Chapter 6

Pathogenesis of TBEV-diseases

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Key points

- In this chapter we describe the pathogenesis of tick-borne encephalitis virus (TBEV).
- To cause infection, TBEV needs to cross three different barriers; the physical, the innate and adaptive and the blood-brain barrier.
- TBEV transmission at the skin interface is pro-inflammatory with a marked increase in immune cell infiltrates at the tick-feeding foci.
- The trigger of innate immune and adaptive immune responses, by TBEV is necessary to clear the infection.
- TBEV employs different strategies to evade the innate immune response.
- Both different animal models and reverse genetics will help us understand TBEV pathogenesis.

Transmission and entry:

Tick vectors and tick -host interface

The *Ixodes ricinus* tick serves as the primary carrier of TBEV-Eu in nature, while the *Ixodes persulcatus* tick is the primary vector for TBEV-Sib and TBEV-FE.[1] I. ricinus is widely spread across Europe, reaching into Turkey and northern Iran, whereas *I. persulcatus* is found in the Urals, Siberia, Far-Eastern Russia, as well as parts of China and Japan.[2,3] A zone of sympatry exists in the northern Baltics, western Finland, and northwestern Russia, where the habitats of *I. ricinus* and *I. persulcatus* overlap, leading to the presence of multiple TBEV subtypes.[3-5] TBEV is maintained within natural transmission cycles involving ixodid ticks and wild-living mammalian hosts. Infected ticks are presumed to remain infected throughout their life cycle.[2] While transovarial transmission of TBEV from an infected female tick to the egg mass is possible, this mode of infection is not entirely efficient in sustaining TBEV within the natural tick population.[6]

The transmission of tick-borne encephalitis virus (TBEV) from an infected tick to a host involves a complex interplay between the tick's feeding process and the immunomodulatory properties of its saliva. This process begins shortly after the tick attaches itself to the host. TBEV is transmitted to the vertebrate host along with the tick's saliva as early as one hour after the tick attaches[7] and POWV is transmitted as fast as 15 minutes after attachment.[8] Tick feeding is a sophisticated process, and successful feeding is facilitated by various components present in the tick's saliva, which possess immunomodulatory properties. Notably, tick salivary factors not only aid in blood feeding but also modulate the host environment, thereby promoting the transmission and establishment of TBEV.[9]

Seminal studies conducted by Labuda et al. (1993) demonstrated the significance of saliva-assisted transmission (SAT) of TBEV.[10] They observed that when naïve guinea pigs were inoculated with a mixture of TBEV and salivary gland extract (SGE) obtained from partially fed uninfected female ticks of species like *Ixodes ricinus*, *Dermacentor reticulatus*, or *Rhipicephalus appendiculatus*, and subsequently, uninfected *Rhipicephalus appendiculatus* nymphs fed on these guinea pigs, there was an increased acquisition of the virus by ticks feeding on animals inoculated with the mixture of SGE and virus compared to those inoculated with the virus alone. This research underscores the crucial role of tick saliva in facilitating the transmission of TBEV and sheds light on the mechanisms involved in the transmission dynamics between ticks and hosts. Observations of pathogens being transmitted from infected ticks to uninfected ticks co-feeding on the same host have offered indirect evidence of what is known as "sequential acquisition of tick-borne pathogens," as noted by Nuttall and Labuda in 2004.[9] It is also referred to as co-feeding transmission. In natural environments, it's common for infected ticks to co-feed alongside uninfected ticks on a single host. Labuda et al. conducted experiments where TBEV-infected *I. ricinus* ticks and uninfected ticks co-fed on naïve, natural host species. Intriguingly, they found that the highest numbers of TBEV-infected ticks originated from susceptible host species with very low levels of viremia, providing compelling evidence that non-viremic co-feeding transmission of TBEV is a primary mechanism for maintaining the virus in natural foci.[11,12]

Tick-host-virus interface during TBEV transmission:

Skin acts as the primary barrier against various forms of damage, including mechanical stress, environmental

factors, and potential infections. It serves as the frontline defense between a tick and its host, making it the first point of contact for both TBEV and tick saliva during feeding. Throughout the feeding process, a tick's mouthparts and saliva interact with the host's blood and lymphatic vessels, as well as various cellular components such as fibroblasts, keratinocytes, Langerhans cells, dendritic cells, macrophages, mast cells, natural killer cells, T lymphocytes, and soluble mediators like cytokines, chemokines, complement proteins, and lectins.[13] These cutaneous immune cells play a pivotal role in initiating the host's immune response and inflammatory reactions against tick feeding and potential pathogen transmission.

The significance of skin infection in the transmission of TBEV is paramount. Skin acts as the primary interface where these viruses establish infection in the host.[9] Labuda et al. thoroughly investigated the initial stages of TBEV replication within the skin of two natural host species: bank voles (*Clethrionomys glareolus*) and yellow-necked field mice (*Apodemus flavicollis*). Their experimental setup mirrored natural conditions, with infected and uninfected Ixodes ricinus ticks placed on specific areas of the host's skin. Their findings revealed a correlation between TBEV detection in feeding ticks and the transmission dynamics from infected to uninfected ticks.[14] Additionally, TBEV exhibited a preference for skin sites where ticks were actively feeding. To characterize TBEV-infected cells, Labuda et al. infested laboratory mice with TBEV-infected ticks and cultured skin explants from the infestation sites. They observed the migration of leukocytes from these explants, with viral antigens present in migrating Langerhans cells and neutrophils, indicating their role in viral dissemination.[14] In vitro studies suggest that dendritic cell populations at the tick feeding site are among the early targets of TBEV infection. Recent research indicates that exposure of bone marrow-derived dendritic cells to tick saliva enhances TBEV replication, partly through activation of the pro-survival Akt pathway.[15]

These results underscore the importance of localized skin infection in the early transmission of the virus from infected ticks and its acquisition by uninfected co-feeding ticks.[11,16] Immune cells infiltrating the skin during tick feeding act as carriers for virus transmission between co-feeding ticks, independent of systemic viremia.[14] Langerhans cells, the primary dendritic cell population in the epidermis, likely play a crucial role in virus dissemination, as evidenced by their migration to draining lymph nodes in response to cutaneous infections with other arthropod-borne viruses.[17] Thus, the presence of TBE viral antigen in emigrating Langerhans cells suggests their involvement in transporting TBEV to the lymphatic system, contributing to overall viral dissemination. The importance of virus-infected cells at the tick feeding site and their contribution to initial viral replication and dissemination was further supported by in vitro experiments where *I. ricinus* tick saliva was shown to modulate TBEV infection of dendritic cells. Specifically, when DCs were cultured with TBEV in the presence of *I. ricinus* saliva, the infection rate of the cells was enhanced and there was a decrease in virus-induced TNF- alpha and IL6 production.[18]

