REVIEW ARTICLE

Zika Virus Overview: Transmission, Origin, Pathogenesis, Animal Model and Diagnosis

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Abstract

Zika virus (ZIKV) was first discovered in 1947 in Uganda. ZIKV did not receive substantial attention until Brazil hosted the 2016 Summer Olympic Games, and ZIKV reached a global audience. ZIKV is a flavivirus transmitted chiefly through mosquito bites, sexual intercourse and, to a lesser extent, breastfeeding. The recent discovery of how ZIKV causes congenital neurodevelopmental defects, including microcephaly, has led to reevaluation of the importance of the interaction of ZIKV with centrosome organization, because centrosomes play an important role in cell division. When ZIKV disrupts centrosome organization and mitotic abnormalities, neural progenitor differentiation is altered, thereby resulting in cell cycle arrest, increased apoptosis and inhibition of neural progenitor cell differentiation; subsequently, abnormalities in neural cell development can result in microcephaly. To aid in the understanding of the importance of ZIKV infection, this review article provides an overview of its history, transmission routes, pathogenesis, animal models and diagnosis.

Key words: Zika virus (ZIKV) centrosome, flavivirus, microcephaly

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BACKGROUND

Zika virus (ZIKV), along with yellow fever, Japanese encephalitis, West Nile virus and dengue virus, is characterized as a positive-sense single-stranded RNA virus of the family *Flaviviridae* and the genus *flavivirus*. An in-depth summary of the family *Flaviviridae*, which includes many other viruses, has been provided in a 2010 review [1]. ZIKV can be classified into distinct African and Asian lineages through phylogenetic analysis; both lines emerged from East Africa during the late 1800s or early 1900s [2]. The Asian line originated during the virus's migration from Africa to Southeast Asia, and was first detected in Malaysia [2]. ZIKV is a mosquito-borne flavivirus typically causing mild illness; its scope has rapidly expanded worldwide,

causing maternal infection and fetal viral transmission [3]. Examination of ZIKV's effects on the health of newborns is crucial for understanding how viral infections affect cellular physiology [4]. ZIKV is associated with microcephaly in newborns whose mothers were infected with ZIKV. Viral microcephaly is a congenital disability wherein the baby's head is smaller than normal according to sex and age [5]. Babies with microcephaly often have smaller brains that hinder their development with respect to that of their peers, thus resulting in a range of health problems [5].

The mechanisms through which ZIKV causes microcephaly have been investigated in detail. Most experimental results support the hypothesis that ZIKV infects neural progenitor cells (NPCs) of the fetal brain and results in microcephaly [6].

Animal models have shown that ZIKV infection causes cell cycle arrest, apoptosis of NPCs, massive neuronal death and axonal rarefaction [7–9]. The human neural organoid model has also shown that ZIKV infection increases cell death and decreases proliferation, thus decreasing neuronal cell-layer volume in a manner resembling microcephaly, although the mechanisms of the biological effects may differ [10–12]. Our studies have demonstrated that ZIKV affects centrosomes and consequently newborn development, because centrosomes play a crucial role in cell division [13]. Therefore, ZIKV causes defects in fetal brain development and microcephaly through multiple mechanisms. However, fewer than 5% of congenitally infected newborns develop microcephaly, whereas more than 95% of congenitally infected cases are normal at birth [4,14,15]. Therefore, the factors involved in ZIKV-associated microcephaly remain to be revealed. This review summarizes five aspects of ZIKV: transmission, origin, pathogenesis, animal models and diagnosis. The future of ZIKV studies is also discussed.

TRANSMISSION OF ZIKV

Mosquito biting

ZIKV is closely related to Spondweni virus, according to phylogenetic analysis, and both are mosquito-borne viruses capable of causing human disease [16]. Arboviruses generally describe hundreds of RNA viruses relying on arthropods, such as mosquitoes or ticks, to transmit infection to humans through biting [17]. Like many other flaviviruses, ZIKV is transmitted in humans through bites by infected mosquitoes, mainly *Aedes aegypti*, predominantly the *Aedes* (*Stegomyia*) genus [18–20]. *Aedes aegypti* mosquitoes usually bite during the day, and their activity peaks during the early morning and late afternoon/evening in tropical and subtropical regions [18–20]. In studies examining ZIKV transmission through infected mosquitoes, ZIKV has been isolated in the two known ZIKV-lineage regions of Africa and Asia from several different mosquito species in the *Aedes* genus (e.g., *A. aegypti*, *A. africanus*, *A. albopictus*, *A. apicoargenteus*, *A. furcifer*, *A. luteocephalus*, *A. opok* and *A. vittatus*); these species may potentially act as vectors for viral transmission in those endemic areas [21–30].

Aedes aegypti primarily transmits the four viruses that have had the greatest effects on human health: yellow fever virus, dengue fever, chikungunya and ZIKV. As shown in Fig 1, mosquitoes require 5–8 days to develop from eggs via larvae to pupae, and another 3 days to become adults [31]. If an adult mosquito bites a ZIKV-carrying primate, the mosquito is likely to acquire the virus from the animal's blood. Then ZIKV infects and replicates in the epithelial cells of the mosquito, circulates in its blood and is released in its saliva. Infectious mosquitoes transmit ZIKV to other non-human primates (NHPs) or to humans. Mosquito transmission expands the infected populations, and subsequently causes outbreaks or epidemics. During epidemics, ZIKV transmission may occur via sexual activity, and to a lesser extent breast milk feeding and vertical transmission.

