Neurovirulence comparison of chikungunya virus isolates of the Asian and East/Central/South African genotypes from Malaysia

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Chikungunya virus (CHIKV), an alphavirus of the family Togaviridae, causes fever, polyarthritis and rash. There are three genotypes: West African, Asian and East/Central/South African (ECSA). The latter two genotypes have caused global outbreaks in recent years. Recent ECSA CHIKV outbreaks have been associated with severe neurological disease, but it is not known if different CHIKV genotypes are associated with different neurovirulence. In this study, the neurovirulence of Asian (MY/06/37348) and ECSA (MY/08/065) strains of CHIKV isolated in Malaysia were compared. Intracerebral inoculation of either virus into suckling mice was followed by virus titration, histopathology and gene expression analysis of the harvested brains. Both strains of CHIKV replicated similarly, yet mice infected with MY/06/37348 showed higher mortality. Histopathology findings showed that both CHIKV strains spread within the brain (where CHIKV antigen was localized to astrocytes and neurons) and beyond to skeletal muscle. In MY/06/37348-infected mice, apoptosis, which is associated with neurovirulence in alphaviruses, was observed earlier in brains. Comparison of gene expression showed that a pro-apoptotic gene (eIF2αK2) was upregulated at higher levels in MY/06/37348-infected mice, while genes involved in anti-apoptosis (BIRC3), antiviral responses and central nervous system protection (including CD40, IL-10RA, MyD88 and PYCARD) were upregulated more highly in MY/08/065-infected mice. In conclusion, the higher mortality observed following MY/06/37348 infection in mice is due not to higher viral replication in the brain, but to differentially expressed genes involved in host immune responses. These findings may help to identify therapeutic strategies and biomarkers for neurological CHIKV infections.

INTRODUCTION

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus from the Togaviridae family, which causes epidemic fever, rash and arthritis. It is an enveloped, linear, positive-sense ssRNA virus with a genome size of approximately 11.8 kb, and two ORFs encoding the nonstructural (nsP1-nsP2-nsP3-nsP4) and structural polyproteins (C-E3-E2-6K-E1) (Powers & Logue, 2007). There are three known genotypes: the West Africa, East/Central/South African (ECSA) and Asian genotypes. Since 2005, CHIKV of the ECSA genotype has caused major outbreaks spreading from East Africa to the Indian Ocean islands, India and Southeast Asian countries, and caused autochthonous outbreaks in many regions for the first time, including Europe and the Middle East. Since 2013, the Asian genotype has been causing epidemics for the first time in the Pacific islands and the Americas (Sam et al., 2015).

While CHIKV infection is generally considered to be benign, the main complication of concern is prolonged arthralgia that may persist for months. However, recent reports during the ECSA outbreaks showed that CHIKV also causes severe neurological complications, particularly in newborns and children. These complications include encephalitis, encephalopathy, meningitis, acute flaccid paralysis, cerebral ataxia, seizures and Guillain–Barré syndrome, and may lead to death or long-term neurological deficits (Gérardin et al., 2008; Robin et al., 2008;
Economopoulou et al., 2009). Although neurological disease was described in historical reports of Asian strain outbreaks (Nimmannitya et al., 1969), it is not clear if the greater number of reports in recent years reflects real differences in neurovirulence between strains of different genotypes, or the greater awareness and diagnostic capabilities during the more recent ECSA outbreaks. This is of relevance to countries like Malaysia, where both Asian and ECSA genotypes of CHIKV circulate (Sam et al., 2009), and is of particular interest as the Asian genotype is currently spreading to new regions.