A study conducted by Thangamani et al. explored the immune response in the skin to TBEV infection. The study involved allowing TBEV-infected ticks to feed on mice, followed by biopsies of the bite sites at one and three hours post-attachment for RNAseq transcriptome and histochemical analysis. The analysis revealed upregulation of various cytokines (Ccl2, Ccl12, Cxcl1, Cxcl2, Cxcl5, IL6, and IL10) and receptors (CCR1, CCR5, and Sell) after just one hour of TBEV-infected tick feeding, indicating an early activation of the inflammatory response and an increase in immune cell accumulation at the attachment site.[19] Immunohistochemical analysis further confirmed the inflammatory microenvironment at the feeding site, showing an influx of inflammatory cells, especially neutrophils, within one hour of TBEV-infected tick feeding. Among these, TBEV antigens were localized in fibroblasts and mononuclear cells, but not in neutrophils.[19] These findings suggest that TBEV-infected ticks induce rapid inflammation at the cutaneous interface, potentially affecting the transmission of flaviviruses to hosts. This study contributes to our understanding of the early immunological events during tick-borne flavivirus transmission, emphasizing the significance of localized skin infection in this process (Figure 1). Together these studies illustrate the important role of localized skin infection during the early stages of tick-borne flavivirus transmission.

Neuroinvasion and neurotropism:

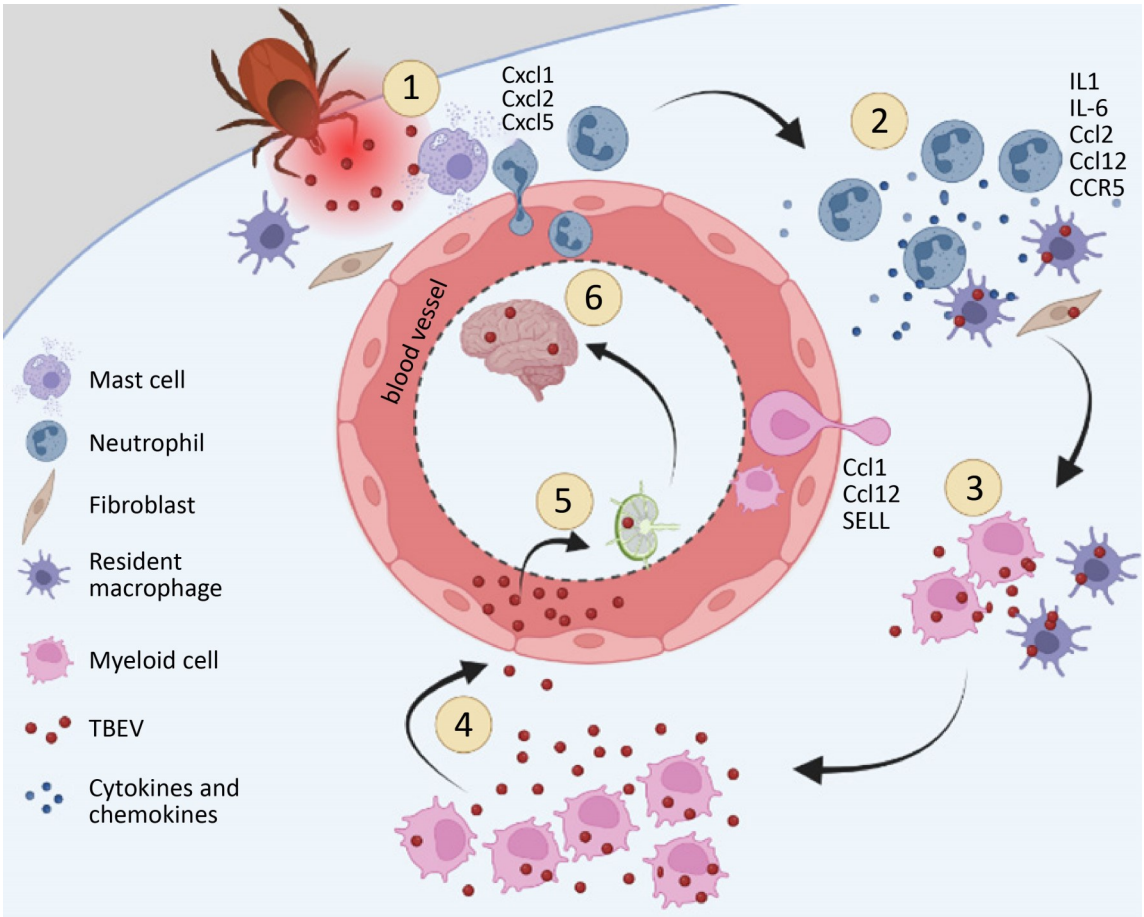
Crossing the brain barriers

It is generally believed that neurotropic flaviviruses can invade the CNS by two main routes; the peripheral nervous system or the hematogenous route via the blood. However, the molecular mechanisms governing the neuroinvasion of TBEV and related tick-borne flaviviruses are not yet clear.

Entry via the peripheral nerves

Some viruses uses the spinal cord to enter the CNS,[20,21] however, during experimental infection of TBEV (strain Torö) and LGTV in mice the spinal cord and brain stem are the last infected areas after sub cutaneous (SC) and intraperitoneal (IP) infection respectively.[22,23] On the other hand, POWV (LB strain) showed spinal cord infection as early as 4 days post-infection and thereafter a caudal to rostral spread within the brain after high viral dose.[24] Indicating that neuroinvasion might depend on the specific virus strain used and the experimental setup. Another report with TBEV (Sofjin) infected mice showed that the autonomic nerves running from the myoenteric plexus were

