

Pathogenesis and Molecular Mechanisms of Zika Virus

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Abstract

Zika virus (ZIKV) is one of the most important emerging viruses of 2016. A developing outbreak in the Americas has demonstrated an association between the virus and serious clinical manifestations, such as Guillain–Barré syndrome in adults and congenital malformations in infants born to infected mothers. Pathogenesis and mechanisms of neurologic or immune disease by ZIKV have not been clearly delineated. However, several pathways have been described to explain viral involvement in brain and immune system as well as other organ systems such as eye, skin, and male and female reproductive tracts. ZIKV activates toll-like receptor 3 and several pathways have been described to explain the mechanisms at a molecular level. The mechanism of microcephaly has been more difficult to demonstrate experimentally, likely due to the multifactorial and complex nature of the phenotype. This article provides an overview of existing literature on ZIKV pathogenicity and possible molecular mechanisms of disease as outlined to date.

Keywords

- ▶ Zika
- ▶ microcephaly
- ▶ congenital malformations
- ▶ pathogenicity
- ▶ mouse models

Zika virus (ZIKV) is an arbovirus belonging to the Flaviviridae family.¹ It was first isolated from the serum of a Rhesus monkey in 1947, followed by the isolation from the solution of ground-up *Aedes africanus* mosquitos and 10% serum-saline, in 1948 from the Zika forest in Uganda.² The first human infection was detected by the presence of neutralizing antibodies against ZIKV in 1952.³ ZIKV has caused sporadic infections in Africa and Asia, and larger outbreaks in Micronesia⁴ in 2007 and in French Polynesia⁵ in 2013. The first major outbreak in the Americas in humans was documented in Chile in 2014, with nucleotide sequencing of the virus suggesting origins from French Polynesia.⁶ In 2015, Brazilian health authorities reported an increasing number of newborns with fetal abnormalities, including abnormal brain development (i.e., microcephaly), in addition to other congenital malformations.⁷ The preponderance of cases of microcephaly in regions with circulating ZIKV lead the United States Center for Disease Control to state that ZIKV

is a likely cause of microcephaly in infants born from mothers infected during pregnancy.⁸ During this time, ZIKV was reported in 30 countries and territories in the Americas,^{9,10} leading to the World Health Organization declaring ZIKV infection a global threat in February 2016. As of June 2016, 755 cases of ZIKV infection has been reported in the United States, of which 3 cases have developed Guillain–Barré syndrome and 6 cases have reported ZIKV-related birth defects or pregnancy losses with birth defects.¹¹

Flaviviruses, including ZIKV, are single-stranded, nonsegmented, positive-sense RNA viruses. Both viral and host cell enzymes are involved in cleaving the single translated polyprotein into three structural proteins (i.e., capsid, pre-membrane/membrane, and envelope) and seven nonstructural proteins (i.e., NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5).¹² The envelope protein functions as a receptor binding and membrane fusion protein, and is the primary target of neutralizing antibodies by the host. The membrane protein

forms a complex to protect the envelope protein from degradation during virion assembly. The nonstructural proteins are involved in viral genome and replication as well as inhibiting cellular innate immune responses.¹³ The NS5 is a multitasking protein, with the C-terminus having RNA-dependent RNA polymerase activity and the N-terminus allowing for methyltransferase activity and RNACapping.^{13,14} Because the ZIKV genome encodes for 10 proteins, posttranslational modifications may expand the functions of the proteins in their altered form.¹⁴

ZIKV is typically transmitted to humans following a bite by an infected mosquito, mainly by *A. aegypti* and *A. albopictus*.¹⁵ Maternal–fetal transmission^{16–19} and sexual transmission^{20–22} have also been observed in humans. ZIKV RNA has been detected in blood,²³ urine,²⁴ semen,²⁵ saliva,²⁶ amniotic fluid,^{16,17} cerebrospinal fluid (CSF),^{27,28} and breast milk,²⁹ suggesting other potential modes of transmission.

