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Tick-borne flaviviruses: dissecting host immune responses and virus countermeasures

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Abstract

The tick-borne encephalitis (TBE) serocomplex of viruses, genus *Flavivirus*, includes a number of important human pathogens that cause serious neurological illnesses and hemorrhagic fevers. These viruses pose a significant public health problem due to high rates of morbidity and mortality, their emergence to new geographic areas, and the recent rise in the incidence of human infections. The most notable member of the TBE serocomplex is tick-borne encephalitis virus (TBEV), a neurotropic flavivirus that causes debilitating and sometimes fatal encephalitis. Although effective prophylactic anti-TBEV vaccines have been developed, there is currently no specific treatment for infection. To identify new targets for therapeutical intervention, it is imperative to understand interactions between TBEV and the host immune response to infection. Interferon (IFN) has a critical role in controlling flavivirus replication. Dendritic cells (DCs) represent an early target of TBEV infection and are major producers of IFN. Thus, interactions between DCs, IFN responses, and the virus are likely to substantially influence the outcome of infection. Early IFN and DC responses are modulated not only by the virus, but also by the tick vector and immunomodulatory compounds of tick saliva inoculated with virus into the skin. Our laboratory is examining interactions between the triad of virus, tick vector, and mammalian host that contribute to the pathogenesis of tick-borne flaviviruses. This work will provide a more detailed understanding of early events in virus infection and their impact on flavivirus pathogenesis.

Keywords

Tick-borne flavivirus; *Ixodes* tick vector; Interferon antagonism; Dendritic cells

Introduction

The tick-borne encephalitis (TBE) serocomplex comprises viruses belonging to the genus *Flavivirus* of the family *Flaviviridae* and includes TBE virus (TBEV), Powassan (POWV), Omsk hemorrhagic fever virus (OHFV), and Kyasanur Forest disease virus (KFDV). These viruses cause encephalitis, meningitis, and/or hemorrhagic fevers and represent a serious public health threat due to high morbidity and mortality rates following infection. TBEV, the prototypical tick-borne flavivirus, has three subtypes: European, Far Eastern, and Siberian. The European subtype has been isolated from most European countries and western Russia, while the Siberian and Far Eastern strains extend from Finland and the Baltic countries through central and eastern Asia to Japan (reviewed in [1]). Viruses within the TBEV serocomplex are considered emerging and re-emerging pathogens due to the recent rise in the incidence of

human infection, the recognition of TBEV in new geographic areas, and the emergence of new viruses such as Alkhurma hemorrhagic fever virus, first isolated in 1995 [2,3].

Transmission of TBEV to humans generally occurs following the bite of an infected tick. Alternative modes of nonvectored transmission include ingestion of unpasteurized milk products from viremic livestock [4] or exposure, presumably through cuts and abrasions, to infected muskrats that are handled as part of the fur trade (reviewed in [5]). Tick-borne encephalitis is a biphasic disease characterized by an initial flu-like illness that develops 1–2 weeks following a tick bite. After resolution of this first phase, 20–30% of patients develop a second phase of disease that generally manifests as a neurological illness ranging from mild meningitis to severe encephalitis often with myelitis and spinal paralysis [5,6]. A third of these patients have long-lasting sequelae that frequently present as cognitive dysfunction and a compromised quality of life. Depending on the TBEV subtype, mortality rates range from 0 to 1.4% for European strains of TBEV to as high as 20–40% for Far Eastern strains of TBEV. An effective vaccine is available and administered throughout Europe as the primary means of prevention. However, treatment of clinically recognized cases is limited to supportive care.

TBEV was originally isolated and shown to cause tick-borne encephalitis in 1937 [6]. Since its discovery, research efforts have primarily focused on defining the epidemiology and clinical features of disease, elucidating the zoonotic cycle and developing diagnostics and effective vaccines. Elegant molecular virology studies have also uncovered important details of the structural features of the virus and its life cycle [7]. However, many aspects of virus maintenance in the environment and mechanisms of disease remain unclear. With 2007 marking the 70th anniversary of the discovery of TBEV, there is still much to learn regarding the virus-vector interface as well as host and viral factors that govern the outcome of infection. Thus, elucidation of the molecular basis of virulence is a present challenge for tick-borne flavivirus research. In a recent initiative, our laboratory is working to better understand the molecular basis of viral pathogenesis by investigating critical interfaces between tick-borne flaviviruses and their tick vector and mammalian hosts.

