MINIREVIEW

The role of viral persistence in flavivirus biology

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This is an excellent comprehensive review on flaviviruses.

Abstract

In nature, vector-borne flaviviruses are persistently cycled between either the tick or mosquito vector and small mammals such as rodents, skunks, and swine. These viruses account for considerable human morbidity and mortality worldwide. Increasing and substantial evidence of viral persistence in humans, which includes the isolation of RNA by RT-PCR and infectious virus by culture, continues to be reported. Viral persistence can also be established *in vitro* in various human, animal, arachnid, and insect cell lines in culture. Although some research has focused on the potential roles of defective virus particles, evasion of the immune response through the manipulation of autophagy and/or apoptosis, the precise mechanism of flavivirus persistence is still not well understood. We propose additional research for further understanding of how viral persistence is established in different systems. Avenues for additional studies include determining whether the multifunctional flavivirus protein NS5 has a role in viral persistence, the development of relevant animal models of viral persistence, and investigating the host responses that allow vector-borne flavivirus replication without detrimental effects on infected cells. Such studies might shed more light on the viral–host relationships and could be used to unravel the mechanisms for establishment of persistence.

Introduction

Defining mechanisms of viral persistence will be critical for understanding vector-borne flavivirus infections. These viral infections account for considerable human morbidity and mortality worldwide. Furthermore, incidence is increasing and infections are being appreciated in previously nonendemic geographic locations. Prominent vector-borne flaviviruses (VBFVs) associated with significant human infections include both tick-borne and mosquito-borne agents. The tick-borne flaviviruses (TBFVs) are exemplified by the tick-borne encephalitis virus (TBEV) sero-complex group, Omsk hemorrhagic fever virus, Kyasanur forest disease virus, Alkhurma virus, Powassan virus (POWV), and deer tick virus (DTV), the latter two occurring in the United States (Holbrook *et al.*, 2005; Brackney *et al.*, 2008b; Ebel, 2010). The mosquito-borne flaviviruses (MBFVs) are perhaps better known and include yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and dengue virus (DENV) serotypes 1–4. Infections with all of these viruses can lead to severe disease, prolonged debilitating neurological sequelae, hemorrhagic fever, and/ or death in some cases (Solomon *et al.*, 1998; Glass *et al.*, 2002; Haglund & Gunther, 2003; Madden, 2003; Van Gerpen, 2003; Carson *et al.*, 2006).

Viral persistence is a hallmark of the ecology of VBFVs. Both TBFVs and MBFVs are cycled between arthropod and vertebrate hosts (Figs 1 and 2), and in many cases, they are maintained without deleterious effects on the hosts. In nature, TBFVs, such as POWV and TBEV, alternately infect small vertebrates such as rodents, hares, some carnivores, and a range of hard-bodied (ixodid) ticks, although recent evidence suggests that the soft-bodied *Ornithodoros* (argasid) ticks can also support TBFV (Rajagopalan *et al.*, 1969; Charrel *et al.*, 2007). Similarly, MBFVs, such as WNV and JEV, primarily alternate in nature between small mammals, birds, and mosquitoes (Fig. 1). In addition, there is evidence that MBFV and TBFV persistence also occurs in humans, and persistence in cell culture is well documented (Poidinger *et al.*, 1991; Lancaster *et al.*, 1998; Bugrysheva *et al.*, 2001; Farfan-Ale *et al.*, 2009; Murray *et al.*, 2010).

Although the hosts for the VBFVs are highly varied, the genomic and structural organization of the viruses
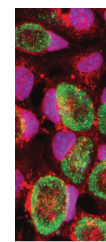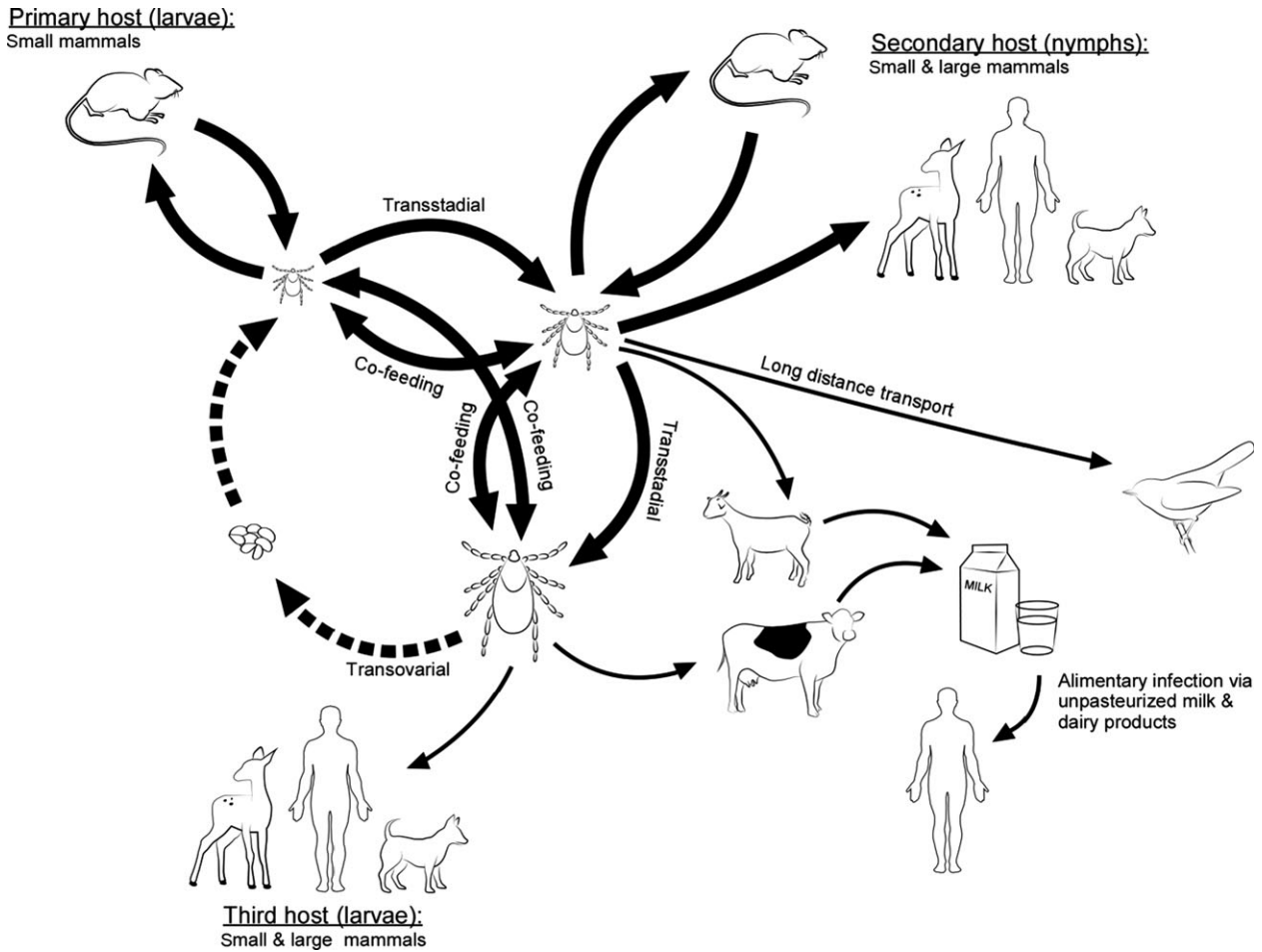
Fig. 1 Flavivirus maintenance and transmission cycle in ticks and vertebrate hosts. Ticks are crucial for viral persistence as they remain infected once they acquire viral infection. Infected ticks are capable of transmitting TBFVs to other ticks when they feed in close proximity on the same animal, as well as the different stages of the tick life cycle. TBFVs persist also in a cycle between small mammals (e.g. rodents) and the ticks that feed on them. Large mammals and humans tend to be incidental, dead-end hosts.
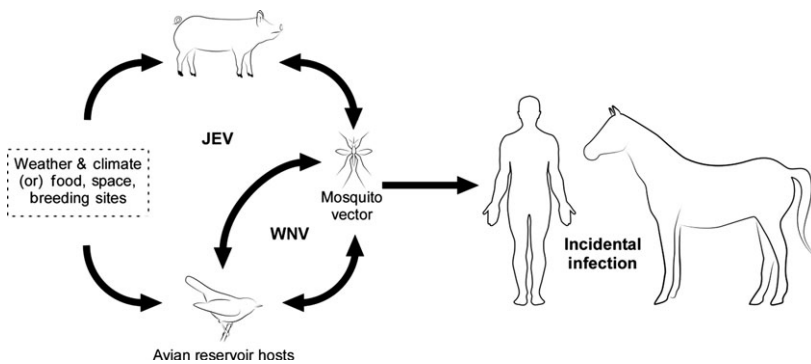

Fig. 2 A representation of a mosquito-borne flavivirus amplification and transmission cycle. WNV is cycled between the mosquito and avian hosts that play an amplification and maintenance role. Swine are important amplifying hosts for JEV, and mosquitoes that acquire blood meals on infected pigs can become infected and transmit the virus. Similar to TBFVs, MBFV infection in humans and large animals, such as horses, is accidental.

themselves is remarkably similar. All flaviviruses are spherical, enveloped particles that contain a genome of (+) ssRNA measuring approximately 11 kb. The genome also functions as mRNA and encodes a single polyprotein that is cleaved into 10 proteins (Table 1). Reasonable progress has been made toward understanding how these viruses replicate (Chambers *et al.*, 1990; Mandl, 2005; Miorin *et al.*, 2008; Tuplin *et al.*, 2011; Pierson & Diamond, 2012; Brinton, 2013; Pierson & Kielian, 2013). We present a simplified overview of the general aspects of a VBFV replication cycle in Fig. 3. The individual proteins are three structural proteins: capsid (C), precursor membrane/mem-

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Table 1 The flavivirus proteins, untranslated regions (UTRs), and their known functions