Virulence differences between CHIKV strains have been previously shown. CHIKV strains which caused clinical myalgia were compared to those that did not, and were found to replicate to a higher titre, cause more cytopathic effect and induce more cytokines in primary myoblast cells (Lohachanakul et al., 2015). An enzootic West African strain caused higher viraemia and more pronounced symptoms in Macaca mulatta compared to an epidemic ECSA strain (Chen et al., 2010). In an arthritis model in adult mice, an epidemic ECSA strain induced more inflammation and foot swelling than a historic Asian strain (Gardner et al., 2010). More recently, a Caribbean strain (Asian) showed reduced mouse joint pathology compared to a La Réunion strain (ECSA), which was associated with reduced pro-inflammatory T helper (T11)1 and natural killer cell responses (Teo et al., 2015). However, there are few data on inter-strain differences in neurovirulence caused by CHIKV.

Suckling mice have been extensively used to study alphavirus neurovirulence, as their central nervous system (CNS) is highly susceptible to infection. CHIKV infection causes necrosis in neurons in the cerebral cortex and spinal cord (White, 1969; Powers & Logue, 2007). CHIKV spreads to the CNS in IFN-α/β R-deficient 129s/v mice through the choroid plexus, and infects ependymal and leptomeningeal cells (Couderc et al., 2008), while mainly infecting astrocytes in OF1 mice (Das et al., 2015).

In this study, suckling mice were intracerebrally infected with CHIKV strains of either an Asian (MY/06/37348) or ECSA (MY/08/065) genotype, and the histopathology and immune-related gene expression were compared to look for differences in neurovirulence. The findings may contribute to understanding the pathogenesis underlying differences in neurovirulence manifestations caused by different CHIKV strains.

RESULTS

CHIKV strain MY/06/37348 causes higher mortality in suckling mice

Suckling mice inoculated with CHIKV showed lethargy, hair loss around the inoculation site on the head, hind limb paralysis and lower weight gain compared to mock-infected mice (Fig. 1a). Mice inoculated with virus isolate MY/06/37348 had significantly higher mortality with 38/42 (90.5 %) dying and a shorter mean time to death (7.2 days) than MY/08/065-infected mice (mortality in 24/40, 60.0 %; mean time to death, 9.0 days). The mortality of the mock-infected mice was 2/33 (6.1 %). Log-rank test analysis showed that total survival of MY/06/37348-, MY/08/065- and mock-infected mice was statistically different ($\chi^2 = 49.2, P < 0.05$) (Fig. 1b).

Infection with either virus isolate led to similar survival rates in the first 5 days post-infection (p.i.), after which the mortality of MY/06/37348-infected mice increased at a faster rate.

![Graph showing weight and survival of suckling mice](image-url)
CHIKV strains MY/06/37348 and MY/08/065 replicated similarly in suckling mice brains

The replication of the two CHIKV strains was found to be similar using virus titration and quantitative real-time PCR (qRT-PCR). Both virus isolates reached peak mean virus titres of approximately 10^5 TCID_{50} g^{-1} (Fig. 2a) at 2 days p.i. and peak RNA levels of approximately 10^{10} copies g^{-1} at 1 day p.i. (Fig. 2b). The only significant difference was a slightly higher titre of MY/08/065 at 8 days p.i. Both RNA and virus titres remained detectable at 8 days p.i., at levels similar to input levels. The similar virus loads of the two CHIKV strains suggest that viral replication alone does not explain the higher mortality and severe disease observed in MY/06/37348-infected mice.

Histopathological changes in CHIKV-infected suckling mice brains

In suckling mice infected with CHIKV, the presence of apoptotic bodies, necrosis, inflammatory cells and perivascular cuffing was mainly observed in theolfactory bulb, thalamus, cerebral cortex (Fig. 3b, c), cerebellum (Fig. 3e) and hippocampus. These were absent in the mock-infected brains (Fig. 3a, d, f). Histopathological changes were also noted in sites beyond the CNS in both MY/06/37348- and MY/08/065-infected mice from 6 days p.i. onwards. Vacuolization and apoptotic bodies were seen in the liver (data not shown), and abundant infiltrates, fragmented myocytes and oedema were observed in thigh skeletal muscle (Fig. 3g).