Currently, it is estimated that about one in five people infected with ZIKV becomes symptomatic with clinical manifestations.³⁰ In most symptomatic cases, ZIKV infection is self-limiting and lasts a few days. In adults, the symptoms usually include a low-grade fever, maculopapular pruritic rash, arthralgia, or nonpurulent conjunctivitis.³⁰ Other symptoms include headaches, myalgias, retro-orbital pain, gastrointestinal disorders, and cervical lymphadenopathy.^{12,31} Currently, there is no evidence to suggest that pregnant women are more susceptible to ZIKV infection than the general population. Interestingly, however, in a preliminary report of 65,726 ZIKV cases in Colombia, 4% tested positive by a reverse transcription polymerase chain reaction (RT-PCR) assay, in which 11,944 were pregnant women where the assay positivity was 12% in that subgroup.³² More serious effects of ZIKV infection include Guillain–Barré syndrome in adults and congenital malformations in fetuses. Vertical transmission associated with several sequelae, including microcephaly and other neurologic abnormalities, ocular abnormalities, fetal growth restriction, hydrops, and fetal loss.^{7,19,27,28,33–37} Fatality from ZIKV infection is rare.^{38,39}

Knowledge regarding the ways that ZIKV uses to exert the clinical effects observed in humans is limited. The purpose of this article is to review the published literature regarding ZIKV and to summarize its pathogenicity and molecular mechanisms, organized by major organ systems.

Brain and Nervous System

Adults

ZIKV infection is associated with Guillain–Barré syndrome in adults.^{40–42} In a case–control study, among 42 patients diagnosed with Guillain–Barré syndrome, 41 (98%) had anti-ZIKV immunoglobulin M (IgM) or immunoglobulin G, and all patients had ZIKV neutralizing antibodies.⁴⁰ The association of neurologic outcomes and ZIKV infection has been demonstrated in animal studies. A mouse model involving intracerebral inoculation of the E/1 (isolated from *A. africanus*) strain of ZIKV in Swiss albino mice of all ages showed neuronal degeneration, inclusion bodies, and areas of softening in the mouse brains.⁴³ Intracerebral inoculation of MP 1751 strain

(isolated from *A. africanus*) of ZIKV in newborn and 5-week-old Webster Swiss white mice resulted in enlargement of astroglial cells and destruction of pyriform cells of Ammon's horn.⁴⁴ Recent studies in mice demonstrate neurologic disease signs in the form of hindlimb paralysis, and high levels of ZIKV RNA in brain and spinal cord tissue.^{45,46} The mechanisms of neurologic or autoimmune disease by ZIKV have not been clearly delineated. One possibility is that the virus may possess common peptides with the human host leading to cross-reactivity. A comparison of penta- and hexapeptides of the ZIKV polyprotein with human proteins potentially related to Guillain–Barré syndrome revealed numerous overlap with proteins linked to demyelination and axonal neuropathies.⁴⁷ Further studies are needed to identify the roles of these proteins and to identify the molecular pathways ZIKV invokes to elicit neurologic disease in adult humans.

Fetus

The potential for ZIKV to cause microcephaly and other congenital neurologic abnormalities in fetuses poses the most serious consequence of ZIKV infection in adults of reproductive age. An increased incidence of microcephaly and other neurologic abnormalities was reported in late 2015 among newborns born in ZIKV infected areas in Brazil.⁴⁸ In one cohort study, abnormal fetal ultrasound findings were noted in 29% of 42 patients with ZIKV, including microcephaly, cerebral calcifications, ventriculomegaly, cerebellar/vermis agenesis, and abnormal middle cerebral artery flow.⁷ Numerous cases have been reported with similar fetal neurologic findings associated with ZIKV infection.^{7,19,27,28,33} ZIKV has been isolated in fetal brain tissue^{19,34} and ZIKV IgM has been detected in the CSF of infants with microcephaly compatible with congenital ZIKV infection.^{27,28}