The flaviviruses

In addition to the tick-borne viruses, the flaviviruses include mosquito-borne dengue virus (DEN, serotypes 1 through 4), yellow fever virus (YFV), Japanese encephalitis virus (JEV), and West Nile virus (WNV). All flaviviruses share a similar virion structure characterized as a small, enveloped particle containing a single-stranded, positive sense RNA genome. The genome is approximately 11 kb in length and is organized as a large open-reading frame flanked by 5'- and 3'- noncoding regions. Following translation, the single polyprotein is cleaved by viral and cellular proteases into three structural proteins, C (capsid), prM/M (membrane and its precursor), and E (envelope), and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (reviewed in [8,9]). The C protein binds viral RNA to form the spherical nucleocapsid, which is enclosed within a host-derived lipid bilayer studded with viral prM and E proteins. The E protein functions in receptor binding and membrane fusion and is the immunodominant antigen of the virus that induces neutralizing antibodies and a protective immune response (review in [10]). The prM protein in immature virus particles protects the E protein from premature membrane fusion. The pr fragment of M is cleaved by furin in the *trans*-Golgi network enabling secretion of mature virions from infected cells [9].

The flavivirus NS proteins form complexes on modified endoplasmic reticulum membranes and function in viral RNA replication and polyprotein processing [9]. NS3 has viral RNA helicase/NTPase activity and, together with its co-factor NS2B, also functions as the viral protease. NS5 is the largest NS protein and most conserved among the flaviviruses. It encodes an N-terminal methyltransferase (MTase) and a C-terminal RNA-dependent RNA polymerase

(RdRP) necessary for viral RNA replication. We have demonstrated that NS5 also has a role in suppressing host interferon (IFN) responses [11]. However, the roles of NS proteins in virus–host interactions are poorly understood.

The big picture of flavivirus pathogenesis

Tick-borne flaviviruses are maintained in nature through a complex enzootic transmission cycle requiring both vertebrate host and ixodid tick vector. Ticks are effective virus reservoirs. Indeed, once infected, virus can persist for the remainder of the tick's life span, enabling virus transmission for years after the initial infection (reviewed in [12]). Virus transmission to a mammalian host is required for maintenance of TBEV in nature although, in contrast to ticks, the mammalian host functions as a short-lived reservoir and infection is generally of limited duration. Small rodents are thought to be the most important mammals in the transmission cycle; humans are an accidental and dead-end host (Fig. 1). Transmission of virus to a mammalian host can occur remarkably fast as transmission of POWV occurs within 15 min of tick attachment to naive mice [13].

Upon inoculation of virus into the skin, initial infection and replication occurs in dendritic cells (DCs) localized in the skin (Fig. 1) and neutrophils [14,15]. Other cells may also be infected since this aspect of viral pathogenesis has not been explored in great detail. DCs are thought to transport virus to nearby lymph nodes. Virus replication at this site leads to viremia and spread to peripheral organs including the central nervous system (CNS) (Fig. 1). The primary cellular targets of infection in the CNS are neurons [16]. However, the mechanisms by which the virus enters and damages the CNS are not defined. Although critical for controlling virus infection in the CNS, the host immune response has been implicated in contributing to neuropathology. Recently, genetic analysis of TBE patients in Lithuania suggested that a deletion within the chemokine receptor CCR5 is a host genetic factor associated with severe TBE [17]. Similarly, a mutation in the CCR5 gene was shown to correlate with severe disease in West Nile virus infections [18]. CCR5 promotes trafficking of leukocytes to the brain facilitating virus clearance [19]. Thus, an impaired immune response may be an important determinant of flavivirus pathogenesis.

Our studies have utilized Langat virus (LGTV), a naturally attenuated member of the TBEV serogroup, as an experimental model. LGTV was tested as a live-attenuated vaccine candidate for TBEV. However, clinical study of the LGTV vaccine was stopped because it caused encephalitis in approximately 1:10,000 vaccine recipients [5]. There are two primary research goals in our laboratory. The first is to elucidate virus–vector interactions important for virus persistence in the tick and transmission to mammalian hosts. The second is to understand the mammalian host immune response to flavivirus infection as it relates to three specific areas: (i) virus recognition and induction of the innate response, (ii) virus-mediated inhibition of innate responses (or virus countermeasures), and (iii) dendritic cell responses that bridge innate and adaptive immunity. In this review we highlight our research accomplishments by discussing them in the context of current knowledge of TBEV ecology and pathogenesis. Presently, we can apply only broad brushstrokes to the picture of TBEV pathogenesis. However, the research efforts of our lab and others are revealing important details of TBEV infection that will provide a more complete picture and facilitate development of novel therapeutics and vaccines.