Figure 1: Proposed overview of the early transmission events of TBEV



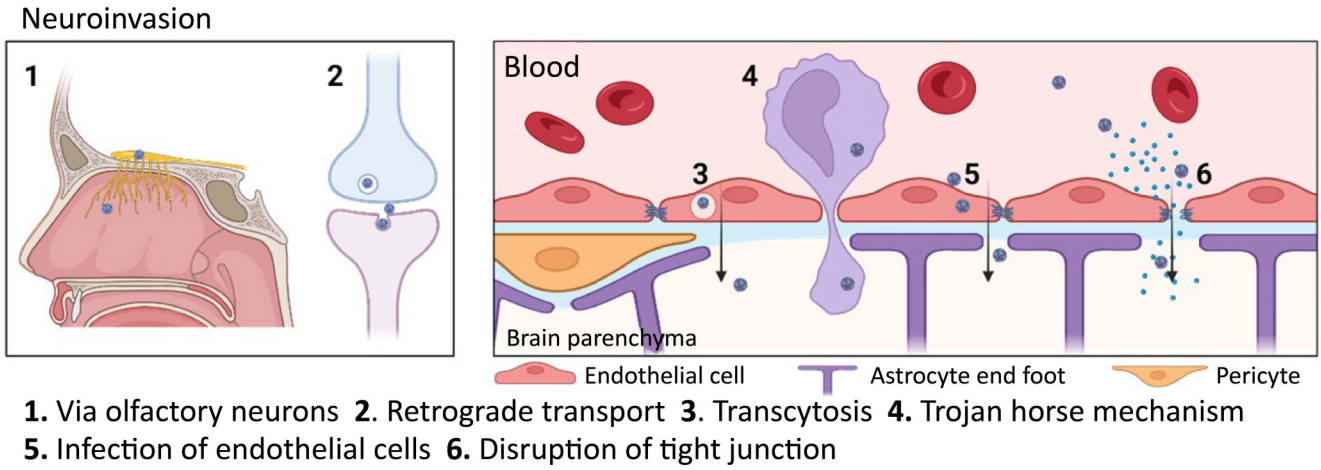
(1) TBEV is transmitted during tick feeding along with tick salivary factors. Mast cells are degranulated as soon as ticks initiate feeding leading to the influx of neutrophils; (2) Release of chemoattractant to recruit immune cells and TBEV establishes infection in permissive cells such as resident fibroblasts, macrophages, and other phagocytes; (3) infiltrating myeloid cells becomes infected with TBEV; (4) replication of TBEV in myeloid cells and release of infectious virus into the blood stream; (5) dissemination of TBEV to the lymphatic tissues; (6) dissemination and establishment of infection in brain. The infographic was generated using Biorender (www.biorender.com).

infected as well as the intestine and intestinal lymph nodes after intravenous infection (IV).[25] There is direct signaling between the gut to the brain via enteroendocrine cells of the mouse gut that form synapses with vagal neurons[26] that may facilitate virus entry. The involvement of the gastrointestinal tract as an important site of infection is supported by the many cases of alimentary TBEV.[27-30] However, in mice the oral route of infection is rather ineffective even in highly immunocompromised interferon alpha receptor (IFNAR) knock out mice[31]. Infection using oral gavage (with feeding needle) is even less efficient.[31] This indicate that the acid environment of the stomach is preventing viral infection, and that the TBEV maybe more likely to establish infection in the mouth or throat. Another possible mechanism for neuroinvasion is via the olfactory sensory neurons in the olfactory bulb. We have seen that the olfactory bulb is the first site of infection after both TBEV (Torö) and LGTV (TP21) after IP and SC infection.[22,32] Also supporting this hypothesis is the reported laboratory-acquired infection with TBEV after high titer exposure of aerosols.[33] However, since a bi-phasic disease course was observed in this case report it indicates viremia before neuroinvasion,[33] and other studies in mice have shown that intranasal infection of mice are less efficient route of infection compared to IP and SC,[31,34] thus neuroinvasion via the olfactory neuron seems less likely for TBEV and LGTV.

Hematogenous route of neuroinvasion

The second plausible route of neuroinvasion is the hematogenous via the blood brain barrier (BBB). The BBB is a very tight barrier that separates the blood from the brain parenchyma and the main function is to prevent free diffusion and toxic molecules to enter the brain. The BBB is lining all capillaries in the brain and to prevent permeability and leakage the endothelial cells have tight junctions. These include the claudines and occludin, which are joined to the cytoskeleton by cytoplasmic proteins, such as zonula occludens (ZO).[35] Lining the endothelial cells are the pericytes and end-feet from nearby astrocytes, and the

Figure 2: Overview of possible routes of TBEV neuroinvasion



The infographic was generated using Biorender (www.biorender.com).

crosstalk between endothelia, pericytes and astrocytes are important to preserve the integrity and function of the barrier. For long it was believed that the breakdown of the BBB was important part of neuroinvasion for TBEV as TBE patients show disruption of the BBB.[36-38] However, virus is detected the brains of mice days before disruption of the BBB,[34,39] and BBB leakage is likely caused by the inflammatory response elicited by the virus in the brain. Microvascular endothelial cells are often used in vitro to mimic the BBB, and infection of these with TBEV (Hypr, Neudoerfl) does not increase permeability or change the key tight junction proteins. Instead the cells become persistently infected and secrete high titers of virus in both directions,[40] indicating that TBEV can cross the BBB via a transcellular pathway without changing permeability. In a more complex in vitro model consisting of both human brain endothelial cells and pericytes POWV (LI9, LI41 linage 2 and LB linage 1) infects both cell types persistently and secrets POWV to the lower chamber without changing the permeabilization.[41] However, no in vivo experiments have verified infection in the vascular endothelial cells of the BBB. Using single nuclei RNA sequencing Chotiwan et al. recently showed that in the cortex of wt mice the pericytes were infected with LGTV but not endothelial cells.[42] The reason for this discrepancy might be that different viral strains and mammalian models were used. Transcytosis is when virus is transported through the cell without productively infecting them. Evidence of transcytosis in vivo through endothelial cells and pericytes has only been shown for Japanese encephalitis (JEV) by electron microscopy.[43] Virus could also traffic through the BBB via so called "Trojan horse" mechanism, where virus infected immune cells infiltrate into the brain. However, even though virus infect different immune cells in the periphery, more research is needed to understand the trafficking behavior of infected cells.[44]

Alternatively, the virus may enter the brain via the blood CSF barrier through the choroid plexus (ChP). ChP is located in the ventricles of the brain and is composed of a monolayer of epithelial cells that contain tight junctions. This epithelial layer rests in a basal lamina surrounding and enclosing a central stroma where dendritic cells, fibroblasts and macrophages can be found. The blood endothelial cells within the ChP central stroma is leaky, thus, the cellular movement of molecules and cells within the CP stroma is not restricted. Both, Zika virus and LGTV have been shown to infect the ChP in vivo, ZIKV targets the pericytes and LGTV targets the ciliated epithelial cells.[34,42,45] However, these observations were made in IFNAR knock out mice and not in WT immunocompetent mice, making these observations difficult to translate into TBEV and human situation. Other factors contributing to neuroinvasion in POWV are, the presence of tick saliva,[24] active replication in macrophages and prolonged viremia, as resistant mice although with similar peak viremia as susceptible mice clear POWV in the periphery.[46]

TBEV tropism in the brain

Viral tropism in the brain is determined by several different factors. First the cellular entry receptor is important for binding and viral entry into cells. For TBEV[47] and LGTV[48] only one entry receptor has been identified, T-Cell Immunoglobulin and Mucin Domain 1 (TIM-1), however it is not likely to be the only one as mice and cells were still susceptible in its absence.[47] We have also seen that cellular tropism of infected wt and IFNAR deficient mice with LGTV is markedly different independent of base line expression of the different brain cells,[42] indicating that host factors, innate immune response and cellular crosstalk are very important for shaping the cellular tropism in the brain.

