2. Review of literature

JEV is a positive sense single stranded (ss) RNA virus belonging to family Flaviviridae, genus Flavivirus, it falls under JE serogroup and comprises of five genotypes. Though outbreaks of encephalitis attributed to JEV were reported in Japan as early as 1871, it was not until 1924 that JEV was isolated from a clinical case in Japan. The disease Japanese encephalitis (JE) is a major seasonal health problem in many rural areas in India and other parts of Asia. As of recent update (i.e. Feb. 2012) on World Health Organization (WHO) website, approximately 20,000 clinical cases with 6,000 deaths are reported annually (http://www.who.int/biologicals/vaccines/ japanese_encephalitis/en). Until 1970, the temperate zone of Asia was the principal site of JE transmission (Tiroumourougane et al., 2002). In the last three decades, the focus of viral epidemics has covered Asia and parts of western Pacific (Fig. 1).

![Global distribution of JEV. Source: CDC, USA](http://wwwnc.cdc.gov/travel/images/map3-8-geographic-distribution-japanese-encephalitis.jpg)

2.1 JE-virion and genome - structural properties

JEV particles are small (~ 50nm) and contain electron dense core/nucleocapsid of size ~30nm. The viral nucleocapsid consists of viral genome and capsid (C) protein, and it is surrounded by a lipid bilayer. The envelope contains two envelop-associated viral structural proteins envelope (E) and membrane glycoprotein (prM/M).
JEV genome is monopartite, linear, size ~11kb, has 5’ type-I cap (m^7GpppAmpN_2), and lacks 3’ poly-A tail. The 5’ and 3’ untranslated region (UTR) is not well conserved amongst various flaviviruses, although several common secondary structures have been found within this region. Inverted complementary sequences at the 5’ and 3’ ends of the genome mediate long-range RNA interactions and RNA cyclization. The circular conformation of flavivirus genomes was demonstrated to be essential for viral RNA amplification (Villordo and Gamarnik 2009).

The open reading frame (ORF) is sandwiched between the 5’ UTR and 3’ UTR of the viral genome. Translation of the single, long ORF produces a large polyprotein that is co- and post-translationally cleaved into at least 10 different proteins (Fig 3).
The N-terminal of the polyprotein, encodes the structural proteins (C-prM-E), followed by the nonstructural proteins (NS1-NS2A-NS2B-NS3-NS4A-2K-NS4B-NS5). Host signal peptidases are responsible for cleavage between C/prM, prM/E, E/NS1, and 2K-NS4B, while the virus encoded serine protease is responsible for cleavages between NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/2K, and NS4B/NS5 junctions.

2.2 Viral replication and morphogenesis:
Flavivirus replication and assembly occurs simultaneously (Fig 4). The flavivirus replicase complex resides in double-layered membrane compartment (Uchil and Satchidanandam 2003). These vesicle packets are result of proliferation of endoplasmic reticulum (ER) membrane in flavivirus infected cells. The flavivirus replicase associates with membranes through interactions involving the small hydrophobic NS proteins, viral RNA, and presumably some host factors. Replication begins with the synthesis of a genome length negative strand RNA, which then serves as a template for the synthesis of additional positive strand RNA. Viral RNA synthesis is asymmetrical and semiconservative. In-situ hybridization studies have identified accumulation of viral RNA in association with vesicle packets in the perinuclear regions (Westaway et al., 2002).

Virion morphogenesis occurs in association with intracellular membranes. Budding intermediates and clearly distinguishable cytoplasmic nucleocapsids have not been frequently observed suggesting that the process of assembly is rapid. The highly basic C protein interacts with the viral genome RNA in the cytoplasm to form a nucleocapsid
precursor that acquires an envelope by budding into the ER lumen. Later stages in virion maturation include glycan modification of E and prM by trimming and terminal addition.

2.3 Mode of transmission

JEV is transmitted in an enzootic cycle among mosquitoes and vertebrates (Fig. 5). Chief JEV amplifying-hosts are pigs and wading birds. The mosquito vector of JEV differs in different regions. The major mosquito vector of JEV in South East Asia is *Culex tritaeniorhynchus*. *Culex vishnui* complex is also incriminated as a vector in India. Humans are considered dead-end host as they exhibit brief periods of viremia, and they do not facilitate further transmission (Tiroumouougane et al., 2002).

![Fig. 5. Enzootic cycle of JEV. Source: CDC, USA](http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5901a1.htm)

2.4 Epidemiology and Phylogenetic distribution

JE occurs in annual epidemics or endemically in many Asian countries including Japan, Korea, Taiwan, China, Vietnam, Thailand, Malaysia, Myanmar, India, Nepal, and Sri Lanka. Since 1995, JEV prevalence has also been recorded in Torres Strait Islands and recently at the tip of far north Queensland Australia (Smith et al., 2011). Thus, the JEV-endemic area has been expanding. JE is no longer only endemic in Asia, but also in Oceania (Oya and Kurane 2007).

In India, first confirmed case of JE was reported in 1955 at Vellore, Tamil Nadu. The next major outbreak of JE occurred in 1973 in Bankura and Burdwan districts of West
Bengal. Subsequently, the outbreaks have occurred in several parts of India - Andhra Pradesh, Assam, Karnataka, Tamil Nadu, Uttar Pradesh, and West Bengal (Fig. 6). One of the devastating JE-outbreaks in India was reported from Uttar Pradesh during 1988 with a total of 4485 cases and 1413 deaths from eight districts with case fatality of 31.5%. During 2005, encephalitis-outbreak from the same area has reportedly affected more than 8000 individuals with 1300 deaths (http://w3.whosea.org/en/Section1226/Section2073.asp). Since 2007, major JE-outbreaks have been reported from regions of Uttar Pradesh, Assam, and Bihar, while only sporadic cases are reported in rest of India. However, during 2009-2010 seasons Karnataka and Tamil Nadu also witnessed a mild outbreak (http://nvbdep.gov.in/jenew.html).

![Fig. 6. JE epidemiology in India. Source: NVBDCP, India (http://nvbdep.gov.in/je8.html).](image)

In India, JE outbreaks usually coincide with monsoons and post-monsoon period when the vector density is high. However, in endemic areas, sporadic cases may occur throughout the year.
In an endemic area, majority of human infections with JEV remains asymptomatic or manifest as mild-febrile illness (www.cdc.gov/ncidod/dvbid/jencephalitis/facts.htm). Most of the JEV-exposed individuals in the endemic area develop beneficial immune response (Kumar et al., 2004) and thus remain protected against JEV throughout their lifetime.

The incidence of JEV infection and encephalitis varies by country-to-country (Endy and Nisalak 2002). The ratio of asymptomatic (sub-clinical) to symptomatic (clinical disease) infection is estimated to range between 100:1 and 1000:1 with an average of 300:1 (Vaughn and Hoke 1992). Few of the JEV infected individuals, i.e. ~ 1:300, develop severe clinical disease, including seizures, meningoencephalitis, or even coma and death; case fatality rate can range from 5 – 30% (http://www.who.int/biologicals/vaccines/japanese_encephalitis/en). In newly affected areas the case fatality rates can range increase from 30% - 40%. Nearly 50% of the surviving patients have significant neurologic sequel (Saxena et al., 2009; Roy et al., 2006).

Recently, with early detection and management of cases (i.e. better health care and mass vaccination strategies) JE incidence has come down to an average of approximately 20% (http://w3.whosea.org/en/Section1226/Section2073.asp) of encephalitic cases in South and South East Asia. For foreign travelers, travelling to these endemic regions, risk acquiring JE is very low but varies based on destination, duration, season, and activities (Tiroumourougane et al., 2002; http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5901a1.htm).

The age distribution of JE patients varies from country to country. In the countries such as India, half of the patients are children under 4 years of age, and most of them are children under 10 years of age. However, in countries like Japan, Korea and Taiwan where mass JE vaccination has been extensively implemented, JE occurs among adult populations rather than children, the majority of the patients being at over 50 years of age (Oya and Kurane 2007).

