Published in final edited form as:

Virus Res. 2018 August 02; 254: 15–20. doi:10.1016/j.virusres.2017.07.025.

Zika Virus Research Models

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Abstract

The 2015 Brazilian Zika virus outbreak sparked a rapid response to control the spread of the virus. What was first understood to be a mild self-resolving infection is now linked to significant neurological defects in both neonates, and adults. The WHO declared the 2016 Zika epidemic a public health emergency and issued an unprecedented recommendation to women in affected regions to delay pregnancy until the risks surrounding Zika virus could be understood, or the epidemic contained. Since that time, considerable effort has been dedicated to understanding Zika transmission and pathogenesis to aid the development of drugs and vaccines. Several models have emerged to study several facets of Zika biology; this review details the various model systems.

Overview of the ZIKV epidemic

Zika virus (ZIKV) was first isolated from a sentinel rhesus macaque from the Zika Forest of Uganda in 1947. Vector-mediated transmission from *Aedes africanus* was assumed to be the likeliest means of acquisition (1). The earliest documented case of human ZIKV infection was recorded in 1952 in a patient with mild disease sequelae and no reported neurological manifestations (2). More than 50 years later, 14 isolated ZIKV cases were reported in Uganda, Nigeria, and Senegal (2–6).

The first major ZIKV outbreak, and first reported cases outside of Africa, occurred in 2007 on Yap Island of Micronesia. Yap disease symptoms included rash, fever, conjunctivitis, and arthralgia. Although it was estimated that only 20% of infections manifested clinical symptoms, acquisition rates approached more than 70% of residents on the island (7). In 2013, a larger ZIKV outbreak occurred in French Polynesia with a remarkable 40-fold increase in the incidence of Guillian-barré syndrome (8, 9).

ZIKV then spread to the Americas, first to Easter Island in Chile (10) and then to Brazil, where local transmission was reported in March 2015 (11). The “first wave” of the ZIKV

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The authors declare no competing financial interests.

outbreak in northeastern Brazil was associated with a peak monthly occurrence of 49.9 cases of microcephaly per 10,000 live births, while the second wave of the outbreak, which was more widespread throughout Brazil, was associated with a reduced peak of 3.9 to 15 monthly cases per 10,000 live births (12). The ZIKV incidence within Brazil to date has been estimated to be up to 1.5 million persons (13). Since that time, ZIKV has spread throughout South America, the Caribbean, Central America, and into North America. ZIKV spread is predicted to continue due to favorable conditions in the Americas that include a large, mobile population, favorable climate for vector transmission, and widespread occurrence of mosquito species capable of ZIKV transmission (14).

ZIKV Pathogenesis in Humans

ZIKV is a mosquito borne Arbovirus that is transmitted primarily by *Aedes* mosquitoes and also has vertical/sexual transmission potential (15, 16). ZIKV efficiently replicates in the salivary glands of mosquitoes, and in various human cells types (17). ZIKV has demonstrated tropism for multiple mammalian cell types including primary dermal fibroblasts, dendritic cells, and epidermal keratinocytes (18). Post-acquisition, ZIKV traffics to the draining lymphatics and thereby disseminates throughout the body. Viral load in the plasma typically peaks and correlates with the onset of symptoms between 3–5 days post infection (19). Viral RNA is detectable in the plasma for approximately 10 days (20). Importantly, ZIKV persists in other compartments and is shed in bodily fluids such as saliva, urine, tears, and semen (19–23). The persistent shedding of ZIKV RNA in several of these fluids is greatly protracted compared to the blood. Several reports have described detectable ZIKV RNA in seminal plasma for months after symptom onset, raising significant concerns for sexual transmission and of a long-lived reservoir in the male genital tract (24–26). Persistence of ZIKV in placental tissues can provide a definitive diagnosis of recent maternal infection during pregnancy (27).

ZIKV may also persist in whole blood and has been localized to erythrocytes, creating a transfusion-mediated acquisition (28). Thus, understanding ZIKV dynamics in anatomic tissue compartments has important ramifications for diagnosis, transmission, and organ or blood donation.

Clinical manifestations of ZIKV are characterized as mild and transient, with presentation of a macular or papular rash, low-grade fever, arthralgia, fatigue, conjunctivitis, and headache (29). Symptoms usually resolve without treatment, except in immunocompromised individuals, which has resulted in fatalities (30, 31). Despite infection being historically characterized as mild, ZIKV has been associated with increasingly severe conditions such as Guillian-barré and microcephaly in neonates that were exposed *in utero* (32). In addition, it is now accepted that a wide range of congenital conditions are possible as a result of *in utero* ZIKV exposure including brain calcifications, arthrogryposis, ophthalmologic alterations, and spinal deformities that do not always present with microcephaly (33–35). It is likely that the full extent of ZIKV congenital syndrome may not be fully described and understood until the affected infants age and begin to reach developmental milestones. Recently, concerns for male fertility and adult neurocognition have been raised, and warrant additional

clinical scrutiny for any patients presenting with even mild neurologic complications (36, 37).