The tick/vector interface

It is important to understand interactions between TBEV and its tick vectors for two main reasons. The first is that a correlation exists between virus pathogenicity and the distribution of specific tick species [20], suggesting that virus adaptation to replication and persistence in individual tick species may influence virus virulence. Hence, identification of virus

determinants required for replication in the tick may lead to a better understanding of viral pathogenesis in humans [21]. The second reason is to identify tick proteins important for virus transmission to the mammalian host. This information may be useful in the development of anti-tick vaccines that block the activity of these proteins and inhibit transmission.

Efficient and biologically accurate methods for infecting ticks in the laboratory are necessary to unravel the mysteries of TBEV–vector interactions. The technical limitations of the two primary methods currently used in laboratory infection of ticks have restricted studies of these interactions. Specifically, infection of ticks by feeding on a viremic host precludes studies of attenuated viruses while infection by microinjection bypasses the natural route of infection. To circumvent these limitations, we developed a technique for synchronously infecting ticks using an adaptation of an immersion protocol developed for *Borrelia burgdorferi* infection of *Ixodes scapularis* larvae [22]. This method essentially involves immersion of *I. scapularis* in tissue culture supernatant containing LGTV and results in approximately 96% of ticks becoming infected following imbibement of the fluid [23]. Virus can then be efficiently transmitted from ticks to mice, and virus is maintained through tick molting into the next life stage (the nymph). Hence, infection by immersion mimics the natural route of infection and recapitulates the natural transmission cycle of LGTV. With the *I. scapularis* genome project nearing completion, we are in a position to utilize the immersion method of infecting ticks in conjunction with modern genomic approaches to identify tick gene products that are affected by virus infection and potentially important in pathogen transmission. In addition, since the immersion technique eliminates the need for both viremic animals and needles, it will greatly facilitate studies of biosafety level (BSL)-3 (POWV) and BSL-4 (TBEV, OHFV and KFDV) flaviviruses.

Important questions that can be addressed using this model are (i) how does the tick respond to infection and (ii) does the virus counteract tick responses? Ticks have a well-developed innate immune system that facilitates recognition and elimination of invading organisms. This system comprises both cellular processes (i.e., phagocytosis and encapsulation) and humoral factors (i.e., defensins, lysozymes, lectins, proteases, and protease inhibitors) (reviewed in [24]). Activation of the tick innate immune response occurs via expression of highly conserved molecules for pathogen recognition such as Toll (similar to mammalian Toll-like receptor), and signal transduction pathways including Imd (homologous to the mammalian tumor necrosis factor), NF- κ B, and JAK-STAT (Janus kinase-signal transducer and activator of transcription).

While different pathogens (i.e., virus vs. bacteria) elicit unique responses in tick vectors, it is noteworthy that the nature of the immune response to invading organisms is also dependent on the tick species. For example, *Dermacentor variabilis* responds to *B. burgdorferi* injection by rapidly releasing defensin peptide, which leads to efficient clearance of the spirochete [24]. In contrast, no defensin peptide is produced in *I. scapularis* following injection with *B. burgdorferi*, which may contribute to establishment of infection. Thus, the innate response of the tick may be an important determinant of vector competence for specific pathogens. Future studies in our laboratory will examine the interactions between TBEV and the tick immune system that affect the vector's ability to support virus infection, replication, and transmission.

To ensure persistence and transmission, tick-borne flaviviruses have likely evolved strategies to modulate or evade specific aspects of tick innate immunity. Due to the conservation of innate defense mechanisms in vertebrates and invertebrates, these virus-encoded evasion strategies may also function in the human host. However, the consequence of virus evasion of innate immunity in humans may be the development of disease, rather than virus persistence. Moreover, given that tick species vary in their innate response to a particular pathogen, it is possible that these responses apply strong evolutionary pressure for selection of viruses with

unique evasive abilities. In turn, this selection may influence the virulence potential of the virus in humans. This fascinating concept could be explored using our immersion protocol, which eliminates mammalian host factors, in conjunction with tick genomics and virus reverse genetics.

