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Animal Models of Zika Virus Infection, Pathogenesis, and Immunity

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ABSTRACT Zika virus (ZIKV) is an emerging mosquito-transmitted flavivirus that now causes epidemics affecting millions of people on multiple continents. The virus has received global attention because of some of its unusual epidemiological and clinical features, including persistent infection in the male reproductive tract and sexual transmission, an ability to cross the placenta during pregnancy and infect the developing fetus to cause congenital malformations, and its association with Guillain-Barré syndrome in adults. This past year has witnessed an intensive effort by the global scientific community to understand the biology of ZIKV and to develop pathogenesis models for the rapid testing of possible countermeasures. Here, we review the recent advances in and utility and limitations of newly developed mouse and nonhuman primate models of ZIKV infection and pathogenesis.

KEYWORDS Zika virus, animal models, flavivirus, viral pathogenesis

Zika virus (ZIKV) is a mosquito-transmitted flavivirus in the *Flaviviridae* family of positive-stranded RNA enveloped viruses. ZIKV is related to several other pathogens of public health importance, including Dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV). ZIKV was isolated in 1947 from the blood of a sentinel rhesus monkey in the Zika forest of Uganda (1). Historically, cases in humans were rare, and ZIKV infection reportedly caused a mild syndrome characterized by self-limiting fever, headache, myalgia, rash, and conjunctivitis (2). However, the magnitude of recent outbreaks, including the 2007 outbreak in Micronesia (3), the 2013-2014 outbreak in French Polynesia (4, 5), and the outbreak from 2015 to the present in the Americas (6–8), revealed that ZIKV infections cause more severe clinical consequences, including Guillain-Barré syndrome (GBS) in adults and microcephaly and congenital malformations in fetuses and newborn infants. Unlike most other flaviviruses, ZIKV has the potential for significant human-to-human transmission through sexual and vertical routes (7, 9–11). The differences in epidemiology and disease presentation during these outbreaks have prompted researchers to develop animal models of ZIKV infection and pathogenesis using contemporary virus strains (Tables 1 and 2). Despite the relatively short time interval, animal models have been established to investigate mechanisms of dissemination, pathogenesis, and host immune response to ZIKV in adults, pregnant mothers, and developing fetuses (Fig. 1). Moreover, these models already are being utilized to evaluate novel therapeutics and vaccines for possible protection and control of ZIKV infection.

ANIMAL MODELS OF ZIKV INFECTION

Mouse models. Prior to the recent epidemics, few animal models of ZIKV infection existed. The first isolated ZIKV strain (MR 766, Uganda 1947) was passaged serially in the

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TABLE 1 ZIKV Strains used in animal models

Strain (reference)	Origin	Lineage	GenBank accession no.	Use(s) in models (reference(s))	cDNA clone (reference)
Dakar 41667	<i>Aedes</i> species, Senegal, 1984	African	None reported	WT C57BL/6 mice ^a (14), <i>ifnar1</i> ^{-/-} mice (14), CD-1 mice (14)	None reported ^b
Dakar 41671 (101)	<i>Aedes taylori</i> , Senegal, 1984	African	KU955595	WT C57BL/6 mice ^a (14), <i>ifnar1</i> ^{-/-} mice (14), CD-1 mice (14)	None reported ^b
Dakar 41519 (102)	<i>Aedes africanus</i> , Senegal, 1984	African	HQ234501	WT C57BL/6 mice ^a (14), Neonatal C57BL/6 mice (14), <i>ifnar1</i> ^{-/-} mice (14), <i>ifnar1</i> ^{-/-} <i>ifngr1</i> ^{-/-} AG129 mice (14), CD-1 mice (14)	None reported ^b
Dakar 41525	<i>Aedes africanus</i> , Senegal, 1984	African	KU955591	WT C57BL/6 mice ^a (26)	None reported ^b
Dakar 41519 (80) (mouse adapted)	2×, <i>Rag1</i> ^{-/-} mice	African	None reported	WT C57BL/6 mice ^a (44, 80, 85)	None reported
FS513025 (103)	Human, Cambodia, 2010	Asian	KU955593	WT C57BL/6 mice ^a (44), <i>ifnar1</i> ^{-/-} mice (15, 104), <i>ifnar1</i> ^{-/-} <i>ifngr1</i> ^{-/-} AG129 mice (15, 75, 104), <i>lr3</i> ^{-/-} <i>lr5</i> ^{-/-} <i>lr7</i> ^{-/-} TKO mice (25), LysMcre <i>ifnar1</i> ^{flac} mice (75), CD-1 mice (15), Pregnant pigtail macaques (60), Cynomolgus macaques (90)	Yes (104)
GZ01/2016 (105)	Human, China (ex Venezuela), 2016	Asian/American	KU820898	Rhesus macaques (38), <i>ifnar1</i> ^{-/-} mice (83)	None reported
H/PF/2013 (106)	Human, French Polynesia, 2013	Asian	KJ776791	WT C57BL/6 mice ^a (14, 28, 42, 80), <i>ifnar1</i> ^{-/-} mice (14, 28), <i>ifnar1</i> ^{-/-} <i>ifngr1</i> ^{-/-} AG129 mice (27), <i>lr3</i> ^{-/-} <i>lr5</i> ^{-/-} <i>lr7</i> ^{-/-} TKO mice (14), Pregnant C57BL/6 mice ^a (61), Pregnant <i>ifnar1</i> ^{-/-} mice (61), Rhesus macaques (87)	Yes (107)
IBH30656 (102)	Human, Nigeria, 1968	Asian	HQ234500, KU963574	Cynomolgus macaques (90)	None reported
MEX_1-44 (108)	<i>Aedes aegypti</i> , Mexico, 2016	Asian/American	KX856011	Chicken embryos (94)	None reported
MP1751 (109)	<i>Aedes africanus</i> , Uganda, 1962	African	None reported	<i>ifnar1</i> ^{-/-} mice (24, 78)	None reported
MR766 ^c (1)	Rhesus macaque, Uganda, 1947	African	AY632535, HQ234498, KU720415, KU955594, KU963573	WT C57BL/6 mice ^a (14, 44), <i>ifnar1</i> ^{-/-} mice (14), <i>lr3</i> ^{-/-} <i>lr5</i> ^{-/-} <i>lr7</i> ^{-/-} mice (14), <i>ifnar1</i> ^{-/-} <i>ifngr1</i> ^{-/-} AG129 mice (30), Rhesus macaques (89)	Yes (110, 111)
P6-740	<i>Aedes aegypti</i> , Malaysia, 1966	Asian	KX377336	<i>ifnar1</i> ^{-/-} <i>ifngr1</i> ^{-/-} AG129 mice (29)	None reported
Paratiba 2015 (ZIKV ^{PRB}) (62)	Human, Brazil, 2015	Asian/American	KX280026	Pregnant SJL mice (62), Pregnant B6 mice (62), WT C57BL/6 mice ^a (28), WT BALB/c mice (13), Neonatal C57BL/6 mice (28), <i>ifnar1</i> ^{-/-} mice (28), Rhesus macaques (92)	Yes (112)
PLCaL_ZV (113)	Human, Canada (ex Thailand), 2013	Asian	KF993678	Rhesus macaques (88)	None reported
PRVABC59 (114)	Human, Puerto Rico, 2015	Asian/American	KU501215	WT BALB/c mice (13, 45), WT SJL mice (13), Neonatal C57BL/6 mice (46), <i>ifnar1</i> ^{-/-} <i>ifngr1</i> ^{-/-} AG129 mice (31), Rhesus macaques (88, 92, 93), Cynomolgus macaques (88, 90)	Yes (31)
SMGC-1	Human, China, 2016	Asian	KX266255	<i>ifnar1</i> ^{-/-} mice (43, 81)	None reported
SPH 2015 ^c (49)	Human, Brazil, 2015	Asian/American	KU321639	Neonatal Swiss mice (47)	None reported
SZ01 (115)	Human, China (ex Samoa), 2016	Asian	KU866423	Pregnant ICR mice (65), Pregnant C57 mice (66)	None reported