Sexual transmission

The first reported case of a sexually transmitted ZIKV infection occurred in Colorado, USA, in 2008 [32]. A female patient who did not have a travel history to any ZIKV-endemic region experienced ZIKV-like symptoms [32]. Her husband was an American scientist who had contracted ZIKV infection through frequent bites from wild *Aedes* spp. mosquitoes in the evenings, while he conducted a routine mosquito-sampling project in villages in Senegal in 2008 [33]. After the infected American scientist returned home from Senegal, the couple had vaginal sexual intercourse, which provided a direct-contact transmission route for a likely sexually transmitted infection [33]. Subsequently, many more sexually acquired ZIKV cases were reported in non-endemic regions. Infected partners often returned from endemic areas and had sexual intercourse, thus increasing ZIKV cases [34]. Public health findings suggested that sexual transmission might have been responsible for the rapid spread of ZIKV, which expanded to various geographical regions and to healthy people worldwide, with unclear effects on fertility and the safety of sperm and egg banks [35]. Therefore, in 2017, the World Health Organization (WHO) constructed a plan to understand ZIKV sexual transmission and determine how to combat the ZIKV epidemic [36]. Notably, ZIKV infects the testis and is present in seminal fluid; to our knowledge, sexual transmission can occur from males to females [32]. Interestingly, sexual transmission has never been observed in any other mosquito-borne flavivirus.

Breastfeeding

ZIKV transmission from mother to child has been shown to cause harm to children during pregnancy or birth; however, less is known regarding transmission through breast milk, given the limited data and publications available [37]. According to the Centers for Disease Control and Prevention [38,39], breastfeeding is an investment in health that generally helps decrease disease risks for infants and mothers. Because of the limited data available regarding the potential risk that human milk might transmit ZIKV, the WHO made a collective decision that the benefits of breastfeeding for the infant and mother outweigh the potential risk of ZIKV transmission. This conclusion was supported by an animal study demonstrating that suckling mice can be infected with ZIKV through ingestion of ZIKV-containing human milk, but human breast milk has potential antiviral activity [40]. Even though ZIKV transmission through breast milk might be possible, the WHO still recommended that mothers with suspected or confirmed ZIKV infection continue to breastfeed their children [41,42]. Fig 1 shows the vertical transmission, sexual transmission and breastfeeding transmission of ZIKV.

ORIGIN OF ZIKV LINEAGES

African lineage of ZIKV

ZIKV was first identified in 1947 in the Zika forest in Uganda by British scientists working in the Yellow Fever

FIGURE 1 | 1. The life cycle of a mosquito. 2. ZIKV infection and transmission from NHPs to other NHPs or humans. 3. Different modes of ZIKV transmission during epidemics.

Research Laboratory, where ZIKV was isolated from a sentinel rhesus monkey [16,21]. Dick et al. [21], on April 18, 1947, placed a rhesus monkey in a cage on a tree platform in the Zika Forest and observed that the monkey developed a fever. The monkey was a species of Rhesus, and the number given to the rhesus monkey by the Rockefeller Foundation's program for research on jungle yellow fever was 766 [43]. After 2 days, Rhesus 766 still showed fever symptoms and was brought into the Foundation's laboratory in Entebbe, Uganda. Its serum was collected and injected into the brains of mice [43]. After 10 days, all mice with cerebellar exposure to the serum had become sick. Dick et al. then isolated the virus from the mouse brains and named it ZIKV; the strain was MR766 [43]. Thus, the first isolated African strain of ZIKV was named MR766 [44]. The British scientists were studying jungle yellow fever when they identified the ZIKV African lineage.

The first known isolation of ZIKV from humans was reported in 1954 [16]. A 10-year-old Nigerian girl presented at a rural dispensary with a complaint of fever and headache [16]. Because scientists were investigating an outbreak of jaundice in the Afikpo Division, Eastern Nigeria, that they suspected of being yellow fever, they assessed whether the girl had yellow fever [16]. The patient's serum was injected into adult Swiss mice, and several days later, ZIKV was isolated from the mice [16]. Two other cases of ZIKV infection were reported in Nigeria in 1954: the first was in a 30-year-old Nigerian man, and the second was in a 24-year-old Nigerian man. Both cases were confirmed to have ZIKV, on the basis of elevated serum neutralizing antibodies [16]. In summary, the results of these investigations of yellow fever contributed to the finding that ZIKV is an arbovirus, which can be transmitted by mosquitoes and infect at least monkeys and humans.

Asian lineage of ZIKV

Outside Africa, the first prototype strain of the Asian lineage of ZIKV was isolated in 1966 from mosquito (*Aedes aegypti*) pools collected in Bentong, Malaysia [29]. Sequence analysis

indicated that this Asian lineage of ZIKV was distinct from the original ZIKV; the Asian lineage of ZIKV is considered part of the African lineage [45]. In Asia, between 1954 and 1976, there were no records of human clinical ZIKV cases according to serological tests or viral isolation procedures [46]. ZIKV cases probably remained undetected because ZIKV remained below the limit for clinical detection [46]. In addition, routine checkups were not conducted in which diagnostic tests for ZIKV could be performed [46].