After neuroinvasion TBEV targets mainly large neurons of the anterior horns, medulla oblongata, pons, dentate nucleus, Purkinje cells, and striatum in humans.[49] Neurons in thalamus, cortex, and Purkinje cells in cerebellum are the main target for TBEV (Hypr) in mice.[50] In POWV lineage-1 the main infected areas are brain stem and spinal cord, and the involvement of spinal cord ventral horn and the brain stem might be the cause of the flaccid paralysis in the mice. Infection can also be detected in the cortex, hippocampus and Purkinje cells in cerebellum.[51] In LGTV infected rats the virus also infects the Purkinje cells, in addition to infection of midbrain, hippocampus, thalamus and frontal lobe.[52] LGTV infection in mice on the other hand does not target the Purkinje cells in the cerebellum but rather excitatory neurons in the entorhinal cortex of the cerebrum.[42] Showing that the experimental systems used are very important. The type I IFN response seem to have a major impact on the cellular tropism in vivo. For LGTV, Lindman et al. showed that RIPK3 is important specifically to restrict infection of the granular cell neurons in the cerebellum. This because it is necessary for upregulation of IFNAR expression and thus upregulation of antiviral Interferon stimulated genes (ISGs).[53] We have shown that both the specific cells and the areas infected with LGTV in the brain is dependent of type I IFN response.[42] In wt mice the excitatory neurons in gray matter of the cerebrum specifically in the entorhinal cortex and audio cortex were infected. Whereas in the absence of IFNAR the tropism shifted to ciliated epithelial cell of the choroid plexus in the ventricles, meninges, and microglia in the white matter tracts of the olfactory.[42] The reasons for this dramatic shift in cellular tropism between the mice are likely to be that the cross talk between cells in the brain, and infiltration of immune cells (CD8 T cells expressing IFNγ) into the brain that activates microglia in WT mice by upregulating CCR1. In the absence of IFNAR the crosstalk between cells are blunted, immune cells are not recruited to the brain, and microglia, which expresses high levels of TIM-1 (Human Protein Atlas), are unable to become activated and thus are susceptible to infection.[42]

Several in vitro studies have shown that primary astrocytes from rat and mouse can be infected with TBEV and they survive and produce virus over many days,[54,55] however, in mice TBEV (Hypr) and LGTV is rarely detected in astrocytes.[42,50] We have also seen that primary mouse astrocytes cultured in vitro become very susceptible to TBEV (Hypr, Aina and Sofjin) in the absence of IFNAR signaling,[56] however, astrocytes are not susceptible in IFNAR knock out mice in vivo,[42] indicating that viral tropism studies should be conducted in vivo not in vitro, as cellular tropism of TBF depends on much more than only the entry receptor.

Immune response to TBEV:

Type I interferon response

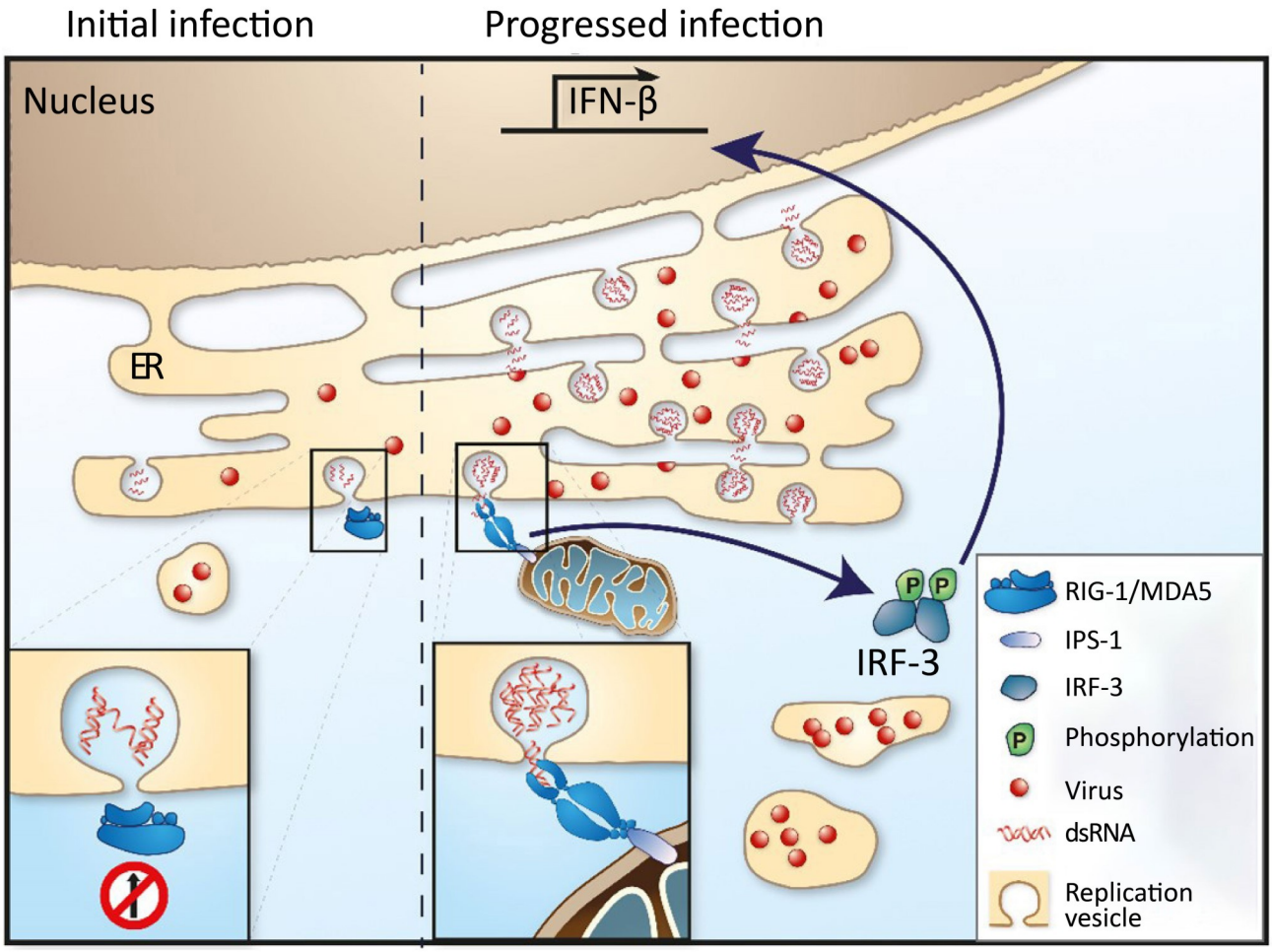
The type I IFN system is the first line of defense against viral infection and an important part of the intrinsic innate immune response that controls virus dissemination and protects against serious disease. This response rapidly detects invading pathogens and upregulates inhibitory effector proteins and cytokines to ensure survival. The detection of pathogens is based on recognition of the non-self pathogen-associated molecular pattern (PAMP) by specific host sensors, the pattern recognition receptors (PRR). This leads to a signaling cascade and the upregulation and secretion of IFN.[57] IFN is a large family of cytokines where the IFNα and -β are type I IFNs and IFNγ is type II IFNs and these are the most studied. Type I IFNs binds to the IFNα receptor (IFNAR), which is expressed on nearly all cell types, in a paracrine and autocrine manner. The IFNAR is composed of a heterodimer of IFNAR1 and IFNAR2. After binding of IFN, the IFNAR activates the Janus kinases, Jak1 and Tyk2, which then phosphorylate the signal transducer and activator of transcription (STAT)-1 and STAT2 proteins, resulting in activation and translocation of the IFN-stimulated gene 3 (ISGF3) transcription factor complex into the nucleus. This ISGF3 induces hundreds of IFN stimulated genes (ISGs), that encode proteins with diverse biological function and some are potent antiviral proteins and part of the response against mammalian viruses.[57]