			Defined functions of viral proteins and untranslated regions of genome	
Flavivirus protein	Nucleotide*, weight, aa	Location in cell	Mosquito-borne flavivirus (MBFV)	Tick-borne flavivirus (TBFV)
5′ UTR	1–132		The 5′ UTR contains conserved RNA stem loops (SL), *cis*-elements located upstream and downstream of the AUG region (UAR and DAR, respectively), and complementary sequence (CS) sponsoring cyclization of the genome by interacting with the 3′ UTR to form a 'panhandle' (Alvarez *et al.*, 2005; Villordo & Gamarnik, 2009; Friebe & Harris, 2010; Friebe *et al.*, 2011). The 'panhandle' structure is essential for certain steps in the flavivirus replication cycle such as replication/translation and viral assembly (Gritsun & Gould, 2007b). SL1-2 or (SL-A and SL-B) is located within the 5′ UTR, whereas the SL3-4 is extended and situated in the N-terminal of the open reading frame ORF (Alvarez *et al.*, 2005; Lodeiro *et al.*, 2009; Villordo & Gamarnik, 2009; Friebe & Harris, 2010; Friebe *et al.*, 2011). The SL1 structure is essential for viral replication and acts as a promoter which is targeted initially by NS5 and then delivered to the 3′ end via cyclization (Filomatori *et al.*, 2006; Zhang *et al.*, 2008; Lodeiro *et al.*, 2009)	Although there is low nucleotide conservation between flaviviruses and different CS homology (Hahn *et al.*, 1987), the 5′ UTRs of TBFVs share the same genomic organization as the MBFVs (Kofler *et al.*, 2006)
Structural proteins Capsid (C)	133–468, 11 kDa, 114 aa	Cytosol/nucleus	The capsid protein is a dimeric alpha-helical (Jones *et al.*, 2003) protein and assembles into an icosahedral structure, measuring 30 nm in diameter, which initiates encapsidation of the associated genomic RNA in virus-induced membrane invaginations of the ER (Welsch *et al.*, 2009). The C protein is also a pro-apoptotic factor in various cell lines (Yang *et al.*, 2002, 2008; Oh *et al.*, 2006; Netsawang *et al.*, 2010; Morchang *et al.*, 2011). C of JEV was reported to limit viral neurovirulence (Mori *et al.*, 2005)	The C-encoding nucleotide sequence of TBEV contains conserved RNA structures that function as replication enhancer elements (Tuplin *et al.*, 2011)[†]
Precursor membrane (prM)	469–972, 26 kDa, 165 aa	ER lumen	During virion assembly, the prM forms a heterodimer with the E protein and acts as a chaperone for correct E protein folding (Kuhn *et al.*, 2002; Lorenz *et al.*, 2002; Zhang *et al.*, 2003). In the final step of virion maturation in the trans-Golgi network prior, to viral release, the precursor is cleaved by furin and only the C-terminal region (M) is retained in the viral membrane (Heinz *et al.*, 1994; Elshuber & Mandl, 2005; Lindenbach *et al.*, 2007). prM protein of JEV also limits viral neurovirulence (Kim *et al.*, 2008)	[†]

Table 1 (continued)

Flavivirus protein	Nucleotide* weight, aa	Location in cell	Defined functions of viral proteins and untranslated regions of genome	
			Mosquito-borne flavivirus (MBFV)	Tick-borne flavivirus (TBFV)
Membrane (M)	748–972, 8 kDa, 90 aa			
Envelope (E)	973–2460, 53 kDa, 495 aa	ER lumen	The E glycoprotein consists of a soluble ectodomain, a stem region, and a transmembrane domain (Bressanelli *et al.*, 2004). The glycoprotein forms 'head to tail' homodimers that completely cover the surface of the mature icosahedral virions. It is also responsible for receptor binding, which leads to virus internalization by clathrin-mediated endocytosis (Lindenbach *et al.*, 2007). The E protein contains three β-sheets domains (DI–DIII): DII includes the membrane fusion peptide (Beasley & Barrett, 2002; Bressanelli *et al.*, 2004; Sukupolvi-Petty *et al.*, 2007); DIII is associated with receptor binding and is also a target for several neutralizing monoclonal antibodies. Crystal structures of the E protein from several flaviviruses all show similar secondary structure and domain organization (Modis *et al.*, 2003, 2005; Zhang *et al.*, 2004; Kanai *et al.*, 2006; Nybakken *et al.*, 2006). The E protein is important in determining neuroinvasiveness and neurovirulence for several MBFVs (Cecilia & Gould, 1991; Hiramatsu *et al.*, 1996; Ni & Barrett, 1996; Sanchez & Ruiz, 1996; Chambers *et al.*, 1998; Beasley *et al.*, 2004; Lee *et al.*, 2004, 2006; Bordignon *et al.*, 2007). For WNV, JEV, and DENV, mutations in the E protein (located in the lateral surface of DIII and the base of DII) affect fusion and receptor binding with target cells (Lee *et al.*, 2004)	The E protein was first described in 1995 and later has been extensively defined and crystalized for many other flaviviruses (Rey *et al.*, 1995). TBFV E protein is important for determining neurovirulence and neuroinvasiveness (Holzmann *et al.*, 1990; McMinn, 1997; Beasley *et al.*, 2005; Mandl, 2005; Engel *et al.*, 2010)
Nonstructural Proteins NS1	2461–3516/46–55 kDa/325 aa	ER lumen/cytosol/ secreted	Low-resolution structural studies found the NS1 structure to be an open barrel configuration with D3 symmetry measuring 10 nm by 7.5 nm and with a central cavity approximately 4.5 nm in diameter (Gutsche *et al.*, 2011; Edeling *et al.*, 2014). All flavivirus NS1 genes share a conserved degree of homology. NS1 is found at different cellular locations such as vesicular compartments associated with the cell membrane or on the cell surface and is also secreted as an extracellular	†

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Table 1 (continued)

Flavivirus protein	Nucleotide* weight, aa	Location in cell	Defined functions of viral proteins and untranslated regions of genome	
			Mosquito-borne flavivirus (MBFV)	Tick-borne flavivirus (TBFV)
			hexamer composed of dimer subunits. In the viral replication cycle, the NS1 glycoprotein has role in early RNA replication where its association with NS4A is critical for formation of the replicase complex (RC; Westaway *et al.*, 1997; Lindenbach *et al.*, 2007). Each subunit forms a homodimer located in the ER lumen, and it has been shown to colocalize with viral dsRNA. Secreted and cell surface associated NS1 is immunogenic and induces an antibody response that can serve as an important diagnosis marker (Falgout *et al.*, 1990; Avirutnan *et al.*, 2006; Sun *et al.*, 2007). NS1 is also implicated in immune evasion functions as it activates human complement and induces host vascular leakage by specific inhibition of the classical and lectin pathways of complement activation. This occurs through a direct interaction with complement components C4 and C1s (Avirutnan *et al.*, 2010). DENV, WNV, and YFV NS1 are shown to limit C4b deposition and C3 convertase activity by enhancing cleavage of C4 through the recruitment of the complement-specific protease C1s (Avirutnan *et al.*, 2010). This effect might explain some of the clinical manifestations of dengue hemorrhagic fever and dengue shock syndrome	
NS2A	3517–4206, 19 kDa, 218 aa	Cytosol	The NS2A protein is highly hydrophobic and is involved in viral RNA replication and capsid assembly as it binds strongly to the 3′ UTR, NS3, and NS5 in the perinuclear regions of cells (Lindenbach *et al.*, 2007; Welsch *et al.*, 2009). Bioinformatic analyses on JEV subgroup revealed an internal RNA sequence and structure located at N-terminal of NS2A that might be important for ribosomal frame shift resulting a NS1of different size (NS1′) (Firth & Atkins, 2009; Firth *et al.*, 2010; Melian *et al.*, 2010). It is also suggested that the size different might be caused by different glycosylation pattern or differently cleavage in	†

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Table 1 (continued)

Flavivirus protein	Nucleotide* weight, aa	Location in cell	Defined functions of viral proteins and untranslated regions of genome	
			Mosquito-borne flavivirus (MBFV)	Tick-borne flavivirus (TBFV)
			NS2A (Blitvich *et al.*, 1995, 1999). Mutations within this pseudo-knot abolish the NS1', suggesting a role in viral neuroinvasiveness and attenuation in mice (Liu *et al.*, 2006; Melian *et al.*, 2010)	†
NS2B	4207–4599, 14 kDa, 130 aa	ER lumen/membrane bound	NS2B is a hydrophobic protein and acts as a cofactor for NS3. Together, they form a serine protease complex essential for processing the virus polyprotein (Chambers *et al.*, 1991; Falgout *et al.*, 1991; D'Arcy *et al.*, 2006; Erbel *et al.*, 2006)	
NS3	4600–6462/69 kDa, 618 aa	Cytosol	The NS3 protein structure is made up of two segregated globular domains (Assenberg *et al.*, 2009). NS3 is a highly conserved and multifunctional protein: The protein has protease activity in one domain, RNA-stimulated NTPase activity and RNA triphosphate activity (RTPase) in the other domain (Chambers *et al.*, 1991; Valle & Falgout, 1998; Lescar *et al.*, 2008). NS3 protein is an essential member of the RC and is activated in association with NS5 to bind the genomic RNA 3' SL prior to replication (Chen *et al.*, 1997; Mackenzie *et al.*, 1998; Lindenbach *et al.*, 2007). Beside its essential role in the viral replication cycle, NS3 can also induce apoptosis (Shafee & AbuBakar, 2003; Ramanathan *et al.*, 2006; Yang *et al.*, 2009; Yiang *et al.*, 2013). NS3 is involved in the inhibition of the transcriptional factor AP1 signaling pathway, which also directly affects apoptosis (Lin *et al.*, 2006). Studies on neurovirulence associated with DENV-1 NS3 have shown that mutations in NS3 (Leu480Ser) enhance the ability of replication in CNS causing leptomeningitis and encephalitis in mice (Bordignon *et al.*, 2007). This mutation was also shown to induce cell death in DENV-1-infected cells (Duarte dos Santos *et al.*, 2000). The NS3 protein can be targeted by antiviral drugs such as ivermectin, ribavirin as inhibitors of the ATPase activity of helicases (Mastrangelo *et al.*, 2012)	The protease domain of LGTV NS3 associates with caspase 8 and induces apoptosis (Prikhod'ko *et al.*, 2002)†