The vertical transmission of ZIKV and effect on fetal neurologic development has been demonstrated in several murine studies, all of which have used animals with immune deficiencies. When pregnant Swiss Jim Lambert (SJL) mice were infected intravenously with a 200 μ L of cell culture supernatant containing between 1×10^3 and 1×10^{12} PFU m^{-1} of a Brazilian strain of the virus between days 10 and 13 of gestation, ZIKV RNA was observed in pup brain tissue compared with that of kidney, liver, or spleen.⁴⁹ Though microcephaly was not able to be reproduced, cortical malformation with reduced cell numbers and thickness and morphology suggestive of cellular death, such as vacuolar nuclei, was noted.⁴⁹ One mouse model involving intraperitoneal injection of 300 μ L (3×10^5 PFU/mL) of ZIKV strain SZ01 (contemporary Asian strain) in immunocompetent C57 mice showed radial glial cells, a type of neural progenitor located in the ventricular zone, as a primary target of infection in fetal mouse brains.⁵⁰ The same ZIKV strain injected directly into embryonic mice brains at E13.5 at concentrations of 6.5×10^4 to 6.5×10^5 PFU/mL was demonstrated to target neural progenitors, including radial glial cells and intermediate/basal progenitor cells.⁵¹ ZIKV was shown to display an inhibitory effect on cortical neural progenitor cell proliferation and differentiation, and the transition of radial glial cells to intermediate progenitor cells.^{50,51} In these mouse models,

genes related to the immune response and apoptosis pathways, including cytokine production and response were shown to be upregulated in ZIKV-infected fetal brains compared with mock-infected brains.^{50,51} Genes involved with cell proliferation, differentiation, migration, and organ development were shown to be downregulated.^{50,51} These animal studies demonstrate the fetal neurotropism of ZIKV, which could explain the subsequent congenital neurologic sequelae of infection.

ZIKV directly targets human neural progenitor cells in vitro.^{52,53} Infection spread to 65 to 90% of the cells within 3 days of inoculation.⁵² In human fetal neural progenitor cells, replication of the virus persisted for 28 days.⁵⁴ ZIKV infection exerted a cytopathic effect on human fetal neural progenitors, characterized by cell rounding, pyknosis, and activation of caspase 3.⁵⁴

Several in vitro studies involving neurosphere and organoid models have been developed to study fetal brain development as a result of ZIKV infection. ZIKV may impair neural differentiation and neurogenesis. When infected with multiplicity of infection (MOI) 1 of MR766 strain of ZIKV, attenuated growth was noted in mouse neurospheres over a day, in human embryonic stem cell derived brain organoids over the course of 5 days.⁵³ When infected with the same strain at MOI 0.0025 to 0.25, morphologic abnormalities, such as apoptotic nuclei, were observed on the sixth day in neurospheres generated by human neural stem cells.⁵⁵ These abnormalities and signs of cell death were also observed 96 hours postinfection from a Brazilian strain of ZIKV at MOI 10 in human neural progenitor cell derived neurospheres and at MOI 0.1 in human pluripotent stem cell derived organoids.⁴⁹ Decreased growth in human induced pluripotent stem cell-derived organoids was noted over the course of 11 days, when infected with 3×10^2 to 3×10^4 PFU of virus on day 35.⁵⁵ A decrease in the population of cortical neurons and dorsal forebrain progenitor cells, as well as an increased apoptotic cells was noted in human pluripotent stem cell derived organoids.⁴⁹ ZIKV appears to impair proliferation and induce death in fetal brain development at a cellular level.

Several pathways have been described to explain the mechanisms at a molecular level. Within the Tyro3, Axl, Mer family of receptor tyrosine kinases, AXL, has been shown to mediate ZIKV infection in human skin cells.⁵⁶ AXL is highly enriched in human radial glial cells, as well as cortical astrocytes, blood microcapillaries, and microglia in vitro.⁵⁷ An increase in AXL was noted in ZIKV infected fetal mouse brain tissue.⁵¹ This could provide a possible mechanism of ZIKV access into the developing fetal brain. In addition, ZIKV infection affects neural cell proliferation and induces cell death. An upregulation of genes associated with autophagy and apoptosis, including *Bmf*, *Irgm1*, *Bcl2*, *Htt*, *Casp6*, and *Abl1*, was demonstrated in the brains of SJL mice pups born to mothers infected with ZIKV.⁴⁹ RNA analysis showed upregulation of genes involved in the apoptotic pathway, such as caspase-3/7 in human neural progenitor cells infected with ZIKV in vitro, and cells in the intermediate zone and cortical plate in embryonic mouse brains infected with ZIKV in vivo.^{51,52,55} Because postmitotic neurons are located in the

cortical plate, these cells may also be a specific target of ZIKV.⁵¹ ZIKV infection was shown to lead to higher caspase-3 activation and cell death.^{51,52} These changes may occur as a direct infection with the virus as well as due to inflammation resulting from the virus. S-phase arrest of human-induced pluripotent stem cells was also seen.⁵²

