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Immunobiology of Japanese Encephalitis Virus

Maximilian Larena and Mario Lobigs
Department of Emerging Pathogens and Vaccines, John Curtin School of Medical Research, The Australian National University, Canberra, ACT Australia

1. Introduction

Japanese encephalitis (JE) is an acute central nervous system inflammatory disease cause by infection with Japanese encephalitis virus (JEV), a small, enveloped, plus-strand RNA virus belonging to the family *Flaviviridae*. It is the leading cause of viral encephalitis in South-East Asia, India and China, where 3 billion people are at risk of contracting the disease (Erlanger *et al*., 2009). Annually, about 35,000 cases of JE are reported, resulting in about 10,000 deaths and a high incidence of neuropsychiatric deficits among survivors. Treatment of JE patients is supportive and in the absence of availability of antiviral compounds the mainstay of protection against JE is vaccination (Halstead & Thomas, 2011). In the past decades there has been an expansion of the geographic distribution of the virus in Asia and the Asia-Pacific region (van den Hurk *et al*., 2009) and there is an urgent requirement for improved human and veterinary JE vaccines. An understanding of the immunological responses that lead to recovery from infection with JEV and account for vaccine-mediated protection is important in the design of rational approaches to new treatments and vaccines against the disease, and will be the focus of this review.

1.1 Clinical manifestations

Infection with JEV starts with a bite of an infected *Culex* mosquito, although the possibility of transplacental transmission has been demonstrated in mice, swine and humans (Chaturvedi *et al*., 1980; Mathur *et al*., 1981; Morimoto *et al*., 1972). The infection is largely subclinical with only 1:50 to 1:10,000 human infections resulting in symptomatic disease (Tsai, 2000). The clinical features of infection range from a non-specific febrile illness, aseptic meningitis, poliomyelitis-like syndrome, to a severe meningoencephalomyelitis (Solomon, 2003; Solomon *et al*., 2000; Solomon *et al*., 1998). The incubation period is from 5 to 15 days before onset of prodromal symptoms, which include fever, generalized weakness, coryza, diarrhoea, and rigors. Afterwards, patients experience headache, vomiting, decreased sensorium, and convulsion. Then a classic presentation ensues, including dull, flat, mask-like facies with wide unblinking eyes, tremor, generalized hypertonia and cogwheel rigidity. Other signs and symptoms found in a subset of patients include generalized tonic-clonic seizures, focal seizures, upper motor neuron facial nerve palsy, extrapyramidal manifestations, asymmetric paralysis, and mental illness. Occasional extrapyramidal symptoms include non-intention tremors, cogwheel rigidity, head nodding and pill rolling movements, opsoclonus, myoclonus, choreoathetosis, and bizarre facial grimacing and lip smacking (Solomon *et al*.,

2000). The case fatality ratio can be as high as 50-60 % (Tsai, 2000), and one half of the survivors have long-term neurologic or psychiatric sequelae (Solomon, 2003).

1.2 Animal models

JE is also a veterinary disease with occasional fatal outcome in horses, and abortions and abnormal births in pigs (Halstead & Jacobson, 2003). While pigs can act as amplifier host in the transmission cycle of the virus, JEV infection of horses, like that of humans, does not generate sufficient viremia for virus transmission. The clinical course of JE in horses resembles that found in humans (Gould *et al*., 1964; Lam *et al*., 2005; Miyake, 1964; Yamanaka *et al*., 2006). Mice have been most extensively used as a model for studies on the pathogenesis of JEV (Kimura *et al*., 2010) and show significant similarity to natural human infection. Notably, extraneural infection of adult mice frequently does not result in detectable viremia or virus burden in extraneural tissues, although some animals will develop CNS infection with mostly fatal outcome (Larena *et al*., 2011). Age, genetic background and route of inoculation are risk factors for severe encephalitis (Grossberg & Scherer, 1966; Larena *et al*., 2011). The pathologic changes seen in mouse brain infected with JEV are similar to those observed in humans, with perivascular cuffs, cellular infiltrates, and mild vascular damage (German *et al*., 2006). Interestingly, there is a lack of a dose response in mortality in mice following virus challenge by an extraneural route (Larena *et al*., 2011). This is thought to reflect, at least in part, induction of more vigorous innate immune responses critical in early control of virus dissemination with increasing amount of virus used for infection (Monath *et al*., 2003).