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Table 1 (continued)

Flavivirus protein	Nucleotide* weight, aa	Location in cell	Defined functions of viral proteins and untranslated regions of genome	
			Mosquito-borne flavivirus (MBFV)	Tick-borne flavivirus (TBFV)
NS4A	6463–6840, 16 kDa, 286 aa	ER lumen/membrane bound	The mature NS4A is a hydrophobic transmembrane protein and associates with most of the RC factors (NS1, NS2A, NS3, NS5, and dsRNA) (Lindenbach *et al.*, 2007). Its interaction with NS1 is essential for viral RNA replication as NS4A acts as a docking site (Bartenschlager *et al.*, 1995; Miller *et al.*, 2007). NS4A interacts with the polypyrimidine tract-binding protein (PTB), with the latter being implicated in translation, transcription, and viral processes for various viruses (Bieleski *et al.*, 2004; Florez *et al.*, 2005; Shi & Lai, 2005) including the role in regulation of HCV replication (Tischendorf *et al.*, 2004; Domitrovich *et al.*, 2005; Aizaki *et al.*, 2006; Chang & Luo, 2006). NS4A-PTB plays an indirect role in stabilization of the viral genome dsRNA intermediates by binding to host cell PTB protein in a similar manner as stabilizing WNV RNA synthesis by the elongation factor 1 alpha (Davis *et al.*, 2007; Jiang *et al.*, 2009). NS4A together with NS4B and NS3 is also responsible for inducing the rearrangement of the host ER membrane leading to the formation of virus-induced membranous spherules and vesicles encasing the dsRNA and RC; this is thought to minimize exposure of the replicating RNA to innate immune sensors such as retinoic acid-inducible gene-I, RIG-I (Mackenzie *et al.*, 1998; Roosendaal *et al.*, 2006; Miller *et al.*, 2007). The mature NS4A can also induce the PI3K-dependent autophagy signaling, which in turn lead to protection from camptothecin- and staurosporine-induced cell death (McLean *et al.*, 2011). The role of NS4A in the up-regulation of the autophagy and ER rearrangement makes the protein a potential target for antiviral drug development	†
2K	6841–6909, 2 kDa, 23 aa		Complete cleavage of the VBFV polyprotein generates an ER-spanning 2K peptide located between NS4A and NS4B (Lin *et al.*, 1993; Roosendaal *et al.*, 2006; Miller *et al.*, 2007). The 2K peptide functions as a signal peptide for NS4B and might play a role in enhancing viral replication by conferring resistance to lycorine, a suppressor of flavivirus RNA synthesis (Zou *et al.*, 2009)	†

Table 1 (continued)

Flavivirus protein	Nucleotide* weight, aa	Location in cell	Defined functions of viral proteins and untranslated regions of genome	
			Mosquito-borne flavivirus (MBFV)	Tick-borne flavivirus (TBFV)
NS4B	6910–7665, 27 kDa, 112 aa	ER lumen/membrane bound	Several studies have shown colocalization of NS4B/dsRNA with NS3, suggesting that NS4B plays a role in RC formation or function (Westaway *et al.*, 2003; Miller *et al.*, 2006). NS4B appears also to be involved in ER rearrangement and modification during viral replication (Westaway *et al.*, 1997). The NS4B protein of WNV, JEV, and DENV inhibits type I interferon (IFN-α/β) response by blocking the phosphorylation of STAT1 (Muñoz-Jordán *et al.*, 2003, 2005; Lin *et al.*, 2004; Liu *et al.*, 2005; Evans & Seeger, 2007). The same is true for KUNV and WNV strain NY99, which blocks the activation of STAT1/ STAT2 and its translocation to the nucleus (Liu *et al.*, 2005). These events disrupt the host immune responses by blocking the IFN-alpha/beta/gamma pathways	†
NS5	7666–10 374, 100 kDa, 900 aa	Cytosol/ER/nucleus	NS5 is the largest and most conserved among the VBFV proteins. NS5 primarily functions as the RNA-dependent RNA polymerase (RdRp) through its C-terminal domain (Lindenbach *et al.*, 2007). The NS5 has been crystalized and contains an N-terminal domain with S-adenosyl methionine methyltransferase activity (MTase), and possibly guanylyltransferase (GTase) for RNA capping (Egloff *et al.*, 2002; Malet *et al.*, 2007; Yap *et al.*, 2007). NS5 is also involved in activating other viral proteins, such as NS3 NTPase and RTPase activity in a dose-dependent manner. DENV NS5 contains both nuclear localization signal (NLS) and nuclear export signal (NES). These facilitate its importation into the nucleus by both importin β1 and the importin α/β complex, and an additional function of the signals could be enhancement of virus replication and hyperphosphorylation of NS5 (Johansson *et al.*, 2001; Brooks *et al.*, 2002). For WNV, NS5 is exported from the nucleus in a chromosome region maintenance 1 (CRM1)-dependent manner (Pryor *et al.*, 2007; Rawlinson *et al.*, 2009). NS5 is involved in different cellular pathways and has a crucial role in the escape from, or blocking, the interferon-alpha/beta (IFN-alpha/beta) signaling pathway. DENV NS5 inhibits host TYK2 and STAT2 phosphorylation and prevents the activation of JAK-STAT signaling pathway (Mazzon *et al.*, 2009)	In addition to the viral RdRp functions of NS5 described for the MBFVs, the TBFV NS5 was the first to be implicated in disrupting innate immune signaling. To suppress critical host responses, LGTV NS5 interacts with IFNAR2 and IFNGR2, two IFN receptor subunits, and antagonizes IFN-dependent responses by suppressing JAK-STAT signal transduction (Best *et al.*, 2005; Park *et al.*, 2007). Cellular responses targeted at restricting viral replication occur via lysosomal degradation of NS5, NS2B, and NS3. The NS5 of LGTV and TBEV interacts with TRIM79-alpha, an IFN-inducible protein, and induces the degradation (Taylor *et al.*, 2011). The cellular antiviral state is also compromised by TBEV NS5 interaction with the scaffold protein Scribble (Werme *et al.*, 2008). This complex blocks the phosphorylation of STAT1 and disrupts the JAK-STAT signaling pathway

Table 1 (continued)

Flavivirus protein	Nucleotide* weight, aa	Location in cell	Defined functions of viral proteins and untranslated regions of genome	
			Mosquito-borne flavivirus (MBFV)	Tick-borne flavivirus (TBFV)
3' UTR	10 375–11 141		The extreme 3' terminal region contains secondary structures and RNA elements important for cyclization of the genome and virus viability. The MBFV 3' UTR can be divided into three regions (Markoff, 2003; Liu *et al.*, 2009); a variable region directly upstream of the stop codon; a second region that is relatively conserved in nucleotide sequences containing hairpin motifs; and a highly conserved core element contains stable SL structures and cyclization motif (Villordo & Gamarnik, 2009)	The 3' UTR of TBFVs exhibits significant heterogeneity in length and sequence even among closely related strains. The TBFV 3'UTR can also be divided into a variable region part and an extremely conserved core element (Gritsun *et al.*, 1997), where the length variability of the variable region has been suggested to be related to the number of laboratory passages (Mandl *et al.*, 1998; Mandl, 2005). A hypothesis interprets the biological importance of the variable region that influences the replication/translation dynamics in mammalian and tick cells (Elvang *et al.*, 2011). The conserved core element contains highly essential sequential and structural motifs defined as SL1-5 for viral maintenance (Mandl *et al.*, 1998; Pletnev, 2001). The pentanucleotide (GAGAG) motif exposed on the SL4 is strictly conserved among all the flavivirus genomes (Khromykh *et al.*, 2003). Other important elements present in the conserved region include the homopurine box, a homopyrimidine box, and cyclization sequences (Mandl, 2005; Gritsun & Gould, 2007a, b)

*Nucleotide numbers are related to the strain Neudoerfl of TBEV.

†Shared function is presumed for both mosquito- and tick-borne flaviviruses. In many cases, no specific studies have been carried out in TBFV.

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brane (prM/M), and envelope (E), and seven nonstructural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Chambers *et al.*, 1990; Ryan *et al.*, 1998; Bollati *et al.*, 2010). The defined functions of these proteins are presented in Table 1. However, the precise function of some of the proteins remains to be elucidated. Furthermore, it should be noted that the bulk of the studies have been carried out in MBFV systems, and it is possible that the functions may not be identical in TBFV.

Recognizing that the VBFVs cycle between vertebrate and arthropod hosts and that both viral and host factors are likely to be involved, it is highly probable that viral persistence is exceedingly complex. The purpose of this review is to evaluate the current literature on flavivirus persistence as well as to suggest ideas for additional research into this interesting and important area.