^aMice treated with anti-IFNAR1 monoclonal antibody.

^bAn infectious cDNA clone for Dakar 1984 strain 41662 has been generated (107).

^cExtensively passaged in mice.

TABLE 2 Animal models of ZIKV infection and disease^a

Model and strain or animal (age)	Virus inoculation route(s)	Pathology(ies) or fetal outcome(s)	Site(s) of virus detection	Reference(s)
Mouse models				
Immunocompetent adult mice				
WT BALB/c	Intravenous	None reported	Blood	13
Male WT C57BL/6	Intratesticular	Testes injury, orchitis	Testicular tissues, persistence in testes	43
Female WT C57BL/6 (diestrus phase)	Intravaginal	None reported	Vaginal tissues	74
Immunocompromised adult mice				
WT C57BL/6 + anti-IFNAR1 Ab	Subcutaneous, intraperitoneal	Uveitis, orchitis, testis injury, reduced sperm count, reduced fertility, encephalitis	Blood, eye tissues, testicular tissues, CNS, persistence in testis	14, 26, 28, 42, 77, 80, 85
<i>Ifnar1</i> ^{-/-} C57BL/6	Subcutaneous, intravenous, intraperitoneal	Weight loss, paralysis, uveitis, orchitis, testis injury, 20–100% mortality	Blood; liver; kidney; spleen; testis; CNS; tears; lacrimal gland; persistence in eyes, CNS, testis	14, 28, 43
<i>Irf3</i> ^{-/-} <i>Irf5</i> ^{-/-} <i>Irf7</i> ^{-/-} TKO C57BL/6	Subcutaneous, intravenous	Weight loss, paralysis, 100% mortality	CNS	14, 25
<i>LysMcre</i> ⁺ <i>Ifnar1</i> ^{fl/fl}	Intravaginal (diestrus phase), intravenous	Weight loss, ruffled fur	Blood, vaginal tissues, liver, spleen, brain	44, 75
A129	Subcutaneous, intraperitoneal	Weight loss, hunched posture, tremors, encephalitis, CNS injury, 0–100% mortality	Blood, ovary, testis, liver, heart, spleen, kidney, muscle, CNS	15, 24, 104
AG129	Intraperitoneal, intradermal, subcutaneous	Weight loss, hunched posture, tremors, encephalitis, CNS injury, ocular disease, 100% mortality	Blood, testis, liver, heart, spleen, kidney, muscle, CNS	15, 27-31
BALB/c + dexamethasone + dexamethasone withdrawal	Intraperitoneal	Weight loss, lethargy, orchitis, encephalitis	Blood, dissemination	45
Immunocompetent neonatal mice				
WT C57BL/6 (1–8 days)	Intraperitoneal, subcutaneous	Ataxia, tremors, seizures, encephalitis, CNS injury, 33% mortality	CNS, spleen, eye tissues	14, 28, 46
Swiss (1 day)	Intracranial, subcutaneous	Ataxia, lethargy, paralysis, gliosis, CNS injury	CNS	47
Infection during pregnancy				
WT C57BL/6 + anti-IFNAR1 Ab	Subcutaneous	IUGR	Fetal head, placenta	61, 80
<i>Ifnar1</i> ^{-/-} C57BL/6, mated with male WT C57BL/6	Subcutaneous	Resorption, IUGR, pallor, placental injury	Fetal body, placenta	61, 74, 80
WT C57BL/6	Intravaginal	IUGR	Fetal brain	74
WT C57BL/6	Intraperitoneal	Brain malformations	Fetal brain, placenta	66
WT C57BL/6	Lateral ventricle of fetal brain	Reduced cortical neural progenitors, brain malformations	Fetal brain	66
WT SJL	Intravenous	IUGR, brain malformations, ocular abnormalities	Neonate brain, neonate spleen	62
ICR	Lateral ventricle of fetal brain	Reduced brain size, brain malformations	Fetal brain	65, 82
Nonhuman primate models				
Immunocompetent adults				
Cynomolgus macaques	Subcutaneous	None reported	Blood, urine, saliva, testis, lymph nodes, CNS	88, 90
Rhesus macaques	Subcutaneous	Weight loss, fever, rash, increased liver enzymes, increased WBCs	Blood, urine, saliva, CSF, semen, vaginal fluid, lacrimal fluid, testis, prostate, intestines, spleen, parotid gland, CNS	38, 87-89, 92, 93
Infection during pregnancy				
Rhesus macaques	Subcutaneous	None reported	Persistent maternal viremia	87
Pigtail macaques	Subcutaneous	Brain malformations, white matter gliosis, white matter hypoplasia	Fetal brain, fetal liver, placenta	60
Chicken embryos	Amniotic	Reduced brain growth, brain malformations, 20–100% mortality	Eyes, crop, heart, intestines, liver, CNS	94