Modeling ZIKV infection

Several systems have emerged to model human ZIKV infection, tropism and pathogenesis. These models have been utilized to address a number of questions that are critical to understanding the recent epidemic. These include: better defining viral dynamics and persistence; evaluating candidate vaccines, therapeutics, and diagnostic tools; assessing neurological risks; and characterizing the various transmission routes. While each model has its own limitations, the information collectively can provide important insights into ZIKV biology.

Cell culture models

Cell culture models have been intensively relied upon to study mechanistic aspects of ZIKV infection, particularly for large-scale antiviral compound screens. Recent advances in stem cell technology fortuitously coincided with the ZIKV emergence. Developing brains and neurons are difficult to study *in vivo*, but with induced pluripotent stem cells (iPSCs), it is possible to study neuronal development at different stages using either 2D monolayer culture models (38) or 3D organoids (39). Certainly, iPSC technology has proven to be a useful tool to determine ZIKV cell targets and to assess the efficacy of candidate therapeutics in neuronal tissues (40).

A critical question still being addressed is precisely when ZIKV infection during pregnancy causes fetal microcephaly and other neurological congenital diseases (41). Tang *et al.* demonstrated that human neural progenitor cells (NPCs) differentiated from human iPSCs were permissive to ZIKV, as were immature neurons derived from NPCs, although to a lesser extent (42). ZIKV infection using this model induced caspase-3, consummately deregulating the cell cycle and ultimately causing cell death. These intriguing findings offered at least a partial rationale for reduced cortical thickness after fetal ZIKV exposure (42). These findings have been replicated with different ZIKV strains (43–46). Qian *et al.* (47) developed a system that modeled 3D brain development in the forebrain. When the organoids were transiently exposed to ZIKV at different stages of development, there was infection of NPCs, and reduced organoid size with expanded ventricular structures (48). NPCs were significantly impacted, although infection of neurons and astrocytes were also observed. Interestingly, nearby uninfected cells stained caspase-3+, suggesting a possible bystander effect. The most dramatic phenotype was observed when the organoid was infected early, although damage was seen with ZIKV exposure at all developmental stages (47). Another study used neurospheres, a 3D culture model that lacks the structural intricacies of an organoid, as an *in vitro* model to demonstrate that a Brazilian ZIKV isolate impacted neuronal growth more than an African strain, and was also species specific to human cells, and not chimpanzee. This finding was replicated using an organoid model and thus the Brazilian strain may display enhanced neurotropism for human cells, compared to African strains (44). Certainly, these studies have established that ZIKV infection can severely damage NPCs and developing neuronal tissue.

Cell culture models have also proven instrumental to identify candidate receptors for ZIKV entry, and selective cell tropism. It was hypothesized that ZIKV infects and replicates in the skin and in neural progenitor cells. Indeed, ZIKV actively replicates *in vitro* within skin fibroblasts and epidermal keratinocytes, as well as skin-derived immature dendritic cells that are highly permissive to both ZIKV and dengue virus (DENV) infection (18). Other cell types are also susceptible to ZIKV infection including placental macrophages, which may serve as a primary cell supporting ZIKV intrauterine infection (49).

Similar to DENV, the TIM and TAM family of receptors have been shown to play an important role in ZIKV entry, particularly AXL, a receptor tyrosine kinase (18, 50). Modulating AXL expression in non-neuronal cell types reduces ZIKV infectivity (18) (51), however genetic ablation of AXL in NPCs did not protect cells from infection and subsequent cell death (52), which has also been confirmed in mouse models (53). ZIKV, like other flaviviruses, likely mediates cellular entry through binding glycoaminglycans on host cell membranes (54). Thus, it is likely that multiple receptors play a cooperative role for cell entry or there may be tissue specific receptors.