To investigate the immunological correlates for recovery and protection from JEV infection, the animal model should resemble the natural infection route and virus dose, and the animals should have a mature immune system and intact blood-brain-barrier. We have shown that in groups of 8- to 12-week-old mice after footpad challenge with 10^3 PFU of JEV (prototype strain Nakayama) ~50% of animals present with clinical signs of infection starting at day 10 post-infection (pi), which included progressive generalized paresis, piloerection and rigidity. Severe neurological impairment demonstrated by ataxia, postural imbalance, and generalized tonic-clonic seizures is evident later in the course of infection, invariably leading to fatality within 24 - 36 h after disease onset (Larena *et al*., 2011). Histopathological examination at day 10 pi with JEV reveals hallmarks of acute viral encephalitis, including microglial nodules surrounding degenerating neurons, meningeal inflammation, and widespread perivascular leukocytic infiltration. Immunohistochemical staining reveals JEV infected neurons in multiple loci, predominantly localized in the following areas: cerebral cortex, hippocampus, thalamus, brainstem and cerebellum. The initial local site of JEV replication following footpad infection probably involves dendritic cells, given the evidence that they support JEV replication (Aleyas *et al*., 2009; Cao *et al*., 2011; Li *et al*., 2010). Local spread then ensues with peak viremia and splenic viral load, detectable only by real time RT-PCR, peaking at day 2 and day 4, respectively (Larena *et al*., 2011). Subsequently, virus enters the brain. Putative mechanisms for virus invasion into the CNS include i) hematogenous spread, ii) entry through olfactory neurons, iii) retrograde axonal transport through peripheral nerves, iv) a "Trojan horse" mechanism through infected monocytes and v) transcytosis through the endothelial cells of the blood-brain-barrier. JEV infection of neurons is accompanied by a local inflammatory reaction. This induces the release of chemokines stimulating CCR5-dependent migration of leukocytes into the brain parenchyma (Larena and Lobigs, unpublished). Virus clearance from the CNS

is complicated by the irreplaceable nature of neurons and the fact that neuronal damage can be caused directly by virus infection or by infiltrating leukocytes in response to the infection (Griffin, 2011).

2. Innate immunity

2.1 Sensing of the pathogen

Host cells detect distinct conserved molecular signatures (pathogen associated molecular patterns: PAMPs) of invading viruses through germ-line encoded transmembrane or cytosolic pathogen recognition receptors (PRRs) (Bowie & Unterholzner, 2008). This initial sensing and recognition is of paramount importance in viral immunobiology, where activating intracellular signalling cascades ultimately lead to the induction of antiviral, inflammatory and adaptive immune responses. Transmembrane PRRs include C-type lectin receptors (CLRs) and the widely studied toll-like receptors (TLRs), both of which are up-regulated after JEV infection (Gupta & Rao, 2011). CLRs contain carbohydrate recognition domains interacting with mannose, fucose, and glucan carbohydrate structures of pathogens (Geijtenbeek & Gringhuis, 2009). A particular CLR, C-type lectin domain family 5 (CLEC5A), is highly expressed after JEV infection and is associated with a proinflammatory profile (Gupta *et al*., 2010). Considering the role of CLEC5A in the immunopathology of dengue hemorrhagic fever (Chen et al., 2008), it can be postulated also to have a key role in the pathologic process of JE neuroinflammation.

TLRs are composed of a leucine-rich repeat-containing ectodomain, a transmembrane domain and an intracellular Toll–interleukin 1 receptor (TIR) domain (Kawai & Akira, 2010). The ectodomain mediates recognition of PAMPs, while the intracellular TIR domain mediates downstream signal transduction. TLR signaling, except that via TLR3, requires the TIR-domain adaptor molecule, MyD88, and therefore can be prevented by MyD88 knock-out (Kawai & Akira, 2010). In the absence of MyD88, bone marrow-derived macrophages and dendritic cells infected with JEV have reduced production of inflammatory cytokines interleukin (IL)-6, IL-10, IL-12, and tumour necrosis factor (TNF)-α (Aleyas *et al*., 2009). This supports a role of TLR signalling through MyD88 in shaping the immune responses to JEV. However, this is not reflected in a markedly altered disease outcome, given that MyD88-/- mice only show a partial impairment in interferon (IFN)-α production and similar susceptibility to JEV in comparison to wild-type mice (Kato *et al*., 2006), suggesting a redundancy in pathways for recognition of JEV infection.

