mice ($p < 0.0001$), while IVIG1 had no therapeutic activity.

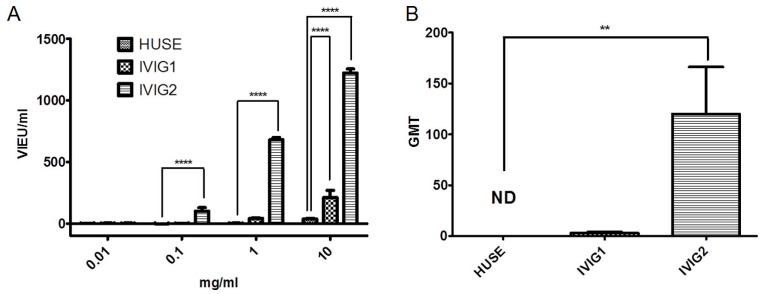

Fig. 1. Specific anti-TBEV antibodies in the pooled human serum (HUSE), IVIG1 and IVIG2 were determined by sandwich-type ELISA (A) and by PRNT (B). For ELISA, the concentrations of HUSE, IVIG1 and IVIG2 of 0.01 mg/ml, 0.1 mg/ml, 1 mg/ml and 10 mg/ml were used. A two way ANOVA-Bonferroni multiple comparison was used for the analysis; the error bars represent standard deviations (****, $p < 0.0001$) (A). PRNT was performed on PS cells with 10 mg/ml of IVIG1, IVIG2 and HUSE, and 1×10^3 pfu of TBEV. The level of TBEV-neutralizing antibodies is expressed as geometric mean titer (GMT) with standard deviation. A Kruskal-Wallis statistics and Dunnís Multiple comparison test was used for the analysis (**, $p < 0.01$; ND, not detected) (B).

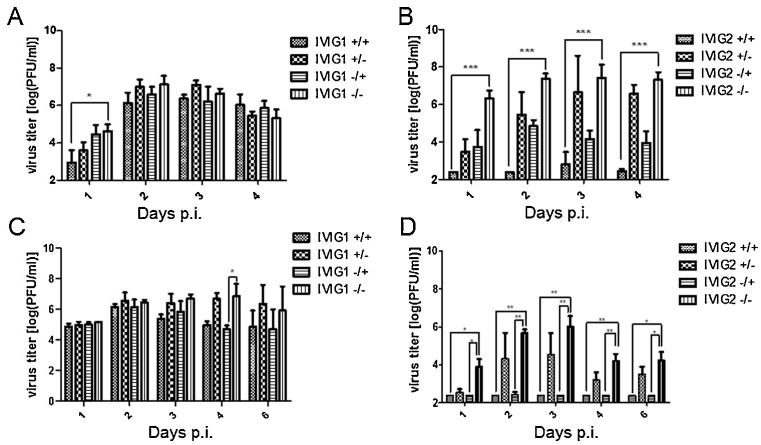

Fig. 2. IVIG1 and IVIG2 were tested for their ability to inhibit TBEV growth *in vitro* in two human neural cell types; i.e., human neuroblastoma (A-B), and human glioblastoma cells (C-D). The experiment was performed with 10 mg/ml of either IVIG1 or IVIG2 and the viral titer was determined by plaque assay as described in Material and Methods. The detection threshold was 2.3794 $\log_{10}$ PFU/ml. The experiments were repeated three times and tested in duplicates. A Kruskal-Wallis statistics and Dunnís Multiple comparison test was used for the analysis (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

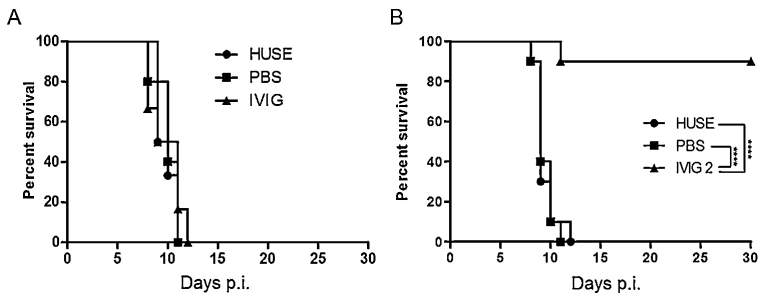

Fig. 3. Survival curve of mice infected with a lethal dose of TBEV. The recipient group of mice was administered with 0.5 ml either HUSE or 50 mg/ml of IVIG1 (A) or IVIG2 (B) intraperitoneally daily from 1 to 5 days p.i. Control mice received PBS at the same time intervals. The mice were monitored daily for 28 days p.i. A log-rank test was used for the analysis (****, $p < 0.0001$).