ZIKV can activate toll-like receptor 3 (TLR3) in human embryonic stem cell derived cerebral organoids, leading to a dysregulation of genes involved in neurogenesis and apoptotic pathways.⁵³ TLR3 inhibits Sonic Hedgehog and Ras-ERK signaling in neural progenitor cells, thereby triggering apoptosis.^{58,59} An increased risk of neuropathological dysfunction resulting from maternal infection of polyinosinic-polycytidylic acid, a synthetic, double-stranded RNA molecular mimic of replicating virus that involves TLR3 signaling, was noted in fetal and neonatal offspring of wild-type mice.⁶⁰ Increased TLR3 gene expression was noted in ZIKV infected fetal mice brains.⁵¹

The mechanism of microcephaly has been more difficult to demonstrate experimentally, likely due to the multifactorial and complex nature of the phenotype. In ZIKV infected fetal mice brains, several microcephaly-associated genes are downregulated, such as *microcephalin*, *Aspm*, *Casc5*, *Cenpf*, *Mcp1*, *Rbbp8*, *Stil*, and *Tbr2*.^{50,51} Many of these genes are involved in regulating the cell cycle and may, therefore, contribute to the impaired proliferation and apoptosis seen in neural cells.⁶¹ In a study of penta- and hexapeptides of the ZIKV polyprotein with human proteins related to microcephaly or altered brain calcification, numerous overlaps were observed.⁴⁷ Cross-reactivity following the immune response by ZIKV may also provide a link between the virus and microcephaly.

Placenta

The placenta acts as both a physical and immunological barriers between the maternal and fetal compartments. Syncytiotrophoblasts cover the surface of the human placenta villous tree and are in direct contact with maternal blood, thus providing the front line of protecting the fetus. Vertical transmission of viruses occur in one or more ways, including direct hematogenous spread into the gestational sac, trophoblastic transcellular or paracellular pathways, transport through infected sperm, pre-pregnancy uterine colonization or transvaginal ascending infection, and invasive procedures during pregnancy.⁶² The association between ZIKV infection in pregnant women and the development of subsequent neurologic, growth, or other congenital sequelae in fetuses suggest the virus has the ability to gain direct access to the intrauterine environment. ZIKV RNA has been detected in amniotic fluid and placental tissue at various gestational ages in humans suggesting that the virus uses mechanisms to directly infect or bypass the placental barrier to gain access to the intrauterine cavity and fetus.¹⁶⁻¹⁹ In a preliminary report of ZIKV cases in Colombia, in 616 patients who were infected with the virus in the third trimester, there were no cases of microcephaly or other ZIKV-related birth defects at the time on data analysis.³² This may support the hypothesis that the

severity of congenital ZIKV disease may have a temporal relationship with gestational age, with more severe cases occurring from infection at earlier gestational ages. The exact mechanism is yet to be explained.

Placental tropism has been demonstrated in mouse models. Subcutaneous inoculation with 50 μ L of phosphate-buffered saline solution containing 10^3 plaque forming units (PFU) of ZIKV strain H/PF/2013 (French Polynesia 2013) in pregnant dams lacking type I interferon (IFN) signaling (*Ifnar1*^{-/-}) crossed with wild-type males on embryonic days 6.5 or 7.5, lead to high levels of ZIKV RNA detected in the placentas and brains of their fetuses when examined at E13.5 and E15.5.⁶³ ZIKV RNA levels almost 1,000 times greater in the placenta compared with maternal serum level.⁶³ ZIKV RNA was also present in different trophoblast cells of the mouse placentas. Pathologic examination of these placentas revealed smaller size, as well as apoptotic trophoblasts, which could allow for disruption of the placental barrier and lead to fetal infection.⁶³ An in vitro study showed that human placental macrophages, and to a lesser extent cytotrophoblasts, were susceptible to ZIKV infection.⁶⁴