Recognition of TBEV and induction of IFN

Rapid detection of the pathogen is crucial for mounting a protective response, and several different PRR families have been identified that recognize numerous ligands. The Toll-like receptors (TLRs) are located on the endosome or the plasma membrane, and the retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs) are in the cytosol. RNA viruses are most likely recognized by TLR3, TLR7, TLR8, or the RLRs (RIG-I and melanoma differentiation-associated gene 5, MDA5), which senses single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA).[58-60]

For TBEV, it is not totally clear which PRRs are dominant. RIG-I, which recognizes short dsRNA and 5’ PPP, has been shown to be important for IFNβ induction in the U2OS (human osteosarcoma) cell line by siRNA depletion,[61] and as MDA5 has been shown to be antagonized by prM of TBEV (Far Eastern subtype) preventing its recruitment to MAVS thus inhibiting IFN upregulation,[62] indicating that both are important for sensing. Both RIG-I and MDA5 bind to the adaptor mitochondria-associated IFNβ promoter stimulator-1 (IPS-1, also called MAVS, VISA or CARDIF) via its caspase recruitment domain after binding to its RNA ligand.[63] IPS-1 is important for IFNβ induction after TBEV (Hypr) infection in mouse embryonic fibroblasts (MEFs); in its absence, no

Figure 3: Viral evasion of IFN induction



TBEV induces vesicles in the Endoplasmatic Reticulum (ER) where the viral RNA synthesis occurs. Early during infection, these vesicles protect the dsRNA from cellular detection by RIG-I and/or MDA5. Later in infection, high amounts of virus particles are produced and the dsRNA leaks out of the vesicles. The pattern recognition receptors (PPRs) RIG-I and/or MDA5 then trigger signalling through IPS-1, phosphorylated IRF3 dimers are transported into the nucleus and IFN-β is upregulated.[64,73]

IFNβ was detected.[64] In addition, mice deficient in IPS-1 succumb to LGTV and TBEV (Hypr) infection earlier. These mice showed lower systemic levels of IFNα, resulting in higher viral titers in the periphery and leading to rapid invasion in the CNS.[23] IPS-1 is also important in the local IFN response within the brain, reducing viral load and spread of LGTV,[23,65,66] indicating an especially important role for RLR in the type I IFN response.

Upon IPS-1 activation, TNF Receptor Associated Factor 3 (TRAF3), TANK Binding Kinase 1 (TBK1) and Inhibitor-κB kinase ε (IKKε) are recruited, leading to phosphorylation and activation of the transcription factor IFN regulatory factor 3 (IRF3). Phosphorylated IRF3, dimerizes and translocate into the nucleus where it binds to the IFNβ gene promoter to initiate transcription and translation.[67,68] IFNβ induction after TBEV infection has been shown to be highly dependent on IRF3 activation in the cells, and IRF3 has been shown to dimerize and translocate into the nucleus after TBEV infection.[64] However, in vivo type I IFN upregulation is not dependent on IRF3 but on IRF7 in the periphery, and IRF7 plays an important role in the CNS to control infection.[69]

Since the type I IFN response is so important in controlling and restricting viral replication, most viruses have developed strategies to prevent upregulation of IFN by antagonizing the different steps in the IFN induction pathway.[74-76] For TBEV (Far Eastern subtype) the prM was recently identified to prevent interaction and signaling between MDA5 and MAVS.[62] TBEV also employ a passive escape mechanism that delays the induction of IFNβ by replicating inside replication vesicles or packets, thereby hiding its dsRNA from RIG-I and other PRRs (Figure 3).[61,64,73,77] Later, during infection, the dsRNA leaks out from the replication vesicles, IRF3 is activated and translocates into the nucleus to transcribe IFNβ, which then is translated and secreted. Thus, the virus is produced and released from

the cell before IFNβ can trigger an antiviral response in neighboring cells (Figure 3).[64,73] Interestingly, different cell types respond to infection in different ways with different kinetic. Primary mouse astrocytes have a very fast type I IFN response and secret IFNs that can protect, astrocytes and primary cortical neurons in culture already 3 to 6 h post infection,[56] and also co-cultured neurons.[78]