**Phylogenetic distribution:** With the advent of molecular techniques in recent times, JE epidemic maps are constructed based on virus genotyping. Genotypic studies based on partial sequencing of C/prM or E-protein sequences have classified JEV into five
different genotypes. There is a loose correlation between genotype and geographic origin of the isolate. Genotype I viruses have been isolated in Japan, China, Taiwan, Vietnam, Nepal, India, and Sri Lanka, whereas genotype II viruses are typically isolated in northern Thailand and Cambodia. Genotype III viruses are found in Indonesia, Malaysia, and southern Thailand. Genotype IV viruses are found in Indonesia/Malaysia regions. The sequence of an Indonesian genotype IV strain suggested that this lineage is the oldest and most divergent. Only the Indonesia-Malaysia region has all genotypes circulating, whereas only more recent genotypes circulate elsewhere (Gubler et al., 2007). A recent study from South Korea shows that circulation of genotype III was predominant until 1993, while genotype I has replaced during recent times (i.e. since 1994) (Yun et al., 2010). Introduction of genotype I in India has also been reported (Fulmali et al., 2011). The term Genotype V was first introduced after the study conducted by Uchil et al in 2001 (Uchil and Satchidanandam 2001). As of 2011, two independent studies from China and South Korea have reported emergence of genotype V JEV in these countries (Li et al., 2011; Takhampunya et al., 2011).

2.5 Prevention

**Vector control:** JEV infection can be minimized by preventing human exposure to mosquito bites. Mosquito repellents, mosquito bed nets impregnated with insecticides are generally used to protect against insect bites. Modalities of spraying antilarval insecticides in and around rice paddy fields are available but are highly expensive. Even though the vector control measure seems basic and cheap, the major drawback is that implementing it in larger areas, especially in rural regions, becomes difficult, due to socio-economical reasons. Reducing the mosquito-pig contact by improving and mosquito proofing piggeries has decreased the incidence in developed countries like Japan and Singapore.

**Vaccination:** In recent years, there is a decrease in the total number of reported JE cases according to the report of WHO. The decrease in JE incidences in China greatly contributes to this decrease. China accounted for the largest number of JE cases in the world. In the last decades, however, China achieved a significant reduction in the number of JE cases because of a nationwide implementation of JE vaccination. In Japan, Korea, and Taiwan, the annual JE case number is less than 10 since the 1990s mainly due to a mass immunization campaign and in part to other ecological factors (Oya and
Kurane 2007). In India, since 2006, mass vaccination against JEV has been implemented at Uttar Pradesh, Assam, and other JEV endemic regions (Kumar et al., 2009). Even though JE is a vaccine-preventable disease, the vaccines are not evenly distributed among the people living in risk prone JE-endemic or -epidemic countries. Major limitations include (i) insufficient production of doses and (ii) highly expensive for usage in developing countries (Oya and Kurane 2007). Some of the available JEV vaccines for humans are listed in the table 1.

<table>
<thead>
<tr>
<th>Type</th>
<th>Example</th>
<th>Age</th>
<th>Dose</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First generation vaccines</strong></td>
<td></td>
<td></td>
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<tr>
<td>Inactivated – Mouse brain derived vaccine</td>
<td>Nakayama-NIH strain or Beijing-1 strain is used as a seed virus</td>
<td>1 yr to all ages</td>
<td>Primary – 3 dose; Booster – 1 yr post primary dose and subsequent 3 yrs</td>
<td>80 – 90 %</td>
</tr>
<tr>
<td>Inactivated – hamster kidney cell-derived vaccine</td>
<td>Beijing-3 strain is used as a seed virus</td>
<td>1 yr to all age</td>
<td></td>
<td>76 – 90 %</td>
</tr>
<tr>
<td><strong>Second generation vaccines</strong></td>
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<td></td>
</tr>
<tr>
<td>Cell-culture derived live attenuated vaccine</td>
<td>SA14-14-2</td>
<td>1 yr to all age</td>
<td>Two doses with an interval of 1 yr, Single dose immunization have also shown comparable efficacy</td>
<td>85 %</td>
</tr>
<tr>
<td>ChimeriVax-JE</td>
<td>All age group</td>
<td></td>
<td></td>
<td>93 – 95 %</td>
</tr>
<tr>
<td>Inactivated – Vero Cell-culture derived</td>
<td>IC51</td>
<td>1 yr to all age</td>
<td>Two doses</td>
<td>83 %</td>
</tr>
</tbody>
</table>

Table 1: Available JEV vaccines for human use.
2.6 Clinical features of JEV infections in humans

JEV infection in humans can range from mild febrile illness to a full-blown mengioencephalomyelitis. It is most often results as asymptomatic febrile illness, and most of JEV exposed individual develop beneficial adaptive response (Kumar et al., 2004).

On an average, 1 in 300 JEV infected individuals produce clinical symptoms (Vaughn and Hoke 1992). JE clinical disease can be divided into three stages, namely, (1) prodromal stage, (2) acute encephalitic stage, and (3) late stage. Prodromal stage is accompanied with non-specific clinical symptoms such as malaise, headache, and fever. It usually lasts for 1-6 days; however, it varies between < 24h to 14 days. Clinical diagnosis is often impossible during this stage of the disease (Solomon 2004).

Clinical symptoms during acute encephalitic stage of JEV infection includes continuous fever, nuchal rigidity, convulsions, and altered sensorium. In severe cases, it might progress into coma. Incidents of polymorphonuclear leucocytosis are also evident in peripheral blood as well as in cerebrospinal fluid (CSF), with slight increase in CSF protein levels. Sugar levels in CSF may increase or may remain normal in different individuals. Case fatality can vary between 5 to 30 % of the cases, with signs of acute cerebral edema or severe respiratory distress.

Late stage of JE disease begins when active inflammation has subsided i.e. when temperature and erythrocyte sedimentation rate (ESR) are completely normal, and neurological signs are tending to improve. If the patient experience a short encephalitic stage then the recovery occurs rapidly, he/she becomes normal within 2-4 weeks of onset of illness. However, if the patient suffers from a prolonged encephalitic stage then the recovery is slow with prolonged convalescence, and they suffer from residual neuronal deficits including acute flaccid paralysis to severe mental retardation in children.

2.7 JE Pathogenesis

Pathogenesis refers to the mechanism by which the disease is caused, and depends on pathogen’s route of entry and final target organ. In neurotropic flavivirus infection, it is hypothesized that these viruses multiply peripherally before reaching central nervous system (CNS). Current knowledge of JE pathogenesis is obtained by studying immune
response to JEV in mice model (in-vivo and in-vitro) studies, or studying human JE patients (survivors vs. non survivors) (Kurane 2002).

The exact mechanism of JEV pathogenesis remains unclear. However, based on in-vitro and in-vivo studies on viral entry, replication, and cell tropism, involving JEV and other related flavivirus, an approximate model of JEV pathogenesis can be built. Two major stages are involved in pathogenesis of any neurotropic virus (Fig. 7).

![Flavivirus infection outline](image)

Fig. 7. Flavivirus infection outline. Source: King and Kesson 2003.

### 2.7.1 Stage-I – Peripheral virus multiplication

Increased viral replication rate depends on the availability of susceptible cells, and augments the chance of viremia, or viral invasion and/or damage into the target organ. Viral replication at the peripheral site forms the primary determining factor of flaviviral disease pathogenesis (Müllbacher et al., 2003).

**Flavivirus replication and cells of myeloid lineage:** Cells of myeloid lineage are known to support several flavivirus replication. After peripheral inoculation, via bite of an infected mosquito, flaviviruses probably do not multiply in epidermal cells of skin but spread to the lymph node by infected dermal dendritic cells (DCs) or Langerhans cells (Chambers and Diamond 2003). Within day one of infection, epidermal Langerhans cells that express viral antigens migrate from the skin to the draining lymph node. These cells also express maturation markers (CD80, CD86, MHC-II, CD11b, and CD83), produce
tumor necrosis factor (TNF) α and interferon (IFN) α, and subsequently become resistant to flavivirus infection. Thus, infected DCs probably serve to promote antigen presentation in the lymph node as well as participate in the spread of infection to lymphoid compartments and CNS using a “Trojan horse” mechanism (King et al., 2007).

After replication in lymphoid tissue, encephalitic viruses are believed to exit via efferent lymphatics and gain access to the circulation, whereby systemic infection is established. Viral antigens have been demonstrated in other peripheral organs including liver, heart, and kidney of infected mice. In humans, the isolations of JEV from the liver of one fatal case and the histological demonstration of damage in the lungs, myocardium, liver, kidney, and reticuloendothelial system support the opinion that JEV also replicates in the periphery in humans before crossing the blood-brain barrier (BBB) (Chambers and Diamond 2003).