Culture models have enhanced our understanding of how ZIKV interferes with the cell cycle and cytopathicity. ZIKV infection results in host cell changes in cellular metabolism, disrupting cell cycle progression (40). Notably, autophagy and cell death pathways are activated following ZIKV infection. NPCs infected with ZIKV *in vitro* underwent caspase-3-mediated apoptosis, downregulated cell cycle related genes, and upregulated genes associated with viral replication, such as those involved in transcription, protein transport, and catabolism (42). *In vitro* infection of human fetal neural stem cells with ZIKV resulted in increased apoptosis, and reduced proliferative capacity. ZIKV proteins NS4A and NS4B were also shown to induce autophagy and impair mTOR signaling, potentiating viral replication, but decreasing neurogenesis (55). Interestingly, ZIKV infection does not induce apoptosis in skin fibroblasts *in vitro*, but does enhance autophagy (18). Curiously, few cytopathic effects were observed in ZIKV-infected Hofbauer cells *in vitro* (49). Collectively, several culture models have demonstrated that ZIKV-mediated disruption of cellular function is cell-specific (49).

Cell culture models have been instrumental in screening compound libraries for potential therapeutics. One study screened 774 FDA-approved drugs for anti-ZIKV activity. Human hepatoma cells (HuH-7 cells) were treated with the drugs for 1 hour prior to infection and screened for effects that inhibited viral entry, translation, or RNA synthesis. 24 suitable drugs were identified using this model and 8 drugs were confirmed to have anti-ZIKV properties in other cells lines (45). Another group screened 727 compounds in the NIH Clinical Compound Collection library in an assay designed to measure cytopathic effects (CPE). This CPE-based assay in Vero 76 cells and identified pyrimidine synthesis inhibitors to have potent anti-ZIKV activity (56). Vero cells are derived from epithelial kidney cells from African green monkeys. This cell line consistently yields high virus titers and is also susceptible to CPE due to its deficiency in type I interferon production (57). Another group developed a culture-based screening assay for ZIKV antivirals, also in Vero cells. The readout of this assay quantifies ZIKV yield using qRT-PCR (58). Culture models provide a useful starting point for screening ZIKV therapeutics. However, as discussed above, ZIKV

metabolism and life cycle may differ in different cell types. Therefore, any drug identified in a cell-culture based assay must be validated *in vivo* to confirm efficacy.

Murine models

Mouse models provide a useful tool to study *in vivo* pathogenesis of ZIKV infection, including fetal infection and vertical transmission. However, wildtype (WT) mice clear ZIKV quickly and do not develop disease due to ZIKV inability to antagonize murine interferon (IFN) signaling. In humans, ZIKV promotes proteasomal degradation of STAT2 and thus blocks IFN signaling (59). Infected murine cells do not show the same block of IFN signaling, which is thought to be why ZIKV is nonlethal and replicates poorly in WT mice. To overcome this limitation and achieve virulence in mice, researchers have developed several models to artificially perturb IFN signaling.

Early studies encountered difficulty to adapt ZIKV to mice, although the virus was neurotropic in mice when infected intracerebrally (60–62). More recently, mice lacking the type I IFN receptor gene (*Ifnar1*^{-/-}), which cannot respond to IFN α/β , develop weight loss and neurological disease consisting of hind limb weakness and paralysis, and ultimately mortality following subcutaneous (SC) or intraperitoneal (IP) ZIKV infection. Sickness in *Ifnar1*^{-/-} mice typically begins a few days following infection and mortality is typically between 7 and 10 days post-infection (63–66). Virus in the serum peaks between 2–3 days post infection and is also detectable in tissues throughout the body including heart, lung, liver, kidney, brain, spleen, muscle, ovary and testis with the highest viral loads typically in the spleen (64–66). In contrast, WT mice infected with ZIKV have low levels of virus in blood and virus is detected in some, but not all, organs observed in *Ifnar1*^{-/-} mice (66). Tissue pathology consisting of mononuclear cell infiltrates and lesions have been detected in the brain and testes in *Ifnar1*^{-/-} mice following ZIKV infection (63, 66, 67). Triple knockout of the *Irf3*, *Irf5*, and *Irf7* genes also resulted in lethal ZIKV infection as these mice produce little IFN α/β . However, single mutants were not susceptible to ZIKV infection, nor were mice lacking MAVS, an adaptor protein for RLR (RIG-1/Mda5) signaling (64).

Mice immunocompromised via other approaches can also enhance ZIKV virulence. The administration of IFNAR-1 blocking antibodies to WT mice prior to infection facilitates systemic viral spread, although disease course was still contracted compared to a complete (genetic) knockout model (53, 64). Immunocompromising mice with dexamethasone prior to infection resulted in systemic, but non-lethal infection. When dexamethasone was withdrawn to reconstitute the full immune system during systemic ZIKV infection, mice quickly lost weight and became lethargic, resulting in mortality (68). These findings highlight the role that host immunity plays in ZIKV pathogenesis, a facet not captured in immunocompromised murine systems.