Flavivirus infection and persistence in humans

Humans are inadvertent targets of VBFV infection (Figs 1 and 2), and infection is often associated with debilitating, acute neurological syndromes or hemorrhagic fever. However, there are several lines of evidence suggesting flavivirus persistence in humans (Murray *et al.*, 2010; Gibney *et al.*, 2011; Baty *et al.*, 2012). The establishment of flavivirus persistence in humans seems to be mainly associated with encephalitic flaviviruses (Diamond, 2003). However, YFV and DENV persistence has been described in eukaryotic cells in culture and in successive generations of *Aedes* mosquitoes (Lodge *et al.*, 1987; Takasaki *et al.*, 2001; Farfan-Ale *et al.*, 2009). YFV and DENV commonly cause a hemorrhagic illness, jaundice, and dengue shock syndrome, and encephalitis is atypical and extremely rare
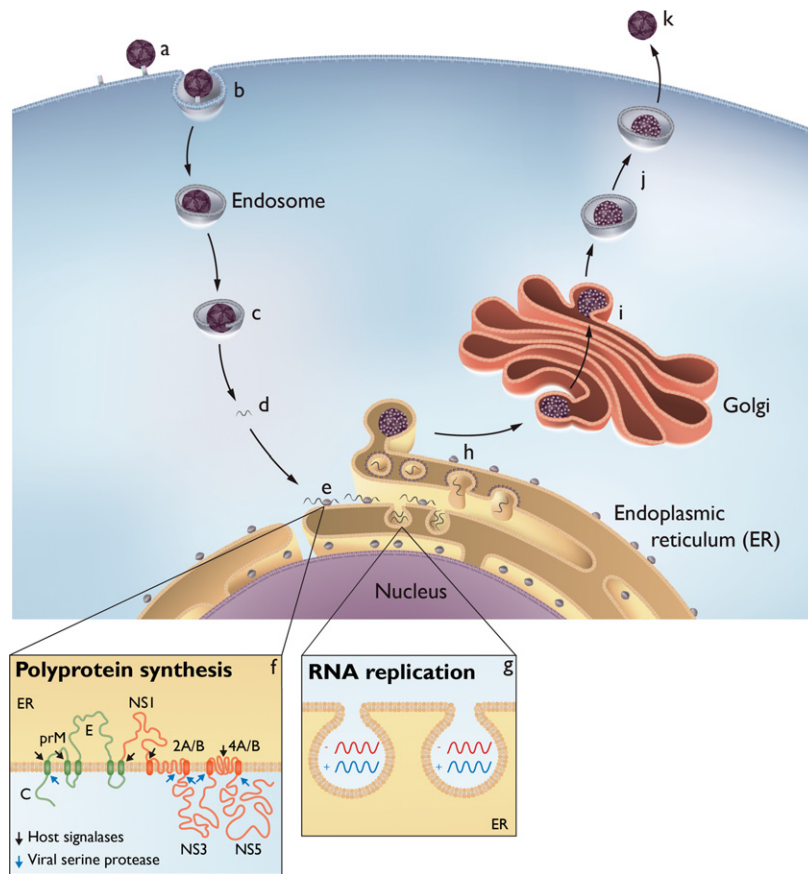

Fig. 3 A simplified overview of the replication cycle for a vector-borne flavivirus. Inbound virions bind to the cell membrane via poorly characterized receptors (a), are captured in vesicles by a clathrin-mediated pathway (b), and delivered to endosomes, where a pH-dependent fusion of the particles with the endosome membrane occurs (c). Subsequent to uncoating, the single-stranded, positive-sense RNA genome (d) migrates to the endoplasmic reticulum (ER) and is translated (e) as a polyprotein traversing the ER membrane several times (f). The polyprotein is cleaved into the viral proteins by viral and cellular proteases, although prM and E remain covalently attached. Through the agency of several viral nonstructural proteins and cellular proteins, there is a proliferation of ER-derived membranes and the formation of spherules that maintain a pore-like connection to the cytoplasm (g); the viral genome is replicated within these spherules by the viral proteins comprising the replication complex. By an as yet uncharacterized mechanism, progeny genomes are delivered to adjacent ER membranes where the capsid protein mediates assembly and inclusion of prM-E into immature virions (h). The immature virions transit the Golgi membrane system (i), and as a mild pH change occurs, the cellular enzyme furin cleaves the prM-E linkage (j), allowing the virus particle to assume its final mature stage prior to release from the cell (k). Defined roles for the individual viral proteins are enumerated in Table 1.

(Tomori, 2004; Gulati & Maheshwari, 2007; Varatharaj, 2010), but persistence cannot be excluded.

Human infection with the encephalitic viruses almost always occurs following a bite from infected ticks or mosquitoes (Figs 1 and 2). Generally, illness tends to be biphasic. The first phase is characterized by a flu-like illness with symptoms such as headache, arthralgia, and malaise. The second phase may present with neurological symptoms, such as mild meningitis to severe encephalitis. Survivors of encephalitis may develop persistent neurological sequelae, suggesting neurological tissue damage and/or viral persistence. Neurological tissue damage, such as loss of neurons, has been reported for WNV infection (Guarner *et al.*, 2004). Long-term morbidity following TBEV infection is known to occur frequently (Haglund *et al.*, 1996; Haglund & Gunther, 2003). A study conducted in Sweden reported chronic postencephalitic syndrome in 36% of patients who had been infected with TBEV (Haglund *et al.*, 1996). In up to 60% of patients who develop encephalitis due to WNV infection, neurological and/or neuropsychological symptoms continue to be reported for up to 7 years following the infection (Murray *et al.*, 2010; Sadek *et al.*, 2010).

The most definitive evidence for viral persistence would be the isolation of viable virus or the demonstration of viral antigens or RNA long after the acute illness. Several reports describe the isolation of infectious VBFVs from persistently infected humans. For example, the Siberian TBEV strain Za was isolated from an individual who had harbored the virus for 10 years (Gritsun *et al.*, 2003). He later died following 2 years of a progressive form of TBEV encephalitis (Gritsun *et al.*, 2003). In addition, isolation of JEV from the cerebrospinal fluid was possible for more than 3 weeks following infection in 19% of individuals that had developed encephalitis (Ravi *et al.*, 1993). JEV persistence was also demonstrated in peripheral mononuclear cells in infected children in northern India (Sharma *et al.*, 1991). In these children, virus could be isolated by culture 8–9 months after acute infection (Sharma *et al.*, 1991). Furthermore, WNV has been transmitted to patients who had received blood transfusions or organ transplants from asymptomatic donors (Pealer *et al.*, 2003; Montgomery *et al.*, 2006; CDC, 2009), suggesting viral persistence in the donors as well as stored blood for transfusion.

Long-term presence of viral nucleic acid is another indicator for persistent infection; however, study results are inconsistent. Viral RNA can be readily sought using molecular biology techniques, such as RT-PCR and transcription-mediated amplification (TMA) (Sánchez-Seco *et al.*, 2005; Muñoz-Jordán *et al.*, 2009; Patel *et al.*, 2013). Using TMA, WNV RNA was detectable in the blood of donors for up to 104 days following the index donation (Busch *et al.*, 2008; Prince *et al.*, 2008). Using RT-PCR, WNV RNA could also be detected in urine of persistently infected individuals for up to 6.5 years following the acute phase of infection (Murray *et al.*, 2010), although virus could not be isolated from the RNA-positive urine samples. In contrast, WNV RNA could not be detected after 6 years in urine in another study reported a year later (Gibney *et al.*,

2011). Baty and colleagues also failed to detect WNV RNA by RT-PCR, but were able to detect viral RNA using TMA (Baty *et al.*, 2012). Similarly for JEV, viral RNA could not be detected by RT-PCR in the individuals who had anti-JEV IgM antibodies (Zhao *et al.*, 2013). In the study by Gibney *et al.* (2011), WNV persistence could not be excluded. Even though the results in these studies are somewhat variable, it seems reasonable to conclude that the presence of viral RNA can also be taken as an indicator of persistent infection.

Viral serology may be an additional surrogate measure for VBFV persistence. In general, infection or vaccination leads to a sterilizing immunity, but long-term persistence of anti-VBFV IgM antibodies has often been assumed to indicate continued exposure to viral antigens or virus particles (Ravi *et al.*, 1993; Stiasny *et al.*, 2012). An exception to this is, of course, DENV where antiviral antibody plays a key role in pathogenesis (Martina *et al.*, 2009; Pierson, 2010). IgM antibodies induced by flaviviruses, such as TBEV and WNV, are known to persist in serum and CSF for 12 months or more (Kapoora *et al.*, 2004; Stiasny *et al.*, 2012). Indeed, IgM antibodies against TBEV persisted for up to 32 months (Stiasny *et al.*, 2012). Furthermore, anti-WNV IgM antibodies persist in previously exposed blood donors for up 16 months (Busch *et al.*, 2008; Prince *et al.*, 2008). However, IgM antibody persistence does not necessarily correlate with infectious virus persistence. For example, persistent IgM antibodies against TBEV can be detected in some cases following vaccination without active viral infection (Rendi-Wagner *et al.*, 2004; Stiasny *et al.*, 2012). For WNV infection, IgM antibodies against the nonstructural protein NS5 cannot be used to distinguish between recent/active infection from past infection (Prince *et al.*, 2008). Thus, serology may be a less useful marker for viral persistence.

A major consideration in VBFV in humans would be identification of sites of viral persistence. As flavivirus persistence seems to be mainly associated with the neurotropic and encephalitogenic viruses, the central nervous system may well be the preferred site for viral persistence. Furthermore, this may account for why patients who recover from encephalitis often have prolonged neurological symptoms. However, other studies by Murray and colleagues postulated kidneys as a preferred site for the establishment of persistent flavivirus infections following the detection of WNV RNA in urine (Murray *et al.*, 2010). Certainly, additional work is required to define the true incidence, the biology, and the pathogenic potential in humans of persistent VBFV infections.

The role of arthropods and arachnids in flavivirus persistence

Arthropod hosts play crucial roles in the biology of both TBFV and MBFV and contribute to persistent infection. In nature, ticks are the important arachnid reservoirs of TBFVs. The persistence of the TBFVs in arachnids is well established, and the role of ticks as long-term reservoirs and vectors for the viruses is clear. Estimates suggest that ticks

transmit about 25% of the known flaviviruses. TBFVs are capable of infecting about 14 tick species, but the ixodid ticks, *Ixodes scapularis* and *Ixodes ricinus*, account for nearly all transmission of TBEV to humans. *Ixodes cookei* may also harbor POWV and DTV, while the soft-bodied *Onithodoros* tick transmits Alkhurma virus (Main *et al.*, 1979; Charrel *et al.*, 2007). To transmit virus, these ticks need only 15 min of attachment to the host (Crowder *et al.*, 2013). The virus is present in the tick salivary glands, and saliva constituents enhance infectivity (Nuttall & Labuda, 2003; Girard *et al.*, 2004). A recent publication revealed that a selected subset of salivary gland genes is expressed when infected ticks feed on naïve mice (McNally *et al.*, 2012).