To determine the spread of CHIKV over time, the presence of CHIKV capsid was observed by immunohistochemical staining (Fig. 4b, d, f). Following intracerebral inoculation of either CHIKV strain in the left parietal area, ipsilateral and contralateral spread of infection was noted within 1–2 days p.i. in the cerebral cortex, cerebellum, hippocampus, thalamus, olfactory bulb, choroid plexus and ependymal cells lining the ventricles. CHIKV antigen was also seen in extracranial sites such as oral mucosal membranes, brown fat cells and skeletal muscle in the head within 2 days p.i., and thigh skeletal muscle from 4 days p.i. (Fig. 4h). There were no pathological changes observed in the spleen and no CHIKV antigen staining in the spleen and liver.

Apoptosis was observed earlier at 2–4 days p.i. for MY/06/37348-infected mice than in MY/08/065-infected mice, in which apoptosis was evident at 4–6 days p.i. Double immunofluorescence staining (Fig. 5) showed that CHIKV localized in some cells with apoptosis activation as indicated by staining of cleaved caspase 3 (Fig. 5c). Necrosis was also observable at 2–4 days p.i. for MY/06/37348-infected mice, while in MY/08/065-infected mice necrosis was present for longer, at up to 6 days p.i. CHIKV was detected in astrocytes using GFAP staining (Fig. 5f) and neuronal cells using MAP-2 staining (Fig. 5i).

Overall, intracerebral inoculation of CHIKV led to rapid spread within the brain, and after a few days, also led to both viral spread and histopathological changes at distant sites outside the CNS. Histopathological changes and spread of CHIKV were broadly similar in mice infected with either virus, although MY/06/37348-infected mice, which had higher mortality, showed apoptosis at earlier stages of infection compared to MY/08/065-infected mice.

Differences in immune-related gene expression in CHIKV-infected suckling mouse brains

Differences in mortality caused by the two virus strains may be due to differences in induced host immune responses, as viral loads were similar, and apoptosis and necrosis were observed at different stages of infection with MY/06/37348 and MY/08/065.
After filtering of the microarray results, the number of genes found to be significantly differentially regulated in both biological replicates of each experiment compared to mock-infected mice were 239 at 1 day p.i., of which 178 (74.5%) genes were upregulated and 61 (25.5%) were downregulated; and 561 at 6 days p.i., of which 454 (80.9%) genes were upregulated and 107 (19.1%) were downregulated (Fig. 6). Following functional annotation to identify immune-related genes, a total of 138 genes in six biological processes involved in immune-related responses were found to be differentially upregulated. The six biological processes were antigen processing and presentation, apoptosis, defence response, immune response, inflammatory response and response to virus (Fig. S1, available in the online Supplementary Material). The majority of these were genes differentially upregulated in both MY/06/37348- and MY/08/065-infected mice at 6 days p.i. \((P<0.05)\).

A total of 45 immune-related genes were selected for quantification by qRT-PCR to confirm the changes observed with the microarray (summarized in Table S1), and then gene expression was directly compared between MY/06/37348- and MY/08/065-infected mice. At 1 day p.i., five genes were found to be upregulated significantly more highly following MY/06/37348 infection than MY/08/065 infection. These were CSF1, eIF2α, FOS, IL-18BP and STAT1 (Fig. 7a). No genes were upregulated at higher levels in MY/08/065-infected mice at 1 day p.i. At 6 days p.i., expression of seven genes was found to be significantly different, six of which were upregulated significantly more highly following MY/08/065 infection.
Fig. 4. CHIKV antigen staining in suckling mice brains and thigh muscle. Mock-infected mice were stained with normal rabbit immunoglobulin fraction as the primary antibody, and sections of the choroid plexus (a), third ventricle (c), cerebellum (e) and thigh skeletal muscle (g) are shown. CHIKV capsid antigen (stained brown, arrows, →) was stained with polyclonal rabbit anti-CHIKV capsid primary antibody, and was detected in various regions of the brain, including choroid plexus (b), third ventricle (d), cerebellum (f) and thigh skeletal muscle (h). Immunohistochemical staining was performed with HRP and haematoxylin counterstain. Magnification: × 40 (g, h); × 60 (a–f). Bars, 10 μm.
(BIRC3, CatS, CD40, IL-10RA, MyD88 and PYCARD),
and one more highly in MY/06/37348 infection (OAS2)
(Fig. 7b).

