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TLR7 is a key regulator of innate immunity against Japanese encephalitis virus infection

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ABSTRACT

Toll-like receptor 7 (TLR7) known to recognize guanidine-rich ssRNA has been shown to mount vital host defense mechanism against many viruses including flaviviruses. Signal transduction through TLR7 has been shown to produce type-1 interferon and proinflammatory mediators, thereby initiating essential innate immune response against ssRNA viruses in hosts. Systemic and brain specific TLR7 knock-down mice ($TLR7^{KD}$) were generated using vivo-morpholinos. These mice were then subcutaneously challenged with lethal dose of JEV (GP78 strain) and were subsequently analyzed for survival. Significant difference in susceptibility to JEV between wild-type and systemic $TLR7^{KD}$ mice was observed whereas, no difference in susceptibility to JEV infection was seen in brain-specific $TLR7^{KD}$ mice. Significant decreases in IFN-α and antiviral proteins were also observed in both $TLR7^{KD}$ mice along with increased viral loads in their brain. Owing to increased viral load, increases in levels of various proinflammatory cyto/chemokines, increased microglial activation and infiltration of peripheral immune cells in brain of $TLR7^{KD}$ mice were also observed. Immunocytochemistry and RNA co-immunoprecipitation performed with JEV-infected N2a or HT22 cells indicated endosomal localization and confirmed interaction between JEV ssRNA with TLR7. Treatment of mice with imiquimod, a TLR7 agonist, prior to JEV infection resulted in their increased survival. Overall, our results suggest that the TLR7 response following JEV infection promotes type-1 interferon production and generation of antiviral state which might contribute to protective effect in systemic infection.

Introduction

Arthropod-borne flaviviruses are an emerging and re-emerging threat to global population and are associated with significant morbidity and mortality. The host's immune response to such viral infections has been under considerable interest, and several studies over the decades have shed light on the intricate balance of innate and adaptive immune responses and also the immune evasion strategies adopted by them to subvert such responses (King et al., 2007; Ye et al., 2013). After gaining entry into a mammalian host, these viruses activate the innate immune system, which detects viral components through pattern-recognition receptors (PRRs) (Akira et al., 2006; Medzhitov, 2007). Viral genomic DNA, single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), RNA with 5′-triphosphate ends and viral proteins are all known to be detected by these PRRs. Presently, three types of PRRs are known to be involved in the detection of such virus-specific components in cells – the Toll-like receptors (TLRs), the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide oligomerization domain (NOD)-like receptors (NLRs). TLRs are a set of evolutionarily conserved molecules consisting of 11–12 members that play crucial role in initiating innate immune responses. Innumerous studies over the years have shown TLRs to be involved in recognition of various pathogens including viruses or viral components (O'Neill et al., 2013).

There has been a growing volume of reports suggesting TLR3- or TLR7-dependent recognition of certain flaviviruses in animal models

Abbreviations: CNS, Central nervous system; IRAK, Interleukin-1 receptor-associated kinase; IKK, Inhibitory Kappa-B kinase; IRF, interferon regulatory factor; IFIT, interferon-induced protein with tetratricopeptide repeats; JEV, Japanese encephalitis virus; MyD88, Myeloid differentiation primary response protein 88; NLR, Nucleotide oligomerization domain like receptors; OASL, 2′-5′-oligoadenylate synthetase-like; PRRs, Pattern-recognition receptors; RIG-I, Retinoic acid-inducible gene I; TBEV, Tick-borne encephalitis virus; TLR, Toll like receptor; TRAF, TNF receptor-associated factor; WNV, West Nile Virus.

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or cultured cells, even though such reports are sometimes contradictory. TLR3 was reported to be involved in West Nile Virus (WNV) pathogenicity as mice deficient in TLR3 were found to be more resistant to WNV infection (Wang et al., 2004). However, studies by another group claimed TLR3-deficient mice to be more susceptible to WNV infection (Daffis et al., 2008). As viral immune evasion strategy, WNV non-structural protein 1 (NS1) had been shown to inhibit TLR3 signaling (Wilson et al., 2008) but, later it was shown not to be involved in this process (Baronti et al., 2010). In studies with other flaviviruses, TLR3 has been recognized as risk factor for human Tick-borne encephalitis virus (TBEV) infection (Kindberg et al., 2011) and is also involved in recognition of dengue virus *in-vitro* (Tsai et al., 2009). TLR7 on the other hand has been shown to be involved in recognition of ssRNA and ssRNA-producing viruses such as vesicular stomatitis virus, influenza virus, human parechovirus 1, and human immunodeficiency virus (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004) and mounting of an innate immune response which is MyD88-dependent. The role of TLR7 in flaviviral infections had been first indicated from studies with dengue virus (de Kruif et al., 2008) and since then, viral recognition and resultant pathogenesis post infections of WNV (Town et al., 2009; Welte et al., 2009), Yellow fever virus (Mandl et al., 2011), and Langat virus (Baker et al., 2013), have been reported.