Another important aspect of ZIKV pathogenesis is a causal link to microcephaly. There are several limitations to modeling fetal microcephaly in mice such as differences in gestation time, neural development, the maternal-placental interface, and in immune regulation during pregnancy (69, 70). Despite these limitations, several groups have developed mouse models of *in utero* ZIKV infection. One group infected pregnant, immunocompetent SJL mice with

a high intravenous dose of a Brazilian ZIKV strain. The high dose of virus most likely allowed for seeding of the placenta despite intact IFN signaling in the mother. Newborn pups displayed signs of intrauterine growth restriction and a high viral load was detected in the brains of the pups (44). Another group crossed *Ifnar*^{-/-} female mice with WT males so that the resulting fetuses would be heterozygotes and have intact IFN signaling. Pregnant mice were infected SC at varying times during pregnancy. Again, ZIKV was able to infect the fetus, resulting in both intrauterine growth restriction and fetal reabsorption. Intrauterine growth restriction was also observed with pregnant WT mice treated with a blocking anti-IFNAR antibody (71). Birth defects have even been observed following IP infection of immunocompetent pregnant mice despite only transient viremia observed in the mother (72). Other studies bypassed the issue of achieving viremia in the mother by directly injecting ZIKV into the cerebroventricular space/lateral ventricle of mouse embryos *in vivo*. This resulted in viral replication in the fetal brain, small brain sizes, and a thin cortical layer, consistent with clinical signs of microcephaly in human newborns (72, 73). Together, these studies demonstrate vertical transmission of ZIKV and definitive evidence that *in utero* infection with ZIKV causes microcephaly. However, it is still unknown what the risk factors for microcephaly are during human *in utero* infection. Unlike these murine models, not all infants borne from ZIKV-exposed mothers develop neurologic abnormalities, and there is a wide spectrum of neurologic complications observed in humans that are not captured in the murine systems (74).

Both the timing and route of infection may affect fetal outcomes. It is now established that ZIKV can be sexually transmitted. Mouse models have demonstrated that intravaginal infection with ZIKV results in systemic and lethal infection when mice were infected during the diestrus phase, but not estrus phase in immunodeficient mice (75). ZIKV can replicate in the vaginal canal of immunocompetent WT pregnant mice resulting in reduced fetal weight. ZIKV was also detected in the brain of WT pups following vaginal infection. Of particular concern in this study was that ZIKV remained undetectable in the mother (76). Future research priority should be allocated to address the fetal risk via sexual exposure of ZIKV.

Nonhuman primate models

Nonhuman primates (NHPs) are a natural host for ZIKV, and likely the major zoonotic reservoir (77). Transmission, cellular tropism, immunologic responses and viral dynamics in NHPs closely resemble that of humans (78). Additionally, pregnancy and neural development in NHPs is near identical to humans compared to other model organisms. Drawbacks to using NHP models include limited animal facilities, a long-life cycle and gestation period, and substantially higher costs compared to small animal models. However, NHP safety and efficacy is often a critical milestone in the developmental path of therapeutic drugs and vaccines.

Several groups have demonstrated that NHPs are suitable models for ZIKV infection. Rhesus macaques rapidly become viremic after SC infection with different ZIKV isolates. So far, there have been no significant differences in viral kinetics amongst the various isolates tested (78–80). Cynomolgus macaques are also susceptible to ZIKV infection (78, 81). NHPs are also susceptible to mucosal infection (vaginally or rectally), providing a

model for sexual transmission of ZIKV (82). Plasma viremia after SC infection is short lived in NHPs, typically lasting less than a week and peaking between 1–2 days post SC infection, when higher infecting doses are used. However, ZIKV is detectable in other bodily fluids including urine, saliva, and semen after clearance from the plasma (78–80). Virus is also present in multiple organs and causes histopathological changes in the CNS, liver, and spleen (78, 80). Recent evidence suggests that ZIKV may persist in certain tissues such as the male and female genital tract, neuronal and lymphoid tissues for at least 35 days post-infection (83). Immune activation peaks between 2–5 days post-infection as well, consistent with rapid clearance of the virus from the blood (78). Additionally, the antibody response generated from primary infection is sufficient to protect against subsequent exposures in rhesus macaques (78–80).