**DISCUSSION**

Strains of different genotypes of CHIKV were hypothesized
to have different neurovirulence effects. Suckling mice
infected intracerebrally with a CHIKV strain of the Asian
genotype (MY/06/37348) had lower weight gain and higher
mortality than mice infected with a strain of the ECSA
genotype (MY/08/065). Viral titres and RNA copies of
each virus within the mouse brains were similar; thus repli-
cation differences were unlikely to be a major contributor
to differences in mortality. However, differences in pathological
changes and differential expression of immune-related genes
may explain the observed inter-strain differences in disease
severity.

Spread of CHIKV to distant organs has been shown
following intradermal or subcutaneous inoculation in
mice (Couderc *et al.*, 2008; Ziegler *et al.*, 2008), and here
we demonstrate that it also occurs after intracerebral inocu-
lation. The virus may have spread systemically following
direct invasion of blood vessels in the brain. It is also
possible that following infection of ependymal cells and
intracranial spread via cerebrospinal fluid, the virus could
have penetrated the blood–cerebrospinal fluid barrier,
entered the bloodstream and spread. CHIKV antigen was
particularly notable in skeletal muscle, for which it shows
a strong tropism in mice (Ziegler *et al.*, 2008) and humans
(Ozden *et al.*, 2007). CHIKV antigen was detected in the
olfactory bulbs in this study. Viral spread into the CNS via
the olfactory nerve is also well described for several viruses,
including CHIKV, showing that viruses have tropism for

In the present study, CHIKV antigen was detected in astro-
cyes, ependymal cells and neurons. This is consistent with

![Fig. 5. Double immunofluorescence staining of the brains of CHIKV-infected mice. Arrows indicate CHIKV capsid present in
cells with either cleaved caspase 3 (indicating activation of apoptosis; a–c), GFAP (indicating astrocytes; d–f) or MAP-2
(indicating neurons; g–i). The brain regions shown are the thalamus at 6 days p.i. (a–c) and 2 days p.i. (d–f), and cerebral
cortex at 2 days p.i. (g–i). CHIKV capsid was stained with Liquid Permanent Red (red), while cleaved caspase 3, GFAP and
MAP-2 were stained with Alexa Fluor 488 (green). DAPI was used to counterstain nuclei (dark blue). Magnification:
× 63. Bars, 10 μm.](image-url)
previous studies in mice showing that CHIKV infects ependymal cells, leptomeningeal cells, astrocytes and oligodendrocytes (Couderc et al., 2008; Das et al., 2010). The infection of mouse neurons has been reported only in some studies (Wang et al., 2008; Das et al., 2015). In human patients, magnetic resonance imaging has shown brain swelling, demyelination and prominent white matter lesions (Ganesan et al., 2008; Gérardin et al., 2008; Robin et al., 2008). A single reported autopsy of a fatal human case revealed oedema, ischaemic changes and focal haemorrhages in the cerebral cortex, with demyelination and oedema in the subcortical white matter (Ganesan et al., 2008). These radiological and autopsy findings of white matter involvement suggest that CHIKV may not be neurotropic in humans. However, the lack of histological reports in humans and inconsistent evidence of neuron infection in animal models indicate that further work is required to confirm the neurotropism of CHIKV (Arpino et al., 2009).

In contrast to our findings of greater mortality in mice due to the Asian strain, there have been numerous reports of severe neurological disease in humans during the recent outbreaks of ECSA strains compared to the limited historical reports of Asian CHIKV (Arpino et al., 2009). This may be due to host differences. However, it is not known whether this truly represents a greater neurovirulence of ECSA strains in humans, or reporting bias due to improved awareness and diagnostics during the worldwide outbreaks in 2005–2010. As the recent epidemic of Asian CHIKV in the Americas unfolds, reports of neurological complications are awaited to shed light on possible differences in clinical disease between the genotypes in humans.