Although several observations suggest encephalitic viruses display tropism for lymphoid tissues; however, the identities of the cell types in such compartments that support replication to the levels needed to generate a viremia sufficient to cause neuroinvasion (viral entry into CNS) have not been determined definitively. Flaviviral replication occurs in various peripheral tissues, but vascular endothelial cells have not necessarily been implicated as important sites of replication. However, it should be noted that dengue virus, JEV, and probably other flaviviruses could enter and probably establish infection in endothelial cells and modulate their activation state and cytokine production. (Chambers and Diamond 2003)

**Cellular receptors required in flaviviral entry:** The cellular receptors that mediate attachment and entry of flaviviruses have been partially characterized. Until date, there has been no definitive identification of the molecules required for flaviviral entry in either peripheral or CNS tissues. Several groups used biochemical approaches to identify candidate JEV-receptor proteins on mammalian cells; however, the physiologic relevance of these candidate proteins remains unclear, as there is heterogeneity in the proteins that bind flaviviruses in different cell types.

Heparin sulphate has been proposed as a flavivirus receptor based on studies showing the dependence of dengue virus infectivity on binding of the E protein to heparin sulphate on
target cells (Chen et al., 1997). Subsequent reports have demonstrated that infectivity of tick borne encephalitis virus (TBEV), YFV, JEV, and Murray valley encephalitis (MVE) viruses is affected by cell surface interaction with glycosaminoglycans. Enhanced binding to glycosaminoglycans is a marker for attenuation of JEV and MVE viruses in mouse model and correlates with rapid clearance of the glycosaminoglycan binding variants from the circulation compared to more pathogenic strains. Wild-type and glycosaminoglycan binding variants may differ, however, with respect to their entry into cells that are permissive for replication or are involved in virus clearance (Chambers and Diamond 2003).

Apart from heparin sulphate, DC-SIGN (the dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin) has been proposed as a cell surface receptor for dengue virus. Additional studies are required to evaluate the in-vivo significance of DC-SIGN as an attachment or entry receptor and whether this is a common determinant of tropism for other flaviviruses (Chambers and Diamond 2003). During recent past, heat-shock protein 70 (HSP70) has been suggested as a putative virus binding receptor for JEV. This molecule has been identified as JEV binding receptor in several in-vitro models including human cell-lines, mouse neuronal cell-line (Neuro2a), as well as in mosquito cell-line C6/36 (Das et al., 2009; Ren et al., 2007). Apart from these receptors, C-type lectin domain family 5 member A (CLEC5A) and mannose receptor have been shown to facilitate dengue virus binding in human macrophages (Watson et al., 2011). However, no such study has been carried out in JEV or WNV.

These studies points out to the fact that flavivirus can interact with several receptors for entry depending on host cell types. Receptor variability in-vivo may be a general mechanism for promoting wide tissue tropisms of arthropod borne viruses, which require cycling in both arthropod and vertebrate hosts. Taken together these observations suggest a possibility of multiple independent cellular receptors molecules being utilized by viruses, either during the spread of flaviviruses within the host or during entry into the CNS.
2.7.2 Stage-II – Virus entry into CNS

The clinical JE disease usually develops when virus breaches the BBB and reaches the CNS. One of the main intriguing questions in JEV immunology is why only 1 in 300 of the JEV-infected individuals develop a clinical disease. In other words, what are the factors that allow virus entry from the blood into the CNS? Even though the exact mechanism of JE disease progression in CNS remains obscure, it can be divided into two major steps.

**Entry into CNS:** A correlation between viremia and viral neuroinvasion is observed in most naturally acquired encephalitic infections. Furthermore, data from a number of studies indicate that factors such as the (i) time of onset, (ii) magnitude and duration of the viremia, as well as (iii) the integrity of the host innate immune system (prior to the onset of virus-specific immune response), influence the viral entry into the CNS.

In flaviviral infection of humans as well as in peripherally infected older mice, a relatively rare stochastic event that permits virus in the circulation to breach the BBB appears to be crucial step leading to mostly fatal encephalitis. The mechanism allowing encephalitic flaviviruses to breach the BBB remains uncertain. Four candidate routes for viral invasion of CNS have been proposed (a) inflammation and damage to vascular integrity (Lossinsky and Shivers 2004), (b) via olfactory bulb (Griffin et al., 2004), (c) Toll like receptor (TLR) mediated entry (Wang et al., 2004), or (d) via transcytosis across vascular endothelial cells (Lossinsky and Shevers 2004).

During JEV infection, entry of virus into CNS is thought to occur via transcytosis. Ultrastructural examination of the brains of mice injected with JEV shows that the virus undergoes endocytosis and transportation across endothelial cells and pericytes without replication (Liou and Hsu 1998). Perivascular cuffing with infiltration of inflammatory cells (T cells and macrophages) is a feature of both human JE and WN encephalitis, though in neither disease do the cells appear to transport viral antigen across the BBB. *In-vitro* WNV infection causes upregulation of cellular adhesion molecules E-selectin, ICAM, and VCAM, which may be important in initiating adhesion and migration of neutrophils and macrophages. The pro-inflammatory cytokines (TNFα) and chemokine (IL8), which are involved in polymorphonuclear cell recruitment, are elevated in the CSF and serum of humans with JE and are higher in fatal as compared to nonfatal cases.
These cytokines may also aid in disrupting the BBB and thus facilitating flavivirus entry into CNS (Solomon and Vaughn 2002).

**Disease associated CNS damage:** Histological examination of fatal human cases of infection with viruses of the JEV serotype show viral antigens in neurons with the greatest involvement in the thalamus and brain stem but relatively little evidence of inflammatory responses (Müllbacher et al., 2003). Macrophages were the predominant inflammatory cells found to invade the brain parenchyma and localized near the virus-infected cells; T and B cells were mainly seen in perivascular regions. Viral antigen is progressively cleared from the brain at later stages of disease (Müllbacher et al., 2003), probably due to the induction of adaptive immune responses but with little evidence of immunopathology in neuronal destruction. A vigorous virus-specific antibody (Ab) response, systemically and within the CNS, appears to be an important factor in recovery from acute encephalitic flavivirus infection; whereas elevated TNFα levels in the serum and CSF (probably reflecting the magnitude of the inflammatory response) is a poor prognostic indicator for recovery. In acute JEV-CNS illness, death results from infection of many neurons and disruption of their function.

Apart from immunopathology associated neuronal damage, JEV infection can directly induce apoptosis in neuroblastoma cells. *In-vitro* studies have shown JEV infection of neuroblastoma cells results in activation of caspase 3 (Tsao et al., 2008) and induces apoptosis via JNK pathway (Gupta et al., 2011). JEV can also bring about apoptosis in caspase 3 and Fas independent pathway (Tsao et al., 2008), *in-vivo* study on rat model propose role of increased oxidative stress, i.e. increased free radicals, in neuronal cell death (Srivastava et al., 2009).

Flavivirus neuropathogenesis involves both neuroinvasiveness (capacity to enter the CNS) and neurovirulence (replication within the CNS). A main principle that applies is the relationship between peripheral virus burden and the propensity to cause neuroinvasion (Chambers and Diamond 2003). In the classical studies of arbovirus pathogenesis, distinctions were made among neuropathogenic phenotypes based on replication efficiency and pathogenic potential in the peripheral tissues versus the CNS, with various phenotypes being distinguished. These phenotypes were related to different
clinical outcomes, which ranges from sub-clinical infection to acute encephalitis of varying severity and are influenced in the mice model by host age and species.

2.7.3 Factors influencing flaviviral pathogenesis

Several risk factors, both viral and host associated are known to play a role in the pathogenesis, disease outcome and severity of JEV infection. The key factors include (1) concomitant condition, (2) short prodromal stage, and (3) deep coma, respiratory changes and decerebrate posturing. Two major concomitant conditions, influencing flavivirus-encephalitis disease outcome, include host immune status (i.e. immunodeficiency and pre-existing flavivirus immunity) and/or co-infection with other infectious agents. Studies on mice model with experimental co-infection of Toxocara canis or Trichinella spirallis have shown mice to be predisposed to JEV encephalitis because of T cell suppression (Gupta and Pavri 1987). Virus intrinsic property such as high replication potential or neurovirulence property of virus could also contribute in virus entry into CNS and manifesting a short prodromal stage. Few of the host-associated and viral-intrinsic factors are discussed in detailed in the following section.