Innate immunity of the mammalian host to tick-borne flavivirus infection

In contrast to ticks, the immune system of mammals is composed of both innate and adaptive responses. The innate response occurs within minutes of infection, providing the first wave of defense against the invading pathogen. Following virus infection, the host cell deploys this rapid response to limit virus replication in both the infected cell and in neighboring cells. Although the innate response is multifaceted, type I interferons (including multiple IFN α molecules and IFN β) have a central role. IFN signaling leads to the upregulation of effector molecules, called IFN-stimulated genes or ISGs, that modulate cellular protein synthesis, RNA-half life, and cell proliferation/survival, all of which directly impact the efficiency of virus replication (reviewed in [25,26]). Type I IFNs can also facilitate anti-viral adaptive immune responses by inducing DC maturation and antigen presentation via the MHC class I pathway [27,28]. Effective IFN responses are critical for recovery from infection with flaviviruses, limiting virus replication in both tissue culture and in vivo models of infection [11,29–31]. The importance of IFN-associated responses is also illustrated by the observation that genetic resistance to both mosquito- and tick-borne flaviviruses in the mouse model is linked to the *Oas1b* gene that encodes a 2'–5' oligoadenylate synthetase (OAS) [32,33]. OAS is an ISG that is upregulated in response to IFN stimulation and together with its effector protein, RNase L, functions to cleave mRNA. Thus, IFNs have multiple, nonredundant roles in suppressing flavivirus replication.

Virus infection is detected by the host cell via pathogen recognition receptors (PRRs) including membrane-bound Toll-like receptors (TLRs) and cytoplasmic RIG-I (retinoic acid inducible gene-I)-like RNA helicases (reviewed in [26]). The primary TLRs involved in detecting RNA viruses are TLR3 and TLR7/8 that recognize double- and single-stranded RNA, respectively. The RIG-I-like helicases include RIG-I and melanoma differentiation-associated gene 5 (Mda5). Both RIG-I and Mda5 recognize double-stranded RNA, whereas 5'-triphosphate single-stranded RNA is recognized by RIG-I alone. Signaling through TLR and RIG-I-like helicases results in the activation of latent transcription factors including NF- κ B, ATF2/c-Jun, interferon-regulatory factor (IRF)-3, and IRF-7, and the early production of IFN β . The expression of PRRs is cell type-dependent. For example, TLR3 is expressed by fibroblasts and conventional dendritic cells (cDC), but not by plasmacytoid DCs (pDCs) where TLR7/8 has an important role in virus detection. The RIG-I pathway is important for viral induction of IFN in cDC and in nondendritic cells, but this pathway is dispensable in pDCs. Recognition of cytosolic dsRNA by protein kinase R (PKR), a third type of PRR ubiquitously expressed at low levels in all cell types, also results in production of IFN [34].

Once produced, IFNs bind to cells in both an autocrine and a paracrine manner, which initiates further signaling that amplifies IFN responses. Cells respond rapidly following stimulation via the JAK-STAT signal transduction pathway [35]. Following IFN α/β binding to the receptor subunits (termed IFNAR1 and IFNAR2), the JAK tyrosine kinases, Jak1 and Tyk2, are auto- and trans-phosphorylated (Fig. 2a). The activated JAKs then phosphorylate the intracellular domains of the receptor subunits, creating a recruitment site for STAT1 and STAT2. The STATs are phosphorylated by JAKs, rendering STATs competent to bind to a third component, IRF-9. The complex of STAT1, STAT2, and IRF-9 forms the transcription factor, IFN-stimulated gene factor 3 (ISGF3). ISGF3 translocates to the nucleus where it binds specific DNA sequences called the IFN-stimulated response element (ISRE) within the promoter regions of ISGs [36]. Finally, these events culminate in the expression of hundreds of ISGs

that confer an anti-viral state within the cell and, together with proinflammatory cytokines and chemokines, orchestrate the adaptive immune response.