Cytosolic PRRs are essential for detecting pathogens invading the cytosol. They are classified into nucleotide binding oligomerization domain (NOD)-like and retinoic acid-inducible gene (RIG)-1-like receptors (Wilkins & Gale, 2010). NOD-like receptors, NOD2 and NLRP3, recognize ssRNA and dsRNA, respectively, and have significant antiviral activity through IFN signalling. Both proteins are expected to recognize flaviviral genomic RNA, although their role in JEV infection remains to be investigated. RIG-1-like receptors, also known as RNA helicases, have a conserved DExD/H box helicase domain and a C-terminal regulatory domain among the three recently identified members, RIG-1, melanoma differentiation-associated antigen 5 (MDA-5) and laboratory of genetics and physiology 2 (LGP2) (Wilkins & Gale, 2010). The C-terminal regulatory domain serves as the recognition site for sensing ssRNA and dsRNA. Kato et al (2006) have shown that RIG-1 receptor signaling, but not that via MDA-5, is critical for the antiviral response against JEV: RIG-1-/-, but not MDA-5-/- mice, display impaired type 1 IFN production and increased susceptibility

to JEV infection. LGP2 was initially reported as a dominant negative regulator of RIG-1 and MDA-5 signalling (Komuro & Horvath, 2006; Murali *et al*., 2008); however, Satoh et al (2010) have demonstrated that bone marrow-derived dendritic cells from LGP2-/- mice infected with JEV have an impaired production of IFN-β, indicating that LGP2 functions upstream of RIG-1 and MDA-5 to potentiate viral RNA-induced signalling as a positive regulator.

2.2 Type 1 interferon induction and signalling

Interferons (IFNs) are a group of cytokines first discovered based on their antiviral activity against influenza (Borden *et al*., 2007). Three families of IFNs, type I, type II and the recently identified type III can be distinguished. Type I IFNs include multiple IFN-α subsets, a single IFN-β, IFN-ω, and the recently discovered IFN-ε (Hardy *et al*., 2004). All members bind to the same cell surface receptor, and are located in a single gene cluster both in humans and in mice. In addition to their antiviral activity, type I IFNs are key to efficient establishment of the adaptive immune responses (Borden *et al*., 2007). Type III IFNs (IFN-λ1, -λ2 and -λ3) are new members of the IFN superfamily first discovered in 2003 and shown to be related to type I IFN (Ank *et al*., 2006). However, they differ by signalling through a receptor complex that is different from that used by type I IFNs. Numerous RNA and DNA viruses induce and are sensitive to IFN-λs, although it remains unclear if type III IFNs are important in the host response against JEV infection. Type II IFN consists of a single cytokine, IFN-γ, and its function in JEV infection is described in Section 2.3.

IFN production after JEV infection was initially documented in mice (Rokutanda, 1969) and later in humans (Burke & Morill, 1987). Early in vitro and in vivo animal studies depicting its significance as an antiviral compound against JEV employed the use of IFN inducers (Ghosh *et al*., 1990; Taylor *et al*., 1980) and recombinant IFN-α (Crance et al., 2003). Furthermore, mice deficient in IFN-α receptor infected with JEV show sustained high viremia and fulminant disease (Lee *et al*., 2004; Lee & Lobigs, 2002; Lobigs *et al*., 2009), demonstrating that type I IFN is a key tropism determinant of JEV.

IFN gene expression is induced by the binding of PRR-activated transcription factors to their promoters (Borden *et al*., 2007). They include IFN regulatory factor (IRF) proteins and NF-κB (Honda *et al*., 2006; Tenoever *et al*., 2007). In the case of JEV infection, RIG-1-dependent IRF-3 and phosphatidylinositol-3 kinase-dependent NF-κB activation is essential for IFN production (Chang *et al*., 2006). NF-κB-dependent and NF-κB-independent mechanisms of IFN induction after JEV infection have been suggested by Abraham et al (2010). Binding of IFN to its cognate receptor at the cell surface triggers a signalling cascade, the Janus kinase - signal transducer and activation of transcription (Jak-Stat) pathway, ultimately triggering IFN-stimulated response element and expression of IFN-stimulated genes (ISGs). ISGs serve as mediators of IFN action directed towards initiation of antiviral and immunoregulatory functions (Borden *et al*., 2007). Antiviral proteins associated with flaviviral infections include double-stranded RNA-activated protein kinase (PKR), the 2′,5′-oligoadenylate synthetases (2′-5′-OAS), ISG15, ISG20, viperin and IFN-induced transmembrane proteins (Brass *et al*., 2009; Hsiao *et al*., ; Jiang *et al*., 2010; Kajaste-Rudnitski *et al*., 2006; Samuel *et al*., 2006). Of these, 2′-5′-OAS proteins are the most widely studied and acts through activation of RNase L, a potent endoribonuclease that cleaves viral RNA (Silverman, 2007). The critical role of 2′-5′-OAS in the control of West Nile virus (WNV) infection was first reported in mice (Mashimo *et al*., 2002; Perelygin *et al*., 2002) and recently in horses and humans, where distinct OAS1a gene polymorphisms were identified as a risk factor (Lim *et al*., 2009; Rios *et al*., 2010). Given the association of OAS with the flavivirus-resistance phenomenon in mice