Host Factors influencing flaviviral pathogenesis: Entry into the CNS represents an important event in both the pathogenesis of disease and the clinical outcome in terms of severity. Based on human studies key factors influencing JE disease severity are decreased levels of humoral response and type-I IFN. However, mode of virus entry in CNS, host age and genetic make-up determine flavivirus pathogenesis.

a) Mode of flavivirus entry into CNS: Several hypotheses are put forth to elucidate the mode of flaviviral entry into CNS.

i. Via leukocytes: As mentioned above, flavivirus replication has been shown to be supported by skin Langerhan’s cells. Flavivirus infection results in increased adhesion molecules levels in endothelial cells, thus aiding in infiltration of various lymphocytes including infected monocytes (which serve as Trojan horse) into CNS (King et al., 2007; King and Kesson 2003).

ii. BBB breach – due cytokines: Increased pro-inflammatory cytokines levels are known to cause BBB breach (Hawkins and Davis 2005). Studies on murine model during WNV infection have shown activation of TLR3 results in increased
TNFα, which in turn increases BBB permeability (Wang et al., 2004). During JEV infection, increased CSF-IL8 levels is correlated with disease severity and poor prognosis (Winter et al., 2004; Singh et al., 2000). IL8 is a strong chemotactic agent of neutrophils, and role of neutrophils infiltration in disease severity has been demonstrated in MVE virus infection of mice (Andrews et al., 1999). Apart from increased pro-inflammatory response, a decreased peripheral type-I IFN response can also contribute to the increased chance of flaviviral entry into CNS (Lazear et al., 2011).

iii. **Entry via endothelial cells:** In JEV infection, apart from above mentioned pathways another mechanism of viral entry into CNS has been reported. Transcytosis of JEV in endothelial cells seems to be possible (Liou and Hsu 1998), even though endothelial cells of brain parenchyma do not support efficient JEV replication. Similar hypothesis have been suggested in WNV and MVE virus infection (King et al., 2007), as presence of viral antigen has been demonstrated in mouse brain endothelial cells *in-vitro*. Therefore, even though flavivirus might not replicate in brain endothelial cells, there is a possibility of flavivirus-endothelial cell interaction might induce cell morphology changes and thus result in increased permeability.

iv. **Axonal transport of virus:** Studies have shown ability of flavivirus to infect spinal cord. Immunohistochemistry of spinal cord showed the presence of WNV antigen, thus suggesting the possibility of WNV entering CNS via anterior grade axonal transport. In the same study, intra nasal inoculation of WNV resulted in fatal encephalitis. The viral infection progress was observed to be from rostral to caudal and then to brain, however the cell tropism was restricted to neurons (King et al., 2007). Similar observation was obtained with St. Louis encephalitis (SLE) and MVE virus infection, but the progress occurred via caudal to rostral (King et al., 2007). In brain, all these viruses infect only neurons.

b) **Pre-existing immunity to flavivirus and Age of Host:** In flavivirus endemic regions, most of individuals (by adulthood), are exposed to natural flaviviral infection and develop a protective immune response. In JEV and WNV infection, anamnestic flaviviral immunity is known to protect against severe disease (Solomon 2004; Libraty et al., 2002). However, in dengue viral infection pre-exposure to different
dengue serotype pre-disposes the individual to more severe disease during secondary dengue infection (Nielsen 2009). Recent study on WNV infection of humans have pointed that host age influences the immune response against WNV, an impaired IFN response was observed in elderly individuals as compared to younger adults (Qian et al., 2011). This is in concordance with the observation that individuals of > 60 yrs of age are prone to develop WN encephalitis.

c) **Host genetic make-up:** The differential response to any infection among individuals within a population could be attributed to their genetic make-up. Genetic variation amongst human population is due to haplotype, copy number variation (CNV), and single nucleotide polymorphism (SNPs). Polymorphism in critical genes that influence viral infection are as follows

i. **Virus resistance genes – IFN stimulated genes (ISGs)**

ii. **Virus Binding receptors – CCR5 for HIV infection**

iii. **Immune response modulating factors – TLRs, natural killer (NK) cell receptors, Cytokine and chemokines**

i. **ISGs polymorphism and Viral infection:** Pioneering studies on flavivirology have shown several strains of the laboratory mice to be resistant to flaviviral infection (Flv'). The gene associated flavivirus resistant phenotype (Flv') was then shown to be 2′-5′-oligoadenylate synthetase (OAS1b) (Perelygin et al., 2002). Recent human genetic studied have shown association of OAS1 polymorphism with disease outcome during WNV (Yakub et al., 2005) and HCV (Knapp et al., 2003) infection. Apart from OAS1, another ISG that associated with host susceptibility or poor prognosis to viral infection is Mx protein. In human population, association of Mx polymorphism has been studied with reference to HCV (Knapp et al., 2003) infection. Its role in other flavivirus infection remains elusive.

ii. **Polymorphism in viral binding receptors:** One of the earliest known studies to associate virus binding receptor polymorphism with viral pathogenesis is based on mouse hepatitis virus (MHV) and SJL mouse strain. Host cell receptor for MHV is biliary glycoprotein (Bgp1). In SJL strain, the Bgp1 allele (Bgp1b) encodes for a functional protein but shows decreased affinity towards MHV. This
property affects the virus replication and thus eventual viral load in the target organ (Brownstein 1998).

Recent studies on HIV and WNV have shown a null-receptor phenotype of CCR5 (Δ32) to influence the disease outcome. In HIV CCR5Δ32 is attributed with increased resistance to virus infection (Galvani and Novembre 2005), while in WNV it is attributed as a risk factor for both early and late clinical manifestations after infection (Glass et al., 2006). This might be because CCR5 acts as receptor for HIV but not for WNV. There are no such studies in JEV.

iii. **Polymorphism in immune response modulating factors:** Immunomodulating factors include TLRs, cytokine, chemokines, NK cell receptors, and others. Detailed studies on effect of TLR (Misch and Hawn 2008) chemokine (Colobran et al., 2007) polymorphism during infectious disease (such as HIV, HCV, HBV, and RSV) have been reported. Until date, there are no reports on association of polymorphism in these receptors with other flavivirus infection other than HCV.

**Viral factors influencing disease pathogenesis:** Viral factors primarily influence viral replication rate and eventually disease pathogenesis. In flaviviruses, role of several viral structural and non-structural proteins have been implicated in affecting virus replication, cell-tropism, and blocking of type-I IFN signaling mechanism.

Flaviviral structural proteins, prM and E play an important role in viral attachment and entry; thereby govern the cell-tropism of these viruses. Mutations in prM protein especially those affecting the glycosylation sites or viral fusion, results in increase or decrease in neurovirulence (Hurrelbrink and McMinn 2003). Single point-mutation in M-protein of ChimeriVax-JE results in increased viral growth kinetics in Vero cell-line, while *in-vivo* neurovirulence remained unaltered (Maier et al., 2007). Likewise, mutation in glycosylation region of prM in JEV led to decreased neurovirulence in mice (Kim et al., 2008). Multiple amino acid substitutions in E-protein of JEV result in attenuation and decreased neurovirulence of the virus (Hurrelbrink and McMinn 2003; Arroyo et al., 2001). WNV strains with glycosylation motif in E-protein showed increased replication potential in DC-SIGN positive cells as compared with non-glycosylated WNV strains (Martina et al., 2008). These glycosylated viral strains are also known to show increased neuroinvasiveness (Shirato et al., 2004). E-protein of flavivirus
forms the primary antigen for neutralizing antibody, therefore, mutations in E-proteins not only modify cell-tropism but also aids the virus in evading adaptive humoral immune response.

The NS-proteins of flavivirus form the core ingredient of replicase complex and protease complex, therefore any mutations on these proteins affect the viral RNA replication, transcription, and translation (Hollidge et al., 2011). 3’ and 5’ UTRs of flavivirus are involved in viral replication. Mutations in these regions are known to destabilize cyclization process and thus influence viral RNA replication (Alvarez et al., 2008). Point mutation that resulted in a substitution of the conserved regions of 3’UTR resulted in much-reduced virus replication (Khromykh et al., 2003). Recent studies have also shown sub-genomic viral RNA to modulate IFN response in mice (Schuessler et al., 2012).

In flaviviral infections of humans, differences in severity of the disease and epidemic potential have been partly attributed to viral virulence. During dengue epidemic, replication potential of strains isolated from patients with dengue hemorrhagic fever (DHF) outcompeted the strains isolated from dengue fever (DF) cases in human DC cultures (Silveria et al., 2011; Cologna et al., 2005). These strains also showed varied cytokine inducing ability in-vitro and correlated disease causing potential among humans (Silveria et al., 2011). In a similar scenario, comparison of genome sequences of lineage 2 WNV strains isolated from patients in South Africa who had mild or severe WNV infections showed major deletions in 3’ UTR and high variability in NS5 region (Botha et al., 2008). Therefore, the multiple point mutations on the viral genome influencing viral replication fitness and virulence would contribute to the overall resistance to host immune response.
2.8 Host immune response during JEV infection

Both innate and adaptive arms of immune response are triggered on pathogen exposure at the site of infection including the skin (Fig 8). Studies based on *in-vitro* cell-culture, *in-vivo* mice model and human clinical samples have demonstrated the importance of immune response in both immunoprotection and immunopathogenesis of flaviviral infection.