Apoptosis, a process of caspase-dependent programmed cell death, correlates positively with neurovirulence due to alphaviruses (Lewis et al., 1996; Griffin, 2005). CHIKV infection activates intrinsic and extrinsic apoptotic pathways in HeLa cells and human fibroblasts (Kreibich-Trotot et al., 2011), and astrocytes and neurons of mice (Das et al., 2015). In this study, CHIKV infection was associated with apoptosis, as shown by cleaved caspase 3 immunofluorescence staining. Numerous non-infected apoptotic cells were also seen and have been previously described (Das et al., 2015), and these may be due to bystander apoptosis via the caspase 8 pathway (Kreibich-Trotot et al., 2011). Apoptosis was observed earlier in the CHIKV-infected suckling mice brains. Numbers of differentially expressed genes following mouse brain infection with either virus isolate MY/06/37348 (black bar) or MY/08/065 (white bar) (a). Venn diagrams showing differentially expressed genes (≥2 or ≤–2 fold change, \( P < 0.05 \)) unique or common to MY/06/37348- and MY/08/065-infected mice at 1 day p.i. (b), and 6 days p.i. (c). Down- and upregulated genes are indicated as green and red, respectively.

**Fig. 6.** Expression profiles of CHIKV-infected suckling mice brains. Numbers of differentially expressed genes following mouse brain infection with either virus isolate MY/06/37348 (black bar) or MY/08/065 (white bar) (a). Venn diagrams showing differentially expressed genes (≥2 or ≤–2 fold change, \( P < 0.05 \)) unique or common to MY/06/37348- and MY/08/065-infected mice at 1 day p.i. (b), and 6 days p.i. (c). Down- and upregulated genes are indicated as green and red, respectively.
MY/06/37348-infected mice, which had higher mortality. This was associated with higher upregulation at 1 day p.i. of a pro-apoptotic gene, eIF2αK2, which phosphorylates α eukaryotic initiation factor-2, a regulator of gene expression. This leads to protein inhibition and enhances apoptosis (Jiang & Wek, 2005). In MY/08/065-infected mice, BIRC3 was upregulated at higher levels at 6 days p.i.; BIRC3 is an inhibitor of apoptosis protein which has an effect in response to peripheral nerve injuries (Wang et al., 2012). Differences in timing and degree of apoptosis may have contributed to the differences in mortality seen in the CHIKV-infected mice.

**Fig. 7.** Comparison of quantification of upregulated immune-related genes in CHIKV-infected suckling mice brains. Mice were infected with either CHIKV isolate MY/06/37348 (black bar) or MY/08/065 (white bar) and gene expression was quantified at 1 day p.i. (a) and 6 days p.i. (b). Relative quantification refers to gene expression of infected mice brains relative to mock-infected mice brains, and is plotted as mean ± range of three independent experiments. *, Significant differences in expression between MY/06/37348- and MY/08/065-infected mice (P<0.01).
Other immune-related genes that were upregulated at higher levels in MY/06/37348-infected mice were CSF1, FOS, IL-18BP and STAT1 at 1 day p.i., and OAS2 at 6 days p.i. CSF1, or macrophage colony-stimulating factor, regulates macrophages and osteoclasts and is associated with persistent arthralgia in CHIKV-infected patients (Chow et al., 2011). FOS is a transcriptional factor involved in Toll-like receptor and TNF signalling pathways, and is increased in Venezuelan equine encephalitis virus (VEEV) infection (Sharma & Maheshwari, 2009). IL-18BP inhibits synthesis of the pro-inflammatory cytokine IL-18, IFN-γ production and early TGFβ cytokine responses, and is found in high levels during acute CHIKV infection (Chirathaworn et al., 2010). STAT1 is a transcription factor of the Jak-STAT pathway. CHIKV nsP2 inhibits IFN-induced STAT1 phosphorylation (Fros et al., 2010) and nuclear translocation of STAT1 (Thon-Hon et al., 2012). OAS2, an IFN-induced antiviral protein important in the RIG-I like receptor and NF-κB signalling pathway, has been observed in CHIKV infection (Bréhin et al., 2009; Fros et al., 2010; Dhanwani et al., 2011). OAS2 also activates endogenous RNase (RNase L), which degrades viral and cellular ssRNA leading to inhibition of viral protein synthesis (Silverman, 2007).