Our laboratory is studying interactions between tick-borne flaviviruses and the innate IFN response by exploring three broad questions: (i) what signal transduction pathways are activated in response to infection, (ii) are these pathways altered by virus replication and if so what is the mechanism, and (iii) how do viral countermeasures contribute to viral pathogenesis? The predominant mechanisms for cellular recognition of flaviviruses and IFN production are through engagement of RIG-I and PKR [37–39]. However, as demonstrated for WNV, IFN production occurs very late in virus replication, approximately 24 h post-infection (hpi) [40]. Consistent with these results, we have found that IRF-3 activation, a requisite event for RIG-I and TLR3-mediated IFN β production, does not substantially occur during early LGTV replication (R.T. Taylor, unpublished results). Taken together, these results suggest that flaviviruses may impose blocks in pathogen recognition or subsequent signal transduction. Alternatively, the RNA replicative intermediates generated during virus replication may be sequestered in cellular compartments such that they are inaccessible to PRR recognition. We are currently working to distinguish between these two possibilities.

LGTV-encoded countermeasures to IFN-mediated JAK-STAT signaling

Another major focus of the lab is investigating interactions between the tick-borne flaviviruses and IFN-mediated JAK-STAT signaling. All flaviviruses examined thus far inhibit JAK-STAT signal transduction (reviewed in [41]). LGTV interferes with the phosphorylation of both Jak1 and Tyk2 in response to IFN β (Fig. 2a), as well as the phosphorylation of Jak1 during IFN γ signaling [11]. Infection with WNV results in the inhibition of JAK phosphorylation in a manner similar to LGTV [42], while JEV replication suppresses Tyk2 activation during IFN α/β signaling, but has only a marginal effect on Jak1 phosphorylation [31]. Replication of DEN may impose two blocks on JAK-STAT signaling in its ability to both antagonize IFN α -mediated Tyk2 phosphorylation and reduce cellular STAT2 expression [43,44]. It is noteworthy that DEN2 infection, but not infection with JEV, leads to the induction of both IRF-1 and IRF-7 that are ISGs expressed in response to IFN α/β signaling [37]. Hence, despite the observation that flaviviruses all target JAK activation for suppression, some viruses such as JEV may be more efficient at inhibiting JAK-STAT signal transduction than others. Furthermore, JEV suppresses JAK-STAT signaling in mosquitoes [31], suggesting that flaviviruses antagonize these pathways in their arthropod vectors.

The flavivirus nonstructural proteins are responsible for inhibiting IFN-mediated JAK-STAT signaling. We examined the ability of individual proteins from LGTV to suppress signaling and found that NS5 is a very efficient IFN antagonist [11]. Thus, in addition to its two known roles in virus replication, i.e., MTase and RdRP activity, NS5 also inhibits IFN signal transduction. It has been subsequently shown that both TBEV and JEV also utilize NS5 as their primary IFN antagonists [45,46]. However, it is thought that DEN2 utilizes NS4B to suppress signaling [47]. The NS2A and NS4A proteins of DEN2 are required to augment the effects of NS4B, with the cumulative effect of all three resulting in robust inhibition of IFN signaling. The NS4B proteins of WNV and YFV show similar abilities to inhibit JAK-STAT signaling as DEN2 NS4B and thus are thought to be the primary IFN antagonists encoded by these viruses [48]. Therefore, while the ability to inhibit IFN-mediated signal transduction is common to flaviviruses, the viral protein responsible, and possibly the mechanism, may differ between viruses.

In further studies to explore flavivirus-mediated inhibition of IFN signaling, we have mapped the IFN antagonist domain within the 903 amino acid residues of LGTV NS5. Using a series of N-terminal and C-terminal truncation constructs of NS5, we determined that the JAK-STAT

inhibitory domain is contained between amino acids 355 and 735, and lies within the RdRP domain [49]. Although DEN NS5 expressed alone is not as efficient as LGTV in suppressing IFN signaling pathways, incorporation of the LGTV inhibitory domain into DEN NS5 was sufficient to render the chimeric protein a potent inhibitor of JAK-STAT signaling. Furthermore, we identified two noncontiguous stretches of amino acids within the RdRP, residues 374–380 and 624–647, as critical for inhibition. Despite considerable separation on the linear NS5 sequence, these residues localized adjacent to each other when modeled on the WNV RdRP crystal structure (Fig. 2b). This finding suggests that the specific residues identified act cooperatively to form a functional site within the RdRP responsible for JAK-STAT inhibition. This work may have implications for the future design of live attenuated vaccines. Selected residues that confer IFN resistance could be mutated to achieve the delicate balance of immunogenicity while reducing the potential for virus virulence.