4. Discussion

Passive transfer of IVIG is a well-established and extensively clinically used procedure. IVIG preparations are successfully used for treatment of patients suffering from a variety of diseases, including myasthenia gravis, idiopathic thrombocytopenia purpura, inflammatory demyelinating polyneuropathy (Ben-Nathan

et al., 2009), but also various viral encephalitides (Caramello et al., 2006, 2007; Makhoul et al., 2009; Ben-Nathan et al., 2009; Golomb et al., 2001; Erlich et al., 1987). In most of these cases, the protective effect of passively administered IVIG has been attributed to the ability of the specific antibodies to neutralize the virus. Based on these data, we suggested to treat severe cases of TBE with IVIG as early in the course of the disease as possible, and proposed to conduct a randomized controlled treatment study focused on IVIG treatment of the severe TBE cases (Růžek et al., 2013). However, there are still only few reports of cases of severe TBE that substantially improved after application of IVIG (Kleiter et al., 2007).

As demonstrated in the present study, the efficiency of the protection by passive antibodies was directly related to the amount of specific antibodies present in the IVIG preparation. In this study, we investigated the titers of antibodies against TBEV in two IVIG lots from the same manufacturer. The levels of anti-TBEV antibodies were at the borderline level in one IVIG lot, but the second lot investigated contained high levels of TBEV-specific antibodies, as demonstrated by ELISA as well as by PRNT. A variability in TBEV-specific antibodies content in IVIG preparations from different geographic origins was shown previously by Rabel et al. (2012) and Goldacker et al. (2015). IVIG preparations originating in Europe had much higher titers than IVIG from Russia, and IVIG from the US contained no detectable titers of TBEV-specific antibodies. It was suggested that the fractionation of IVIG from plasma donations collected around the world would produce IVIG with universal content of antibodies against a broad range of pathogens (Bayry et al., 2003), which is, unfortunately, not feasible at present. The US represent the most important source of human plasma for IVIG production, and the IVIG manufacture in Europe largely depends on plasma donations from the US (Rabel et al., 2012). With respect to differences in the specific antibody content between individual IVIG lots, each lot should be marked with the details on the geographical origin of the plasma or this information should be available on request from the respective manufacturer. A preparation of a "tailor-made" pathogen-specific IVIG enriched with antibodies against specific infectious agents was also suggested (Bayry et al., 2003). In case of TBE, IVIG preparations not containing specific anti-TBEV antibodies might be spiked with chimeric/humanized anti-TBEV monoclonals (Baykov et al., 2014).

Inhibition of TBEV growth by the two IVIG lots was investigated *in vitro* in two human neural cell lines; i.e., human neuroblastoma and human glioblastoma cells. In both cell lines, a significant reduction of TBEV growth was seen when the cells were treated with the higher TBEV neutralization titer IVIG lot, while treatment with IVIG lacking neutralizing levels of TBEV-specific antibodies had no or little effect on virus growth. IVIG2 probably neutralized virus particles released from the infected cells; however, the growth of the virus in the culture was inhibited as well, as demonstrated by the reduced numbers of viral RNA copies in the culture supernatant.

A more convincing evidence for a potentially beneficial clinical effect of IVIG for the therapy of TBE needs to be provided by the direct demonstration of the efficacy *in vivo* (Planitzer et al., 2007). In our experiments, mice were inoculated with a lethal dose of TBEV. No control mice or mice treated with IVIG lacking neutralizing levels of TBEV-specific antibodies survived the virus challenge. By contrast, 90% of TBEV-infected mice treated with the higher TBEV neutralization titer IVIG lot survived the otherwise lethal TBEV challenge. These results demonstrated a good correlation between the *in vitro* and *in vivo* IVIG neutralization activity. Similarly to our study, Planitzer et al. (2007) showed a good correlation between WNV neutralization as measured by a functional *in vitro* assay and protection against an otherwise lethal WNV infection *in vivo* and suggested that the *in vitro* assays can be useful in revealing important functional parameters of individual IVIG lots.