Type I IFN signaling and response against TBEV

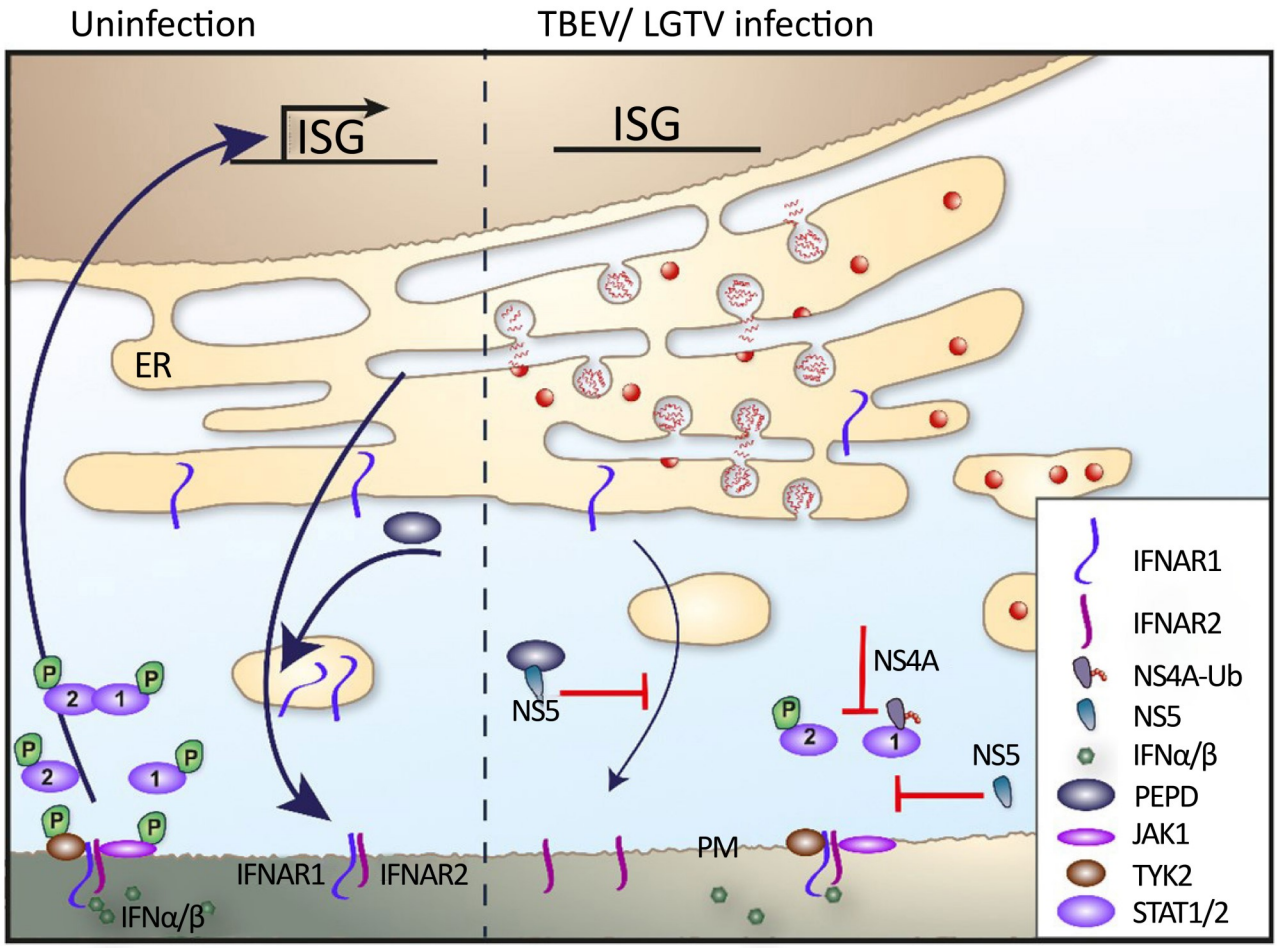
After infection and secretion of IFN, the IFN binds to its receptor the IFNAR1/2 which stimulates the upregulation of hundreds of ISGs that can limit the infection. The ISGs encode for PRR, adaptors and transcription factors to ensure a rapid response after infection. Cytokines and chemokines are also produced which activate and recruit immune cells to limit the infection, as well as antiviral proteins that can target viral replication directly in the cell.[79] The IFNAR is therefore a key molecule in the type I IFN response. The importance of this molecule has been demonstrated for many viruses. For LGTV the type I IFN response determines tropism and can protect mice from lethal infection. In the absence of this response, the virus replicates uncontrollably in all organs, induces a rapid opening of the blood-brain barrier, and the mice succumb very quickly. This research also has shown that IFNAR is important in all cell types; hematopoietic, stroma, neuroectodermal and cells in the periphery.[34]

Most steps in the viral "life" cycle are targeted by 1 or several antiviral proteins encoded by the ISGs. Several ISGs have been identified to have antiviral effect on TBEV the Interferon-induced transmembrane proteins (IFITMs) 1, 2, 3, the rodent tripartite motif (TRIM) protein, TRIM79α, and viperin (virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible).[80-82] Although all three IFITM proteins are antivirally active IFITM3 is the most potent one and can protect against virus induced cell death, and IFITM proteins are most effective against cell free virus and not against cell to cell virus spread.[80] The antiviral mechanism of TRIM79α is direct targeting of the viral polymerase, the non-structural protein 5 (NS5), an essential component of the replication complex, for lysosomal degradation. TRIM79α seems to be specific for TBEV and LGTV, because mosquito-borne flaiviviruses; WNV and Japanese encephalitis virus (JEV), were shown not to be restricted by this protein.[81]

Viperin, on the other hand, is a highly conserved protein with broad spectrum antiviral activity, which has been shown to restrict a diverse range of viruses from different families. For the Flaviviridae family, viperin restricts hepatitis C, DENV, WNV and TBEV. However, the antiviral mechanism seem to depend on the specific virus. For TBEV, viperin selectively target the positive stranded RNA synthesis. The intracellular location to the ER via viperins N-terminal amphipathic alpha helix is important as it coincides with viral replication. The antiviral activity is depending on the radical S-adenosyl methionine (SAM) domain and the proper iron-sulphur maturation of the protein.[82,83] Recent studies have identified several viral and cellular interaction partners to viperin.[32,83-87] Viperin is able to target TBEV in multiple ways mediating antiviral activity in a cell type-specific manner. Viperin interacts with several TBEV proteins; prM, E, NS2A, NS2B and NS3. The interaction between NS3 and viperin results in proteasome-dependent degradation of NS3[86]. The stability of prM, E, NS2A and NS2B are affected by viperin, but only in the presence of NS3.[86] Interestingly, although viperin do not directly interact with the TBEV C protein, viperin expression induce C particle formation and release from virus infected cells and disturbing the assembly process of TBEV.[87] Viperin mediates this effect by interacting and sequestering the cellular protein Golgi brefeldin A-resistant guanine nucleotide exchange factor 1 (GBF1),[87] which is involved in the vesicular trafficking of the secretory pathway[88,89] and is a pro-viral factor for many different viruses.[90-93] Thus, viperin may target other viruses via its interaction with GBF1. The in vivo importance of viperin during TBEV infection was recently shown in the viperin-/- mice .[32] This study show that specific regions of the brain rely differentially on the antiviral activity of viperin for protection against LGTV. Viperin is important in the olfactory bulb and cerebrum, while viral replication were unchanged in cerebellum and brain stem in the absence of viperin. This effect is due to the different neuronal subtypes, viperin expression is very important in cortical neurons but not at all in granular cell neurons isolated from the cerebellum.[32] Looking at polymorphisms in human TBE have identified several ISGs associated with TBE disease for example Interferon Induced Protein With Tetratricopeptide Repeats 1 (IFIT1),[94] 2'-5'-oligoadenylate synthetase (OAS)2 and OAS3.[95,96]