Despite the identification of the region within LGTV NS5 responsible for inhibition, the precise mechanism of antagonism remains unknown. In LGTV-infected cells, NS5 associates with IFN receptor complexes, an interaction that is most likely responsible for the failure of JAKs to activate in response to IFN stimulation. However, it is unknown whether the receptor subunits are targeted directly or if other molecules are recruited or activated to suppress IFN responsiveness. JEV NS5 may subvert the function of host cell protein tyrosine phosphatases (PTPs), which are normally involved in negative regulation of JAK-STAT signaling by dephosphorylating activated JAKs [45]. However, the exact PTP involved is also undefined. We are actively working to determine the precise protein–protein interactions required for IFN resistance. This understanding is essential to clarify the role of IFN antagonism in pathogenesis of the different flaviviruses. In addition, type I IFN has been tested as a potential therapeutic for treatment of flavivirus infection although it has had limited success. This may be due to the ability of flaviviruses to suppress IFN-mediated signaling. Hence, understanding the molecular mechanisms involved in inhibition would aid in the design of novel therapeutics that, when delivered with type I IFN, could potentiate its action for treatment of infection.

Dendritic cells: bridging innate and adaptive immunity

Host defense against infection requires both innate and adaptive immune responses working together to mediate clearance of invading pathogens. DCs are the key cellular component that bridges these two responses. As elements of the skin, DCs are strategically located at the site of arthropod-borne flavivirus entry into the mammalian host. This places them in position to initiate an immediate innate immune response and to serve as early targets of flavivirus infection. The first demonstration of flavivirus infection of Langerhans cells, a migratory DC population in the skin, came from studies of mice naturally infected with TBEV via tick feeding [14]. Subsequently, other members of the flavivirus family including DEN and WNV have been shown to infect DCs in vivo and in vitro [15,50–52]. In the context of flavivirus infection, DCs potentially carry out several important functions that include: (i) detecting virus-derived products via PRRs and producing interferon, (ii) transporting virus to draining lymph nodes that likely promotes virus dissemination, and (iii) initiating and shaping the adaptive immune response to infection. Hence, we are interested in exploring both flavivirus-DC interactions at a molecular level and the role DCs have in the outcome of disease.

As mentioned above, production of IFN α/β is critical for controlling flavivirus infection. In a recent report, WNV was shown to be a potent inducer of IFN α secretion by pDCs [52], a subset of DCs particularly tuned to sense virus infection and produce large amounts of IFN α . Although WNV infection was undetectable in human pDCs exposed to WNV in vitro, these cells produced high levels of IFN α . In contrast, monocyte-derived DCs were productively infected by WNV infection, but produced only low levels of IFN α . Similarly, LGTV exposed mouse pDCs showed no detectable levels of virus replication, but high levels of IFN α expression (S.

J. Robertson, unpublished data). Thus, DC subsets may carry out different functions in the course of flavivirus infection: pDCs functioning as particularly keen sensors of flavivirus infection and prominent producers of IFN, and myeloid DCs supporting productive virus infection.

The ability of DCs to initiate and shape the adaptive immune response to virus infection is dependent on their maturation and activation state (reviewed in [53,54]). Immature DCs in the periphery take up viral antigens resulting in their activation and migration to local lymphoid tissues. While in transit, DCs undergo a maturation program that confers their ability to activate naive T-cells into T helper type 1 (Th1), Th2, and cytotoxic T lymphocyte (CTL) effector cells. The maturation program requires DC recognition of virus via PRRs. This interaction results in activation of signaling pathways that lead to high expression of major histocompatibility complex (MHC) class II (required for antigen presentation), T-cell co-stimulatory molecules (i.e., CD80 and CD86), and proinflammatory cytokines, such as type I IFN, interleukin-6 (IL-6), and IL-12, which drive anti-viral Th1 responses (reviewed in [55]).