ZIKV is unable to replicate efficiently in primary human trophoblast cells derived from late-pregnancy placentas, which undergo fusion to form syncytiotrophoblasts during in vitro culturing similar to their natural in vivo differentiation process.⁶² The innate immune response, involving IFNs, is the primary host defense strategy used to combat viral infections. Induction of IFN-stimulated genes was enhanced with increased fusion of primary human trophoblasts (PHT) cells, and attenuated when primary human trophoblast cell fusion was disrupted.⁶² The conditioned medium isolated specifically from primary human trophoblast cell was shown to protect nonplacental recipient cells from infection as well by causing the production of IFN-stimulated genes.⁶²

Type I IFNs, including IFN α and IFN β , are produced by many cell types, with immune cells, such as dendritic cells, serving as the primary producers of the antiviral response.⁶² In contrast, cells of epithelial origin produce type III IFNs, including IFN λ 1–4, in response to viral infection.⁶² To date, mouse models have demonstrated that the absence of IFN α / β signaling in mice (i.e., use of *Ifnar1*^{-/-} or *Ifr3*^{-/-} *Ifr5*^{-/-} *Ifr7*^{-/-} mice aged 506 weeks), cause them to be more susceptible to ZIKV infection (MR 766 and H/PF/2013 strains) compared with wild-type mice, as measured by weight loss, hindlimb paralysis, and a 100% lethality in 10 days after both subcutaneous and intravenous infection.⁴⁵ Knockout mice between 3 and 11 weeks in age lacking type I IFN receptor (IFN α / β), and type I and type III IFN receptors (IFN α / β / γ), show viremia, and signs of neurologic disease, such as “toe walking,” tremors and imbalance when infected subcutaneously or in the footpad with 1×10^4 PFU of FSS13025 (Asian) strain of ZIKV.⁴⁶ Thus, IFN signaling plays a key role in ZIKV pathogenesis.

Syncytiotrophoblasts constitutively produce type III IFNs, particularly IFN λ 1, to protect against ZIKV in vitro.⁶² The paracrine effects of trophoblast-derived IFN λ 1 confer protection to nonplacental cells.⁶² Placental macrophages infected with MOI 1 of ZIKV PR 2015 strain activated IFN α pathways

and other proinflammatory cytokines, such as interleukin (IL)-6 when observed 24 hours after infection.⁶⁴ Therefore, ZIKV may access the fetal compartment by evading type I and type III IFN antiviral signaling pathways in placental macrophages and villous syncytiotrophoblasts in late pregnancy, or bypass these cells using a different mechanism.^{62,64} Once such mechanism of evasion was depicted in vitro, in which the ZIKV encoded NS5 protein inhibited type I IFN signaling in human 293T cells, by binding and degrading STAT2.⁶⁵ Both type I IFN and type III IFN use JAK-STAT signaling, including STAT2; therefore, ZIKV may overcome the IFN antiviral response through STAT2 degradation by NS5.⁶⁵

Growth Restriction and Fetal Demise

Other serious sequelae of ZIKV include intrauterine growth restriction (IUGR), hydrops fetalis, and fetal demise.^{17,19} Abnormal umbilical artery flow and placental insufficiency, as well as IUGR and fetal demise, have been diagnosed by ultrasound in patients infected with ZIKV.⁷ Murine studies in SJL mice as well as *Ifnar1*^{-/-} mice crossed with wild-type males have demonstrated IUGR in mouse pups and fetuses, respectively,^{49,63} and fetal demise.⁶³ Mouse models of ZIKV infection demonstrate placentas with vascular injury, such as reduced fetal capillaries and destruction of placental microvasculature and a large number of nucleated fetal erythrocytes, an indicator of fetal stress.⁶³ Decreased weight gain and weight loss and lethality have also been documented in knockout mice infected between age 3 weeks and 6 months.^{45,46} These findings may be related to direct effects of ZIKV on growth and development, or due to the impaired neurologic function associated with the virus.

Eye

Ocular findings associated with ZIKV have been described in infants with congenital ZIKV. These include macular abnormalities such as pigment mottling and chorioretinal atrophy, and optic nerve abnormalities such as hypoplasia with double-ring sign, pallor, increased cup-to-disk ratio.^{36,37} Among infants with microcephaly and presumed diagnosis of congenital ZIKV, ocular abnormalities were present in 34.5% of 29 children, and 29.3% of the eyes examined displayed ocular abnormalities.³⁵

Ocular abnormalities associated with ZIKV infection have also been observed in vivo in SJL mice pups born to infected mothers.⁴⁹ It is unclear whether these ocular lesions are a direct result of ZIKV, or part of the spectrum of microcephaly-associated abnormalities. The expression of AXL, a flavivirus entry receptor, was enriched in ciliary marginal zone, adjacent to neural retina, in vitro.⁵⁷ The isolation of ZIKV in ocular cells, however, has not yet been demonstrated.