Genes with higher upregulation in MY/08/065-infected mice at 6 days p.i. were CatS, CD40, IL-10RA, MyD88 and PYCARD. CatS is a lysosomal protease that helps prepare peptides for presentation by class II MHC to activate immune responses, and it is produced in response to Sindbis virus (SINV) infection (Johnston et al., 2001). CD40 is a costimulatory molecule found on antigen presenting cells including microglia (O’Keefe et al., 2002), which is involved in a wide range of T-cell and B-cell immune responses, including those in response to VEEV (Taylor et al., 2012) and CHIKV infection (Das et al., 2015). IL-10RA is a receptor for anti-inflammatory IL-10, which is produced in glial cells such as astrocytes and oligodendrocytes, and leads to activation and release of NF-κB (Zhou et al., 2009), a central pro-inflammatory transcription factor. IL-10 also promotes neuroprotection following trauma, possibly by its anti-apoptotic effects (Bachis et al., 2001), and is part of the neuroinflammatory responses in Semliki Forest virus (SFV) infection (Morris et al., 1997). MyD88 is involved in key signalling pathways involved in clearing viruses, including CHIKV (Kam et al., 2009). MyD88 interacts with Toll-like receptors to activate IRF3 and IRF7, which leads to type I IFN production essential for antiviral responses (Rudd et al., 2012). MyD88 is cytoprotective in the CNS against alphaviruses such as VEEV (Sharma & Maheshwari, 2009), SINV (Lewis et al., 1996) and Western equine encephalitis virus (Peltier et al., 2013), and also protects joints from Ross River virus (Assunção-Miranda et al., 2013). PYCARD expression is seen during VEEV infection (Sharma et al., 2008), and it mediates inflammation as part of the inflamasome, a cytosolic multi-protein complex (Osawa et al., 2011). PYCARD can be activated by ligands, DNA and RNA (van de Veerdonk et al., 2011).

No neurovirulence determinants have been identified within the CHIKV sequence yet, although these have been described in other alphaviruses and reviewed (Voss et al., 2010). Amino acid differences between the virus isolates MY/06/37348 and MY/08/065 used in this study were previously compared to corresponding residues in other alphaviruses reported to affect neurovirulence (Sam et al., 2012). A notable difference between the two Malaysian viruses was the deletion of 21 nt or seven amino acids at positions 376 to 382 of nsP3 of MY/06/37348 (Sam et al., 2012). In SINV strain AR86, an 18 amino acid deletion at positions 386 to 403 of nsP3 was found to be a neurovirulence determinant (Suthar et al., 2005). The underlying differences in viral sequences encoding neurovirulence determinants would be of interest for future research.

In conclusion, a difference in neurovirulence was seen between suckling mice infected with CHIKV strains from Asian or ECSA genotypes. As replication of the two virus strains was similar, this difference in neurovirulence may be due to differences in induced host immune responses. In MY/06/37348-infected mice, which had higher mortality, there was greater upregulation of pro-apoptotic Bax and earlier onset of apoptosis, which has been associated with neurovirulence. However, in MY/08/065-infected mice, there was greater upregulation of genes involved in anti-apoptosis, antiviral responses and CNS protection from cell death. This study has provided novel information on important host responses against intracerebral CHIKV infection in a mouse model, which may be potentially used to develop therapeutic and prophylactic strategies against CHIKV infection, and identify biomarkers for severity of neurological infections.