![Immune response in skin](image)

**Fig. 8.** Immune response in skin. Modified from source: Nestle et al., 2009.

2.8.1 Adaptive immune response

Adaptive immune response during flavivirus infection is well studied in both *in-vivo* murine model and humans. Both humoral and cell-mediated response plays an important role in curtailing flaviviral infections. Anamnestic anti-flavivirus immunity is known to prevent severe JEV disease (Libraty et al., 2002). Passive administration of immunoglobulins, before or shortly after infectious challenge, has been shown to protect mice from lethal changes of WNV (Ledizet et al., 2007; Ben-Nathan et al., 2003; Engle and Diamond. 2003; Diamond et al., 2003) and JEV infections (Beasley et al., 2004; Kimura-Kuroda and Yasui., 1988). Recent study on murine model has also shown importance of humoral response over CD8⁺ response in curtailing JEV encephalitis (Larena et al., 2011). In JEV infection of humans, a correlation between host survival, and increased IgM and IgG levels in CSF have been noted (Winter et al., 2004). Patients
with immunosuppression are more prone to WNV infection and increased disease severity (Chan-Tack and Forrest, 2006). However, during dengue infections in humans, role of humoral response remains controversial as both immunoprotective and possibility of antibody dependent enhancement is been postulated (Wahala and Silva 2010).

Anti-flaviviral cell-mediated immune response comprises of both CD4+ and CD8+ cells. As mentioned above, during JEV encephalitis models, CD8+ T cells do not play a pivotal role in host protection as compared to humoral or CD4+ T cells (Larena et al., 2011; Biswas et al., 2009). In humans, induction of virus specific cytotoxic T cells post JEV-vaccination has been documented (Konishi et al., 1998). In JEV infected patients, the Th1 response was impaired and it correlated with the severity of the post-encephalitic neuro-sequelae (Kumar et al., 2004).

In WNV infection, CD8+ cells play a key role in immunoprotection of the host along with CD4+ cells. This phenomenon has been documented in both murine models (Kitaura et al., 2011; Stewart et al., 2011; Kim et al., 2010; Brien et al., 2008; Shrestha and Diamond 2004) and humans (Parsons et al., 2008; Lanteri et al., 2008). Impaired T cell function has been attributed as one of the major determinants of WNV disease severities in elderly patients (Brien et al., 2009). In contrast, Wang et al., showed CD8+ cells contributed in immunopathogenesis of WNV disease rather than immunoprotection if the mice were challenged with higher WN-viral dose (Wang et al., 2003).

During dengue virus infection, T cells play a role in both immunoprotection as well as immunopathogenesis. On secondary dengue virus infection, there is an enhanced intracellular cytokine production by T cells and is correlated with sub-clinical secondary infection (Hatch et al., 2011). However, other studies have shown correlation between T cell response and DHF immunopathogenesis (Duangchinda et al., 2010; Dung et al., 2010; Appanna et al., 2007; Loke et al., 2001). Recent study has shown that memory CD8+ T cells obtained due previous exposure to dengue virus is highly cross-reactive against other dengue serotypes (Friberg et al., 2011). Thus, it is postulated that increased T cell activation during secondary heterologous dengue-virus infection result in increased cytokine storm, leading to increased vascular leakage and disease severity (Kurane et al., 2011). Therefore, during flaviviral infections, viral dose and functional impairment of T cells would facilitate increased disease severity.
2.8.2 Innate immune response during flaviviral infection

Innate immune response is non-specific, and is triggered immediately on pathogen exposure. The major components of innate immune response include physical barriers (mucosal membranes, skin etc), cells (phagocytes/ APCs, lymphocytes), cytokines, chemokines, complements, and several other inflammatory mediators (e.g. acute phase proteins). Host’s innate immune response also forms the scaffold on which adaptive response is shaped and/or skewed during an infection. Severity of flaviviral infection is reflected in adaptive response impairment, indirectly indicating a possible inefficient innate immune response in the host.

Antigen presenting cells (APCs) act as a bridge between innate and adaptive immune response. These include peripheral monocytes, macrophages and DCs. These cells are capable of recognizing invading pathogens through pattern recognition receptor (PRR) such as TLR, retinoic-acid inducible gene (RIG), melanoma differentiation-associated protein 5 (MDA5), and nucleotide oligomerization domain (NOD)-like receptors (NLRs). Upon pathogen recognition, APCs undergo functional activation resulting in expression of phenotypic maturation markers and soluble pro-/anti- inflammatory cytokines as well as chemokine, and potentiate adaptive response (Fig 8). Therefore, pathogens can indirectly manipulate adaptive response by preventing APCs from functional activation (Guermonprez et al., 2002).

Apart from APCs, innate lymphocytes also play an important role in early anti-viral response. Innate lymphocytes include NK cells, natural killer T (NKT) cells, and γδ T cells. NK and NKT cells establishes primary defense against viral invasion and tumors before adaptive response sets-in. Recent studies have also shown these cells are capable of interacting with DCs and exert immunomodulatory effects, thereby controlling / fine-tuning the immune response cascade (Moretta 2005).

Primary innate cytokine to impart anti-viral effect is interferon (IFN). In-vitro and murine in-vivo studies have shown the requirement of functional type-I IFN response to curtail flavivirus (Muñoz-Jordán and Fredericksen 2010; Samuel and Diamond 2005; Lobigs et al., 2003). In humans, decreased IFN response is observed in elderly patients infected with WNV (Qian et al., 2011). Similarly, in dengue virus infection a blunted plasmacytoid DCs (pDCs) response is noted in patients developing DHF (Pichyangkul et
al., 2003). Role other pro-inflammatory cytokines and chemokines in flaviviral disease have also been reported. Increased levels of IL8 in human clinical samples have been reported in JEV (Winter et al., 2004). In-vivo studies on WNV have shown inflammatory monocytes (Getts et al., 2008) and CCR2 levels (Lim et al., 2011) contribute in disease pathogenesis. Therefore, innate immune response is known to play a crucial role in flavivirus disease pathology. A more detailed review on role of innate immune response during flaviviral infection is presented in following section.

**Role of Macrophages:** Although a wide range of immune effectors is induced by virus infection, the predominant antiviral mechanism appears to be correlated with, or depends on the interaction of virus with APCs at the primary multiplication site.

Macrophages have essential functions in the innate immune system and have multiple roles in host defense. Mature, resident macrophages differentiate from circulating monocytes and occupy peripheral tissues and organs where they are most likely to encounter pathogens during the early stages of infections. Upon encounter with infectious agents, macrophages can employ a broad array of anti-microbial effector mechanisms. Many of the effector functions of macrophages are strongly augmented by IFNγ, which comes from either NK cells or Th1 cells. IFNγ also induces the antigen-presenting functions of macrophages by turning on the expression of a battery of genes involved in antigen processing and presentation.

Macrophages support various virus replication including HIV, influenza, dengue (Chen and Wang, 2002), WNV, and JEV (Aleyas et al., 2009; Mathur et al., 1988; Kedarnath et al., 1986). These cells activate anti-viral mechanisms during early hours of infection (Ellermann-Eriksen 2005), via cytokines (type-I IFN, TNFα, etc) and other mediators (such as, nitric-oxide). Viruses on the other hand, depending on their virulence, are known to modulate the effector functions of macrophages. For example Congo fever virus (Peyrefitte et al., 2010) and arenavirus (Groseth et al., 2011) evade macrophage activation, while influenza H5N1 (Lee et al., 2009) and YFV (Woodson et al., 2011) enhance immune activation, resulting in decreased immune response or immunopathogenesis respectively.
Interaction between virus and macrophages has been more thoroughly studied on HIV model as it establishes persistent infection in these cells. The ability of the macrophages to carry HIV into the CNS has been pointed out as possible cause for AIDS associated dementia (Amor et al., 2010), while coronary artery disease of the AIDS patients has been linked to impaired lipid metabolic functions of macrophages (Crowe et al., 2010). Recent study has pointed out the importance of sinusoidal macrophages in preventing viral invasion of CNS from periphery (Iannacone et al., 2010). Apart from viral diseases, macrophages are also known to contribute for other neurodegenerative disorders (Amor et al., 2010). Therefore, macrophages appear to play an important role in viral pathology by regulating both viral replication as well as immune response.