(Brinton & Perelygin, 2003), this ISG most likely also plays an important role in recovery from JEV infection. ISG-15 is an additional ISG recently documented to be involved in the control of JEV infection (Hsiao *et al*., 2010).

Considering its antiviral action, the therapeutic potential of recombinant IFN in human cases of JE has been investigated. While an initial study suggested a benefit (Harinasuta *et al*., 1985), a subsequent randomised double-blind placebo-controlled clinical trial did not (Solomon *et al*., 2003). The failure to observe a benefit in the larger scale study posed the question of clinical relevance of IFN treatment. It remains to be seen, whether the outcome might differ if higher doses were given, given earlier in the course of infection, or given in combination with other drugs. It is likely that the failure of IFN therapy after an established JEV infection can be attributed to the IFN-antagonistic mechanisms of the virus itself. JEV counteracts the effect of IFN by blocking tyrosine kinase 2 (Tyk2) and Stat activation (Lin *et al*., 2004). This is mediated by the viral NS5 protein through the activation of protein tyrosine phosphatases (Lin *et al*., 2006). Additionally, JEV NS4A protein is reported to block IFN action through inhibiting phosphorylation of Stat 1 and Stat 2 (Lin *et al*., 2008a). Moreover, aside from IFN antagonism at the level of Jak-Stat signalling, JEV is also able to inhibit a downstream antiviral molecule, viperin, by promoting its degradation via a proteasome-dependent mechanism (Chan *et al*., 2008).

2.3 Cellular factors, chemokines and cytokines

Neutrophil leucocytosis is a unique feature in human cases of JE (Chaturvedi *et al*., 1979; Singh *et al*., 2000). A neutrophil chemotactic factor derived from JEV-stimulated macrophages has been reported to induce neutrophilia (Khanna *et al*., 1991). Additionally, an increased level of the neutrophil chemoattractant, IL-8, found in CSF and serum of JEV infected individuals is significantly associated with neutrophilia and an elevated level of IL-8 in CSF and plasma is linked with adverse clinical outcome (Singh *et al*., 2000; Winter *et al*., 2004). This contrasts with a potentially beneficial role of neutrophils in the control of JEV infection by a mechanism involving the degradation of virus via triggering a respiratory burst and the generation of toxic radicals (Srivastava *et al*., 1999).

Cells of the monocytic lineage and the release of soluble factors thereof have been implicated in JEV pathogenesis. Macrophages predominate the inflammatory cells infiltrating the brain parenchyma of individuals with Japanese encephalitis (Johnson *et al*., 1985). They are permissive for JEV replication, and provide a putative mechanism for JEV entry into the CNS (Aleyas *et al*., 2009; Hasegawa *et al*., 1990; Mathur *et al*., 1988; Yang *et al*., 2004). Cathepsin L-mediated processing of the capsid protein appears to play a role in JEV replication in macrophages, since mutant virus resistant to cleavage by the protease has impaired growth in macrophage but not fibroblast or mosquito cell lines (Mori *et al*., 2007). Microglia are a brain-resident macrophage cell population, which can be infected with JEV for prolonged periods without morphological alteration, suggesting that microglia might serve as a reservoir for viral persistence in the CNS (Thongtan *et al*., 2010). Local immune responses initiated by microglial cells may provide protection against JEV infection of the CNS. However, microglial activation resulting in elevated levels of proinflammatory cytokines (IL-6, TNF-α, IL-1β) and chemokines (IL-8, RANTES, MCP1) in the CSF and plasma may give rise to irreversible neuronal damage and correlates with an increased mortality rate (Chen *et al*., 2004; Chen *et al*., 2010; Ghoshal *et al*., 2007; Ravi *et al*., 1997; Saxena *et al*., 2008; Winter *et al*., 2004).