Even though different ISGs can potently restrict TBEV replication if induced before infection,[56,81,82,98] IFN treatment after infection has limited effect in vitro.[98] The reason for this is the expression of an IFN antagonist, NS4A[100] and NS5.[98,99] TBEV NS4A blocks the phosphorylation and dimerization of STAT1/STAT2 to reduce the type I and type II IFN-mediated signaling.[100] The NS5 protein of LGTV interferes with the phosphorylation of Jak1 and Tyk2 in response to IFNβ, which leads to failure of STAT1/2 phosphorylation and subsequent ISG expression.[98,99] Werme et al. showed that the interaction between Scribble and NS5 is important for plasma membrane targeting and IFN antagonist activity; however, the exact target of NS5 is unclear.[99] In addition, NS5 was shown to block IFN signaling by selectively reducing the level of IFNAR1 expression on the cell surface. This reduction was dependent on NS5 binding to prolidase. Prolidase is needed for IFNAR1 intracellular trafficking, maturation, activation of IFNβ-

Figure 4: Interferon signaling and inhibition



The active IFN receptor is composed of 2 subunits, IFNAR1 and IFNAR2. Prolidase (PEPD) is required for IFNAR1 maturation and intracellular trafficking to the plasma membrane (PM). Once IFNα/β binds to the IFNAR1/2, JAK1 and TYK2 becomes phosphorylated, which then results in phosphorylation of STAT1 and 2. This leads to dimerization of STAT and a signaling cascade that results in upregulation of ISG expression (left panel). In TBEV- and LGTV-infected cells (right panel) the IFN antagonist NS5 binds to PEPD, thus preventing IFNAR1 transport to the PM, and IFNα/β signaling.[97] NS5 also interferes with JAK1, TYK2, and STAT1 phosphorylation upon IFNα/β stimulation, thereby inhibiting ISG production.[98,99] Ubiquitinated NS4A binds to STAT1 and prevent STAT1/STAT2 dimerization and phosphorylation.[100]

stimulated gene induction, and IFN-I-dependent viral control (Figure 4).[97] The relationship between NS5 function and virulence has not been observed for tick-borne flaviviruses, such as TBEV and the low virulence LGTV NS5; both exhibited the same degree of p-STAT inhibition. However, there are most likely other viral proteins that are important for pathogenicity and suppression of innate immune responses, as this has been shown for other flaviviruses. However, for TBEV these mechanisms have yet to be identified.

Adaptive immune response against TBEV

Humoral immunity is an important component of the immune response. As with other flaviviruses, a functional humoral immune response is critically important in controlling infections.[101] Depleting B cells with immunosuppressive treatment of Rituximab lead to severe and fatal TBE.[102] On the other hand, passive transfer of monoclonal or polyclonal TBEV-specific antibodies protects mice in vivo and protection correlates with in vitro neutralization.[103-107] No infectious virus could be detected in the blood or brain of passively protected mice subsequent to TBEV challenge. However, in a vaccination study the antibodies response protected against disease but did not from neuroinvasion, as viral RNA was detected in the CNS.[50] However, antibodies protect not only by neutralization; therefore, because limited virus replication does occur, this indicates that mechanisms of protection from disease exist other than sterilizing immunity.[108]

In addition to effective humoral immunity, the activation of cellular immunity is usually required for clearance of established infection. Distinct T cell subsets play a key role in the induction of protective immune response against TBEV infections. CD4+ T cells are essential in priming the

TBEV-specific antibody response and sustaining the CD8+ T cell response.

For more details about the interplay between TBEV and the humoral immune response, cellular immune response, and different innate immune cells please visit Chapter 7 Immunology of TBEV infection by Zens and Ackermann-Gäumann.

Tools to study pathogenesis:

Overview of relevant animal models

Animal models are pivotal in comprehending the pathogenesis, transmission dynamics, and potential interventions for tick-borne encephalitis virus infection. An optimal animal model should closely emulate the human condition in terms of disease symptoms and underlying mechanisms. Tick-borne viruses exhibit minimal host specificity due to ticks' feeding habits, which vary as they mature and can encompass hosts of various sizes or species without preference. Humans typically become infected incidentally when ticks venture beyond their natural habitats or human ventures into the habitat of ticks. The diverse array of hosts that ticks can feed on renders many tick-borne viruses amenable to investigation using laboratory animals.

Both large and small animal models have been utilized to explore the fundamental aspects of TBEV infection, disease progression, and neuropathogenesis. Early investigations in sheep resulted in a better understanding of the differential neurovirulence and pathogenesis of TBEV.[109] Several species of non-human primates, such as Macaca mulatta (rhesus macaques), Cercopithecus aethiops (African Green monkeys), Macaca fascicularis (Crab-eating macaques), Macaca cynomolgus, and Macaca sylvanus, have been employed to study TBE neuropathogenesis. Though non-human primate models do not mimic human clinical outcomes, they are a good model to understand TBEV infections and to evaluate vaccine efficacy.[110-113]

Small mammals such as Syrian golden hamsters,[114] moles[115] have been used to understand TBEV pathogenesis and disease progression. However, they show reduced susceptibility. Laboratory mice such as ICR, C57BL/6 or BALB/c mice serve as a promising animal model for advancing research into the mechanisms underlying tick-borne virus infections and their pathogenesis.[22,116-120] Due to their closer phylogenetic relationship with humans and notable genomic similarities, especially evident in knock-out mice, where specific genes are deleted to elucidate mammalian genetic factors in infection and disease progression, they offer valuable insights.[23,119] Mice are susceptible to TBEV isolates, resulting in fever and neurological symptoms resembling human encephalitis. Histological examination of infected mice has unveiled substantial brain inflammation and damage, aligning with clinical manifestations observed in human cases.[116,117,119,120]

Kurhade et al. (2018) used C57BL/6 mice to characterize the pathogenesis of TBEV isolated from 2 different transmission foci.[22] The investigators compared the neuroinvasiveness, neurovirulence, and immune response of two European strains (HB171/11 from Germany and Toro-2003 from Sweden) in mice, uncovering distinct differences that enhance our understanding of TBEV pathogenesis. The HB171/11 is low virulent tick isolate from a focus where TBE patients only show gastrointestinal and constitutional symptoms.[121] The Torö-2003 strain is an infectious clone from an island where 32 neurological TBE cases[122] occured. The strain HB171/11 was found to be a low virulent phenotype with low or delayed neuroinvasiveness, and the Toro-2003 strain was found to be highly pathogenic.[22]

In addition, mice have also been used to investigate viral genetic determinants of infection and pathogenesis, and E protein, NS2B, NS3, NS5 protein, and the variable region of the 3' untranslated region have been shown to be important for determining pathogenicity in mice.[118,122-127] However, more studies are needed to fully understand the reason for the different clinical outcomes. Some strains of TBEV and POWV have been suggested to become persistent or chronic however, the mechanism is not clear, but it is interesting that in experimental models of TBEV and related viruses, the virus RNA is found in the brain of rodents[128-132] and in non-human primates[110,113,133,134] for a long time even in the absence of severe disease in the acute phase, although it is not clear if the virus RNA is infectious.