^aAbbreviations: CNS, central nervous system; IUGR, intrauterine growth restriction; WBCs, white blood cells; CSF, cerebrospinal fluid.

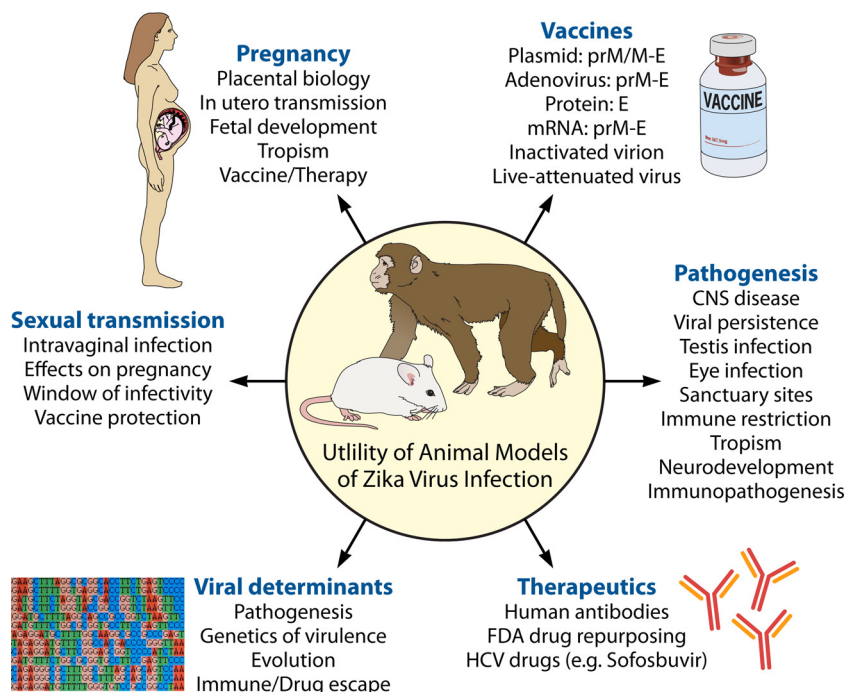


FIG 1 Use of newly developed mouse and NHP models of ZIKV infection and pathogenesis. The utility of currently described animal models of ZIKV infection is illustrated. Models are being employed for the investigation of basic features of ZIKV infection and pathogenesis and to investigate unusual features of ZIKV biology, including sexual transmission, transplacental transmission, and congenital malformations in developing fetuses. In addition, these models are being used to evaluate candidate vaccines and therapeutics for the prevention and treatment of ZIKV disease in individuals and in fetuses during infection of pregnant hosts.

brains of mice more than 100 times (1). Inoculation of ZIKV MR 766 via an intracranial route caused neurological disease in suckling or adult mice (12). In comparison, infection of adult immunocompetent inbred or outbred mice with ZIKV MR 766 via a peripheral inoculation route did not cause disease. The extensive passage history of ZIKV MR 766 has raised concern about the utility of this strain and its relationship to contemporary clinical isolates due to the likely accumulation of mutations that adapt the virus to specific cell types.

Within the last 2 years, efforts have focused on generating new mouse models with more contemporary ZIKV isolates (Tables 1 and 2). Initial peripheral inoculation studies showed no disease signs and little to no infectious virus or viral RNA in tissues of wild-type (WT) C57BL/6, BALB/c, or CD-1 mice infected with African and Asian ZIKV isolates, including strains from French Polynesia, Brazil, or Puerto Rico (13–15). Consistent with these mouse experiments, biochemical analysis showed that ZIKV antagonizes the human type I interferon (IFN) response, in part through its NS5 protein, which promotes degradation of STAT2 (16, 17), a transcription factor that mediates signaling by the type I IFN receptor, IFNAR. However, ZIKV NS5 did not promote degradation of mouse STAT2 (16), which may explain why immunocompetent strains of mice generally are resistant to ZIKV infection and disease.