Astroyctes were originally classified as a subclass of glial cells with pleiotropic functions for maintainance of CNS homeostasis, and only recently were they shown to be immunocompetent cells (Dong & Benveniste, 2001). JEV infected astrocytes are an important source of chemokines (CCL5 and CXCL10) for migration of leukocytes into the CNS (Bhowmick *et al*., 2007; Chen *et al*., 2010).

Lastly, natural killer (NK) and γ/δ T cells form the cytotoxic arm of the innate immune pathways. Both exhibit cellular cytotoxicity by causing apoptotic lysis of virally infected cells, either through a direct cell-cell contact mechanism or, indirectly, by release of soluble cytokines, IFN-γ and TNF-α. An in vitro study has demonstrated the antiviral activity of IFN-γ against JEV (Hasegawa *et al*., 1990) and we have confirmed a critical role of IFN-γ in recovery from JEV infection using IFN-γ-/- mice, which demonstrate significantly increased mortality relative to wild-type mice (Larena and Lobigs, unpublished). IFN-γ mediates its antiviral effect, at least in part, through induction of nitric oxide (NO) synthase (Karupiah *et al*., 1993) and an inhibitory effect of NO on JEV growth has been documented (Lin *et al*., 1997; Saxena *et al*., 2000). IFN-γ, derived from γ/δ T cells is necessary for the early control of dissemination of WNV, which is closely related to JEV (Wang *et al*., 2003a). γ/δ T cells may play a protective role at the interface of innate and adaptive immunity, since TCRδ-/- mice display higher susceptibility after secondary challenge with WNV compared to wild-type mice (Wang *et al*., 2006). It will be interesting to uncover whether γ/δ T cells are also important in experimental models of JEV.

3. Adaptive immunity

Adaptive immunity represents the second wave of immune responses and is characterized by specificity, high potency, and development of memory. For it to become activated, it requires signals from antigen presenting cells of the innate immune system. This can be directly through cell-to-cell communication or, indirectly, by recognition of soluble cytokines. Adaptive immunity is composed of the humoral and cell-mediated immune responses mediated by B and T lymphocytes, respectively. The essential contribution of the adaptive immune responses in recovery from viral infections has been evident from empirical observations in people with defective B cell or T cell development (Fulginiti *et al*., 1968; Wilfert *et al*., 1977; Wyatt, 1973).

3.1 B cells

Humoral immunity has paramount protective function in primary JEV infection. The importance of a vigorous, virus-specific, humoral immune response in ameliorating and preventing illness has been documented in human cases of JE (Burke *et al*., 1987; Libraty *et al*., 2002; McCallum, 1991) and in animal models by administration of antibody prior or subsequent to infection with JEV (Goncalvez *et al*., 2008; Gupta *et al*., 2003; Kimura-Kuroda & Yasui, 1988; Zhang *et al*., 1989). We have shown that mice genetically defective in B cells and antibody (μMT-/-) develop uncontrolled viremia, viral persistence in peripheral tissues, rapid and widespread viral dissemination into the CNS, and early uniform mortality (Larena *et al*., 2011). Additionally, transfer of purified JEV-immune B cell fully protects recipient wild-type mice from lethal JEV challenge (Larena *et al*., 2011).

The early IgM response against JEV is independent of T cell help (Larena *et al*., 2011), most likely due to the highly ordered and repetitive surface structures of the virion particle

(Spohn & Bachmann, 2008). Neutralizing anti-JEV IgM antibodies are most important in recovery from primary infection. This is supported by the finding that B cell-deficient mice develop detectable virus in both serum and spleen as early as day 4 pi, a time point when neutralizing anti-JEV IgM antibody start to appear in wild-type mice. In addition, CD4+ T cell-deficient mice (MHCII-/-), which have truncated IgM and blunted IgG antibody responses, present with undetectable early viremia, indicating that even suboptimal anti-JEV IgM antibody levels provided a beneficial effect (Larena *et al*., 2011).