Ticks acquire the virus while feeding on infected rodents, typically by a process called 'cofeeding' (Fig. 1; Labuda *et al.*, 1993a, b), and the virus persists throughout several life stages. Ticks at the larvae, nymph, and adult life stages can become infected by feeding on infected animals (horizontal transmission) or can transmit virus vertically across instars (transstadial transmission) and through the eggs (transovarial transmission; Fig. 1a; Labuda *et al.*, 1993a, 1996; Nuttall & Labuda, 2003). Indeed, results of a carefully controlled study in our laboratory show that transstadial transmission seems to be very high (Mitzel *et al.*, 2007). The blood meal remains in the midgut for long periods of time, allowing infection of the epithelial cells lining the midgut with subsequent translocation into the hemocoel (Nuttall & Labuda, 2003). Infection of the midgut cells may be facilitated by the heterophagous nature of ticks (i.e. blood meal digestion is principally an intracellular process). In the open circulatory system, tissues and organs are bathed in hemolymph, which acts as a medium for transporting nutrients, hormones, and immune effector molecules. Therefore, the hemolymph likely serves as a viral dissemination medium. TBE virus was found in the esophagus and subesophageal ganglion in *Dermacentor marginatus* larvae and in columnar epidermal cells of *Dermacentor reticulatus* nymphs (Nuttall & Labuda, 2003). In *D. reticulatus* nymphs, TBE virus was demonstrated in epidermal cells and in vacuoles in the region of Golgi complexes of salivary gland cells (Nosek *et al.*, 1984). The precise mechanisms by which TBFVs traverse various tissues in the tick and reach the salivary glands remain to be fully elucidated.

Prevalence surveys indicate that 0.5–5% of ixodid ticks carry the virus in Europe, but prevalence of up to 40% has been reported in Russia (Ustinova *et al.*, 1997). In north-central USA, up to 4.9% of *I. scapularis* ticks are infected by Powassan or DTV (Brackney *et al.*, 2008b; Dupuis *et al.*, 2013). Interestingly, in a study carried out in Chicago, *I. scapularis* was found to infest 2.8% of wild birds, which figure prominently in the MBFV cycle (Fig. 1b; Hamer *et al.*, 2011). However, identification of TBFV was not attempted in this study. In brief, TBFV persistence in ticks is well established, and the essential role of ticks in the biology of these viruses is not in doubt.

As is the case for TBFVs, the role of mosquitoes in the maintenance of the MBFV cycle is also well characterized. Mosquitoes are thought to account for transmitting approximately 50% of more than 70 known flaviviruses (Gould, 2001). Diverse mosquito species can be infected by MBFVs. However, the two major mosquito vectors are *Aedes aegypti* (e.g. transmission of YFV and DENV) and *Culex* species (e.g. transmission of WNV and JEV). Adult female mosquitoes become infected when they obtain blood meals from flavivirus-infected animals, and virus replication in the mosquito has been well described (Girard *et al.*, 2005; McGee *et al.*, 2010; Colpitts *et al.*, 2012). Girard *et al.* (2004) used immunohistochemistry to demonstrate that WNV infects epithelial cells of the *Culex pipiens* midgut and that viral antigen can be detected as early as day 2 postinfection. Viral antigen staining becomes more intense in the cells of the midgut over time until day 14 to 21 following infection (Girard *et al.*, 2004). Using DENV-2, virus titer in the midgut peaks to about 9×10^3 pfu mL^{-1} by 10 days postinfection, but declines to about 7.4×10^2 pfu mL^{-1} by day 12 (Sánchez-Vargas *et al.*, 2009). Dissemination of virus into various tissues occurs at various time points, and the amount of antigen also varies. Similar to TBFVs in ticks, the hemolymph serves as a vehicle for viral dissemination. However, virus also spreads in a cell–cell fashion to the muscle of the posterior midgut from 6 to 27 days postinfection (Girard *et al.*, 2004). Studies with DENV-2 showed that infected mosquitoes mount an RNA interference (RNAi)-mediated antiviral response, but impairing the vector RNAi resulted in increased viral replication (Sánchez-Vargas *et al.*, 2009). This mechanism could be associated with observations of flavivirus-related RNA that persists as DNA in mosquitoes (Crochu *et al.*, 2004). As mosquitoes are such efficient vectors for the transmission of the MBFVs, these reports suggest that the viruses have evolved a mechanism of evading the host response to persist in the vector.

Mosquitoes also acquire and deliver virus horizontally during blood meals and are competent to transmit vertically to progeny by transovarial passage (Rosen *et al.*, 1983). WNV can persist overwinter in mosquitoes that hibernate in cold months. Cool temperatures also facilitate persistence of flaviviruses in adult female mosquitoes. For example, St. Louis encephalitis virus (SLEV) survived for more than 100 days of winter in *Culex quinquefasciatus* (Kramer & Ebel, 2003). Similarly, JEV and WNV have been transmitted by mosquitoes that carried the viruses at cold temperatures when exposed to temperature increases equal to ambient levels (Kramer & Ebel, 2003). However, at low temperature, mosquitoes are less likely to acquire new infections (Colpitts *et al.*, 2012). At 18 °C, low WNV dissemination to the legs of the *C. pipiens* was observed, and rapid viral dissemination occurred at higher temperatures (Colpitts *et al.*, 2012). Furthermore, higher temperatures increase vector population growth rate and the rate of viral evolution in the mosquito (Girard *et al.*, 2005).

The prevalence and maintenance of WNV across landscapes is mediated by environmental factors, such as local effects of agriculture on vector and host communities (Crowder *et al.*, 2013). Results of investigations carried out in the states of Oregon and Washington showed that the prevalence of WNV in both *C. pipiens* and *Culex tarsalis* was similar at 14.5% or 13.5%, respectively (Crowder *et al.*,

2013). However, a study conducted in Stratford, Connecticut, showed that the *C. pipiens* was the most dominant mosquito captured in this WNV focus area (Anderson *et al.*, 2004). In the captured mosquitoes, more than 85% of WNV isolations were from the same species, whereas *Culex salinarius* accounted for between 5% and 12% (Anderson *et al.*, 2004). In a similar study conducted in Mexico, *C. quinquefasciatus* was more common at 48.3% and MBFV RNA was detected in 70% of the pools (Farfan-Ale *et al.*, 2009). It is evident that mosquitoes are important as viral hosts and vectors. Furthermore, it can be concluded that the MBFVs have evolved mechanisms of evading the host innate responses to persist in the MBFVs.

Flavivirus infection and persistence in animals

The principal vertebrate hosts for VBFVs are small mammals, marsupials, and birds (Fig. 1), but the viruses can also infect reptiles (Mackenzie *et al.*, 2002, 2004; Steinman *et al.*, 2003; Jacobson *et al.*, 2005; Root *et al.*, 2005; Marschang, 2011). In most instances, larger animals, such as cervids, goats, and sheep, are incidental hosts. The 1999 outbreak of WNV in humans in New York is thought to be associated with WNV infection in birds (Strausbaugh *et al.*, 2001). The mosquito-borne Wesselsbron virus could also be cycled between mosquitoes and birds, but not much is known about the vertebrate host(s). Thus, in nature, numerous species are susceptible to VBFVs and might serve as reservoirs or secondary amplifying hosts. In this section, we will survey the literature and the three potential markers for viral persistence: isolation of virus, identification of viral RNA or protein, and viral serology.

Small mammals, particularly rodents, are the principal vertebrate hosts and reservoirs for TBFVs (Mansfield *et al.*, 2009; Dobler *et al.*, 2012). In Europe, the yellow-necked mouse (*Apodemus flavicollis*) and bank vole (*Myodes glareolus*) are implicated as the most common hosts, and they develop sufficient viremia to infect ticks that feed on them (Weidmann *et al.*, 2011; Dobler *et al.*, 2012; Knap *et al.*, 2012). In various wild rodent species captured in Brandenburg, Germany, an average TBEV infection rate of 15% was reported (Achazi *et al.*, 2011). In the captured rodents, TBEV RNA was detected by RT-PCR in the brains and spleens. In North America, deer mice (*Peromyscus*), squirrels, and the striped skunk (*Mephitis mephitis*) are important reservoirs of POWV and DTV (Main *et al.*, 1979; Telford *et al.*, 1997). Based on serological studies, a 6.2% DTV prevalence in the red-backed voles (*Myodes rutilus*) was found in Siberia and Alaska (Deardorff *et al.*, 2013). DTV in New Mexico was serologically prevalent in *Peromyscus truei* and *Peromyscus maniculatus* at 22.2% and 6.0%, respectively (Deardorff *et al.*, 2013). Therefore, it seems likely that persistent infection of these small mammals occurs.

Deer may stabilize and maintain TBFVs at levels that are important for transmission (Pugliese *et al.*, 2007; Carpi *et al.*, 2009; McGee *et al.*, 2010; Dobler *et al.*, 2012). While deer are important sources of blood meals for ticks, they develop low virus titer and are therefore not competent in transmission of TBFVs. However, in a broader sense, deer are important in viral persistence because they help sustain tick populations (Cagnacci *et al.*, 2012).

The situation for domesticated animals is also less clear than for rodents. Dogs, horses, and monkeys can be infected with TBFVs, but case reports in veterinary practice are relatively infrequent (Jaenson *et al.*, 2012). Large domesticated animals, such as goats, sheep, and cattle, become viremic for a short while and develop antibodies following infection with TBEV, but do not show any specific clinical signs of illness (Mansfield *et al.*, 2009; Klaus *et al.*, 2012). However, transmission of TBEV via milk from these domesticated animals (Fig. 1) has been documented, suggesting that virus may persist following the acute viremia in at least some instances (Vereta *et al.*, 1991; Caini *et al.*, 2012; Hudopisk *et al.*, 2013).

Birds and primates are considered to be the primary reservoirs of MBFVs. Other mammals are generally accidental hosts, but this may not always be the case, and when viremic, these domestic animals are able to infect mosquitoes.

For JEV, the major amplifying hosts are birds and pigs, which attain high levels of viremia. They provide a source of infection for the mosquito species that subsequently transmit JEV to humans (Hukkanen *et al.*, 2006; van den Hurk *et al.*, 2009). JEV control has been achieved through vaccination of pigs and humans in Korea, Japan, and Taiwan (Igarashi, 2002) while horses are considered dead-end hosts of JEV infection due to a very low level of viremia.