(i) Pathogenesis in immunocompromised adult mice. Mice with genetic deficiencies in the type I IFN signaling pathway display enhanced susceptibility to infection by flaviviruses (18–23). Accordingly, after the failure of immunocompetent mice to sustain ZIKV infection, several groups evaluated the capacity of mice with innate immune deficiencies to support ZIKV replication and disease. Mice lacking the *Ifnar1* gene, including A129 mice and *Ifnar1*^{-/-} C57BL/6 mice, or mice deficient in *Irf3*, *Irf5*, and *Irf7* (*Irf3*^{-/-} *Irf5*^{-/-} *Irf7*^{-/-} triple knockout [TKO]) transcription factors developed severe disease, including hind limb weakness, paralysis, and death, following subcutaneous,

intraperitoneal, or intravenous inoculation of African (MR 766 or Dakar 1984), Asian (H/PF/2013), or American (Brazil Paraiba_2015) ZIKV strains (14, 15, 24, 25). Similar results were observed following intraperitoneal inoculation of ZIKV Dakar 1984 in WT C57BL/6 mice treated with a blocking anti-IFNAR1 monoclonal antibody (MAb) at time points both prior to and after virus inoculation (26).

ZIKV-infected A129 *Ifnar1*^{-/-} mice of all tested ages supported infection and developed disease (15, 24). However, lethality was age dependent, with 100% of 3-week-old, 50% of 5-week-old, and 0% of 11-week-old A129 mice succumbing to infection (15). Consistent with these data, the outcome of ZIKV infection in *Ifnar1*^{-/-} C57BL/6 mice also was age dependent, with 3-, 4-, and 6-month-old mice displaying enhanced survival rates compared with those of 5- to 6-week-old mice (14).

Mice lacking both the type I and type II IFN receptors (AG129) showed greater susceptibility and more severe disease following ZIKV infection (15, 27–31). By intradermal, subcutaneous, or intraperitoneal inoculation routes, infection with contemporary ZIKV strains from Cambodia, French Polynesia, or Puerto Rico was uniformly fatal in AG129 mice (15, 27, 31). Subcutaneous inoculation of as little as one PFU of ZIKV resulted in 100% lethality in three- to four-week-old AG129 mice (27). The severe outcomes of ZIKV infection in A129 and AG129 mice, including tremors, ataxia, and paralysis, were associated with extensive pathology in the central nervous system as well as high viral loads in the brain, spinal cord, spleen, and testes (15, 24, 27, 30, 31).

ZIKV infection in adult humans results in conjunctivitis in ~50% of symptomatic infections (32–34), with uveitis having been diagnosed in multiple patients (35, 36). ZIKV replication in eye-associated tissues has been reported in infected humans, with confirmation of viral RNA and infectious virus in conjunctival fluid (37). ZIKV RNA also was detected in lacrimal fluid of infected rhesus macaques (38). *Ifnar1*^{-/-} mice infected with French Polynesian or Brazilian ZIKV strains developed conjunctivitis and panuveitis, and these disease manifestations were associated with ZIKV RNA in the cornea, iris, optic nerve, and ganglion and bipolar cells in the retina (28). Thus, *Ifnar1*^{-/-} mice may be useful for investigating the pathogenesis of eye disease associated with ZIKV infection.

Hematospermia and prostatitis have been described in ZIKV-infected men (39–41). Recently, mouse models have investigated the consequences of ZIKV infection in the male reproductive tract (42, 43). In one of these studies, male WT C57BL/6 mice were treated with a single dose of a blocking anti-IFNAR1 MAb, followed by subcutaneous inoculation of a mouse-adapted African strain (Dakar 41519) of ZIKV (42). ZIKV infection was detected in several cells of the male reproductive tract, including spermatogonia, spermatocytes, mature sperm, and Sertoli cells, and ZIKV infection in the testis and epididymis persisted for weeks. Infection in the seminiferous tubules of the testis was associated with inflammatory cell infiltrates, cell death of Sertoli and male germ cells, reduced production of male sex hormones, diminished sperm counts and mobility, and decreased male fertility (42). Similar studies were performed by another group in male *Ifnar1*^{-/-} C57BL/6 mice inoculated intraperitoneally with a contemporary ZIKV strain isolated in China (43). Here, ZIKV infection also led to inflammation and injury in tissues of the male reproductive tract, including the testes and epididymis, and ZIKV infection was detected in spermatogonia and testicular peritubular-myoid cells. Prostate infection by ZIKV was not observed in this model. *Ifnar1*^{-/-} mice surviving acute ZIKV infection displayed severe damage in the testes out through day 60 postinfection (43). Similar outcomes, including testicular inflammation and injury, were observed following direct testicular inoculation of ZIKV of WT C57BL/6 mice; while this inoculation model was nonphysiological, it bypassed the restriction of ZIKV replication and dissemination by the innate immune response (43). Collectively, these models will allow for further investigation of ZIKV pathogenesis and persistence in the male reproductive tract.

In addition to facilitating the investigation of ZIKV pathogenesis, blockade of type I IFN signaling in WT C57BL/6 mice, by administration of a blocking anti-IFNAR1 MAb prior to infection, also has been used to map H-2^b-restricted CD8⁺ T cell receptor

epitopes across the proteomes of both African (MR766) and Asian (FSS13025) strains (44).

ZIKV infection also has been performed in BALB/c mice with acquired immune deficiencies (45). Mice were treated with the immunosuppressing steroid dexamethasone for 3 days prior to and 9 days after intraperitoneal inoculation with a Puerto Rican ZIKV strain (PRVABC59). ZIKV infection of dexamethasone-treated mice resulted in weight loss, viremia, and a disseminated infection, with viral RNA and antigen detected in many tissues, including the brain, kidney, testis, and spleen. Dexamethasone withdrawal 9 days after infection led to rapid deterioration of the mice that was associated with inflammation and injury in the brain, kidney, and testis (45). Using this model, the authors showed that administration of exogenous type I IFN could improve clinical outcome. This dexamethasone-induced immunosuppression model may have utility for investigating mechanisms of host immune response-associated damage and countermeasures for ZIKV infection.