Mechanistically, antibodies elicited against flaviviruses exhibit their action directly by neutralization of infectivity, or indirectly by antibody-dependent cell-mediated cytotoxicity, Fc-γ-receptor-mediated clearance, or complement-mediated cytotoxicity (Pierson *et al*., 2008). Neutralizing antibodies predominantly target the E protein of the virion, although protective antibodies against prM and NS1 proteins have also been documented (Dewasthaly *et al*., 2001; Kolaskar & Kulkarni-Kale, 1999; Konishi *et al*., 1991; Konishi *et al*., 1992a; Konishi *et al*., 1992b; Lin *et al*., 2008b; Lin *et al*., 1998; Nam *et al*., 1999; Seif *et al*., 1995; Wu et al., *2003*; Wu & Lin, *2001*; Xu et al., *2004*). The latter can control JEV infection by their complement-mediated cytolytic potential (Krishna *et al*., 2009; Lin *et al*., 2008b; Lin *et al*., 1998). Antibody neutralizes flavivirus infectivity with high efficiency mainly by interfering with early steps of the viral entry pathway, including attachment, internalisation, and fusion (Butrapet *et al*., 1998; Crill & Roehrig, 2001; Goncalvez *et al*., 2008; Nybakken *et al*., 2005).

3.2 T cells

T cells can be classified phentoypically, on the basis of their antigen receptor usage (α/β vs γ/δ) and their co-receptor expression (CD4 vs. CD8), or functionally (cytotoxic vs helper). Generally, cytotoxic T lymphocytes (CTLs) are predominantly of CD8+ and helper T (Th) cells of CD4+ phenotype. T cells of the γ/δ phenotype recognize non-classical major histocompatibility complex (MHC) antigens and form part of the innate immune response as described earlier. On the other hand, CD8+ and CD4+ α/β T cells recognize MHC-I and MHC-II plus peptide antigen, respectively, and serve as mediators of adaptive immune responses.

3.2.1 CD4+ T cell immune response

Exposure to JEV induces effective CD4+ T cells immunity, characterised by T cell proliferation, production of Th1 and Th2 cytokines, and immunoglobulin class switching (Konishi *et al*., 1995; Ramakrishna *et al*., 2003). Putative Th epitopes that elicit virus-specific and flavivirus cross-reactive proliferative responses in immune splenocytes have been mapped in E protein (Kutubuddin *et al*., 1991). In humans, exposure to live JEV infection or vaccination similarly induces JEV-specific and flavivirus cross-reactive CD4+ T cell responses (Aihara *et al*., 1998; Konishi *et al*., 1995). A region of NS3 protein (residues 193 – 324) has been identified as the dominant source of peptide determinants for CD4+ T cells in a healthy JEV-endemic cohort (Kumar *et al*., 2004a; Kumar *et al*., 2004c). Patients with severe encephalitis had impaired NS3-specific CD4+ T cell responses, indicating a critical protective role of these immune cells in the pathogenesis of JE (Kumar *et al*., 2004b).

Multifaceted CD4+ T cells contribute to controlling infection by various mechanisms, including antiviral cytokine production, antibody class switching, direct cytotoxicity, and maintenance CD8+ T cell activity (Zhu *et al*., 2010). The protective value of JEV-immune CD4+ T cells has been explored in adoptive transfer experiments and genetically deficient

mice (Biswas *et al*., 2009; Larena *et al*., 2011). The lack of CD4+ T cells in MHCII-/- mice results in a truncated JEV-specific IgM response and significantly blunted immunolgobulin class switching to IgG (Larena *et al*., 2011). As a consequence, anti-JEV neutralizing activity in MHCII-/- mice increases marginally up to day 8 pi and drops significantly thereafter. This results in an increased viral burden in the CNS late in the course of infection and uniform mortality. Thus, the beneficial effect of JEV-immune CD4+ T cells predominantly involves effective antibody production, thereby preventing virus entry into the CNS.

3.2.2 CD8+ T cell immune response

Early reports demonstrated JEV-specific CD8+ T cell proliferative responses and cytolytic activity in humans and mice after vaccination or exposure to live JEV infection (Konishi *et al*., 1995; Konishi *et al*., 1997; Konishi *et al*., 1998; Murali-Krishna *et al*., 1995a; Murali-Krishna *et al*., 1994; 1995b). Peptide determinants recognized by JEV-immune CD8+ cells are starting to be identified: they include a H-2Kd-restricted E protein-derived peptide (CYHASVTDI) (Takada *et al*., 2000) and a H-2Db-restricted NS4B protein-derived peptide (SAVWNSTTA) (Larena *et al*., 2011; Trobaugh *et al*., 2010). The humans CD8+ T cell response against JEV appears to be biased to peptide determinants derived from the NS3 protein (Kumar *et al*., 2004c; Kumar *et al*., 2003), as was first reported for the CTL response against the closely related Murray Valley encephalitis virus (MVEV) in mice (Lobigs *et al*., 1994). This response against determinants in NS3 protein is broadly flavivirus cross-reactive and paradoxically recognises disparate epitopes from JEV and distantly related flaviviruses, but ignores more similar peptides from "self" and other virus families (Regner *et al*., 2001), suggesting that primary sequence homology is not always the crucial factor in peptide recognition in the cross-reactive cellular immune responses against flaviviruses.