**METHODS**

**Virus isolates.** The Malaysian CHIKV isolates used in this study were MY/06/37348, of the Asian genotype, isolated from a patient during the Bagan Panchor outbreak in 2006 (GenBank accession number FN295483); and MY/08/065, of the ECSA genotype, isolated from a patient in Johor in 2008 (GenBank accession number FN295485). Both patients had uncomplicated CHIKV disease. Both isolates had been passaged three times in Vero cells (African green monkey kidney, ATCC CCL-81).

**Mouse neuroinfection.** Institute for Cancer Research (ICR) mice were used with ethical approval from University of Malaya Animal Care and Use Committee (MP/14/07/2010/ICS(R)), which follows guidelines set by the Institute of Animal Technology, UK. Two-day-old suckling mice were anaesthetized with CO2 then inoculated intracerebrally in the left parietal area at a depth of 2 to 3 mm, with 20 μl of viral suspension of 5.5 log10 TCID50 ml-1. Mock-infected mice were inoculated with serum-free media (SFM). Animals were observed for 14 days for morbidity and mortality. The day that a moribund mouse was euthanized with CO2 was considered the day of death for calculation of mean time to death. Another group of mice was inoculated and euthanized at 0, 1, 2, 4, 6 and 8 days post-infection (days p.i.). Some brains were kept in 10 % neutral buffered formalin fixative for histology studies. The remaining brains were placed in 2 ml tubes with 1.4 mm ceramic beads, and either SFM (for
virus titration or qRT-PCR) or TRIZol reagent (Invitrogen; for total RNA extraction) was added. Homogenization was performed twice for 15 s at 5000 r.p.m. in a Precellys 24 homogenizer (Bertin Technologies). The virus suspension was collected from the supernatant after centrifugation of 1000 g for 10 min at 4 °C.

**In vivo growth kinetic assay.** The virus titration assay was performed with 70 % confluent Vero cells in 96-well plates as described previously (Chiam et al., 2013). For the RNA quantification assay, CHIKV RNA was extracted from 140 µl of virus suspension with a QIaAmp Viral Mini kit (Qiagen), qRT-PCR was performed with nsP3 primers nsP3-F (5’-GGCGGTAGTCCAAAGGGAAT-3’) and nsP3-R (5’-AGCATCGAGTTCTGACGGG-3’), as described previously (Chiam et al., 2013).

**Histology staining.** Brain sections were stained with haematoxylin and eosin (H&E). For immunohistochemistry, antigen retrieval was performed by immersing sections in boiling Tris-EDTA buffer (pH 9.0) for 20 min. Sections were pre-treated for 20 min each with 3 % H2O2 in methanol to neutralize the endogenous peroxidase enzyme activity, followed by incubation with goat serum (diluted 1 : 20) (Dako) to mask non-specific antibody binding. Tissue was then incubated overnight at 4 °C with primary antibody, rabbit anti-CHIKV capsid antibody (diluted 1 : 5000; a gift from Professor Andres Merits of University of Tartu, Estonia), followed by incubation with the secondary antibody, EnVision/HRP Rabbit (Dako), for 30 min at room temperature. The antigen was revealed by DAB chromogen reagent (Dako) before counterstaining with eosin. As a negative control, normal rabbit immunoglobulin fraction (Dako) was used to replace primary antibody.

For double immunofluorescence staining, overnight incubation with the first primary antibody was instead followed by incubation with goat anti-rabbit IgG alkaline phosphatase (diluted 1 : 500) and then Liquid Permanent Red (Dako) in a moist dark chamber at room temperature for 30 min each. The slides were soaked again in boiled Tris-EDTA buffer for 10 min for antigen retrieval before overnight incubation with a second primary antibody, either 1 : 200 dilution of rabbit anti-cleaved caspase 3 (Asp175, Cell Signalling), 1 : 1000 dilution of mouse anti-GFAP (GA5, Cell Signalling), or 1 : 2500 dilution of rabbit anti-MAP-2 (H-300, Santa Cruz) at 4 °C. This was followed by incubation with either goat anti-rabbit or goat anti-mouse Alexa Fluor-488 conjugate (Invitrogen) for 30 min at room temperature. Slides were examined with a confocal microscope (Leica).