Role of type-I IFN in viral infection and viral counter measures: Type-I IFN were discovered in 1957 based on their ability to establish antiviral activity. Type-I IFN includes five subfamilies namely IFNα, IFNβ, IFNλ, IFNω, and IFNГ. The IFNαβ members are best characterized and are known to be induced during viral infections. In humans, there are multiple genes encoding IFNα, while a single gene encodes IFNβ. On viral infection, transcriptional activation of IFNα/β genes occurs due to coordinated activation of PRRs (e.g. TLRs, RIGs, and MDA), receptor binding adaptor molecules (such as MyD88, TRIF), cytoplasmic kinases (IRAK1, IKK), and various transcription factors (NF-κB, IRFs).

Type-I IFN mediate anti-viral effect due to their ability to induce several gene products namely the ISGs that can destabilize viral replication, transcription, assembly, and maturation. Type-I IFN induced genes include OAS, RNaseL, Mx, protein kinase R (PKR), and several other ISGs. In mice, innate resistance to flavivirus infection is related to the OAS gene (Perelygin et al., 2002). IFN induced anti-viral mechanism involves signaling via IFN specific binding receptor (IFNR1 and IFNR2) and JAK-STAT pathway.

In-vivo and in-vitro cell-line based studies have shown that during flavivirus infection, type-I IFN induction is dependent on viral replication (Pichyangkul et al., 2003), involving MyD88 (Aleyas et al., 2009) and PI3K pathway (Chang et al., 2006). Functional type-I IFN response is known to control the tropism of viral spread and viral load. Mice lacking IFN receptor or STAT proteins are more susceptible to flaviviral
infection, and exhibited an increased tissue viral load and mortality as compared to wild-type mice (Perry et al., 2011; King et al., 2007; Samuel and Diamond 2005). In humans, a blunted pDC (major type-I IFN producing cells *in-vivo*) response was observed during acute dengue virus infection (Pichyangkul et al., 2003). In contrast, increased IFNα levels were observed in non-survivors of JEV infected patients (Winter et al., 2004). Susceptibility of flaviviruses to anti-viral effects also depends on the virus virulence. Several *in-vitro* studies have shown pathogenic viral strains to be less susceptible to type-I IFN anti-viral effect as compared to attenuated non-pathogenic / vaccine viral strains (Keller et al., 2006; Aguilar et al., 2005).

Like other RNA viruses such as influenza (García-Sastre 2011), flaviviruses circumvent the IFN response primarily by inhibiting IFN signaling pathway. Flaviviruses mediate deregulation of JAK-STAT pathway by either inducing degradation of STAT proteins (Ashour et al., 2009; Jones et al., 2005) or by inhibiting the phosphorylation of JAK/Tyk tyrosine kinases, or STAT1/2 proteins. NS5 is the primary flaviviral protein that acts as STAT1/2 phosphorylation antagonist (Mazzon et al., 2009; Lin et al., 2006). Other flaviviral proteins, which act as IFN antagonist include NS3, NS2A/B, and NS4A/B (Hollidge et al., 2011; Muñoz-Jordán and Fredericksen., 2010; Liu et al., 2005).

Study on Kunjin virus has shown that NS2A protein is capable of inhibiting IFNβ transcript levels. Site directed mutagenesis resulting single amino acid substitution in NS2A protein resulted in increased IFNβ transcript levels as compared to wild-type NS2A protein (Liu et al., 2004). Apart from viral proteins recent study has also shown sub-genomic viral RNA of WNV can over-ride IFN response in murine model (Schuessler et al., 2012).

Recent *in-vitro* analysis of NS5 protein of the highly pathogenic NY99 strain of WNV showed enhanced inhibition of STAT1 phosphorylation as compared to less pathogenic Kunjin virus NS5 protein (Laurent-Rolle et al., 2010). Therefore, the ability of flavivirus to evade IFN response is influenced by virus virulence also.

**Role of Nitric-oxide:** Nitric-oxide is a gaseous nitrogen centered free radical produced by a variety of cells due to catabolism of L-arginine by action of group of enzymes called nitric-oxide synthetase (NOS). Nitric-oxide acts as intracellular messenger
molecule and mediates a variety of physiological processes throughout the body (including in CNS) (Pacher et al., 2007; Conti et al., 2007); and forms a critical component of innate immune response. It has been demonstrated that nitric-oxide can exert both protective and detrimental effects in several disease states.

During viral infection, IFNγ induces activation of inducible NOS (iNOS) thus resulting in increased production of nitric-oxide (Akaike and Maeda, 2000). In flaviviral infection, nitric-oxide production has been documented in murine primary macrophages (Kreil and Eibl, 1996) and cell-lines of myeloid lineage (RAW 264.7) (Lin et al., 1997). However, two independent studies have demonstrated that dengue virus infection does not induce nitric-oxide in primary human monocyte/macrophage cultures (Espina et al., 2003; Chen and Wang 2002). Likewise, there was no alteration in nitric-oxide levels during JEV infection of murine microglial cells (Thongtan et al., 2010). In humans, no correlation between nitric-oxide levels and JEV infected patients survivability was observed (Winter et al 2004).

Nitric-oxide is known to contribute in viral load decrease in several flaviviral infections. The anti-viral activity of nitric-oxide is more thoroughly studied using in-vitro culture and external nitric-oxide donors. Presence of external nitric-oxide donors during JEV infection of both neuronal cell-lines (N18, NT-2) and epithelial cell-line (BHK21) resulted in viral load reduction at both infectious virion and viral RNA level (Lin et al., 1997). Similar observation has been reported during dengue virus infection of neuroblastoma cell-line (Charmsilpa et al 2005) and is partly due to inhibition of viral RNA dependent polymerase activity, which then down regulates viral RNA synthesis (Takhampunya et al 2006).

Apart from contributing in direct anti-viral effect, studies based on murine models, have shown nitric-oxide to impart both immunoprotection as well as immunopathogenesis. During TBEV infection, blocking of iNOS by chemical inhibitor resulted in increased average survival time of the animal (Kreil and Eibl, 1996). However, contrasting results have been reported during dengue virus infection. In iNOS knockout murine model, dengue disease severity increased (Fagundes et al., 2011), while iNOS inhibition by chemical agent resulted in decreased hemorrhage development and the severity of hemorrhage (Yen et al., 2008). Earlier study has also shown that levels of iNOS
expression in mouse CNS to correlate with neutrophil recruitment and thus disease severity in MVE virus infection (Andrews et al., 1999). However, in iNOS knockout model, no such drastic variation in disease severity was observed as compared to wild-type animal (Lobigs et al., 2003). The major difference between these two studies on MVE virus infection is that Andrew et al had used weanling SWISS model, while Lobigs et al had used adult (6 week old) B6 mice. It is therefore possible that nitric-oxide contribution in immunoprotection or immunopathogenesis during flaviviral infection could be influenced by host age and flaviviral type.

Role of pro-inflammatory cytokine and chemokine: Cytokines / chemokines are soluble low-molecular weight molecules, which exert immunomodulation. On viral infection, both cytokines and chemokines are induced from a variety of immune cells. These factors are known to impart both immunoprotection as well as facilitate immunopathogenesis during viral infections. Since, cytokines and chemokines act as the connecting bridge between innate and adaptive response, viruses can deregulate either arm of immune response by modulating cytokine/chemokine levels. In-vivo murine model and in-vitro cell culture studies had demonstrated the ability of flaviviruses to induce a spectrum of cytokines/chemokines. Few of such studies are represented in table-2.