In 1977, 11 years later, a serological study reported that a ZIKV outbreak in Asia occurred in central Java, Indonesia [47]. These reports of ZIKV infection began to occur in the latter half of the rainy season that year, when *Aedes aegypti* usually flourishes. Seven patients were hospitalized in Tegalyoso Hospital, Klaten, in central Java; each had high fever, malaise, stomachache, dizziness and anorexia [47]. The data from these seven patients indicated that the clinical characteristics of ZIKV infection are usually mild and non-fatal [47]. ZIKV is highly likely to be transmitted by *Aedes aegypti*, which is a known possible vector in Malaysia [29].

Both the African and Asian ZIKV lineages are related and can be distinguished through detailed analysis of the genomic RNA sequence [45]. The primary difference between strains is the number of available potential glycosylation sites [17]. The distribution area of ZIKV increased to include French Polynesia (FP), given that Zika fever was an emerging infectious disease [48]. As of October 2013, in FP, a total of 8,700 suspected and 400 laboratory-confirmed cases had been reported [48]. The complete coding sequence of the virus was obtained from a 51-year-old woman in November 2013 [48]. After returning from FP, she was hospitalized in metropolitan France because she was feeling ill with fever, headache, myalgia, arthralgia and a rash [48]. Coding sequence analysis revealed that the patient had been infected with the ZIKV Asian lineage, which had approximately 99.9% nucleotide and amino acid sequence identity with the strain that spread during the 2000s in South Asia and the Pacific Islands [17]. These findings

suggested the existence of a second lineage of ZIKV: the origin of the ZIKV lineage from Uganda probably spread to Malaysia around 1945 and subsequently continued to spread and reached Micronesia around 1960, thereby forming the Asian cluster [45,49]. Therefore, two known lineages of ZIKV exist: an African lineage and an Asian lineage [50]. The African lineage is ancestral to the Asian lineage, and both share similar amino acid sequences.

The data overlaid on a world map suggest that distinct modes of ZIKV transmission among humans, animals and mosquitoes have occurred throughout the tropical and sub-tropical regions of Africa and Asia for more than 70 years. The timeline of events in ZIKV history is summarized in Fig 2.

PATHOGENESIS OF ZIKV

ZIKV infection of permissive cells

Little information is available regarding ZIKV pathogenesis, as research is ongoing; however, understanding the life cycle of ZIKV development will aid in the understanding of ZIKV pathogenesis. As described earlier in the section on ZIKV transmission through bites from infected mosquitoes, the primary mosquito vector is *Aedes aegypti*, predominantly the *Aedes (Stegomyia)* genus. *Aedes* mosquitoes lay eggs in high-moisture areas; the eggs then grow from larvae to pupae and finally become adults [Fig 1 and [51]]. The cycle is completed in approximately 1.5–3 weeks [51]. A female adult mosquito can produce a massive quantity (average of 100–200 eggs per batch) after consuming a blood meal, and produces an average of five batches of eggs in her lifetime [51]. Each female produces a total of 500–1,000 eggs in her lifetime. Adult female mosquitoes usually bite humans and

animals to obtain blood to enable egg development. As the sylvatic cycle transitions into the urban cycle, humans are affected, because *Aedes* mosquitoes must bite humans to obtain blood [51].

Aedes mosquitoes infected with ZIKV pierce through the human skin, and the infected mosquito's saliva lubricates the skin and directly contacts human blood. The surface of ZIKV has envelope proteins, which are primarily involved in attachment to host cell membranes as the virus is internalized through endocytosis [51]. As shown in Fig 3, the attachment depends on the interaction between the viral envelope protein and a receptor. AXL, Tyro3, TIM1 and DC-sign have been identified as receptors allowing ZIKV to infect permissive cells [52,53]. However, further investigation is necessary, because recent results have been disputed [54,55]. Viral entry is completed through endocytosis via endosomes. As the capsid breaks apart in the cytoplasm, viral RNA is released into the endoplasmic reticulum [51]. The positive-sense RNA genome is translated by host ribosomes attached to the endoplasmic reticulum, and structural and non-structural polypeptides are processed through proteolytic activity [51]. When all their parts are assembled correctly, virions are transported out of cells via endosomal sorting complexes, moving from the endoplasmic reticulum–Golgi intermediate compartment to the Golgi apparatus [51]. Through exocytosis, mature virions exit the cell, and a single ZIKV can rapidly yield amounts of virus that can take over the host immune defense system [51].

ZIKV and microcephaly

In September 2015, multiple reports indicated an increase in the numbers of neonatal microcephaly cases among pregnant women giving birth in northeastern Brazil [56,57].

Zika virus from 1947 to 2017

FIGURE 2 | World map showing the timeline of events of ZIKV epidemics. As Zika virus travels from one country to another, the world map is labeled with numbers, years and colors indicating how Zika virus arose and evolved over time.