**Gene expression study.** Total RNA was extracted from mouse brains at 1 and 6 days p.i. using the TRIZol protocol until phase separation. Two biological replicates were analysed for each experiment. RNA was isolated using an RNaseasy Mini kit (Qiagen) with DNAse I (Qiagen) for DNA removal. The extracted total RNA was eluted with 40 µl of RNA Storage Solution (Ambion) and stored at −80 °C. Total RNA concentration, quality and integrity were determined with an RNA 6000 Nano LabChip on a 2100 Bioanalyzer (Agilent). Total RNA of 200 ng from each of two biological replicates that fulfilled the quality control (A260/A280 ≥ 1.8 and A260/A230 ≥ 1.8) and with the highest RNA integrity number (≥ 8) was selected for the gene expression study. Double-stranded cDNA was synthesized and hybridized onto a SurePrint G3 Mouse GE 8K microarray (Agilent) and scanned with a microarray scanner G2505C (Agilent). Data were analysed with Genespring GX 12.5 (Agilent), using one-way ANOVA to compare gene expression between samples. Genes in CHIKV-infected mice were considered to be differentially regulated if expressed at either ≥ 2 or ≤ −2 fold changes compared to mock-infected mice, in both biological replicates (P<0.05). Genes were uploaded to the Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resources 6.7 (http://david.ncifcrf.gov/tools.jsp) for functional annotation.

**qRT-PCR confirmation.** The gene expression changes observed in the microarray data were confirmed for 45 selected genes using a customized TaqMan array microfluidic card (Applied Biosystems), based on previous immune-related gene expression and proteomic studies of neurovirulence due to SINV (Ryman & Klimstra, 2008; Johnston et al., 2001), SFV (McKimmie et al., 2006), VEEV (Kotserski et al., 2007; Sharma et al., 2008; Sharma & Maheshwari, 2009), Japanese encephalitis virus (Saha & Rangarajan, 2003; Gupta & Rao, 2011; Yang et al., 2011), West Nile virus (Venter et al., 2005), rabies virus (Prosniaik et al., 2001; Saha & Rangarajan, 2003; Wang et al., 2005) and dengue virus (Fink et al., 2007; Bordignon et al., 2008; de Kruijf et al., 2008). Total RNA of 400 ng from each of three biological replicates was selected for confirmation, while 18S, GAPDH, GUSB and HPRT1 genes were used as endogenous controls. The cDNA was synthesized using a High Capacity RNA-to-cDNA kit (Applied Biosystems) and mixed with TaqMan Fast Advanced Master Mix (Applied Biosystems) before being loaded into the TaqMan array microfluidic card. The qRT-PCR was performed in a Quant Studio 12K Flex Real-Time PCR System (Applied Biosystems) and analysed with Expression Suite Software 1.0.3 (Applied Biosystems) with the Benjamini–Hochberg false discovery rate test. Immune-related genes with fold changes ≥1.5 in either MY/06/37348- or MY/08/065-infected mice or both were selected for direct comparison of expression between MY/06/37348- and MY/08/065-infected mice using the independent t-test (P<0.01).

**Data analysis.** The Mann–Whitney test was used to compare medians of non-parametric results for in vitro and in vivo replication assays. Survival of mice was analysed using the Kaplan–Meier survival curve. One-way ANOVA was performed to compare gene expression of CHIKV-infected mice with mock-infected mice, while the independent t-test was used to compare qRT-PCR measurements of gene expression between MY/06/37348- and MY/08/065-infected mice. All statistical analyses were performed using SPSS 20 (IBM) and a P-value <0.05 was considered statistically significant unless stated otherwise.

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d's roles in the disease, and diagnostics.