We have begun to examine the effect of LGTV infection on DC maturation using murine bone marrow-derived DCs as an in vitro model. While LGTV-infected DCs upregulate MHC class II, they failed to upregulate the T-cell co-stimulatory molecules CD80 and CD86 (S. J. Robertson, unpublished data). LGTV infected DCs also did not produce IL-12, despite upregulating TNF α in response to infection. This finding suggests that the inhibition of DC maturation is selective (i.e., inhibition of IL-12, but not TNF α). The lack of IL-12 induction in response to LGTV infection may skew development of the adaptive immune responses away from a Th1-type response.

Other flaviviruses interfere with DC maturation in vitro. For example, DEN-infected human monocyte-derived DCs fail to upregulate MHC and co-stimulatory molecules and have an impaired ability to stimulate T-cell proliferation [56]. Interestingly, infected DCs do not respond to subsequent TNF α stimulation, suggesting that DEN actively blocks DC maturation. These findings contradict earlier in vitro studies demonstrating that DEN infection of DCs induce maturation and production of IFN and proinflammatory cytokines [50,57]. The reason for these disparate findings is unclear. They might be explained by different means of generating DCs in vitro and/or variability in virus isolates. Because the flavivirus-DC interface is likely a critical factor in determining the outcome of infection, flavivirus-mediated modulation of DC phenotype and function warrants further investigation.

The failure of DCs to mature in response to flavivirus infection has important implications for induction of effective anti-viral T-cell-mediated immunity. Low expression of MHC and co-stimulatory molecules reduces the capacity of DCs to appropriately present antigen and activate T-cells [55]. This DC phenotype, in turn, may lead to an aberrant antiviral immune response that is ineffective in virus clearance. In addition, phenotypically immature DCs have been shown to induce immunosuppressive regulatory T (Treg) cells (reviewed in [58]). While Treg cells are essential for maintaining homeostasis and controlling the immune response, aberrant activation of these cells may contribute to virus survival and/or pathogenesis. Treg cells have been investigated in many persistent infectious diseases, but their role in acute viral infections is poorly defined. Recently, it has been reported that Treg cells are expanded in blood of patients with acute DEN infection [59]. Interestingly, patients with mild febrile disease have more Treg cells than patients with severe hemorrhagic fever. This observation suggests that Treg cell expansion functions to suppress the production of vasoactive cytokines, such as IFN γ , TNF α , and IL-6, all of which contribute to the immunopathology associated with DEN infection. The possible involvement of Treg cells during LGTV infection represents an interesting avenue of future research.

The precise mechanisms by which LGTV modulates DC maturation have yet to be defined. However, one possibility is that the impairment of DC maturation involves the interferon antagonist activity of LGTV. Following virus infection, innate responses control the magnitude and quality of the adaptive immune response by modulating the function of DCs (reviewed in [60]). Specifically, both the type I IFN induction pathway and secreted IFN are important for phenotypic and functional maturation of DCs in response to virus infection [61]. LGTV infection inhibits IFN-induced STAT1 phosphorylation in both human monocyte-derived DCs and mouse bone marrow-derived DCs [11]. Therefore, we can speculate that the lack of DC maturation following LGTV infection involves NS5-mediated IFN antagonism. Recently, respiratory syncytial virus (RSV)-mediated inhibition of DC maturation has been shown to be dependent on the IFN antagonist activity of NS1 and NS2 proteins [62,63]. Thus, DC suppression may represent a particularly important consequence of IFN antagonist activity of many viruses.

In regard to tick-borne flavivirus infection, it is also noteworthy that tick saliva contains an array of proteins with immunomodulatory activities that may directly affect DC responses. Tick saliva can inhibit several functions of the immune system including phagocytosis, production of proinflammatory cytokines by macrophages, and NK cell activity (reviewed in [64,65]). In addition, tick salivary proteins can bind and neutralize chemokines that normally recruit cells of the innate immune system [66]. Termed evasins, these chemokine-binding proteins inhibit neutrophil recruitment and anti-inflammatory activity in a murine model of skin infection. The effect of tick saliva on DCs is beginning to be explored in more detail. Tick saliva from *I. ricinus* was shown to inhibit DC maturation upon stimulation with virus-specific TLRs (i.e., TLR3, TLR7, and TLR9) [67]. Moreover, DCs from saliva-treated mice show impaired antigen presentation and Th1 polarizing capacity, and strongly induce development of an anti-inflammatory Th2 type response. Thus, in the context of TBEV, it will be important to consider the contribution of the tick vector to virus infection and DC function in the mammalian host.