To enter the brain, immunoglobulins must cross the blood-brain barrier (BBB). Using mouse model, it was demonstrated that a significant fraction of systemically administered IVIG enters the brain through a saturable transport across the BBB. This suggests that therapeutically relevant IVIG concentrations can be reached in cerebral tissues even in the absence of BBB disruption (St-Amour et al., 2013). It was found that TBEV infection induces considerable breakdown of the BBB in mice (Růžek et al., 2011), and the integrity of the BBB is also damaged in severe cases of TBE in humans (Palus et al., 2014; Chekhonin et al., 2002; Kang et al., 2013). Breakdown of the blood–brain barrier thus allows increased access of IVIG into the brain.

The use of specific anti-TBEV immunoglobulins for TBE therapy was discontinued in Europe due to ADE concerns. However, in Russia, specific immunoglobulins are still in use and the immunotherapy ensures high level of protection (Pen'evskaia and Rudakov, 2010). ADE of flavivirus infection, with an exception for dengue (Pinto et al., 2015), is still without a clear proof *in vivo*, and a subject to debate (Kreil and Eibl, 1997). For TBEV, ADE has been demonstrated *in vitro* in infected macrophages (Kopecký et al., 1991; Phillpotts et al., 1985). However, it was demonstrated that the same antibodies that were able to enhance TBEV replication in mouse peritoneal macrophages *in vitro* were protective against lethal TBEV infection in mice (Kreil and Eibl, 1997). Neither sublethal TBEV challenges nor suboptimal dilutions of the immunoglobulins, even if applied together, could provide any indication of ADE occurring *in vivo* (Kreil and Eibl, 1997). In the present study, we did not see any indications for ADE caused by IVIG containing TBEV-specific antibodies at neutralizing or sub-neutralizing levels in both human neural cells *in vitro* as well as in TBE murine models *in vivo*. Even if the ADE phenomenon might occur in rare cases due to the exclusive administration of specific anti-TBEV immunoglobulins, it is likely that the ADE effect may be suppressed by the immunomodulatory effects of the high amounts of generic immunoglobulins contained in the IVIG preparations.

In summary, this study shows that (i) individual IVIG lots, even from the same manufacturer, have significantly different content of TBEV-specific antibodies, (ii) IVIG lots containing TBEV-specific antibodies effectively neutralize the virus in vitro as well as in mice, while IVIG lots without TBEV-specific antibodies have no TBEV-neutralizing activity (iii) IVIG lots lacking TBEV-specific antibodies exhibit no immunomodulatory effect leading to improved survival of TBEV-infected mice, and (iv) no ADE of TBEV infectivity was observed after treatment with IVIG, regardless on the amount of TBEV-specific antibodies present in the preparation.

Based on the results of the present study, we can conclude that IVIG lots with high TBEV-specific antibody titers might represent a useful post-exposure prophylaxis or first-line effective therapy in TBE. These findings are particularly important for patients with high risk of severe TBE, including those with primary immunodeficiency or those with failed anti-TBEV vaccination, who depend on the presence of protective levels of antibodies present in IVIG preparations.

Conflict of interest

None.

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Summary and future perspectives:

Scientific interest in arboviruses has a long tradition. Based on historical descriptions, tick-borne encephalitis (TBE) had been referred to by various local designations, including Russian spring-summer disease, Far Eastern encephalitis, Taiga encephalitis, Central European encephalitis, Kumlinge disease, Biphasic milk fever, Czechoslovak encephalitis and Frühsommer meningoencephalitis (Kunz & Heinz, 2003). References to the existence of the disease date back to 18th century Scandinavia; medically the disease was first described in the early 1930s by the Austrian physician H. Schneider (Schneider, 1931). Most of the foundation research on the disease was carried out in Russia, the former Czechoslovakia and Austria. In Europe, the virus was first isolated in 1948 in the former Czechoslovakia by Dr. Gallia, who isolated the virus from an infected patient and from the *Ixodes ricinus* ticks. This suggested that the tick served as a vector for the disease, just as the *Ixodes persulcatus* tick had been found to do in Far Eastern Russia (Gallia, Rampas & Hollender, 1949).