Cytotoxic CD8+ T cells exert their function by lysing virally infected cells directly through Fas-FasL interaction or the perforin-granzymes exocytosis mechanism, and indirectly by release of soluble cytokines, IFN-γ and TNF-α. A dominant protective role of CD8+ T cells was initially reported by Murali-Krishna et al (1996); however, this involved co-injection of a large number of splenocytes with virus into the brain and required co-transfer of CD4+ T cells. In contrast, we have found only a subsidiary role of CD8+ T cells in recovery from JEV infection in a murine model (Larena *et al*., 2011). Thus, in vivo depletion of CD8+ T cells does not significantly increase susceptibility of mice to virus infection and genetic deficiencies in cytolytic effector pathways of T lymphocytes does not exacerbate the pathogenesis of JEV. However, CD8+ T cells contribute to a significant level to reducing viral load in the CNS of infected Fas-/-, granzymeA/B-/- and CD8+ T cell-depleted mice. Thus, although CD8+ T cells apparently do not provide a significant advantage in terms of survival following JEV infection, they demonstrate a beneficial role in controlling virus growth in the CNS, with the proviso that the latter may occur at the cost of increased immunopathology (Larena *et al*., 2011).

3.2.3 The contribution of CD8+ T cells to recovery from infection differs between JEV and closely related flaviviruses

Our work and that of others has uncovered a conflicting role of CD8+ T cells in recovery from infection with encephalitic flaviviruses. While essential for virus elimination from the CNS and survival in mouse models of West Nile encephalitis (Shrestha & Diamond, 2004; Shrestha *et al*., 2006; Wang *et al*., 2003b; 2004), and a disease-potentiating effect of CTLs was

documented in mice infected with MVEV (Licon Luna *et al*., 2002), the CD8+ T cell response does not markedly affect outcome of infection with JEV (Larena *et al*., 2011). Notably, mice genetically deficient in the Fas- or perforin-dependent pathways of cytotoxicity show greatly increased susceptibility to virulent lineage I WNV infection (Shrestha & Diamond, 2007), but do not differ from wild-type mice in susceptibility to infection with JEV (Larena et al., 2011) or lineage II WNV strain, Sarafend (Wang *et al*., 2004), and are more resistant to infection with MVEV (Licon Luna *et al*., 2002). These findings highlight a difference in pathogenesis between even closely related flaviviruses belonging to the JEV serocomplex (Mullbacher *et al*., 2004) that most likely involves a difference in the capacity of the cellular immune response to resolve the virus infections in the CNS.

3.2.4 Modulation of MHC-I

MHC-I is expressed on virtually all mammalian cells and the cell surface expression of this class of restriction elements for CTLs is up-regulated as a consequence of infection with JEV and other flaviviruses in a diverse range of cell types from different species (Kesson *et al*., 2002; Lobigs *et al*., 2003). Flavivirus-induced up-regulation of MHC-I cell surface expression is, at least in part, IFN-independent (Abraham *et al*., 2010; Kesson & King, 2001; Mullbacher & Lobigs, 1995), and also includes that of non-classical MHC-I (Abraham *et al*., 2008). Although the physiological relevance of this phenomenon in virus transmission remains unclear, it has been proposed that the process may contribute to reduced NK cell activity, which is inhibited by engagement with MHC-I by NK cell inhibitory receptors (Hershkovitz *et al*., 2008; Momburg *et al*., 2001). It has also been hypothesised that flavivirus-induced up-regulation of MHC-I leads to transient T cell autoimmunity (given the increase in "self" antigen presentation), followed by subsequent suppression of "self"-reactive T cell activity, and that flavivirus infection or live vaccination of humans in the tropics could contribute to the observed lower incidence of overt autoimmunity in the tropics than in temperate climates, where flaviviruses are not endemic (Lobigs *et al*., 1996).