(ii) Pathogenesis in immunocompetent neonatal mice. ZIKV infection has been studied in WT neonatal mice (14, 28, 46, 47). Neonatal mice may be useful models as key brain developmental processes in rodents occur postnatally, in contrast to the case for humans, where they occur during the third trimester of fetal development (48). Infection of 7- to 8-day-old WT C57BL/6 mice with ZIKV Dakar 41519 or ZIKV H/PF/2013 by subcutaneous or intraperitoneal injection resulted in central nervous system pathology and partial lethality (14, 28). In comparison, subcutaneous inoculation of 1-day-old WT C57BL/6 mice with ZIKV PRVABC59 resulted in nonfatal neurological disease characterized by tremors, ataxia, and seizures that developed 2 weeks later (46). These disease signs were associated with ZIKV infection in the brain, neurodegeneration in the cerebellum, and infiltration of brain tissue with CD4⁺ and CD8⁺ T cells. One-day-old neonatal outbred Swiss mice inoculated via a subcutaneous or intracranial route with ZIKV strain SPH 2015, a Brazilian clinical isolate (49), also displayed lethargy, ataxia, and paralysis with evidence of ZIKV infection in the brain (47). Thus, ZIKV infection of neonatal mice can be used to define mechanisms of pathogenesis as an alternative to studying immunocompromised adult mice. In addition, ZIKV infection of neonatal WT mice, in which a subset survive, may permit the evaluation of long-term neurodevelopmental and behavioral sequelae associated with ZIKV infection of the maturing brain.

(iii) Infection during pregnancy and fetal pathogenesis. ZIKV infection of pregnant women can lead to fetal microcephaly and other disorders, including fetal growth restriction, ocular disorders, and fetal demise (8, 11, 50–57). To date, 29 countries and territories have confirmed cases of ZIKV congenital syndrome, with the bulk of these occurring in Brazil (58). Accordingly, significant effort has been made to develop models of ZIKV pathogenesis in developing fetuses. ZIKV infection of pregnant mice and nonhuman primates (NHPs) has been reported to cause pathological changes in the placenta and brains of developing fetuses, which is consistent with many of the congenital malformations observed in humans (59, 60).

For experiments in mice, ZIKV has been inoculated into pregnant mice or directly into the brain of the developing fetus using WT mice or mice with genetic deficiencies in innate antiviral responses. Infection of pregnant *Ifnar1*^{-/-} C57BL/6 mice at embryonic day 6.5 (E6.5) and E7.5 with ZIKV strain H/PF/2013 via subcutaneous inoculation led to placental infection, fetal brain injury with accompanying neuronal cell death, and fetal demise (61). For these experiments, *Ifnar1*^{-/-} female mice were mated with WT sires, resulting in fetuses that were heterozygous for *Ifnar1*. Thus, despite the fetuses having the ability to respond to type I IFN, severe outcomes still were observed, suggesting that a type I IFN response in the fetus is not sufficient to protect from ZIKV-induced injury. In a parallel set of experiments, pregnant WT C57BL/6 mice were treated with anti-IFNAR1 MAb prior to ZIKV inoculation (61). Fetuses from these anti-IFNAR1-treated pregnant mice displayed intrauterine growth restriction and high levels of ZIKV infection in the placenta and fetal head. This study also detected ZIKV infection in trophoblasts and endothelial cells at the maternal-fetal interface (61),

suggesting that this model can be exploited to define cellular tropism and mechanisms of transplacental transmission of ZIKV to the fetus.

In contrast to data obtained after intravenous and subcutaneous inoculation of ZIKV into pregnant WT C57BL/6 mice, where no fetal defects occurred in the absence of blockade of innate immunity, intravenous inoculation of pregnant WT SJL mice with a Brazilian ZIKV strain caused intrauterine growth restriction of developing fetuses, cortical malformations, a reduction of cortical neurons in fetal brains, and fetal ocular abnormalities (62). These effects were associated with the presence of ZIKV RNA in the brains of the fetuses. Although this model required inoculation of unusually high doses of ZIKV (4×10^{10} PFU per animal) via an intravenous route (which technically is challenging to achieve), it may have utility for investigating mechanisms of ZIKV teratogenicity in more immunocompetent animals. Another limitation is that SJL mice are not fully immunocompetent, as they have elevated levels of certain T cell subsets, a higher susceptibility to experimental autoimmune encephalomyelitis and several different viral infections, and an increased incidence of Hodgkin's lymphoma (63, 64).

Other models have assessed the impact of ZIKV infection on the developing fetus in mice. In contrast to the experimental model systems described above, which result in transplacental transmission, some groups have injected ZIKV directly into the developing fetus. Direct injection of ZIKV (Asian lineage strain SZ01) into the cerebroventricular space of fetuses developing in WT ICR or C57BL/6 mice at E13.5 resulted in decreased brain size, thinning of cortical layers, reduced numbers of cortical neural progenitors, and death of immature and mature neurons within 3 to 5 days postinfection (65, 66).