In geographic regions where pig populations are low, swine may not be important for zoonotic transmission of JEV. Herons and egrets are important JEV-amplifying hosts and source of infection for mosquito species that transmit JEV to humans (Nemeth *et al.*, 2009). Wild-caught pigeons were shown to have antibodies that persist for up to 15 months. It is not clear whether the antibody persistence is associated with viral persistence. Although various bird species do play a prominent role in JEV infection, evidence of birds as a source of persistent infection is less certain. In some instances, birds can acquire JEV viremia and then fail to develop or lose neutralizing antibodies.

Birds are also excellent amplifying hosts for WNV, and migratory birds can move the viruses to different areas because they become viremic for several days. Near 100% mortality is associated with natural or experimental WNV infection in birds, such as American crows (*Corvus brachyrhynchos*), blue jays (*Cyanocitta cristata*), and greater sage-grouse (*Centrocercus urophasianus*; Steele *et al.*, 2000; Komar *et al.*, 2003; Clark *et al.*, 2006), but some birds are able to carry the virus for a longer time before they develop antibodies or succumb to disease (McKenzie & Goulet, 2010). For instance, a combined 37% of house sparrows that were either naturally or experimentally infected with WNV tested positive for WNV RNA by RT-PCR (Wheeler *et al.*, 2012). In the same study, Wheeler *et al.* (2012) showed that 97% and 100% of WNV-infected house sparrows and finches, respectively, seroconverted following exposure to WNV. Birds that develop neutralizing anti-WNV antibodies are protected from reinfection (Nemeth *et al.*,

2009), suggesting total virus clearance in seroconverted birds, and antibodies against WNV have been found in wild birds, suggesting exposure to WNV.

Early demonstration of WNV persistence was described in blue-gray pigeons, from which virus was isolated up to 100 days postinfection, and viral antigen could be detected in liver tissue for up to 180 days (Ruiz *et al.*, 2010). American robins (*Tardus migratorius*) are known to be competent reservoirs of WNV and SLEV (Kilpatrick *et al.*, 2006). WNV persistence was also demonstrated by the detection of viral RNA in the presence of antibody for up to 36 weeks in spleens of naturally infected house sparrows and finches (Wheeler *et al.*, 2012). Taken together, it seems that there is decent evidence for persistent infection by WNV in some bird species. However, many details about the biology of persistence remain to be studied.

Antibodies to various flaviviruses, such as SLEV, POWV, JEV, and WNV, have been identified in chelonians, snakes, and crocodiles in different geographic locations (Steinman *et al.*, 2003; Marschang, 2011), and it is known that alligators and crocodiles can be infected with WNV. However, there are no reports suggesting that reptiles host persistent MBFV infection.

In summary, it is apparent that various animals and birds can sponsor persistent VBFV infection. Nevertheless, the precise role that these persistent infections play in larger scheme of viral maintenance merits additional study.

Experimental models for persistent flavivirus biology

There are several animal models of persistent mosquito-borne flavivirus infection, including WNV and SLEV (Charlier *et al.*, 2004; Kimura *et al.*, 2010). As shown in Table 2, mice and hamsters have been the main study models. In a nonhuman primate study, WNV was isolated from central nervous system tissues more than 5 months post-intra-cerebral inoculation (Pogodina *et al.*, 1983), suggesting that these species might also be relevant system.

Persistent WNV has also been studied experimentally in the golden hamster. The clinical outcomes of WNV infection in hamsters vary and depend on animal age and immune competence, viral dose, and route of infection. WNV infection can lead to asymptomatic illness, encephalitis, severe paralysis, and acute death (Xiao *et al.*, 2001; Morrey *et al.*, 2004; Tesh *et al.*, 2005). Adult golden hamsters infected with WNV develop chronic infection, characterized by shedding of virus in urine for up to 8 months (Xiao *et al.*, 2001; Ding *et al.*, 2005; Tonry *et al.*, 2005). The chronically infected animals exhibit antigens in tubular epithelial cells, interstitial cells, and macrophages of distal renal tubules (Tesh *et al.*, 2005). Interestingly, naïve hamsters inoculated intraperitoneally with WNV-containing urine from persistently infected hamster do not develop clinical disease, but the hamsters become viremic and develop antibody responses (Ding *et al.*, 2005). This suggests possible viral genetic changes, which may facilitate persistent infection, although other explanations cannot be totally excluded.

Hamsters have also been used as experimental models to study the persistence of SLEV and are among the natural vertebrate hosts of the Banzi flavivirus, a member of the YFV group of flaviviruses (Grard *et al.*, 2010). Golden hamsters infected with SLEV did not develop clinical signs of illness, but they developed viruria and antibodies against SLEV at 28 days postinfection (Siirin *et al.*, 2007).

Table 2 Experimental animal models developed for various flaviviruses

Vector	Flavivirus	Experimental models for VBFV persistence
Mosquito	WNV	C57BL/6 (B6) mice and C3H/HeN (C3) mice (Appler *et al.*, 2010; Pierson & Diamond, 2012): WNV RNA persisted in a pantropic manner in 12% of infected mice for up to 6 months. Infectious virus could be isolated in 12% of mice for up to 4 months. C3H mice survival rate was lower and 22% when compared to the survival rate of B6 mice, which was 78%
		Macaque rhesus (Pogodina *et al.*, 1981): Virus persisted in asymptomatic animals for 5½ months and could be isolated from cerebellum, cerebral subcortical ganglia, lymph nodes, and kidneys
		Golden hamster (Mesocricetus auratus; Tesh *et al.*, 2005; Tonry *et al.*, 2005): Chronic renal infection with persistent shedding of virus for up to 8 months. Virus could be recovered by culture, and genotypic and phenotypic changes were identified
		House sparrow (*Passer domesticus*; Nemeth *et al.*, 2009): infectious virus persisted in tissues for up to 43 days, but was not detectable in sera after 6 days. WNV RNA persisted in tissues for up to 65 days
	SLEV	Golden hamster (Siirin *et al.*, 2007): Infected animals remained asymptomatic, but virus could be cocultivated in various organs for up to 185 days
	JEV	Swiss albino mice (Mathur *et al.*, 1986a, b): Persistence was demonstrated by reactivation in 41% of congenitally infected pups. In adult mice, viral persistence was shown to last longer (16 weeks) in pregnant mice compared with 4 weeks in nonpregnant mice
Tick	TBEV	*Macaque rhesus* (Pogodina, 1983; Pogodina *et al.*, 1984, 1981): Monkeys recovered from encephalitis and virus persisted for at least 738 days. In asymptomatic animals, virus persisted for 302 days
	LIV*	Immunosuppressed guinea pigs (Zlotnik *et al.*, 1971): LIV was lethal in young animals, but older animals acquire a nonapparent infection with viral replication in the brain and spleen
	POWV	Deer mouse (*Peromyscus leucopus*; Telford *et al.*, 1997): not a well-characterized model, but adult mice appear to survive infection

*Louping ill virus infects sheep.

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Hamsters that become persistently infected with SLEV shed virus in their urine for up to 185 days postinfection. The shedding of virus in the urine of infected hamsters also suggests that the kidneys could be an organ preferred for viral persistence.

C57BL/6J mice maintained a persistent WNV infection in the face of robust neutralizing antibody levels for more than 6 months (Appler *et al.*, 2010; Stewart *et al.*, 2011). In this study, infectious virus was recovered in the skin, and viral RNA was identified in the skin, as well as the spinal cord and brain (Appler *et al.*, 2010). In contrast, WNV persistence was uncommon in kidneys and rare in the heart (Appler *et al.*, 2010). However, in another study in which C57BL/6J mice were infected with WNV derived from the urine of persistently infected hamsters, the kidney was found to be the preferred organ for viral persistence (Saxena *et al.*, 2013). The hamster-derived WNV was also found to be highly attenuated in both neuroinvasiveness and neurovirulence in infected mice (Saxena *et al.*, 2013). These results also suggest that the virus acquires phenotypic changes to be able to persist.

While the inbred laboratory mouse models are patently useful for understanding some aspects of flavivirus persistence, very few natural rodent hosts have been established as experimental animal models. Evidence of exposure to WNV has been reported in rodent species, such as rats (*Rattus*), bank voles (*M. glareolus*), and deer mice (*Peromyscus*; Molnar *et al.*, 1976; Root *et al.*, 2005; Docherty *et al.*, 2006; Gomez *et al.*, 2008). However, infectious virus was only identified in the bank vole, whereas antibodies were detected in all of the species (Molnar *et al.*, 1976; Root *et al.*, 2005).

Although persistence in mammals obviously plays a significant role in TBFV infections, little attention has been devoted to experimental studies of this aspect of TBFV biology. While disease or therapy models have been established for TBFVs (Kreil *et al.*, 1997; Holbrook *et al.*, 2005; Hayasaka *et al.*, 2010; Palus *et al.*, 2013), none of these specifically investigated viral persistence. Experiments to determine the susceptibility of several wild and domesticated mammals to POWV showed that viremia lasted for 0–3 days in the goat, pig, skunk, red fox, and gray fox (Yiang *et al.*, 2013). In another study, adult deer mice (*Peromoyscus leucopus*) survived challenge with POWV without apparent illness, but evidence of viral persistence was not sought (Telford *et al.*, 1997). Development of suitable experimental models for persistent TBFV infection would clearly provide useful information about this aspect of these important pathogens.

Mechanisms of flavivirus persistence

The previous sections have looked at flavivirus persistence in humans, animals, and arthropod vectors, as well as some relevant animal models. In this section, we will survey information related to the initiation and maintenance of persistence.

When mammalian cell cultures are acutely infected with TBFV, a legion of general cellular defense and antiviral systems are triggered, as are specific factors designed to limit or restrict virus reproduction. Some of these include type I interferon (IFN-α/IFN-β), type III interferon (IFN-λ), mitochondrial activated signaling, and the induction of inflammatory factors, such as interleukins (Tam & Messner, 1999; Madden, 2003; Van Gerpen, 2003). For example, the IFN-induced tripartite motif protein, TRIM79α, has been shown to restrict TBEV replication by degrading NS5 (Taylor *et al.*, 2011). The unimpeded deployment of these antiviral factors and systems would lead to cell death. Cell death is thought to be mediated primarily through apoptosis (Růžek *et al.*, 2009), and programmed cell death induced by various TBFVs has been described in neurons, epithelial cells, hepatocytes, Kupffer cells, and neuroblastoma cells (Ramanathan *et al.*, 2006).