FIGURE 3 | The viral life cycle in host cells.

Afterward, an increase in neonatal microcephaly cases was reported in southeast Brazil [58,59]. ZIKV has been isolated from the amniotic fluid of mothers pregnant with infants with confirmed microcephaly [56,58–60]. ZIKV has also been isolated from the brain of a fetus with congenital central nervous system (CNS) abnormalities [4]. In Brazil, a systematic study has linked ZIKV to microcephaly; the investigators followed patients in Rio de Janeiro, Brazil, and reported the clinical manifestations of acute ZIKV infection in mothers and consequent fetal effects [61]. The study enrolled pregnant women who had rash develop within the prior 5 days. The pregnant women's blood and urine samples were obtained to test for ZIKV through reverse transcriptase-polymerase chain reaction (PCR) assays. The women were prospectively enrolled and followed to collect data on the pregnancy and infant outcomes. A total of 345 women were enrolled from September 2015 through May 2016, among whom 182 women (53%) tested positive according to ZIKV PCR in the blood, urine or both. The timing of acute ZIKV infection ranged from 6 to 39 weeks of gestation. In ultrasound studies in a total of 153 women, 59 women were found to be positive for ZIKV during pregnancy. The remaining 75 women who were positive for ZIKV declined imaging analysis because either the facility was too far away or they feared knowing whether their child had fetal abnormalities associated with ZIKV infection. The women who tested negative for ZIKV infection still received regular prenatal care. Regarding infant outcomes, 49 of the 117 live-born infants (42%) were infected with

ZIKV in utero, and the infants during the first month of life showed abnormal findings primarily affecting the CNS. Microcephaly was observed in infants whose mothers were infected with ZIKV in weeks 8, 12, 30 and 38 of gestation. Although only mild clinical symptoms were observed in mothers with ZIKV infection during pregnancy, this finding should not be taken lightly, because ZIKV harms fetuses, causing fetal death, fetal growth restriction and many CNS abnormalities [61].

A review by Wen et al. lists the approaches used during 2016–2017 to investigate ZIKV-caused microcephaly and summarizes the mechanistic information on how ZIKV causes microcephaly [62]. The modeling approaches included 1) two-dimensional (2D) cell culture of NPCs [63]; 2) three-dimensional (3D) culture of NPCs; 3) mouse models and 4) NHP models. First, the 2D studies in human NPCs revealed that ZIKV infection dysregulates cellular gene expression, mitigates NPC growth, induces autophagy or apoptosis, and increases cell death [63–66]. Second, studies using 3D models indicated that ZIKV decreases neuronal-cell-layer thickness and overall volume, attenuates organoid growth, and impairs cortical growth and folding [12,64,67]. Moreover, in IFN-deficient mouse models (A129 or AG129), ZIKV has been detected in the placenta and brain, and found to be transmitted to the fetus, thus resulting in CNS neurodegeneration [68–71]. NHPs are clearly the best model for ZIKV infection and pathogenesis. ZIKV infection in Rhesus macaques causes symptoms similar to those in humans, and viral particles are detected in the

saliva, urine and cerebrospinal fluid. Many studies using the in vitro and in vivo models have demonstrated that ZIKV is directly associated with microcephaly.

Given that ZIKV can affect newborns' health before they even have a chance of living a normal healthy life, further understanding of how ZIKV infection affects cellular physiology is crucial [4]. Microcephaly is believed to be related to abnormal centrosome function [17]. Centrosomes are cellular organelles that serve as the primary microtubule-organizing centers of animal cells. Centrosomes have roles in mitosis and vesicle migration, polarization and trafficking [17]. Studies have demonstrated that in vitro ZIKV infection alters cellular functions including mitosis, and causes spindle misorientation and increased centrosome numbers [66,72–74]. Greater numbers of centrosomes correlate with microcephaly. A delay in mitosis and an increase in apoptosis can occur when the centrosome number increases [17]. Cells infected with ZIKV have disrupted centrosome organization and mitotic abnormalities, thus leading to altered neural progenitor differentiation [66,72–75]. Our recent studies have also demonstrated that ZIKV infection disrupts the structure and function of pericentromeric materials [13]. Therefore, when centrosomes and neural progenitor differentiation are affected by ZIKV, microcephaly may develop after cell cycle arrest, increased apoptosis and halted NPC cell differentiation result in abnormal neural cell development.

ZIKV and Guillain-Barré syndrome

The characteristics of ZIKV include not only microcephaly in neonates but also Guillain-Barré syndrome (GBS) in adults [76,77]. Although a link has been reported between ZIKV and GBS, more research is needed. During the ZIKV outbreak in FP, an unexpectedly high number of GBS cases was observed. In December 2013, the first patient presenting with “Zika-like syndrome” symptoms, including low fever, myalgia, rash and conjunctivitis, was diagnosed with GBS and hospitalized [78]. Patients with ZIKV-associated GBS are mostly men and exhibit neurological symptoms. During the epidemic of ZIKV in Brazil, cases of GBS increased by 20-fold. Collectively, these epidemiological data reinforce the hypothesis of a relationship between ZIKV and GBS. Ocular complications in adults have been reported [79]. The Centers for Disease Control and Prevention is actively investigating the association between ZIKV and GBS. Thus, ZIKV is known to cause congenital neurodevelopmental defects, including microcephaly in children and possibly GBS in adults.