(iv) Models of sexual transmission. Unlike most flaviviruses, which are transmitted virtually exclusively by mosquito vectors, sexual transmission of ZIKV has been reported and is estimated to explain about 3 to 23% of the current infections (10, 39, 40, 67–71). In most of these reported cases, transmission has occurred from infected males to female partners, which may be due to the long-term persistence of ZIKV in testis and semen (67, 72, 73), as modeled by studies in mice (42, 43). To investigate the consequences of sexual transmission of ZIKV to females and developing fetuses, three groups have reported intravaginal ZIKV transmission in mice (74–76). Following intravaginal inoculation of contemporary Cambodian (FSS10325) or Puerto Rican (PRVABC59) ZIKV strains into WT C57BL/6 mice, synchronized to the diestrus phase of the estrous cycle by injection of the hormone medroxyprogesterone acetate, replication of ZIKV was detected in vaginal washes or tissues, demonstrating that the female reproductive tract of immunocompetent mice can support ZIKV replication (74, 76). Intravaginal ZIKV inoculation of *Ifnar1*^{-/-} mice pretreated with medroxyprogesterone acetate resulted in a disseminated infection with high viral burdens in the vagina, uterus, and ovary, as well as the spleen and brain (74). Intravaginal inoculation of *Tlr7*^{-/-};*Mavs*^{-/-} or *Irf3*^{-/-};*Irf7*^{-/-} double knockout (DKO) mice also resulted in elevated viral loads in the female reproductive tract (74), indicating that these innate immune recognition and signaling pathways control ZIKV replication in these tissues. Although mice with genetic defects in type I (e.g., *Ifnar1*^{-/-} mice) or type I and II (e.g., AG129 mice) IFN signaling display markedly enhanced susceptibility to lethal ZIKV infection following subcutaneous, intraperitoneal, or intravenous inoculation routes, severe ZIKV infection following intravaginal inoculation of immunocompromised mice occurred only during the diestrus phase (75). Additionally, mice lacking expression of *Ifnar1* only in myeloid cells (*LysMcre*⁺*Ifnar1*^{fl/fl} mice) developed disseminated infection when inoculated during diestrus. Thus, through a mechanism that remains to be elucidated, type I IFN signaling in myeloid cells contributes to the control of ZIKV infection following intravaginal inoculation.

Some of these models have been used to investigate the impact of sexual transmission of ZIKV on the developing fetus. Intravaginal inoculation of pregnant WT C57BL/6 mice with ZIKV strain FSS13025 resulted in viral antigen in fetal brain that was associated with a small reduction in fetal weight (74). Intravaginal infection of pregnant

Ifnar1^{-/-} mice at E4.5 or E8.5 resulted in severe consequences for the fetus, including resorption (74). These intravaginal infection models likely will have additional utility for testing whether candidate countermeasures can prevent congenital malformations or fetal injury in the context of sexual transmission of ZIKV.

(v) Use of murine models for the evaluation of vaccines and therapeutics. The rapid development of mouse models of ZIKV pathogenesis has facilitated preclinical studies assessing the protective efficacy of candidate therapeutics and vaccines against ZIKV infection and disease. Studies designed to evaluate the utility of therapeutic murine and human MAbs for the prevention and treatment of ZIKV-induced disease have been an intense area of investigation. For example, mice deficient in type I IFN signaling by treatment with anti-IFNAR1 MAb (14) or by genetic deletion of type I, or type I and type II, IFN receptor subunits (14, 15, 24) have been used to evaluate the protective capacity of human and mouse anti-ZIKV MAbs (77–81). When administered as prophylaxis, neutralizing mouse MAbs targeting the lateral ridge of E domain III (77) or human MAbs targeting interdimer and intradimer epitopes in the E domain II (ZIKV-117 and Z20, respectively) (80) or separate epitopes in domains I, II, and III (Z3L1 and Z23) (81) all protected vulnerable mice from lethal ZIKV infection. Analogously, a cross-reactive human MAb targeting the envelope dimer epitope of dengue virus (EDE1 C10) (79) and an Fc mutant form of a ZIKV-specific human MAb (ZKA64) targeting E domain III, which cannot bind FcγRs or C1q (78), also protected mice from lethal ZIKV infection. Although MAb prophylaxis protected against ZIKV-induced morbidity and mortality, viremia, although reduced, remained detectable (77). These susceptible mouse models also have been used to demonstrate that human MAbs can protect from lethal ZIKV infection when administered as a postexposure therapeutic (78, 80, 81). The Fc mutant form of human MAb ZKA64 or WT forms of the neutralizing human MAbs Z23 and Z3L1 (81) protected *Ifnar1*^{-/-} mice against lethality when administered 1 day after ZIKV inoculation (78, 81), and a single dose of human MAb ZIKV-117 protected WT C57BL/6 mice treated with anti-IFNAR1 MAb from lethal mouse-adapted ZIKV-Dakar infection when administered as late as 5 days after virus inoculation (80).

Antibody-based therapies also have been evaluated for their capacity to protect against ZIKV-induced teratogenicity. Treatment of *Ifnar1*^{-/-} pregnant dams mated to WT sires with human MAb ZIKV-117 1 day prior to subcutaneous inoculation of ZIKV-Brazil (Paraiba 2015) reduced maternal infection and fetal mortality (80). In addition, therapeutic administration (at day +1) of ZIKV-117 to WT pregnant dams treated with anti-IFNAR1 MAb reduced ZIKV infection levels in the fetal placenta and brain, and these effects were associated with transport of ZIKV-117 across the maternal-fetal placental barrier (80). Consistent with a possible role for antibody-based therapies for the treatment or prevention of ZIKV fetal disease, human convalescent-phase serum injected on day 1 and 2 after direct inoculation of ZIKV into the fetal brains of pregnant ICR mice reduced the number of ZIKV-infected cells and the thinning of the cortical plate and ventricular zone/subventricular zone in the fetal brain (82).

In addition to antibody-based therapeutics, immunocompromised mice are being used to evaluate small molecules for antiviral efficacy. Three studies have used susceptible AG129 or A129 mice to demonstrate that compounds targeting the viral RNA-dependent RNA polymerase (RdRp) can reduce the viral burden in tissues and reduce or delay virus-induced morbidity and mortality following inoculation of ZIKV (29, 30, 83). In addition, treatment for 1 week with sofosbuvir, an RdRp inhibitor approved by the Food and Drug Administration for the treatment of hepatitis C virus infection (84), beginning 1 day after infection with a mouse-adapted strain of ZIKV-Dakar 41519 improved the survival rate of C57BL/6 mice treated with an anti-IFNAR1 MAb (85).