4. Implications for vaccination against JE

Current vaccines against JE include non-adjuvanted and alum-adjuvanted inactivated vaccines that are licensed internationally, and an attenuated live vaccine predominantly used in China (Beasley *et al*., 2008; Halstead & Thomas, 2011). There is a clear need for development and licensing of new JE vaccines, which raises the question of immunological correlates for protection against JEV infection that should be targeted by an effective JE vaccine. Our understanding of immune pathways essential for recovery from primary JEV infection emphasises the critical role of neutralising antibody against E protein and the requirement for effective B and CD4+ T cell immune responses, while suggesting a subsidiary contribution of CD8+ T cells to recovery. TLR signalling and type I IFN are also expected to play an important role in induction of effective B cell immunity (Hou *et al*., 2011; Kasturi *et al*., 2011; Le Bon *et al*., 2001). Similar to recovery from primary JEV infection, neutralising antibody against E protein is also key to vaccine-induced protective immunity, with no or only partial protection provided by JEV-immune CD8+ T cell memory (Chen *et al*., 1999; Konishi *et al*., 2003; Pan *et al*., 2001). This evidence highlights that induction of potent and durable memory B cells that produce high-affinity, neutralising antibody against E protein is the prime criterion for efficacy of a vaccine against JEV, in addition to safety and tolerability.

Factor	Outcome after JEV infection
Age	↑ disease severity in younger animals
Route of Infection	intracranial and intranasal = high mortality extraneural = ↓ disease severity
Mouse strain background	impacts on disease outcome
Innate Immunity	
Pathogen Recognition	
Myd88	absence = ↓ production of inflammatory cytokines, no effect on disease severity
RIG-1	absence = ↓ production of type 1 IFN; ↑ disease severity
MDA-5	absence = no effect on production of type 1 IFN; no effect on disease severity
LGP2	absence = ↑ production of type 1 IFN
Interferon Induction and Signalling	
NF-κB	↑ PI3K dependent production of type 1 IFN
IRF-3	↑ RIG-1 dependent production of type 1 IFN
IFN-α	inhibits virus, no effect with treatment of human cases
IFN-α receptor	absence = ↑ disease severity
Jak-stat	inhibited by viral NS4A and NS5 proteins
ISG15	inhibits virus
Viperin	inhibited via proteosome-dependent mechanism
Cellular Factors	
Neutrophils	neutrophilia, intracellular degradation of JEV ↑ release of inflammatory cytokines; ↑ pathology
Macrophage	monocytosis; ↑ migration of monocytes to CNS; ↑ inflammatory cytokines
Microglial Cells	microgliosis; ↑ release of inflammatory cytokines; ↑ pathology
Astrocytes	↑ release of inflammatory cytokines
Other cytokines and chemokines	
IFN-γ	absence = ↑ disease severity
CCR5	absence = ↑ disease severity
Adaptive Immunity	
Effector cells	
B cells	absence = ↑ disease severity
CD4+ T cells	absence = ↑ disease severity
CD8+ Tcells	absence = ↑ CNS viral burden without impact on mortality rate
Cytolytic Pathways	
Perforin	absence = no effect on disease severity
Granzymes A/B	absence = no effect on disease severity
Fas	absence = no effect on disease severity

(↑) increased, (↓) decreased

Table 1. Factors affecting outcome of JEV infection

5. References

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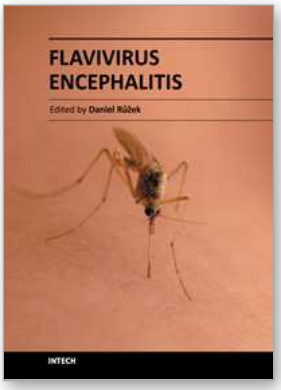

Flavivirus Encephalitis

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Encephalitis is an inflammation of the brain tissue associated with clinical evidence of brain dysfunction. The disease is of high public health importance worldwide due to its high morbidity and mortality. Flaviviruses, such as tick-borne encephalitis virus, Japanese encephalitis virus, Murray Valley encephalitis virus, or St. Louis encephalitis virus, represent important causative agents of encephalitis in humans in various parts of the world. The book Flavivirus Encephalitis provides the most recent information about selected aspects associated with encephalitic flaviviruses. The book contains chapters that cover a wide spectrum of subjects including flavivirus biology, virus-host interactions, role of vectors in disease epidemiology, neurological dengue, and West Nile encephalitis. Special attention is paid to tick-borne encephalitis and Japanese encephalitis viruses. The book uniquely combines up-to-date reviews with cutting-edge original research data, and provides a condensed source of information for clinicians, virologists, pathologists, immunologists, as well as for students of medicine or life sciences.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821