Impeding or evading the antiviral response is one characteristic of VBFVs, which plays an important role in viral persistence. During flavivirus infection, IFN production is induced within hours, but viral RNA replication complexes are enclosed in vesicles, which may offer protection from recognition by pathogen recognition receptors, thus delaying IFN production (Welsch *et al.*, 2009; Gillespie *et al.*, 2010; Overby *et al.*, 2010; Offerdahl *et al.*, 2012; Ye *et al.*, 2013). In addition, some VBFVs can directly affect IFN secretion by inhibiting IFN gene transcription, suppressing IFN signaling or impairing the functions of interferon-stimulated genes (Best *et al.*, 2005; Robertson *et al.*, 2009; Ye *et al.*, 2013). The humoral and cell-mediated immune responses are also prone to inhibition by VBFVs. Studies with WNV in C57BL/6 mice suggest that WNV-specific antibodies correlate with decreased spread to the CNS (Diamond, 2003). Antibody escape mutants could also evade the T cell recognition (Diamond, 2003), but a precise role of these in viral persistence is yet to be defined.

The E protein is thought to be responsible for provoking the cytopathic effect (Isaeva *et al.*, 1998; Prikhod'ko *et al.*, 2001). However, the NS3 protease and NS2B-NS3 protease precursor (Table 1) are also known to induce apoptosis by binding to caspase 8 (Shafee & AbuBakar, 2003; Ramanathan *et al.*, 2006; Safronetz *et al.*, 2013). In addition, NS2A has also been implicated in causing IFN-independent cytopathic effect (Chang *et al.*, 1999; Melian *et al.*, 2013). Observations in our laboratory (L. Mlera, D. Offerdahl & M.E. Bloom, unpublished results) and those of others (Lancaster *et al.*, 1998) indicate that TBFV infection in mammalian cell culture is characterized by an acute phase, which kills most infected cells; however, a small number of cells survive the initial cytolytic phase, and the culture is repopulated with cells almost all of which are infected. Clearly, VBFV induces an acutely cytopathic infection in mammalian cells, and thus, the development of a persistent infection implies that cell death factors must be evaded or modulated, although the precise mechanisms are still obscure.

The implication of specific viral proteins, domains, or sequences that combat cytolytic cell death has been rather limited, but any viral determinants that limit cell death in the face of acute infection might also enhance the initiation of persistent infection. For example, viral NS4A (Table 1) was shown to induce phosphatidylinositol 3-kinase

(PI3K)-dependent autophagy and thereby leading to protection from cell death (McLean *et al.*, 2011). The manipulation of apoptosis by JEV, which activates PI3K in infected cells, was also reported (Lee *et al.*, 2005). JEV triggers apoptosis during the late stages of infection, and the activation of PI3K is thought to provide protection from early cell death. Limited replication implies a low level of viral protein expression and may favor viral persistence.

Over the years, significant attention has been focused on defective interfering (DI) virus particles, which limit replication of the wild-type virus (Schmaljohn & Blair, 1977; Debnath *et al.*, 1991; Blitvich *et al.*, 1999). The DI particles represent truncated genomes that can be replicated and encapsidated and compete with wild-type viral particles when they infect cells. In vesicular stomatitis virus (VSV), DI particles are thought to modulate virulence (Cave *et al.*, 1985). However, the role of VBFV DI particles in persistence is not completely certain. For TBEV, the C protein is reported to tolerate internal deletions ranging from 4 to 21 amino acids, and the deletions seem to favor attenuation and immunogenicity (Kofler *et al.*, 2002). DI particles of VBFVs, such as Murray valley encephalitis (MVE), TBEV Sofjin strain, and WNV, are known to generate truncated NS1 proteins, following infection at high multiplicity of infection (Debnath *et al.*, 1991; Poidinger *et al.*, 1991; Chen *et al.*, 1996; Bugrysheva *et al.*, 2001). Persistent infection of Vero cells with MVE was associated with a truncated NS1, whereas this form of NS1 was not noted in the acute infection (Brinton, 1982; Lancaster *et al.*, 1998). The truncated NS1 in MVE virus was a result of the presence of DI RNA, which contained a large internal deletion (Lancaster *et al.*, 1998). In this case, MVE DI particles reduced the wild-type MVE titer by 75–95% (Poidinger *et al.*, 1991). However, a causal role for the MVE DI particles in maintaining persistent infection was not conclusively demonstrated. Studies of the Far Eastern Sofjin strain of the TBEV complex identified a 39-kDa truncated NS1 in both acutely and persistently infected human kidney RH cells (Bugrysheva *et al.*, 2001). For WNV, naturally occurring DI particles interfere with transmission in mosquitoes and minimally impact pathogenesis in mice (Pesko *et al.*, 2012). Furthermore, truncated DENV RNA species, suggesting the presence of DI particles, have been identified in acute human infections (Li *et al.*, 2011), but have not been described in other acute VBFV infections in humans. Similarly, Banzi virus DI particles seem to have been generated in resistant C3H/RV mice (Smith, 1981). Although DI particles and the truncated NS1 may be frequently observed in persistent infections, it is not at all clear that they are independently sufficient for the establishment and maintenance of a persistent infection *in vitro* or *in vivo*. Stable expression of truncated NS1 failed to render persistent infection with JEV, suggesting that truncated NS1 is a consequence rather than a cause for viral persistence (Liao *et al.*, 1998). The role of DI particles merits further investigation, but the role of other aspects of virus biology should also be scrutinized.

Restricted expression of the envelope (E) protein may also favor development of persistence. In KN73 cells that were persistently infected with JEV, the E protein was found to be expressed at markedly low levels compared with acutely infected cells (Feng *et al.*, 2002). In these studies, the expression of NS3 was found to be unchanged in the acute and persistent phases of JEV infection (Feng *et al.*, 2002). As the E protein is important for pathogenesis and immunity (CDC, 2009), low-level expression could result in immune tolerance and contribute toward viral persistence.

In addition to low expression levels, mutations in the E protein of JEV, YFV, and WNV might also play a role in VBFV persistence (Ding *et al.*, 2005; Farfan-Ale *et al.*, 2009). A 138E→K mutation in the E protein of JEV and WNV was shown to inhibit cell–cell spread of the virus and to contribute toward the development of a small-plaque phenotype (Carson *et al.*, 2006). While this mutation leads to viral attenuation (Carson *et al.*, 2006) and is key in the attenuation of JEV SA14-14-2 vaccine (Monath *et al.*, 2002), further elucidation of mutations that may play a role in persistence is required.

Observations that hamsters and mice infected with MBFVs obtained from urine of other MBFV-infected animals do not suffer severe disease and become persistently infected indicate that the virus is attenuated (Rosen *et al.*, 1983; Ding *et al.*, 2005). A number of amino acid-changing mutations in C, E, NS1, NS2A, NS2B, and NS5 were reportedly associated with persistence of WNV in serially passaged hamsters (Sánchez-Vargas *et al.*, 2009). These mutations are thought to have attenuated the virus (Sánchez-Vargas *et al.*, 2009). However, the same mutations have not been reported by other groups, suggesting that the mutations may not be specific to development or maintenance of viral persistence.

Mechanisms not directly involving viral proteins may also play a significant role in persistence. For instance, JEV delays the exposure of dsRNA to innate sensors and inhibits phosphorylation of IRF3 via noncoding viral short flaviviral RNA (Espada-Murao & Morita, 2011; Chang *et al.*, 2013). Similarly, WNV delays recognition by pathogen recognition receptors by activating IRF3 in a RIG-I-dependent manner without antagonizing the host IFN response (Fredericksen & Gale, 2006). The mechanism of evasion is currently not clear, but membrane-bound vesicles that enclose the RIG-I-activating dsRNA of TBEV have been described (Overby *et al.*, 2010). As the delayed recognition of viral pathogen-associated molecular patterns is only temporary, these manipulations are probably just among the suite of mechanisms deployed for the establishment of viral persistence. The specific viral genes and how they manipulate apoptosis at a cellular level needs further examination.

Specific host genes that may contribute toward the development of flavivirus persistence in the vertebrate host have not been defined, but there are some suggestions. For instance, the overexpression of the proto-oncogene *Bcl-2* was reported to prevent apoptosis and promote persistence in JEV-infected BHK and CHO cells (Liao *et al.*, 1998), suggesting that the control of apoptosis is likely to be implicated. In addition, some inbred laboratory mice strains can carry the 2′-5′-oligoadenylate synthetase gene, *Flv*ʳ, which confers flavivirus resistance (Urosevic *et al.*, 1997).

The animals are productively infected with flaviviruses, but produce low virus titer (Brinton & Perelygin, 2003; Barkhash *et al.*, 2010). Mice that are susceptible to flavivirus infection carry the *Flv^s* allele. The *Flv^r*-like allele has also been characterized in wild mice and could be a partial explanation of flavivirus persistence in rodents in nature (Urosevic *et al.*, 1997). Additional vertebrate genes, apart from the *Flv*, could play a role in varied susceptibilities of different mouse strains to flavivirus infection. This was suggested from recent observations that TBEV-infected BALBc mice were moderately resistant, STS mice are highly resistant, whereas the BALBc/STS recombinant mice were highly sensitive to infection (Palus *et al.*, 2013).

Immunosuppression may also be a potentiating factor for the establishment of flavivirus persistence in animal hosts. For example, WNV persistence was demonstrated by the detection of WNV RNA and immunohistochemistry in brain tissue of an immunosuppressed 57-year-old man 4 months after the initial diagnosis (Penn *et al.*, 2006). The follicular B cell lymphoma, from which he had suffered, may have facilitated, or aggravated, the persistence of WNV. In mice, transient immunosuppression with cyclophosphamide leads to WNV recrudescence (Appler *et al.*, 2010), an observation suggesting that some aspects of the immune system operate to restrict WNV replication during persistence. However, the situation is complicated because WNV is able to persist for up to 16 months in the face of a robust humoral immune response in C57BL/6 mice (Appler *et al.*, 2010). Furthermore, WNV persistence was reported in the brains of CD8+ T cell-deficient rodents (Shrestha & Diamond, 2004), but the CD8+ T cell deficiency did not affect the antibody response in this mouse model. Knowledge about specific immune system components that could facilitate or control viral persistence remains to be characterized.