Although efforts are under way to develop a ZIKV vaccine (reviewed in reference 86), limited studies have evaluated ZIKV vaccine candidates in mice, perhaps due to the general resistance of immunocompetent strains to ZIKV infection and disease. Nevertheless, immunocompetent mice have been used to assess the immunogenicity of

vaccine candidates as well as their protective efficacy against viremia. For example, intramuscular immunization of BALB/c and SJL mice with plasmid DNA encoding the ZIKV M and E proteins prevented viremia following intravenous inoculation of Brazilian (Paraíba 2015) or Puerto Rican (PRVABC59) ZIKV strains (13). In analogous studies, intramuscular or subcutaneous immunization of BALB/c mice with a purified, inactivated ZIKV vaccine also prevented viremia. In both cases, protection was mediated by ZIKV-specific antibodies, as efficacy correlated with the levels of ZIKV E protein-specific antibody, and passive transfer of purified IgG from vaccinated animals prevented viremia following an intravenous challenge (13).

Collectively, these studies illustrate that mouse models of ZIKV pathogenesis in immunocompromised and immunocompetent neonatal, adult, and pregnant mice can be utilized to evaluate rapidly and in a cost-effective manner candidate therapies and vaccines for efficacy against ZIKV replication and control of spread, persistence, lethality, and teratogenicity.

NHP models. (i) Pathogenesis in immunocompetent adult macaques. Nonhuman primates (NHPs) also are being used to evaluate aspects of ZIKV biology and pathogenesis (38, 87–90). Several groups have characterized ZIKV infection in pregnant and nonpregnant rhesus, cynomolgus, and pigtail macaques. In each study, either the African ZIKV strain MR 766 or more contemporary ZIKV strains were administered subcutaneously at doses comparable to those inoculated by infected mosquitoes, and a breadth of clinical, virological, and immunological parameters were assessed. Inoculation of rhesus macaques with an Asian lineage ZIKV strain (H/FP/2013) resulted in mild weight loss, development of a mild rash around the injection site, and elevated serum creatine kinase and alanine aminotransferase in some animals (87). Although weight loss and rash were not observed across all studies, elevated liver enzymes at early times postinfection were a consistent feature of ZIKV infection of rhesus macaques (38, 87, 88). In some experiments, ZIKV infection also resulted in elevated body temperature for up to 10 days postinfection (38, 88). ZIKV-infected rhesus macaques developed viremia that peaked at 2 to 6 days after infection and typically became undetectable by day 10 (38, 87–89). ZIKV RNA was detected in the urine, saliva, and cerebrospinal fluid of some animals, suggesting that dissemination can occur. ZIKV RNA also was detected in the seminal fluid and vaginal secretions, albeit more sporadically (87, 88). Using multiple approaches, including *in situ* hybridization for the ZIKV genome, immunohistochemistry with a cross-reactive flavivirus-specific MAb, and quantitative reverse transcription-PCR (RT-PCR) analysis for viral RNA in tissues, ZIKV infection was detected in several tissues of rhesus and cynomolgus macaques, including secondary lymphoid organs, the male reproductive tract, the intestines, and the brain and spinal cord (38, 88, 90). These studies support the use of rhesus and cynomolgus macaques as models for improving our understanding of the cellular and tissue tropism of ZIKV infection. Infected rhesus macaques also developed ZIKV-specific humoral and cell-mediated immune responses (38, 87–89) that protected against challenge with homologous and heterologous viruses (87–89), indicating that this model will be useful for the evaluation of ZIKV adaptive immunity.

(ii) Infection during pregnancy and fetal pathogenesis. ZIKV infection studies in pregnant NHPs are deemed particularly important because the placental barrier and gestational development more closely resembles those of humans relative to mice, where there are differences in morphological, spatial, and temporal placentation and *in utero* brain development (59). Pregnant rhesus macaques infected with ZIKV strain H/PF/2013 developed viremia lasting 30 to 55 days (87). As persistent viremia has been reported in pregnant women (91), these observations suggested that rhesus macaques mimic at least some features of ZIKV infection of pregnant humans. A successful NHP model of *in utero* transmission was established after subcutaneous inoculation of a pregnant pigtail macaque with a Cambodian ZIKV strain (FSS13025) at a time point corresponding to ~28 weeks of human pregnancy (60). Infection resulted in reduced growth of the fetal brain, white matter deficiency and gliosis, and axonal damage. ZIKV

RNA was detected in the chorionic villous tissue of the placenta as well as the fetal brain and liver, suggesting transplacental transmission followed by ZIKV invasion and injury to the fetal brain. Although clinically apparent infection of the pigtail macaque dam was not observed, ZIKV RNA was detected in the maternal brain, eyes, spleen, and liver (60). This study suggests that pregnant pigtail macaques can serve as a primate model to investigate ZIKV pathogenesis in the developing fetus and possibly that asymptomatic infected mothers can still transmit ZIKV to fetuses *in utero*.