NHP models are also able to closely mimic certain aspects of human pregnancy, such as remodeling of the villous structure that supports blood flow to the fetus (70, 84). ZIKV infection in rhesus macaques during the first or third trimester results in prolonged maternal plasma viremia compared to non-pregnant macaques, similar to what has been reported during human pregnancy (48, 79). The virus is able to efficiently reach the fetus after maternal SC infection and ZIKV was readily detected in maternal and fetal tissues after maternal infection during all phases of gestation. ZIKV was detected in fetal tissues at the time of necropsy (end of third trimester), although the tissue distribution of virus was variable and did not seem to associate with pathology (85). In a pig-tailed macaque model, fetal brain lesions and microcephaly develop after SC infection of the mother during pregnancy. In this study, the mother was challenged SC in 5 locations at day 119 of gestation (corresponding to approximately week 28 of human pregnancy) (86). These data underscore the need for additional studies to be conducted in pregnant NHPs to assess the duration of neurologic risk to a developing fetus.

Vaccines and Therapeutics

Currently, there are no approved vaccines or drugs to prevent or to treat ZIKV, although the pace of development of both has been striking. Multiple ZIKV vaccine platforms have already been tested in pre-clinical NHPs models with promising results. The success of vaccines modalities against related flaviviruses such as yellow fever (YFV), tick-borne encephalitis (TBEV), and Japanese encephalitis (JEV) have demonstrated that a vaccine-induced antibody response against the viral E protein is sufficient for complete protection (87, 88). Antibodies generated from prior exposures are also sufficient to protect against subsequent re-exposure (78). Several platforms have proven successful in mouse or NHPs including DNA vaccines, inactivated virus vaccines, adenovirus vector-based vaccines, mRNA vaccines, and live-attenuated viruses (89–92). Several of these platforms have already moved into human clinical trials although the ultimate deployment of a ZIKV vaccine is expected to take several years (93).

Several groups have raised a concern for the potential of antibody-dependent enhancement (ADE) in response to ZIKV vaccination. ADE has been a major concern for vaccinating against DENV, a closely related flavivirus that is being actively transmitted in many of the same regions as ZIKV. DENV antibodies have been shown to lead to ADE and enhance

ZIKV infectivity *in vitro* (94–96). However, a study conducted in NHPs with past DENV infection showed that ZIKV infection was not more severe in this cohort, compared to DENV-naïve animals. In fact, animals with DENV immunity recovered from ZIKV faster than naïve macaques (97). However, a concern remains for vaccine safety in individuals with past DENV exposure or the possibility that ZIKV antibodies generated from infection can result in potentially severe ADE.

An approved vaccine strategy to combat the ZIKV epidemic is likely years away and emergency treatment options in pregnant women and their partners is needed urgently to prevent viral transmission to the fetus and resultant microcephaly. Studies have aimed to repurpose already approved broad-spectrum antivirals to shorten the duration of ZIKV infection and to stop prolonged viral replication in fetal neural tissue. Suramin is one example of several FDA approved drugs potentially available for repurpose for another indication. Suramin is an approved anti-parasitic drug that can prevent enterovirus-71 and CHIKV infections in animal models (98, 99) and has demonstrated anti-ZIKV properties in cell culture (100, 101). Sofosbuvir, an RNA-dependent RNA polymerase inhibitor, is currently approved for treatment of hepatitis C virus has also shown limited efficacy against ZIKV *in vitro* and in murine systems (102, 103). BCX4430, an adenosine nucleoside analog, has been shown to inhibit viral RNA-dependent RNA polymerases and has broad-spectrum anti-viral properties against several viruses, including ZIKV in mouse models (104). BCX4430 is currently in phase I trials for Ebola virus treatment (105). Cholesterol-25-hydroxylase (CH25H) is a natural oxysterol that is involved in the innate immune response. Administration of CH25H to fetal mice and macaques appear to inhibit ZIKV replication (106). Re-purposing approved and validated drugs (or using a naturally occurring small molecule like CH25H) could accelerate clinical approval for ZIKV treatment. Additional research on the safety and efficacy of these compounds in pregnancy models is urgently needed.

Conclusions

Since the untimely re-emergence of ZIKV, the scientific community has made enormous advancements. Both culture and animal models have been the mainstay of the rapid developmental pace, and have not only facilitated understanding of the basic ZIKV-host interaction, but also enabled the timely development of vaccines and therapeutics. To date, multiple vaccine candidates have already entered clinical evaluation. Because we still do not completely understand when the fetus is at the greatest risk, ethical concerns for testing vaccines and therapeutics in pregnant women in endemic regions remain. Research that utilizes pregnant NHPs provide the major impetus to enable continued development, allowing for vaccine and therapeutic deployment to areas of the greatest unmet need.

Acknowledgments

This work was supported in part by NIAID grant AI127089-02 awarded to J.B.W.

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Highlights

- Zika virus (ZIKV) has been linked to neurological defects in newborns and adults.
- The success and limitations of ZIKV model systems in ZIKV pathogenesis research are discussed.
- These model systems have proven critical to develop drugs and vaccines.