Arbovirology has also a long history at the Academy of Sciences in České Budějovice, Czech Republic. The study of the pathogenesis of tick-borne encephalitis virus (TBEV) was brought to the Academy by Dr. Doubravka Málková in the 1960s, originating from her work at the Military Institute of Hygiene, Epidemiology and Immunology. Dr. Málková's research focused on studying the entry of the virus into the host, looking at local replication and spread of TBEV to the lymph nodes, which was followed by induction of viremia (Málková & Fraňková, 1959), (Málková & Kolman, 1964), (Málková & Filip, 1968). In addition, Dr. Málková's team described the immune reaction to TBEV infection in mice in terms of temporary leucopenia and evaluated immune cell type proportions during viremia in mice (Málková, Pala & Sidak, 1961). The ecological and epidemiological interaction of vector and TBEV was studied by Dr. Rudolf Benda and Dr. Vlasta Danielová together with Dr. Milan Daniel (Danielová, 1990), (Daniel et al., 2011). Based on this knowledge, Dr. Jan Kopecký and Dr. Libor Grubhoffer concentrated further on vector and host interactions that affect the host immune system (Kopecký & Staňková, 1998), (Kopecký & Kuthejlová, 1998), (Kopecký, Kuthejová & Pechová, 1999), (Kovář,

Kopecký & Říhová, 2001), and on improving detection methods by monoclonal antibodies dealing with potential antigenic epitopes and the host cell receptor, which is utilized by the virus (Kopecký et al., 1991), (Grubhoffer, Kopecký & Tomková, 1992). Moreover, they described the effect of antibody dependent enhancement of TBEV in cultured mouse macrophages (Kopecký, Grubhoffer & Tomková, 1991). Finally, the Laboratory of Arbovirology was established under the supervision of Dr. Daniel Růžek, who extended the research focus to include perspectives derived from clinical work, molecular biology and phylogenetics.

My PhD thesis, built on collaboration with my colleagues, follows these foundations. Despite great interest in and comprehensive study of the subject to date, many questions concerning TBEV have not yet been answered. The submitted work, in collaboration with my colleagues, aims to shed light on the clinical aspects of TBE including pathogenesis and immune reaction to the virus infection. It was made possible with the help of various approaches, including primary cell cultures, animal models and, thanks to collaboration with České Budějovice Hospital, data and samples from patients suffering from TBE.

Following on the historically important findings of Dr. Málková's team on the impact of a host's immune reaction to TBEV infection, the research contributing to this thesis has confirmed the impaired cell count of mononuclear and polynuclear cells in cerebrospinal fluid (CSF) and serum, together with age-related physiological changes of blood-brain barrier integrity in CSF of TBE patients (**CHAPTER I**; Elsterová et al., in prep.).

The age of a TBE patient is one of several negative predispositions for the severity of the disease (Kaiser & Holzmann, 2000), (Logar et al., 2006), (Lenhard et al., 2018), (Elsterová et al., in prep.). Our research has shown that this is linked to the decreased fitness of BBB and further to a probable increase in the immunopathological reaction in terms of a higher production of intrathecal antibodies in CSF. Therefore, since locally synthetized intrathecal antibodies are the main B-cell byproducts, the immunopathological potential should be taken into account due to their cytotoxic effect. Age also corresponds to the alteration of a patient's immune status, where altered cell migration and cell function might profoundly affect the severity of TBE (Shah & Mooradian, 1997), (Montgomery, 2016). This is supported by the fact that elderly patients had

lower infiltration of cells to CSF in our study as well as in other published research (Logar et al., 2006), (Elsterová et al., in prep.).

In another of our studies, age arose as an important factor in impairing various host immune regulators during TBE. Younger patients had lower levels of GM-CSF (granulocyte macrophage colony stimulating factor) and MCP-1 (macrophage chemotactic protein-1) than older TBE patients. This suggests a stronger downregulation of innate immune responses in younger patients than older ones (Palus et al., 2015). On the other hand, increased MCP-1 production corresponded with disease severity in mice (Palus et al., 2013). In addition, increased MCP-1 and GM-CSF were found in the brains of mice during the late phase of neuronal progress of TBE (Formanova Pokorna et al., 2019).

Nevertheless, TBE patients had lower concentrations of GM-CSF and MCP-1 levels in serum than healthy control groups (Palus et al., 2015). These findings were further analysed with a special focus on the kinetics of cytokine and chemokine production by a study carried out by my colleagues *in vitro* on human cell lines of neural origin and *in vivo* on mice (Formanova Pokorna et al., 2019). The roles of both factors are critical for homeostasis and immune reaction in CNS. MCP-1 plays an essential role in several peripheral and CNS-inflammatory disorders that are characterized by mononuclear cell infiltrates (Thompson, Karpus & Eldik, 2008). GM-CSF, together with the macrophage colony stimulating factor, are the principal microglial growth factors (Cosenza-Nashat et al., 2007).