Only one study has reported the detailed analysis of cytokine and chemokine profile in JEV infected patients (Winter et al., 2004). This study showed correlation between an increased IL6, CCL5 levels in serum and CSF obtained from non-survivors as compared to survivors. However, no study has been reported, either by using murine knockout models or siRNA in-vitro culture models, to confirm the exact role of these cytokines/chemokines in JEV disease severity. Meanwhile, importance of CCL5 (Crawford et al., 2011) and its corresponding receptor CCR5 (Glass et al., 2005), has been proven to play protective role via recruiting and maintaining functional effector CD8+ T cells during WNV infection in mice. Protective role of CCR5 in WNV infection has also been observed in humans also. Individuals with homozygous CCR5Δ32 shows an increased susceptibility to develop more sever symptomatic WNV disease (Lim et al., 2008; Glass et al., 2006).
<table>
<thead>
<tr>
<th>Virus Name</th>
<th>Model</th>
<th>Pro-inflammatory Cytokine induced</th>
<th>Chemokine induced</th>
<th>Ref</th>
</tr>
</thead>
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<tr>
<td><strong>DENV</strong></td>
<td>HepG2 cell line (in-vitro)</td>
<td>IL6</td>
<td>IL8, CCL3, CCL4, CCL5</td>
<td>Conceição et al., 2010, Medin et al., 2005</td>
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<td></td>
<td>Lung cancer cell lines (in-vitro)</td>
<td>IL6</td>
<td>CCL5</td>
<td>Lee et al., 2007</td>
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<td></td>
<td>B6 mice</td>
<td>IFNγ, TNFα</td>
<td>CCL2, CCL5, CXCL1, CXCL2, CXCL10</td>
<td>Amaral et al., 2011, Chen et al., 2006</td>
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<td></td>
<td>human primary monocytes, Bcells, DCs</td>
<td></td>
<td>CCL8, CXCL10</td>
<td>Becerra et al., 2009</td>
</tr>
<tr>
<td></td>
<td>human MDDCs</td>
<td>IL1β, IL12</td>
<td>IL8, CCL5</td>
<td>Chen and Wang 2002</td>
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<tr>
<td></td>
<td>human monocytes, macrophages - primary (in-vitro)</td>
<td>IL6, IL17</td>
<td>CCL2, CCL5, CXCL10</td>
<td>Becquart et al., 2010</td>
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<td></td>
<td>human - patient plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
<td>mouse macrophages, bmDCs - primary cells (in-vitro)</td>
<td>IL6, IL12, TNFα</td>
<td>CCL2</td>
<td>Li et al., 2011, Cao et al., 2011, Aleyas et al., 2009</td>
</tr>
<tr>
<td></td>
<td>mouse brain, sera</td>
<td>TNFα</td>
<td></td>
<td>Biswas et al., 2009, Fujii et al., 2008</td>
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<tr>
<td></td>
<td>rat microglial cells (in-vitro)</td>
<td>IL1β, IL6, TNFα</td>
<td>CCL5</td>
<td>Chen et al., 2010, Chen et al., 2004</td>
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<td></td>
<td>rat astrocytes (in-vitro)</td>
<td>IL6</td>
<td>CCL2, CCL5</td>
<td>Chen et al., 2000</td>
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<td></td>
<td>human astroglial cell-line (in-vitro)</td>
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<td>IL8, CCL2, CXCL9, CXCL10</td>
<td>Mishra et al., 2008</td>
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<td></td>
<td>human - patient plasma</td>
<td>IL6, TNFα</td>
<td>IL8, CCL5</td>
<td>Winter et al., 2004, Ravi et al., 1997</td>
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<td><strong>JEV</strong></td>
<td>B6 mouse sera and brain</td>
<td>TNFα</td>
<td>CCL2</td>
<td>Wang et al., 2004, Getts et al., 2008</td>
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<td>Tobler et al., 2008</td>
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<td>Khaiboullina et al., 2005</td>
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<td>human Kuffer cells - primary cells (in-vitro)</td>
<td>TNFα</td>
<td>IL8, CCL5</td>
<td>Woodson et al., 2011</td>
</tr>
</tbody>
</table>

Table 2: List of various pro-inflammatory cytokines and chemokines induced by flavivirus.

TNFα is one of the most prominent pro-inflammatory cytokine that is correlated with immunopathogenesis of several diseases, both infectious as well as autoimmune.
However, during flavivirus infection role of TNFα remains controversial. Earlier study on murine model during WNV infection, have shown a potential TNFα role in BBB breach and WNV entry into CNS (Wang et al., 2004). However, recent studies have shown TNFα to impart protective role in WNV disease by recruiting and activating monocytes (Shrestha et al., 2008) and microglia (Szretter et al., 2009); as well as by inhibiting neuronal apoptosis triggered by CXCR3 pathway (Zhang et al., 2010). Virulent dengue virus induced strong TNFα and apoptosis in human DCs (Silveira et al., 2011). In dengue-infected patients increased plasma TNFα levels is observed in DHF cases (Azeredo et al., 2010; Wang et al., 2007). Likewise, during JEV infection of humans, correlation between increased serum- and CSF- TNFα levels with poor disease prognosis has been reported (Winter at al., 2004; Ravi et al., 1997). However, study based on microarray analysis of differential gene expression changes in whole blood obtained from children with severe dengue virus infections showed downregulation of TNFα gene along with other NF-κB regulated genes (de Kruif et al., 2008). Therefore, TNFα appears to play both immunoprotective as well as immunopathogenic role during flavivirus infections.

Other chemokines studied thoroughly during flaviviral infections include CCL2, CXCL10 and CXCL9. CCL2 is reported to have immunopathogenic role in WNV infection by mediating increased infiltration of Ly6c+ inflammatory monocytes into CNS from periphery (Getts et al., 2008). However, mice lacking cogent receptor of CCL2 (i.e. CCR2ΔΔ) had decreased infiltration of Ly6c+ inflammatory monocytes, but showed increased virus burden in brain and mortality (Lim et al., 2011). CXCL10 is an IFN induced chemokine. Earlier study has shown increased accumulation of CXCL10 mRNA in NY-99 WNV strain infected mouse brain (Shirato et al., 2004). Neuronal CXCL10 levels have been shown to regulate CD8+ effector T cells recruitment (Klein et al., 2005) via CXCR3 (Zhang et al., 2008) and thus control WNV infection.

In dengue virus infection, CXCL10 appears to mediate anti-viral effect by preventing viral binding with host receptor and thus inhibiting viral entry (Ip and Liao, 2010). CXCL10−/− mice showed increased dengue viral load even though T cell recruitment in brain was comparable to that wild-type mice. However, in clinical dengue infection, based on human samples, increased CXCL9 and CXCL10 levels in patient’s plasma has been correlated with disease severity (Dejnirattisai et al., 2008), Therefore, role of
various chemokines and cytokines in flavivirus disease pathogenesis varies according to different flavivirus and study-model used.

**Role of DCs:** DCs are professional APCs, reside in peripheral tissues, and exhibit active macropinocytosis and receptor-mediated endocytosis. DCs express a number of PRRs, including phagocytic receptors and TLRs. DCs are best known for their role in the initiation of adaptive immune responses, but these cells can also contribute to direct antimicrobial products leads to the induction of several antimicrobial effector responses, such as type-I IFN, nitric oxide production.

Viruses are known to modulate DC activation and thus regulate adaptive response activation and indirectly contributing in disease pathogenesis (Freer and Matteucci, 2009). Simultaneously, certain viruses such HIV, use DC as “Trojan horse” to facilitate viral spread into various organs (Rinaldo and Piazza, 2004). Recent study has shown that peripheral DCs contribute in both innate and adaptive response, against neurotropic virus (VSV), in CNS (Steel et al., 2009). Therefore, DCs play a crucial role in viral pathogenesis.

First report to show the importance of dendritic cells in flavivirus infection dated back to 1990s. In the preliminary studies by Dr.King’s group showed mouse splenic DCs were capable of inducing WNV specific CD4⁺ T cells proliferation *in-vitro* (Kulkarni et al., 1991) and WNV infection led to Langerhans cell maturation (Johnston et al., 1996). However, proof of flavivirus infection and replication in human DCs came only at 2000. This study reported that monocytes derived DCs were susceptible to dengue virus infection and replication, and confirmed the ability of Langerhans cells to support dengue virus infection using human skin explants - *ex-vivo* model (Wu et al., 2000).

Interaction between flavivirus and human DCs has been more thoroughly studied in dengue virus infection as compared to other flaviviruses. Several studies have shown that dengue virus infection result in activation of human monocyte derived DCs, *in-vitro*. Dengue virus infection was capable of stimulating various co-stimulatory markers, such as CD11b, CD40, CD80, CD83, CD86, HLA-ABC, and HLA-DR in DCs and induced production of TNFα and IFNα (Sun et al., 2009; Ho et al., 2001; Libarty et al., 2001). Dengue virus induced DCs maturation and type-I IFN production was dependent on
virus replication (Sun et al., 2009; Pichyangkul et al., 2003). Studies on dengue virus infection have documented an increased DCs maturation in the by-stander DCs as compared with dengue virus infected DCs (Dejnirattisai et al., 2008; Nightingale et al., 2008; Palmer et al., 2005; Sanchez et al., 2005; Libraty et al., 2001).

Impairment of DCs’ functional activation can contribute to increased disease severity. During dengue virus infection, a blunted blood pDCs response has been demonstrated resulting in increased viral load and altered innate immune response, and it has been associated with dengue disease severity (Pichyangkul et al., 2003). In-vitro experimental model has shown dengue virus has the potential to impair DC functional status (Dejnirattisai et al., 2008; Sanchez et al., 2005; Libraty et al., 2001). Dengue virus infection is also known to inhibit type-I IFN (Rodriguez-Madoz et al., 2010) and IL12p70 (Nightingale et al., 2008; Libraty et al., 2001) production from human DCs.