The variety of animal models utilized in research on TBEV underscores the comprehensive strategy needed to grasp and fight this virus, with mice being pivotal in revealing the mechanisms of infection and the progression of the disease.

Reverse genetics systems

Reverse genetics of viruses is the generation and manipulation of viral genomes to investigate the direct effects of changes on virus biology and pathogenesis. For flaviviruses, the first reverse genetic system was developed in 1989 for YFV.[135] Since the genome of flaviviruses is positive stranded, they are infectious if introduced into susceptible cells.[136] There are several different approaches to generate infectious virus. One important step is the generation of a complementary DNA (cDNA) to the RNA genome. The cDNA is often cloned into a plasmid under a specific promoter, which enables the in vitro transcription of viral RNA. This DNA clone enables the introduction of mutations into the genome, and subsequent analysis of the resulting phenotype. Reverse genetics have been used to study virulence, replication, host range, vaccines, and functions of the coding and non-coding regions. However, these clones are laborious and difficult to generate due to

instability and toxicity of some viral sequences in bacteria.[137]

For TBEV 2 separate approaches were used in the beginning; plasmid-based infectious clones[138] and the PCR based methods for constructing recombinant virus.[139,140] Both rely on *in vitro* transcription and transfection of RNA. The most recent technique for generating TBEV clones is the infectious-subgenomic-amplicon (ISA) method. Three PCR amplicons are produced that have a CMV promoter at the 5' non-coding region (NCR) and 70-100 bp overlapping regions; the hepatitis delta ribozyme is followed by the simian virus 40 polyadenylation signal. The amplicons are mixed and introduced into the cells where they recombine and produce infectious virus.[141]

Infectious clone systems have been very useful in studying determinants of replication and biological characteristics as well as to identify pathogenicity factors of TBEV. Two advantages of this approach are that the genome is defined and can be manipulated. In contrast, natural viral isolates of positive stranded RNA viruses are present as a population of different viral types also called quasispecies. This is due to the error prone RNA dependent RNA polymerase. In addition, manipulating natural viral isolates with specific mutagenesis inducing drugs is a very nonspecific approach.

With this technique, several determinates of pathogenicity have been identified. Specifically, the envelope protein responsible for receptor mediated entry,[126] the function of the membrane protein in virus budding,[142] and the importance of different regions in the 3'NCR. Neurovirulence in mice was shown to be dependent on specific amino acid residues in the upper lateral surface of domain III in the envelope (E) protein of TBEV (residues E308, E310 and E311), possibly due to disruption of the receptor binding.[126] The residues S267L, K315E, N389D in LGTV E protein and K46E in the NS3 protein, were shown to be crucial for neuroinvasiveness in immunodeficient mice.[143] The 5' and the 3' NCR contain complementary sequences that help genomic cyclization to form panhandle structures. The NCRs have several conserved structural stem loops that are important for replication, translation initiation and packaging.[144,145] At the beginning of the flavivirus 3' NCR, a secondary structure forms a pseudoknot that protects the terminal 300 to 500 bases from exoribonuclease XRN1 degradation, generating a subgenomic flavivirus RNA (sfRNA).[146-148] The sfRNA has been shown to be critical for WNV induced cytopathic effects[149] and pathogenicity in mice,[149] and is involved in viral subversion of type I IFN response by a yet unknown mechanism.[150] The TBEV sfRNA has been shown to specifically interfere with the RNAi system of ticks.[151] The 3' NCR of TBEV can be divided into a highly conserved core element and a variable region that is both heterogenic in length and sequence.[152] Several European TBEV strains contain an internal poly(A) tract in the variable region of the 3' NCR, which was considered dispensable for replication and virulence in mice.[127,153] However, studies recently showed that the variable region and the poly(A) tract can modulate virulence of the Far Eastern TBEV.[123,154] We have also detected different lengths of the poly(A) tract in a blood feeding tick indicating that the poly(A) might be important for the switch between invertebrate to vertebrate.[155] To investigate this further a long poly(A) Torö-38A and a TBEV Torö with a short poly(A) were cloned and rescued. We were able to show that the viruses with long poly(A) were attenuated in cell culture but more virulent in mice compared with the short poly(A), and the genome with short poly(A) was much more stable compared with the long version, which developed a high quasispecies diversity.[122]

Ongoing challenges and areas for future investigation

Important advances in the identification of molecular and cellular mechanisms of TBEV-induced pathogenesis have been made in recent years. Skin is the interface between a feeding TBEV-infected tick and a host; consequently, the cutaneous immune cells likely play a crucial role in virus transmission. In the earliest stages of TBEV-infected tick feeding, a complex, inflammatory micro-environment exists in the mammalian host's skin, with increased recruitment, migration, and accumulation of Langerhans cells, mononuclear phagocytes, and neutrophils. The dynamic secretion of tick salivary factors at the infected tick feeding foci modulates the cutaneous micro-environment to facilitate TBEV transmission, establishment, and dissemination from the skin to the terminal organs. However, many unanswered questions remain about the function of immune cells at the feeding site of a TBFV-infected tick. Modern single-cell and spatial transcriptomics techniques will allow us to investigate these early transmission events. They will enable us to understand immune processes at a single-cell level. In addition, gaps exist in our current understanding of the dissemination of viruses from the skin to the central nervous system. A better understanding of the virus transmission, establishment, neuroinvasion, dissemination and cellular tropism within the brain will allow us to develop novel countermeasures to prevent TBEV transmission, treat TBEV infections, and reduce disease burden. The interactions between the virus and the innate and adaptive immune response are not fully understood. The use of reverse genetics, specific knock out mouse models, new technologies like whole brain imaging, single cell sequencing and spatial transcriptomics will greatly advance our understanding of TBEV pathogenesis in the future.

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