Mechanistic studies of ZIKV pathogenesis in the CNS

To understand the mechanisms underlying how ZIKV causes microcephaly and other CNS disorders, multiple models have been applied. As described above, ZIKV-associated disorders occur primarily in congenitally infected neonates, as shown in Fig 4A. The in vitro models have included 2D and 3D cell cultures. The animal models have primarily included

mouse and NPH models. Primary human NPCs (Fig 4B), hiNSC-derived NPCs, microglial cell lines and human dermal fibroblast cells have been used to investigate the effects of ZIKV on cellular function, growth, death, apoptosis and innate defenses (Fig 4C). Moreover, 3D NPCs, such as hiPSC-derived cerebral organoids, hiPSC-derived forebrain organoids and hiPSC-derived cerebral neurospheres, have been used to study the viral effects on neural cell differentiation. ZIKV has a wide range of permissive cell types [80], thus supporting its efficient infection and replication. ZIKV infection causes increased cell death, disrupted cell cycle progression, dysregulated gene expression, attenuated human NPC growth and apoptosis [55,63–66,81–83]. ZIKV replicates in primary human neural progenitors and induces cell death, inhibition of the Akt–mTOR pathway, activation of autophagy, centrosomal depletion and mitochondrial sequestration of phospho-TBK1 [83,84]. ZIKV preferentially infects NSCs, astrocytes, oligodendrocyte precursor cells and microglia [85]. ZIKV infects NPCs in the VZ, impairs cell cycle progression, activates immune responses [86] and induces antiviral gene expression [86]. AXL mediates ZIKV entry and modulates innate immune responses [52,87]. ZIKV can infect and replicate in primary human trophoblasts [88,89], which resist ZIKV infection and release IFN λ 1 [89,90]. ZIKV infects and replicates in Hofbauer cells and induces IFN, proinflammatory cytokine and antiviral gene expression [89]. ZIKV infects cells via AXL and other factors and induces the transcription of TLR3, RIG-I and MDA5. Increased cell death, decreased proliferation, and decreased neuronal-cell-layer thickness and overall cell size have been observed in ZIKV-infected 3D culture models [10–12,64,74].

The first animal models for ZIKV were immunocompromised mice, such as A129 or AG129, which lack IFN α 1 or IFN α 2 responses (Fig 4B). Those studies have revealed how ZIKV is transmitted from mother to fetus. Although wild type (WT) adult mice are important for ZIKV study, they are resistant to ZIKV infection [7,91,92]. The mice used as models for ZIKV studies to date have deficiencies in either the IFN or the IFN receptor, induced either genetically or via antibody treatment [93]. The A129 mouse has genetically knocked out receptors for type I IFN (IFN- α and IFN- β) and hence is not susceptible to IFN- α and IFN- β stimulation, and is vulnerable to ZIKV infection [70]. The AG129 mouse lacks the IFN- α , IFN- β and IFN- γ receptors [70,94]. Another mouse strain with triple knockout of three genes (*ifn3*^{-/-}, *ifn5*^{-/-} and *ifn7*^{-/-}) produces little IFN- α and IFN- β and is also fatally susceptible to ZIKV infection [94]. The importance of IFN- α /IFN- β signaling in preventing ZIKV infection has also been confirmed with anti-IFN- α receptor antibodies. Blockade of the type I IFN response with the anti-IFN α 1 antibody in pregnant WT mice enables ZIKV to cross the placenta and infect the fetus [95]. Beyond IFN- α , IFN- β and IFN- γ receptors, elevated ZIKV replication has recently been observed in the placenta and fetus when the fetus lacks IFN- λ receptor [92]. With these mouse

FIGURE 4 | In vitro and in vivo models for investigating ZIKV-associated CNS disorders and outcomes.

models, ZIKV has been found to decrease proliferative zones, disrupt cortical layers, interfere with the differentiation of NPCs, and impair neurogenesis and folding of neural progenitor cells. Studies in other animal models have used NHPs, such as Rhesus macaques, to confirm the information obtained from mouse models. ZIKV has been detected in the brain and found to cause signs of neurologic disease in NHPs. In NHPs, ZIKV is present in the saliva, urine and cerebrospinal fluid, and causes symptoms similar to those in humans [96–98]. The effects of ZIKV infection on cells and animals are summarized in Fig 4C.

ANIMAL MODELS OF ZIKV

ZIKV has continued to spread worldwide for many years, but ZIKV pathogenesis and its mechanisms are not yet fully

understood, as research continues. Using animal models of infection and disease is necessary to provide preliminary understanding of ZIKV pathogenesis and mechanisms that could enable development a vaccine [99]. Animal models are crucial for understanding ZIKV pathogenesis and mechanisms to enable vaccine development.