Infection by VBFVs of arthropod cells has not received the same degree of study; however, acute infection is not accompanied by the same cytopathic response observed in mammalian cells. In addition, very little is known about how or in what tissues TBFVs persist in the ticks, but the viruses likely evolved mechanisms of modulating or evading the tick immune system over the millenia (Robertson *et al.*, 2009). Persistent infection of the *I. scapularis* cell line ISE-6 (Munderloh *et al.*, 1994) was readily established and was noncytopathic (Offerdahl *et al.*, 2012). Detailed studies of persistent TBFV infection in arthropod cell lines using contemporary techniques and methods are certain to yield useful and interesting information.

The mechanism(s) of how MBFVs persist in mosquitoes also remains a suitable topic for investigation. SLEV persists in the midgut of *C. pipiens* for hours before infecting the midgut epithelium (Whitfield *et al.*, 1973; Brackney *et al.*, 2008a), but this is a short time. Interestingly, approximately two-thirds of flavivirus-related sequences were reportedly detected as integrated dsDNA form in laboratory-bred and wild *Aedes* mosquitoes (Crochu *et al.*, 2004), although detection of a complete flavivirus genome was not reported. Furthermore, the finding of partial flavivirus-like sequences in DNA form is not clear. Clearly, research into the biology of flavivirus persistence in mosquitoes and ticks has been limited and is worthy of extensive additional research.

In summary, it should be apparent that both viral and host factors play a role in the initiation and maintenance of persistent infection at both the cellular and organismal levels. In addition, successful persistence in nature almost certainly depends upon ecological and environmental forces, but these latter factors are wholly beyond the scope of this limited review.

Lessons from other viral systems

Multiple RNA and DNA viruses are known to establish persistence in culture as well as in humans and animals. Consideration of several may provide insight, if not direct parallels, useful in the study of biology of persistent VBFV infections.

The *Hepacivirus* genus, containing hepatitis C virus (HCV) species, belongs to the *Flaviviridae* family, together with the *Flavivirus* genus under which VBFVs are classified. Despite the virus-specific cytotoxic T lymphocytes and antibodies, persistent HCV infections, which are established with high efficiency, are known to occur in humans and animals, such as chimpanzees (Main *et al.*, 1979; Caini *et al.*, 2012; McNally *et al.*, 2012). HCV persistence is associated with various strategies, such as the high genetic variability that facilitates passive immune evasion. *In vivo*, HCV fails to activate CD4+ T cells, leading to exhaustion of CD8+ T cells. At cellular level, HCV can block interferon induction by blocking RIG-I and mitochondrial antiviral signaling (MAVS) using its NS3-NS4A protease, which cleaves the IFN promoter-stimulator 1 (Gould, 2001; Muñoz-Jordán *et al.*, 2005; Baril *et al.*, 2009; Hudopisk *et al.*, 2013; Perera-Lecoin *et al.*, 2013).

Hantavirus infections are another interesting example of viral persistence. Hantaviruses are segmented, RNA viruses that cause lifelong infections in their reservoir rodent hosts, despite high levels of neutralizing antibodies (Botten *et al.*, 2000; Meyer & Schmaljohn, 2000; Easterbrook & Klein, 2008). Pathogen recognition receptors, such as RIG-I and TLR7, are not elevated in the lungs of infected rats, suggesting that evasion of viral recognition may contribute toward the establishment of a persistent infection. Perhaps, the reason for noninduction of RIG-I is the fact that hantaviruses do not produce detectable amounts of dsRNA (Wang *et al.*, 2011). IFNs, such as IFN-β, IFN-λ, MxA, and pro-inflammatory chemokines, cytokines, and transcription factor genes are elevated midway in the infection followed by a down-regulation that favors the expression of TGF-β (Schountz *et al.*, 2012). A continued up-regulation of the cytokines could be detrimental to the cell, and the virus would fail to persist. Results of a recently established animal model also show that host-adapted SNV achieves prolonged and disseminated infection, with no disease in hamsters (Safronetz *et al.*, 2013). Therefore, hantaviruses may have evolved mechanisms of manipulating target genes, which establishes persistence when induced.

One of the best-studied models of persistent RNA virus infection is the rodent-borne arenavirus, lymphocytic choriomeningitis virus (LCMV). In LCMV clone 13 (Cl13), a glutamine at position 1079 in the glycoprotein (lysine in the ARM 53b strain of LCMV) is important for persistence, but its precise function is unknown (Salvato *et al.*, 1991; Moskophidis *et al.*, 1995). The mechanisms of LCMV persistence are linked to the down-regulation of MHC and costimulatory molecules, inflammatory cytokines, as well as virus-induced production of immunosuppressive cytokines (Ng *et al.*, 2011). The inability of CD134-deficient mice to control LCMV infection over time was reported to be a result of CD4 and CD8 responses (Boettler *et al.*, 2012). In addition, OX40 also has a role in the establishment of persistent LMCV infection (Boettler *et al.*, 2012). A recent report showed that IFN blockade of type I IFN signaling results in a CD4 T cell-dependent clearance of LCMV Cl13 (Teijaro *et al.*, 2013). This is an interesting observation considering that IFN induction is part of the antiviral response and that its induction can lead to apoptosis. However, the precise mechanism of persistence is not completely clear (Easterbrook & Klein, 2008), but it also demonstrates 'clever' viral manipulation of the host system to establish persistence.

Coxsackievirus infections are another system from which lessons might be drawn. Coxsackievirus B3 has been implicated toward the development of certain chronic muscle diseases, such as chronic inflammatory myopathy (Lodge *et al.*, 1987; Tam & Messner, 1999). Coxsackievirus persistence in cell culture can take two forms: (1) an incurable steady state characterized by nonlytic virus infection and (2) an antiviral-curable carrier culture system (Brinton, 2013; Pierson & Kielian, 2013). While mechanisms of coxsackievirus persistence are not completely understood, down-regulation of the coxsackievirus receptor (CAR) has been suggested to play a role in viral persistence (Varatharaj, 2010). Down-regulation of CAR in coxsackievirus-infected HL-1 cells occurs rapidly from 60% following three passages to 90% at passage 8 (Varatharaj, 2010). The importance of CAR down-regulation is emphasized by the fact that *in vitro* CAR knockout results in reduced viral replication as well as virus-induced cell lysis (Tomori, 2004; Gulati & Maheshwari, 2007).

These selected examples simply highlight the diversity of possible mechanisms that the VBFV might harness in initiating and maintaining persistence and provide concepts that might be useful to investigate.

Avenues for future studies

In the preceding sections, we surveyed a substantial literature with relevance to various aspects of flavivirus persistence. Elucidation of how flaviviruses persist in humans could help toward the development of therapeutic interventions that could alleviate morbidity and budgetary burdens associated with neurological sequelae. Despite the current knowledge, a relative dearth of knowledge still exists, and additional research is merited on these significant human pathogens. For instance, the definition of

specific viral proteins and cellular factors and their interactions in the establishment and maintenance of persistent infection is very limited. As noted, for flaviviruses to persist in infected cells in culture or *in vivo*, specific host defenses need to be evaded or controlled.

The role of NS5 as an interferon antagonist (especially in TBFVs) has been established (Best *et al.*, 2005), but its IFN antagonism in the context of VBFV persistence has not been fully explored. This is also true for MBFV NS4B, which impairs IFN-α/β induction via JAK/STAT signaling (Muñoz-Jordán *et al.*, 2005). Furthermore, mutations in NS2A of Kunjin virus result in increased IFN levels, suggesting an IFN antagonistic role of NS2A (Liu *et al.*, 2004). Intriguing is the fact that VBFVs antagonize IFN even in the cells that will eventually die in the acute phase of infection. As IFN antagonism seems to be important for HCV, interrogating the role of these VBFV proteins to ascertain their role in the establishment of viral persistence will be critical.

The specific mammalian host immune responses that are evaded or controlled also need to be identified precisely. Although overexpression of Bcl-2 in BHK and CHO cells resulted in the inhibition of apoptosis and JEV persistence, a direct viral effect on Bcl-2 was not determined (Liao *et al.*, 1998). It would be useful to understand which, and how, VBFV proteins interact with the Bcl-2 pathway. Indeed, other host pathways could be involved and need to be elucidated further.

The exact correlates of persistent flavivirus infection in the arachnid or insect vector host also need to be determined. The biology of ticks and that of mammals is completely different. For ixodid tick vectors, identifying genetic or biological targets that could play a role in viral persistence is imperative. These vector mechanisms might find application, when known, in efforts to control natural flavivirus persistence cycles in ticks. The mechanism(s) could involve viral genetic changes, such as specific mutations that may render the virus less detectable in infected cells. An important question for transmission and viral evolution dynamics is why arthropod vectors do not appear to be killed by viral infection.

The down-regulation of the CAR, as a coevolutionary development that favors persistence, is intriguing (Fechner *et al.*, 2007; Pinkert *et al.*, 2011). For flaviviruses, various receptors have been identified as possible virus-entry mechanisms (Smit *et al.*, 2011; Perera-Lecoin *et al.*, 2013). Investigations into whether there is a down-regulation of flavivirus receptors, as in Coxsackievirus persistence, could be useful in defining flavivirus persistence mechanism(s).

Finally, the establishment of relevant animal models of VBFV persistence will also be crucial for understanding the dynamics of viral persistence and host responses. Animals that serve as the natural host reservoirs will be key in developing these models. Some models have been established, but may have failed to answer ecologically important question. Thus, future work will combine studies encompassing the biology of VBFVs, molecular cell biology, animal models, and eventually virus–host ecology.

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Acknowledgements

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