Our research has found a strong proinflammatory immune reaction in terms of elevated levels of cytokines in TBE patient serums, such as IL-6 (interleukin 6), IL-8 and IL-12 (Palus et al., 2015). These cytokines promote the recruitment of neutrophils and T cells into CNS and balance the immune response to Th1 type response. Moreover, these cytokines induce the expression of MMP9, which was shown to have increased in TBE patient serums in our study (Palus, Žampachová, et al., 2014) and confirmed in CSF (Kang et al., 2013). MMP-9 together with its inhibitor, TIMP-1, plays an important role in the functioning of BBB, which might be associated with the pathogenesis of TBE, since higher levels of MMP-9 and TIMP-1 lead to BBB disruption in neurodegenerative diseases (Rosenberg, 2009).

Moreover, increased levels of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) were found in TBE patient serums compared to healthy controls. Elevation of HGF functions as mitogen and morphogen and its increased levels have been found in CNS injury (Nayeri et al., 2000), (Tsuboi et al., 2002). VEGF has been shown to enhance MMP9 activity (Valable et al., 2005), which has association with disease severity in TBE patients (Kang et al., 2013), (Palus, Žampachová, et al., 2014). VEGF is also derived by astrocytes, causing BBB disruption in CNS inflammatory disease (Argaw et al., 2012), where it plays a role in neuroinflammation during TBEV infection (Palus, Bílý, et al., 2014).

To understand the penetration of the virus into CNS in greater depth, the work contributing to this thesis characterized different human primary cells of the neurovascular unit in terms of TBEV infection (**CHAPTER II**). BBB is a physical barrier between the blood stream and the brain parenchyma and is composed of brain microvascular endothelial cells which form so-called tight junctions (Cardoso, Brites & Brito, 2010). Post mortem studies of CNS damage in TBE patients have revealed changes in the formation of perivascular infiltration and degradation of neurons, which are the primary targets of TBEV (Gelpi et al., 2005), (Gelpi et al., 2006), (Hirano et al., 2014), (Bílý et al., 2015). These are influenced by other cells within the microvascular unit such as astrocytes, which are the source of pro-inflammatory molecules causing neuroinflammation and neurodegeneration (Ramesh, Maclean & Philipp, 2013). Moreover, they control water and ion homeostasis at the vessel–neuron interface. As shown in our research and that of others, astrocytes are capable of a persistent TBEV infection, producing various proinflammatory cytokines and chemokines (Palus, Bílý, et al., 2014), (Potokar et al., 2014), (Formanova Pokorna et al., 2019), (Potokar, Jorgačevski & Zorec, 2019). However, even microvascular endothelial cells are capable of a persistent infection, though in relatively low numbers. Our *in vitro* model further confirmed the crossing of TBEV through BBB without compromising its integrity, which suggests a transcellular pathway as the TBEV entry to CNS via BBB (Palus et al., 2017). Similar results were obtained during infections from other flaviviruses (Verma et al., 2010), (Papa et al., 2017). To fully understand the effect of TBEV infection on BBB, the role of various cell types of the neurovascular unit – such as

microglia as the resident immunocompetent cells of the brain and pericytes as regulators of endothelial transcytosis and regulators of astrocyte polarization – should be further studied to complete the framework of the TBEV neuroinvasion and the role of BBB (Abbott et al., 2010), (Armulik et al., 2010).

In response to the work presented in this thesis, it is arguable whether a host's immune reaction to TBEV infection is profitable for the host, or whether the immunopathology overcomes immune reaction benefits and is in fact one of the agents of the spread of the virus to CNS and the neurological sequelae. There is no targeted therapy for treating TBE at this time. As there has been an increasing number of case reports on the successful treatment of arboviral infections with human pooled intravenous immunoglobulins (IVIGs), our team tested the immunomodulatory effect of IVIG on an infection arising from TBEV (**CHAPTER III**, (Elsterová et al., 2017)), (Růžek, Dobler & Niller, 2013). An important factor in the use of IVIG is the specific combination of antibodies, which differs geographically (Rabel et al., 2012). We used two lots of IVIG from the same manufacturer and the levels of TBEV-specific antibodies differed significantly. We did not observe the nonspecific immunomodulatory effect of IVIG on TBEV replication *in vitro* in human cell lines of neural origin and *in vivo* on mice, but we demonstrated a strong neutralization of TBEV by IVIG containing specific anti-TBEV antibodies.