(iii) Use of NHP models for the evaluation of vaccines and therapeutics. Rhesus macaques have been used to evaluate the immunogenicity and protective efficacy of active ZIKV immunization, including inactivated virus, DNA plasmid-based, and vector-based vaccines, as well as the protective efficacy of passive immunization against ZIKV challenge (92, 93). In these studies, the primary endpoints of protective efficacy were virological in nature. Rhesus macaques were immunized twice (weeks 0 and 4) with a formalin-inactivated ZIKV strain PRVABC59 adjuvanted with alum (ZPIV), a DNA plasmid vaccine encoding the M and E proteins from ZIKV strain BeH815744, or a rhesus adenovirus M-E vectored vaccine (92). Each of these vaccines generated ZIKV-specific neutralizing antibody responses and completely protected against subcutaneous challenge with 10^3 PFU of Brazilian (Paraiba 2015) or Puerto Rican (PRVABC59) ZIKV strains administered 4 weeks after the final immunization (92). Furthermore, passive transfer of purified IgG from ZPIV-vaccinated rhesus macaques to naive WT BALB/c mice or rhesus macaques conferred dose-dependent protection against viremia following intravenous (mice) or subcutaneous (macaques) inoculation of ZIKV Paraiba 2015 (92). In analogous studies, rhesus macaques also were used to evaluate the immunogenicity and protective capacity of DNA plasmid vaccines carrying full-length prM-E genes derived from ZIKV strain H/PF/2013 with the prM signal sequence replaced with one from the related Japanese encephalitis virus (JEV) to improve expression of subviral particles (93). In these experiments, rhesus macaques immunized intramuscularly with two doses (weeks 0 and 4) developed ZIKV-specific neutralizing antibody responses, and 17/18 animals were protected completely against viremia following a subcutaneous challenge with ZIKV strain PRVABC59. These studies highlight how NHP models of ZIKV infection and immunity are being utilized to evaluate ZIKV vaccine candidates, several of which already are in phase I clinical trials in humans.

Other models. While efforts have focused on the development and characterization of models of ZIKV pathogenesis in mice and NHPs, less work has been performed with other model animals. One group evaluated the utility of chicken embryos (94). In these experiments, different doses of ZIKV strain MEX1-44 were injected through the chicken amniotic membrane on day E2.5 or E5. Injected embryos became infected with ZIKV, and dose-dependent mortality was observed by day 3 after infection. Inoculation of embryos at a later stage of development, E13, resulted in active ZIKV infection but no mortality. Magnetic resonance imaging (MRI) of ZIKV-infected chicken embryos at E15 and E20 revealed brain malformations, reduced brain growth, and increased ventricular volumes, particularly in the cerebral cortex regions. Thus, experimental infection of chicken embryos may provide a complementary system to investigate mechanisms of ZIKV pathogenesis in developing embryos and test possible interventions.

LIMITATIONS OF ANIMAL MODELS AND FUTURE DIRECTIONS

Using contemporary ZIKV strains, a variety of animal models that mimic aspects of ZIKV infection in humans have been developed in mice, NHPs, and other species. Their development has led to new knowledge regarding the biology and pathogenesis of ZIKV. However, each of these systems has limitations that must be considered in the design and interpretation of experimental findings.

Limitations of mouse models. Limitations of mouse models include the following. (i) The mouse placenta is structurally and immunologically distinct from the human placenta, and efficient transmission may require higher maternal viremia (95), which is achieved experimentally by a deficiency of type I IFN signaling or the use of exceptionally high inoculating doses. (ii) As ZIKV is not naturally adapted to replicate in

immunocompetent mice, likely due to an absence of species-specific immune evasion mechanisms, most pathogenesis models in adult animals have required the use of some type of acquired or genetic immunodeficiency, which affects the relevance of the findings to humans. (iii) Some disease manifestations associated with ZIKV infection in humans have not been observed in mice. For example, GBS is an autoimmune disorder characterized by weakness, sensory abnormalities, and autonomic dysfunction due to damage to peripheral nerves. Although ZIKV infection of humans has been linked to GBS (5, 96, 97), no animal model (mice or NHPs) to study this aspect of ZIKV pathogenesis has yet been described. (iv) In contrast to many other mammalian species, mice lack expression of the neonatal Fc receptor (FcRn) on their trophoblasts in the chorioallantoic placenta (98). Instead, FcRn is expressed in the mouse yolk sac endoderm (98), and the transfer of IgG in mice occurs predominantly at the suckling stage (99). As reduced levels of transport of maternal or exogenous IgG into the fetus occur in mice, protection by a given antibody or vaccine may be underestimated.

Limitations of NHP models. Limitations of NHPs include the following. (i) The cost is high, and throughput is low. Experiments with individual NHPs under animal biosafety level 2 (A-BSL2) or A-BSL3 (in European countries) conditions cost greater than \$15,000 per animal, including purchase, housing, infection, bodily fluid sampling, and tissue analysis. Studies with pregnant NHPs are even more expensive (>\$25,000 per animal). Beyond the availability issue, the cost limits the number of animals that can be infected and observed at a given site and thus impacts the statistical power of the study and the ability to resolve differences in a given experimental parameter. (ii) Although the placenta and fetal development of NHPs more closely resemble those of humans than those of mice, accordingly, the gestational period is much longer (e.g., 164 and 183 days for rhesus and pigtail macaques, respectively), which lengthens the time of experiments and subsequent analysis. (iii) There are only a limited number of NHP colonies that have the expertise and size to perform experiments with enough animals to show vaccine or antiviral protection.

CONCLUSIONS

An intensive effort during the past year by the global scientific community has resulted in the rapid development, characterization, and deployment of animal models for the study of multiple aspects of ZIKV biology. Although great progress already has been made, it is anticipated that the use and refinement of these animal models will lead to greater knowledge of the many remaining questions, including (i) the mechanisms of ZIKV persistence in the male reproductive tract, eyes, and other sites, (ii) the molecular basis for cellular tropism *in vivo*, (iii) the viral and host factors that facilitate or restrict transplacental transmission, invasion of the fetal brain, and fetal developmental malformations during ZIKV infection of pregnant hosts, (iv) the relationship between the timing of ZIKV infection of pregnant hosts and effects on the infection and injury to the developing fetus, (v) the immune mechanisms that mediate ZIKV clearance and correlates of protection, (vi) possible roles for immunopathogenesis in disease severity, and (vii) the roles of strain variation and sequence polymorphisms in ZIKV pathogenesis. The continued development and characterization of animal models, including models that may reflect better the influence of host genetic variation (e.g., Collaborative Cross mice) (100), immune status, and comorbidities on the diverse clinical manifestations of ZIKV infection, remain an important priority.

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