Other flaviviruses such as WNV (Silva et al., 2007; Johnston et al., 1996) and YFV (Querec et al., 2006; Barba-Spaeth et al., 2005) have been shown to stimulate both murine and human DCs in-vitro. Studies on JEV – DCs interaction started very recently from 2009. Aleyas et al was the first report to show JEV infection and DCs activation based on in-vitro model in murine bone marrow derived DCs (bmDCs) (Aleyas et al., 2009). Wild-type JEV strain infection of murine bmDCs resulted in functional impairment (Cao et al., 2011; Aleyas et al., 2010, 2009), while infection with SA14-14-2 (live attenuated JEV vaccine strain) resulted in DC functional activation (Li et al., 2011). Differential gene expression analysis have shown that chimeric dengue 1–4 vaccine strains, wild-type dengue 3 virus, and attenuated dengue-3 vaccine varied in their ability to regulate signature innate gene expression levels in human myeloid DCs. The chimeric vaccine dengue strains induced strong type-I IFN and associated genes, together with chemokine and other mediators involved in the initiation
of adaptive responses; while, wild-type dengue-3 virus induced a predominantly inflammatory profile, and the attenuated dengue-3 vaccine strain infection led to a blunted response (Balas et al., 2010).

Flavivirus uses DC-SIGN molecule to gain entry into DCs (Lozach et al., 2005; Tassaneetrithep et al., 2003). A recent study has shown polymorphism in DC-SIGN is associated with severity of dengue disease (Wang et al., 2011). Therefore, apart from virus mediated DC functional impairment, host genetic polymorphism that affecting DCs markers can also influence flavivirus disease pathogenesis.

**Role of NK/NKT cells:** NK/NKT cells are innate lymphocytes and have cytolytic potential. These have important role in both anti-viral as well as immunomodulatory immune response during viral infection.

a) *NK/NKT mediated anti-viral response:* Both, NK and NKT cells provide first line anti-viral defense (Lanier 2008; Biron and Brossay, 2001). NK cells are known to impart direct-cytolysis of virus-infected cells (via perforin-granzyme or Fas-FasL pathway) and contribute in antibody-dependent cell-mediated cytotoxicity (ADCC). NK and NKT cells can also impart anti-viral effect through secretion of IFNγ. Extensive studies on NK role during disease pathology have been reported during CMV, HIV, and HCV infections. Studies on role of NK/NKT cells in flavivirus pathology have been very limited and primarily reported with respect to dengue, WNV, and YFV infection.

In several flavivirus infections such as WNV (Zhang et al., 2010) and dengue virus (Shresta et al., 2004), NK and NKT cells are known to reduce virus load and curtail viral spread. In humans, an increase in early NK and NKT activation is associated with mild dengue disease (Azeredo et al., 2006). Recent study has also shown dengue virus and WNV E-protein to interact with NK cell receptor NKp44 thereby resulting NK cell activation (Hershkovitz et al., 2009). IL12 dependent induction of IFNγ in NK cell population of human PBMCs was observed on dengue virus antigenic stimulation (Suwannasaen et al., 2010).

Earlier studies during 1980’s have demonstrated non-adherent population of human PBMCs to mediate ADCC and caused significant lysis of dengue virus infected Raji cell-
line (Kurane et al., 1984). During dengue virus infection of murine model, at three days post-infection an increase in activated NK cell was observed in spleen (Shresta et al., 2004) and it is shown to control dengue virus load (Shresta et al., 2005). Likewise, increased NK cell levels in lymph node and infiltration in brain has been reported during WNV infection (Bréhin et al., 2008). In contrast, another study have shown that NK cells do not play a significant role in controlling WNV infection, as depletion of NK cells did not affect the course of WNV encephalitis (Shrestha et al., 2006).

In humans, changes in NK cell sub-population frequency have been observed in peripheral blood post YF17DD vaccination. Vaccinated individuals showed increased frequency of pre-NK cells (immature), while cytotoxic/mature NK cell frequency dropped (Martins et al., 2008). Few studies have reported NK cell activation levels to correlate with disease severity. Presence of NKT cells at the site of inflammation was observed in the liver tissue samples obtained from patients who succumbed to yellow fever (Quaresma et al., 2006). Chau et al., reported a prominent increase in NK cell activation phenotype in infants with acute DHF (Chau et al., 2008). This was in contrast to the study reported by Azeredo et al, where they have shown that in adult cases early activation of NK cells lead to mild dengue clinical manifestations (Azeredo et al., 2006). This contradiction might be due to age difference of host and hence the corresponding functional activation of NK cells.

In murine models, CXCL10 and CCR5 levels appears to influence recruitment of NK cells into affected organs, during dengue virus infection (Chen et al., 2006) and WNV infection (Glass et al., 2005). However, no such reports have been documented in human studies involving flavivirus infection.

Flaviviruses induce MHC-I molecules on the target cells (Abraham et al., 2010; Abraham and Manjunath 2006; Cheng et al., 2004; King and Kesson, 1988). Therefore, it is hypothesized that flaviviruses might use this mechanism to evade NK cell recognition (King et al., 2007). A recent study on dengue virus has confirmed this hypothesis using in-vitro cell-line model. Human peripheral NK cells failed to lyse NK-sensitive cell-line (K562) when it was transfected with dengue virus replicons. Dengue virus induces MHC-I in K562 and THP1, and thus rendering them insensitive to NK lysis (Hershkovitz et al., 2008). Therefore, NK cells appear to influence disease
pathology in flavivirus infection, meanwhile flavivirus have also evolved mechanisms to evade NK cell response.

b) Immunomodulatory effect of NK/NKT cells: Apart from imparting / inducing innate anti-viral response, NK and NKT cells are also known to play an immunomodulatory role. These cells interact with DCs, hence influence adaptive response cascade. Therefore, NK/NKT cells bridges the gap between innate and adaptive immune response. Preliminary studies on NK-DC interaction have been based on tumor model; in recent years, more reports have provided evidence for impairment NK-DC interaction, or NK/NKT functional activation in viral diseases.

NK/NKT-DC interactions are bi-directional and can occur at various sites including the site of inflammation (Moretta 2005; Cooper et al., 2004). Recent study on murine model has demonstrated that, during corneal HSV-1 infection, the ability of NK cells to migrate and impart viral clearance at the site of infection was facilitated by corneal DCs (Frank et al., 2012). Therefore, interaction of DCs with innate lymphocytes represents a major control mechanism in limiting the virus infection at the periphery before adaptive response sets-in.

The cross-talk between innate lymphocytes and DCs leads to functional activation of DCs and NK/NKT cells, and is dependent on direct cell-to-cell contact as well as soluble factors (Reschner et al., 2008; Gerosa et al., 2005, 2002; Piccioli et al., 2002). NK/NKT induced DC maturation is dependent on TNFα (Piccioli et al., 2002), CD40-40L interactions (Caielli et al., 2010; Kitamura et al., 1999), and several other unknown mechanisms. In-vitro study have also shown that during NKT cell induced DCs maturation, presence of additional DCs activating ligands (i.e. TLR ligands) along with NKT cells can skew DC maturation to proinflammatory from tolerogenic DCs (Caielli et al., 2010).

DC-induced NK/NKT activation is dependent on type-I IFN, IL12, IL18, and on IL15 levels. Studies based on human DCs and NK interaction, in-vitro models, have shown type-I IFN (Prestwich et al., 2009; Benlahrech et al., 2009) and IL15 (Fawaz et al., 1999) to enhance NK cell cytolytic ability. Likewise, in murine model type-I IFN is shown to
activate NKT cell, however type-I IFN induced NKT cells impaired DCs priming ability with CD8\(^+\) T cells (Bochtler et al., 2008).

Major outcome of NK/NKT – DC interaction is DC editing, i.e. removal of functionally defective immature DCs and retaining of mature DCs due to NK/NKT cell mediated cytolysis. Thus, NK/NKT cells, indirectly favor development of beneficial and specific adaptive immune response to viral antigens. In several chronic viral infections of humans, impairment of DC-NK interaction has been observed (Tjwa et al., 2012; Benlahrech et al., 2011; Mavilio et al., 2006; Jinushi et al., 2004). Therefore, NK/NKT-DCs interactions are multifactorial bringing about both positive and negative regulation of host immune response during viral infection and it remains unexplored in flavivirus infection.