Small-animal models

Immunocompetent mouse models

Animal models of ZIKV infection did not gain traction until the recent ZIKV epidemic. From the first isolation of ZIKV in 1947 until 2015, only three animal-model studies were published, in which researchers tested viral pathogenic potential [22,100,101]. This lag in research may be one reason why ZIKV pathogenesis and mechanisms are not yet fully understood. In the first publication of a mouse

model for ZIKV, the prototype MR766 strain of ZIKV was injected into the skulls of mice and found to cause neurological disease, such as motor weakness and paralysis of the limbs in suckling or adult mice [22]. However, when adult immunocompetent mice were intraperitoneally infected with ZIKV, no sign of disease was observed, thereby indicating that the infection route through the skull is essential to initiate successful infection [99]. Recently, researchers have examined ZIKV infection and disease in non-pregnant and pregnant immunocompetent mice infected with contemporary ZIKV strains [99]. After ZIKV injection, some mice notably showed no clinical disease, and very few or no signs of ZIKV were detected in WT C57BL/6, Swiss Webster, BALB/c and CD-1 mice [69,70,94,102]. During these outbreaks, researchers developed animal models of ZIKV infection and pathogenesis by using different strains of ZIKV; similar results have been reported in a 2017 review [93]. ZIKV does not naturally adapt to replicate in immunocompetent mice, because it is missing part of the species-specific immune evasion mechanism; consequently, the use of immunocompetent mice in ZIKV research has been limited. Nevertheless, immunocompetent mice still have important roles in research and have been used to evaluate vaccine immunogenicity and efficacy in protecting against viremia [93,102].

Immunocompromised mouse models

Although immunocompetent mouse models cannot support ZIKV replication and disease naturally, several groups have examined the possibility of developing ZIKV infection and disease in immunocompromised adult mice. The innate immune system plays a key role in identifying viral infections through the recognition of pathogen-associated molecular patterns (PAMPs) by innate-immune-system receptors called host pattern-recognition receptors (PRRs) [103]. When the PRRs and PAMPs interact, signaling cascades ultimately increase the production of cytokines, including type I interferon (IFN), via the transcription factors NF- κ B and interferon regulatory factors (IRFs) [104]. In animal models, various groups have altered IFN responses, which are important for antiviral defense [99]. The receptors of IFN- α and IFN- β , downstream signaling molecules and signal transducer and activator of transcription 1 (STAT-1) are crucial in protecting against viral infection [105,106]. The IRFs, particularly IRF3 and IRF7 are crucial in ensuring that the type I IFN response follows viral infection [106]. Type II IFN or IFN- γ communicates through a distinct receptor, IFNGR1, the canonical cytokine in adaptive Th1 immunity, which is required to respond to intracellular pathogens [107]. A129 mice (129S2 Ifnar1tm1Agt) and Ifnar1^{-/-} C57BL/6 mice or mice deficient in transcription factors IRF3/5/7^{-/-} lack the receptor for type I IFN and consequently are vulnerable to both the African and Asian lineages of ZIKV and have high viral doses in the brain [70,94,95,108,109]. When these animals are injected with ZIKV, they develop symptoms including hind-limb weakness, paralysis and

death. The disease severity in these immunocompromised mice depends on their age: older mice (11 weeks old) are less prone to infection than younger mice (3–5 weeks old) [70,94]. Mice lacking both the type I and type II IFN receptors (AG129, 129/Sv Ifnar1tm1Agt Ifngr1tm1Agt) show greater disease severity after ZIKV infection than their counterpart A129 mice [70,110–112]. Analysis of tissues after ZIKV infection in A129 and AG129 mice has revealed that ZIKV is present primarily in the testes and brain. The presence of ZIKV in the testes is consistent with reports of ZIKV's mode of transmission including sexual transmission. In addition, signal transducer and activator of transcription 2 (Stat2)^{-/-} mice are highly likely to be infected with ZIKV. After injection with ZIKV, Stat2^{-/-} mice show neurological symptoms, and ZIKV is found in their CNS, gonads and other visceral organs [113]. Stat2^{-/-} mice lack both type 1 and type III IFN signaling. ZIKV infection from an Asian strain in gestation in pregnant Ifnar1^{-/-} C57BL/6 mice has been found to last from days 6.5 and 7.5, during which some fetal death occurs; survivors of ZIKV infection show uterine problems such as growth restriction and growth impairment [68]. In these experiments, Ifnar^{-/-} mice were mated with WT sires, thus resulting in fetuses heterozygous for IFNAR1. Although the fetuses respond to type I IFN, some severe outcomes have been observed, thus implying that a type 1 IFN response in the fetus does not protect the fetus from ZIKV induced injury [68].

Overall, these immunocompromised mouse models have demonstrated ZIKV's ability to cause fetal abnormalities and deterioration of gonadal tissue, and to be sexually transmitted [35,68,91,93,114,115]. In addition, these mouse models have been extensively used to understand the pathogenesis and mechanisms to eventually develop a vaccine for ZIKV [93,116–120]. Although these immunocompromised mouse models are helpful for understanding ZIKV's pathogenesis and mechanism, and eventually creating a vaccine, the models have limitations that must be considered in experimental design and data analysis.