Conclusions

Tick-borne flaviviruses present a fascinating triad of virus, tick vector, and mammalian host. As highlighted in this review, these interactions (a ménage à trois, of sorts) greatly influence virus maintenance in nature. Moreover, the long-term relationship of virus with tick vectors places evolutionary constraints on virus genotype and hence, its potential virulence in mammalian hosts. Thus, it is difficult to evaluate the actions of one participant without careful consideration of the others. The present challenge is to identify factors that allow virus persistence in nature and promote human infection and pathogenicity. New experimental approaches and modern genomic tools provide a prime opportunity to uncover some of these factors and to gain a better understanding of the interactions between virus, tick, and mammalian host.

Considerable progress has been made recently in defining host defenses to virus infection and virus-encoded countermeasures. Flavivirus infection in the mammalian host is a fine balance between two ultimate outcomes: (i) eliminating virus while causing minimal tissue damage and (ii) virus survival and immune-mediated pathology. Given the potential adverse effects these viruses have on dendritic cells, and consequently the host immune response, dissecting protective from pathogenic responses is important. The IFN antagonist activity of LGTV NS5 is an exciting example of such a factor that counteracts the host immune response and may play an important role in the outcome of infection. It is tempting to speculate on a future therapeutic strategy to boost anti-viral responses that is rationally designed around small-molecule inhibitors that disrupt the interactions between flavivirus IFN antagonists and the host cell signaling pathways.

Although not specifically explored in our current studies, both humoral and cell-mediated immune responses are clearly important factors in determining the outcome of flavivirus infections. Neutralizing antibodies, primarily directed against the E protein, are critical for protection against flavivirus infection including TBEV and WNV (reviewed in [68]). Moreover, prospective clinical studies of TBE patients have revealed a correlation between low levels of neutralizing serum antibodies with severe acute encephalitic syndrome [69]. Thus, compromised antibody responses early in the course of TBEV infection may lead to more severe disease. T-cell responses also play an important role in viral clearance, prevention of virus dissemination, and resolution of CNS infection (reviewed in [70,71]). However, pathogenic effects of T-cell responses have also been demonstrated in animal models of WNV and DEN infections. Because DCs are central to the development of adaptive humoral and cell-mediated immunity, modulation of their function by flavivirus infection is likely important for shaping host immune responses. Thus, future work will examine how flavivirus subversion of DC responses affects both protective and pathogenic responses.

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Fig. 1.

Transmission cycle of tick-borne encephalitis virus. Upper panel: The tick life cycle progresses through four developmental stages (egg, larva, nymph, and adult) (dashed, blue arrows). Engorgement on a suitable host is required for ticks to transition to each developmental stage, and for adult females to produce eggs. Tick-borne encephalitis virus (TBEV) infection can be maintained throughout the tick lifespan (solid, red arrows). Transmission to naive ticks occurs primarily from nymphs to larvae while co-feeding on the same rodent host (thick arrows). Lower panel: Transmission of TBEV to humans generally occurs via the bite of an infected tick (red arrows). Dendritic cells (as well as neutrophils) are believed to be early cellular targets of TBEV infection. These cells may function in transporting the virus to draining lymph nodes.

Virus replicates within the lymph node leading to viremia and spread to other organs including the CNS. The transmission cycle was reproduced with modifications from Lindquist et al. [6] with permission from the publisher

Fig. 2.

Overview of the IFN signaling pathway and inhibition by flaviviruses. **a** IFN signaling is initiated by the ligation of IFN receptors by IFN- α/β leading to the phosphorylation of JAKs (JAK1 and TYK2) and STATs (STAT1 and STAT2). The phosphorylated STATs form a heterodimer and bind to IRF-9 to form the transcription factor, ISGF3. ISGF3 then translocates to the nucleus and binds to the ISRE sequences within the promoter regions of hundreds of interferon-stimulated genes to induce their expression. All arthropod-borne flaviviruses examined thus far inhibit the activation of JAKs. DEN also affects the cellular levels of STAT2. **b** The two amino acid stretches identified as important for the JAK-STAT inhibitory activity of LGTV NS5 are modeled on the WNV RdRP three-dimensional structure. LGTV residues 374–380 and 624–647 (pink) and the polymerase active site (blue) are shown