Skin

Human skin is the primary physical and immunological barrier used in defense of external pathogens. In the case of ZIKV infection, this barrier is breached by the bite of an infected mosquito. In vitro and ex vivo experiments of human

skin cells, including fibroblasts, keratinocytes, and dendritic cells illustrate that these cell types are early targets of ZIKV infection. In a study of human skin cells, several entry/adhesion factors were shown to permit ZIKV entry, including AXL, DC-SIGN, Tyro3, and TIM-1 to a lesser extent.⁵⁶ ZIKV induced transcription of TLR3, RIG-1, and MDA5, as well as several IFN stimulated genes such as *OAS2*, *ISG15*, and *MX1*.⁵⁶ Enhanced IFN α and IFN β gene expression and IFN stimulated genes, cytokines, and other immune modulators such as CCL5, CXCL10, AIM2, and IL-1 β were seen.⁵⁶ The replication of ZIKV was decreased by type I and type II IFN antiviral effects, whereas formation of autophagosomes was associated with enhanced viral replication.⁵⁶

Testes

Male-to-female (as well as female-to-male) sexual transmission of ZIKV in humans has been reported.^{20–22} While high viral load was detected in semen, blood samples showed undetectable levels by real time RT-PCR.²¹ The prolonged presence of ZIKV in human semen 27 to 62 days after onset of febrile illness, compared with other body samples, has also been demonstrated.²⁵ In *Ifnar1*^{-/-} knockout mice, the highest level of ZIKV RNA was found in the testes compared with brain, spinal cord, liver, spleen, kidney, and serum.⁴⁵ Knockout mice lacking type I IFN receptors, and type I and III IFN receptors, demonstrated high viral titers in the testes compared with brain, heart, lung, liver, spleen, and muscle.⁴⁶ ZIKV has not been isolated in ovarian tissue. The link between sexual transmission and congenital disease is unclear.

Discussion

Although knowledge and data regarding ZIKV is continuing to emerge, certain observations can be made from existing literature. The structure of ZIKV proteins may exhibit cross-reactivity with a variety of proteins involved in cell growth and immune processes, resulting in the variety of downstream signaling effects leading to disease. ZIKV may involve direct or indirect effects on neuronal stem cell proliferation, differentiation, and migration and interactions. At the level of the placenta, it may bypass the strong IFN-mediated defense through a variety of mechanisms to overcome the antiviral response. The ability of ZIKV to access immune privileged sites, such as the blood-brain barrier or maternal-fetal placental barrier may account for its tropism to these tissues and subsequent clinical effects. It remains unclear why congenital malformations affect a subset of infected pregnant women. A temporal relationship likely exists, with more serious fetal sequelae developing as a result of early embryonic or fetal infection. There may also be individual genetic differences involved that affect the impact of ZIKV infection. It is also important to address whether genetic changes in currently circulating strains of ZIKV may facilitate the induction of fetal abnormalities by altering aspects of virus replication, pathogenesis, or cell tropism.

Currently, the treatment of ZIKV infection is supportive. Given the emerging evidence for concerning sequelae of ZIKV,

including congenital disease, the need for vaccines and therapeutics is great. Although several mouse models are available, nonhuman primate models will likely be needed for vaccine development. Various vaccine approaches are in the preclinical development phase, including inactivated virus, virus-like particles, live vectored vaccines, subunit vaccines, live recombinant vaccines, nucleic acid-based vaccines. Many of these therapeutics are expected to enter Phase I clinical studies in 2017. Given success of live vectored vaccines including yellow fever, Japanese encephalitis, and dengue fever, which are all closely related to ZIKV, this may be an avenue of study.^{66,67} Understanding the molecular mechanisms of ZIKV may allow for development of therapeutic targets and vaccines to combat the neurologic, fetal, and other clinically significant sequelae of ZIKV infection.

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