Postnatal ZIKV infection in mice

Recent *in vivo* studies in mouse models and human case studies have revealed that ZIKV can pass through the placental barrier and infect fetuses, which may die or develop microcephaly or other malformations of the brain [94,112,121]. Most mouse models have been used to study congenital infection of ZIKV; whether ZIKV infection in newborns (a postnatal infection) could cause severe disease remains unknown. The results from our group and others have shown that ZIKV not only replicates in neonatal mice (WT) but also causes their death [6,71,94,95,122–124]. Our recent studies [124] have indicated that 1) several strains of WT neonatal mice are susceptible to ZIKV infection via different infection routes, and 2) ZIKV infection causes 100% mortality of 1- to 3-day-old mice, 50–70% mortality of 5- to 7-day-old mice, and no deaths of 14-day-old or older mice. However, how ZIKV infection causes death in neonatal

mice remains unknown. A neonatal mouse model might be useful to uncover whether and how ZIKV can infect newborns and cause disease. ZIKV infection in neonatal mice has been reported by several groups [6,71,94,95,122,123]. Our unpublished data show that ZIKV infection in neonatal mice results in heart diseases accompanied by abnormal EKG and degradation of gap junction protein alpha (Cx43, also known as GJA1). The EKG data exhibit 1) extended P-R, suggesting a block of atrioventricular conductance; 2) widened QRS, suggesting an intraventricular conductance block; and 3) S-T elevation, indicating myocardial injury, all of which are characteristic of myocardial infarction [125] resulting from necrosis. We hypothesize that ZIKV infection impairs the function of the neonatal heart and leads to sudden death.

Large-animal models of ZIKV infection

Non-human-primate models

Researchers are also using NHP models for studying ZIKV, because NHPs should aid in understanding the pathogenesis and mechanism of ZIKV and eventual vaccine creation because of their genetic similarities to humans. Because monkeys have close genetic similarity to humans, some researchers are studying Rhesus macaques, which are susceptible to both the African and Asian strains of ZIKV [93,96,126–128]. When Rhesus macaques are infected with ZIKV, they develop viremia 2–6 days post-infection, and by 10 days, the viremia is undetectable. ZIKV is present in various parts of the body, such as in the organs, urine, saliva and cerebrospinal fluid in some animals [96,126]. Interestingly, several tissues of cynomolgus macaques have been found to be infected with ZIKV, including the male reproductive tract, intestines, brain and spinal cord [129,130]. Pregnant ZIKV-infected Rhesus macaques develop viremia, which lasts from 30 to 55 days [96,126,127]. Subcutaneous injection of an Asian-lineage strain of ZIKV into a pregnant pig-tail macaque has been found to decrease brain growth in her offspring [131,132]. In the fetal brain, ZIKV infection damages the CNS. ZIKV has been detected in the placenta, fetal brain and liver, and maternal brain, eyes, spleen and liver [132]. ZIKV infection prompts a T-cell response to protect NHPs against ZIKV reinfection and heterologous infection [96,129]. Therefore, NHPs are valuable in testing to develop a vaccine and to assess its efficacy before its distribution. In summary, NHP models are crucial in understanding the pathogenesis and mechanism of ZIKV and eventually developing a vaccine, because the DNA of NHPs and humans is highly similar. Although NHP models are excellent for studying ZIKV, they are expensive to maintain and require more space than other models such as small-animal models.

ZIKV DIAGNOSTICS

Finding the best diagnostic test for detecting ZIKV depends on the stage of the disease, which is divided into two phases: acute and convalescent [133]. During the acute phase, infection occurs during early stages wherein the viruses

replicates in the infected cells and causes the host to develop viremia. When the clinical manifestations are complete, an initial response to the infection is mounted, and IgM antibody production increases to fight the virus. This immune response is also part of the acute phase. The convalescent phase is the opposite of the acute phase; it begins during the late stage of infection, when IgG antibodies are more specific in their response to the developing virus. An ideal diagnostic test should have high sensitivity and specificity to correctly identify individuals positive for the virus without detecting those negative for the virus [133]. The diagnostic tests include molecular and serological assays. Molecular assay is mainly used for the detection of genetic variants, whereas serological assays can be used to detect previous circulating virus and measure patient immune response to the virus by detecting antibodies to the virus in the serum [133]. Clinical evaluation alone is not sufficient to truly understand ZIKV diagnosis, because of excessive cross-reactivity with other arboviruses. Therefore, the Centers for Disease Control and Prevention established guidelines for ZIKV diagnosis consisting of a reverse transcriptase reaction assays followed by real-time polymerase chain reaction (RT-PCR), IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) and plaque reduction neutralization testing (PRNT).

The RT-PCR assay is a reliable diagnostic method but is suitable only when the infection remains in the acute phase, because the viral RNA can still be identified in body fluids [133]. RT-PCR assay sensitivity is important to avoid false-negative results. However, limitations exist, owing to the emergence of variants of ZIKV. As many as ten nucleotide mismatches have been identified between the oligonucleotide sequences reported in published assays and the consensus sequence of the Asian-lineage ZIKV strain. In addition, as many as five mismatches have been found in individual primers or probes, thus potentially limiting the RT-PCR sensitivity, given the genetic variability of the Asian-lineage strain of ZIKV [134,135]. In summary, continual research is necessary to detect new ZIKV variants and update the primer and probe sequences to avoid confusion; such an update would improve the detection sensitivity.