There is a concern about antibody dependent enhancement for the use of specific immunotherapy in TBE patients, since there have been reports of severely pronounced neurological complications after specific cases of immunotherapy in childhood TBE (Kluger et al., 1995), (Waldvogel et al., 1996). With the exception of secondary DENV infection or crossreactivity of DENV and ZIKV infection (Littaua, Kurane & Ennis, 1990), (Dejnirattisai et al., 2016), (Katzelnick et al., 2017), antibody dependent enhancement of flavivirus infection does not have any systematic *in vivo* proof and is therefore still under debate. However, the benefits derived from the use of IVIG as described in severe flavivirus encephalitis case reports outweigh the outcomes of experiments on mice models, which study the immunomodulatory effect of these substances in comparison to the specific therapeutic benefits of antibodies contained in IVIG (Kreil & Eibl, 1997), (Ben-Nathan et al., 2009),

(Srivastava, Ramakrishna & Cantin, 2015), and the outcomes of clinical randomized studies, which have not proven this effect (Rayamajhi et al., 2015), (Gnann et al., 2019).

A more targeted antibody therapy is currently a popular topic for the treatment of various viral infections. Eliciting a neutralizing antibody response is a goal of many vaccine development programs and commonly correlates with protection from the specific disease. Neutralizing antibodies with the most protective functionalities may be a rare component of a polyclonal, pathogen-specific antibody response, which further complicates efforts to identify the specific elements of a protective immune response. In spite of these challenges, a successful effort for identifying potent highly specific neutralizing antibodies has been carried out in terms of viral and also flaviviral infections (Burton et al., 2012), (Escolano et al., 2016), (Robbiani et al., 2017), (Young et al., 2020). To elucidate the potential of neutralizing antibodies as a therapy for TBE, we screened antibody responses in TBE patients and selected individuals with a high response in collaboration with Prof. Robbiani from The Rockefeller University, New York, and our collaborators from České Budějovice Hospital. Based on data from selected TBE patients, we intend to select potent neutralizing clones of antibodies and to test them for their potential therapeutic effect. The anticipated results could be used further to study flavivirus crossreactivity and reaction to antibody dependent enhancement.

In response to proposed results of current clinical research including findings mentioned in the thesis, it is important to emphasise that each TBE patient requires an individual approach, since the host's genetic background may play a role in the resultant pathological effect of TBE in *in vivo* experiments (Palus et al., 2013), (Palus et al., 2018). Different polymorphisms in immune regulating genes have been proposed as risk factors for the development of clinical TBE, having an impact on the severity of TBE (Barkhash et al., 2010), (Mickiene et al., 2014), (Barkhash et al., 2016). However, comprehensive clinical work on the study of a genome-wide search in TBE patients is still needed.

To improve the clinical status of a TBE patient, timely and specific diagnostic tools are required. Current diagnosis is based on the specific immune response

by detecting specific IgM and IgG antibodies and the clinical and anamnestic status of the patient suffering from TBE, since the viral genome is mostly undetectable once neurological symptoms occur (Reusken et al., 2019). There has been some research, however, that suggests the detection of viral nucleic acid even during the second phase of TBE (Saksida et al., 2005), (Nagy et al., 2018), (Veje et al., 2018). It is important to question the conditions of virus multiplication and antibody responses in individual patients suffering from TBE. The presence of the virus, or viral products, and specific antibodies in urine is still under the discussion and represents a potential noninvasive diagnostic tool. In our future work, we will concentrate on the overall clinical picture and hope to propose new insights for the diagnostics of TBE and its prevention.

The following future tasks build on the work presented in this thesis:

- using an *in vitro* model to characterize the role of each cell type that forms a microvascular unit, such as the role of pericytes within BBB disruption during TBEV infection, or the role of microglia and its immunomodulatory cytokine and chemokine production during TBEV infection.

- expanding the collaboration with colleagues from České Budějovice Hospital and University Hospital Brno, focussing on improving the diagnosis of TBE by using noninvasive methods and specifying their methodology, as currently the main diagnostic tool is a serological examination of specific IgM and IgG antibodies and the patient's clinical status. The nuclear acid of the virus together with specific antibodies (anti-envelope protein E and nonstructural protein NS1 of TBEV) will be examined in patient specimens. Moreover, a retrospective survey will examine the epidemiological situation in an endemic area such as South Bohemia.

- following the clinical study of antibody response in TBEV patients in collaboration with Dr. Robbiani from the Rockefeller University, we will continue to research elite neutrilizing specific antibodies, which have the potential to act as therapeutics. Moreover, flavivirus antibody enhancement and antibody crossreactivity will be studied, together with a comparison of immune responses in vaccinated individuals and TBE patients.

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