Japanese encephalitis virus (JEV) is a common mosquito-borne flavivirus which predominantly infects neurons. Upon entry in the human body, JEV induces transient systemic viremia, crosses blood–brain barrier and infects neurons in brain which results in massive uncontrolled inflammation in the central nervous system (CNS) (Dutta et al., 2010). Even though the infected neurons eventually die, we hypothesized that a type-1 interferon-mediated innate immune response is generated by these cells. Contrary to the general understanding that resident glial cells and/or infiltrating peripheral monocytes or lymphocytes are the only cellular components of the immune response in the CNS post viral infections, recent studies have shown that neurons can be active participants in such processes (Chakraborty et al., 2010; Peltier et al., 2010). Our earlier studies based on *in vitro* and murine models of Japanese encephalitis (JE) suggest a possible neuronal innate immune response against the virus is mounted following viral recognition through retinoic-acid-inducible gene I (RIG-I) and its downstream adapter STING (Nazmi et al., 2011; Nazmi et al., 2012). However, on ablating the PRR or its adapter by anti-sense oligonucleotides, we were not able to completely block the immune responses which made us to suspect the involvement of other mechanisms at play. In this current study, we aim to elucidate the contribution of TLR7 in mounting an innate immune response against JEV, using *in-vivo* TLR7 knock-down models.

Materials and methods

Ethics statement

All animal experiments were approved by the Institutional Animal and Ethics Committee of the National Brain Research Centre (approval no. NBRC/IAEC/2011/66). The animals were handled in strict accordance with good animal practice as defined by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment and Forestry, Government of India.

Virus and cells

JEV (GP78 strain) was propagated in suckling BALB/c mice as described earlier (Nazmi et al., 2010). Mouse neuroblastoma (Neuro2a) cells were procured from National Centre for Cell Science, Pune, India and mouse hippocampal neuronal HT22 cell is a kind gift from Dr. Shiv Kumar Sharma, National Brain Research Centre. HT22 cells were used for our experiments with prior permission from Dr. Dave Schubert of Salk Institute from whom these cells were originally obtained. These cells were grown at 37 ° C in Dulbecco's modified Eagle medium (DMEM) supplemented with 3.7% sodium bi-carbonate, 10% fetal bovine serum and penicillin/streptomycin.

Primary cortical neuron culture

Cortical neurons were cultured following published protocol (Nazmi et al., 2010). Briefly, cortices of P2 BALB/c mice pups were dissected aseptically in calcium-magnesium-free (CMF)-Tyrode solution following decapitation. The meninges were removed, tissues were chopped into smaller pieces and collected in CMF-Tyrode. These were treated with trypsin DNAse and then dissociated in the same solution by triturating to make single cell suspension, pelleted and resuspended in neurobasal media. Neurobasal media were supplemented with L-glutamine (2 mM), 30% glucose, 5% fetal calf serum, 10% horse serum and penicillin–streptomycin. Cells were plated at a density of 5×10^3 cells/cm^2 onto poly-D-lysine-coated Labtek chamber slides (Nunc, Roskilde, Denmark). After 48 h of incubation at 37 °C, the serum containing medium was removed. Cells were incubated with serum free media for 4 h with antibiotics alone. For experimental treatments, the resting medium was exchanged for DMEM with N2 and B27 supplements, 25 mM KCl and antibiotics. Arabinoside (2×10^{-5} M) was used for the inhibition of astrocyte multiplication.

Imiquimod treatment of animals

To analyze the beneficial effect of TLR-7 signaling in mice model of JEV, adult mice (BALB/c, 4–6 weeks) of either sex were divided into four groups – sham, JEV-infected, JEV + vehicle and JEV + imiquimod. There were 8 animals in each group initially. Mice belonging to JEV + imiquimod groups received imiquimod (1 mg/kg body weight, once daily) whereas mice of JEV + vehicle group received PBS (once daily) for 7 days continuously before JEV infection. Then animals belonging to all groups, except sham, were infected with 3×10^5 plaque forming units (PFU) of JEV (GP78 strain) subcutaneously. Mice of sham group received equal volume of filtered MEM.

Systemic knock-down of TLR7 in BALB/c mice

In order to assess the role of TLR7 in mice model of JE we used anti-TLR7 vivo-morpholinos (octaguanidium dendrimer-conjugated antisense oligonucleotide) for knock-down. All morpholino sequences were custom-synthesized and made commercially available by Gene Tools LLC, (Philomath, OR, USA). The TLR7 morpholino sequence was screened with BLAST (www.ncbi.nlm.nih.gov/BLAST/) against primate and murine mRNA sequences. Mice were divided into four groups – sham, JEV-infected, JEV + Sc-Mo and JEV + TLR7^{KD}. Mice belonging to JEV + TLR7^{KD} were first injected (i.p., 12.5 mg/kg body weight, once per day for 5 consecutive days) with vivo morpholino against TLR7 (5′ TCC GTG TCC ACA TCG AAA ACA CCA T 3′) and subsequently infected with JEV (subcutaneous, 3×10^5 PFU). Mice of JEV + Sc-Mo group in parallel, received scrambled morpholino (Sc-MO; 5′ GAT AAT TCT GGT TTT AAA TTC 3′) in same dosage, with subsequent JEV infection by the same route. Mice in only JEV-infected group were administered 1 × sterile PBS i.p. for 5 days prior to being infected with JEV; sham group received only PBS injections for 5 days and were neither infected with JEV nor treated with morpholino.

Knocking-down TLR7 in BALB/c mouse brain

As efficiency of vivo morpholinos in crossing the blood–brain barrier is quite low, to achieve significant knock-down in brain we devised a slightly different strategy based on a previously published method (Reissner et al., 2012). Four week old mice were divided into four similar groups as described above. JEV + TLR7^{KD} group mice were first injected intra cranially (single dose; 12.5 mg/kg body weight) with