MAC-ELISA, a serologic test, qualitatively detects antibodies in the serum or cerebrospinal fluid. One drawback is that MAC-ELISA results can be complex to understand, owing to possible non-specific antibody reactivity. Therefore, tests that are positive, ambiguous or inconclusive should be confirmed with PRNT—serological testing that can use a specific antibody to neutralize a virus by preventing plaque formation in a cell monolayer. PRNT is currently considered the “gold standard” in differentiating flavivirus serological diagnosis, because of its high specificity. Although this assay performs well, it is expensive, requiring highly specialized laboratories that have the proper equipment to perform cell culture and crucially have permission to work with the active virus. In addition, training in safe use and handling of this assay is necessary, because it is complex to perform;

moreover, the results usually are not available for 5–10 days [135,136].

Several commercial serological tests are available, and the most impressive are those from Euroimmun AG (Germany) and InBios (USA). The first available serological commercial test for ZIKV detection was the Euroimmun assay, which has been extensively evaluated in the literature [137–140]. The anti-ZIKV IgM/IgG/IgA ELISA is based on an ELISA using the NS1 protein of ZIKV to detect IgM, IgG and IgA in serum samples. According to Huzly et al. [137], this test has shown high specificity in testing of various samples from patients with previous exposure to flavivirus infections. To understand which tests are best, the tests must be compared. L'Huillier et al. [138] have conducted a comparative study using Euroimmun IgM and IgG ELISAs and MAC-ELISA, and subsequent PRNT to confirm whether the results were positive or inconclusive. Notably, the Euroimmun's combined IgG/IgM test had good specificity (95%), better than that of the MAC-ELISA. However, the sensitivity of this test was far lower than that of the MAC-ELISA (39.5%).

The InBios assay, also referred to as the ZIKV Detect™ 2.0 IgM Capture ELISA Kit, is for in vitro diagnostic use only. The ZIKV Detect™ 2.0 IgM Capture ELISA Kit is used for qualitative detection of ZIKV IgM antibodies in human sera. This assay was the first commercial serology kit to receive FDA Marketing Authorization in the USA, on May 23, 2019. The ZIKV Detect™ 2.0 IgM Capture ELISA Kit has recently replaced the ZIKV Detect™ IgM Capture ELISA Kit, and numerous significant changes were made from the original. According to Basile et al. [141], the average sensitivity increased from 90.4% to 92.5% with the ZIKV Detect™ 2.0 IgM Capture ELISA Kit, and the specificity increased from 79.5% to 97.4%—an important 17.9% difference. In addition, the accuracy of the updated kit was 89%, compared with 63.9%, with a significant 25.1% difference from the original kit. The agreement among laboratories increased from 79.5% to 97.4%, a significant 17.5% difference. In summary, these data have shown that the ZIKV Detect™ 2.0 IgM Capture ELISA Kit is a successful in vitro diagnostic test that should be further investigated, given its potential to decrease the number of PRNT confirmation tests. Overall, all these assays have great potential for routine diagnostic laboratories if they pass each stage of a systematic clinical evaluation.

CONCLUSIONS AND PERSPECTIVES

Although the ZIKV epidemic has put the world on notice, clear understanding of ZIKV pathogenesis, prevention and treatment has been lacking. In the near future, such understanding will be essential to elucidate ZIKV pathogenesis and enable the development of clear prevention and treatment plans. The mechanisms of how ZIKV infection causes congenital microcephaly and GBS require further investigation. Animal models will continue to be an essential resource for researchers studying ZIKV pathogenesis and progression. When ZIKV pathogenesis is understood,

creating a vaccine will be a logical next step, as with any other viruses. As they develop ZIKV vaccine, researchers may need to examine the ZIKV family and genus to build on prior successful experiences in combatting those viruses. After a ZIKV vaccine is developed, researchers must test the vaccine on the animal models to ensure that it is effective before it is approved to be distributed to the general population.

Some critical questions concerning ZIKV still remain. First, only 5% of the neonates born to ZIKV-infected mothers during pregnancy develop microcephaly. What are the non-viral factors that enable ZIKV to cause neurodegeneration? Second, although the Asian strain has been demonstrated to be more pathogenic than the African strain, both strains exhibit similar effects on cells. A larger number of strains should be applied in infection studies in both cells (2D or 3D cultures) and animals to assess the pathogenic effects of ZIKV and obtain more definitive answers. Third, although ZIKV infection is widely accepted to induce impaired growth and NPC death, which may be the major pathway underlying ZIKV-associated microcephaly, ZIKV-mediated defects in mitosis may play a role, given that we and others have consistently found that ZIKV causes degradation of pericentriolar material 1 (PCM1) or structural damage to centrosomes. These experimental results still lack support from animal studies. In addition, whether ZIKV infection is associated with functional failures of systems other than the CNS should be investigated. For example, can ZIKV infection affect heart development and cause heart diseases? Finally, the ZIKV epidemic has been over for many years, and ZIKV infections are now rarely reported. Should it be expected to recur? A long-term prospective investigation in previously infected patients should now be conducted to determine whether ZIKV has long-term effects on humans.

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COMPETING INTERESTS

The authors have no other competing interests to disclose.

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