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Overview

Animal Models of Zika Virus

Michael P Bradley[1,*] and Claude M Nagamine[2]

Zika virus has garnered great attention over the last several years, as outbreaks of the disease have emerged throughout the Western Hemisphere. Until quite recently Zika virus was considered a fairly benign virus, with limited clinical severity in both people and animals. The size and scope of the outbreak in the Western Hemisphere has allowed for the identification of severe clinical disease that is associated with Zika virus infection, most notably microcephaly among newborns, and an association with Guillian–Barré syndrome in adults. This recent association with severe clinical disease, of which further analysis strongly suggested causation by Zika virus, has resulted in a massive increase in the amount of both basic and applied research of this virus. Both small and large animal models are being used to uncover the pathogenesis of this emerging disease and to develop vaccine and therapeutic strategies. Here we review the animal-model–based Zika virus research that has been performed to date.

Abbreviations: dpi, days postinfection; E, embryonic day

The history of Zika virus dates back to 1947, when the virus was initially discovered during experiments aimed at identifying the arboreal vector of yellow fever virus in the Zika forest of Uganda.[18] One sentinel rhesus macaque (*Macaca mulatta*) developed a slight fever (peaking at 40 °C) of 4 d duration. No other signs of illness were seen. Serum taken during the 3rd day of the fever was inoculated intracerebrally and intraperitoneally into 5- to 6-wk-old Swiss mice and subcutaneously into a second rhesus. Only intracerebrally inoculated mice became sick, and the first Zika virus strain (MR766) was subsequently isolated from brain suspensions of the sick mice.[16,17] This virus caused minimal to no symptoms when introduced into macaques, although they developed neutralizing antibodies against Zika virus.[16] A second Zika virus strain (E/1) was subsequently isolated from *Aedes africanus* mosquitoes trapped in this forest, suggesting that this insect may be the arthropod vector.

Zika virus is a single-stranded positive-sense RNA virus in the genus *Flavivirus,* which includes a variety of arthropod-vectored viruses such as dengue, yellow fever, Japanese encephalitis, West Nile, and tick-borne encephalitis.[31] Throughout the 1950s and 1960s, serologic data from humans suggested widespread distribution of Zika virus throughout Africa and Southern Asia.[92] Zika virus was subsequently isolated from *A. aegypti* mosquitoes, in Malaysia, the first isolation of this virus outside of Africa.[50] Despite the widespread seroprevalence, only 13 documented cases of natural Zika virus infection existed prior to 2007,[92] although the current thought is that this number grossly underrepresents the actual number of Zika virus infections, given the absence of Zika-virus–specific clinical signs and diagnostics, and the confounding effect of antigenic crossreactivity among flaviviruses.[31] In 2007, an outbreak of Zika virus occurred in the Yap Islands in the Federated States of Micronesia, and subsequent outbreaks occurred in several South Pacific islands including French Polynesia (2013), Cook Islands (2014), New Caledonia (2014), and Easter Island (2014), which led into the current outbreak in the Americas.[12,35,92] This outbreak began in coastal Brazil (2014) and has spread rapidly throughout South and Central America and the islands of the Caribbean, primarily due to 2 factors: the prevalence of *A. aegypti* mosquitoes and an immunologically naïve, dense, urban population.[12] Sequence analysis of Zika virus has revealed 2 lineages, African and Asian, and analysis has shown that the recent outbreaks in the Pacific and the Americas are of the Asian lineage.[31] Comparison of isolates from Brazil and French Polynesia show 87% to 90% sequence similarity to the original MR766 strain from Uganda,[13,24,25] and the severity of clinical and research outcomes due to Zika virus infection may vary between these lineages.

The recent outbreaks have greatly increased our understanding of the clinical signs associated with Zika virus. An estimated 75% to 80% of Zika virus infections are asymptomatic.[35,40] When clinical signs are noted, the infection is generally associated with mild symptoms such as fever, rash, arthralgia, headache, conjunctivitis, and lethargy. Recently, Zika virus has been associated with increases in Guillian–Barré syndrome and microcephaly, as well as case reports of other manifestations of CNS and ocular disease.[9,56,92] There is some concern that microcephaly is the extreme manifestation of infection during pregnancy and that milder neurologic impairments might emerge as the affected infant population ages.[62] Furthering the concern is that Zika virus can be transmitted not only by mosquitoes and vertically but also through sexual transmission,[27,60] and blood transfusions;[59] in addition, there is a single case report of Zika virus transmission through a monkey bite,[41] although mosquito transmission could not be ruled out.

As a result of the widespread outbreak of Zika virus associated disease, the World Health Organization declared Zika virus a

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[1]Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, Michigan, and [2]Department of Comparative Medicine, Stanford University School of Medicine, Stanford, California
**Corresponding author. Email: mpbradle@med.umich.edu*

Public Health Emergency of International Concern on 1 February 2016. In addition, several branches of the US Department of Health and Human Services have published a notice to prioritize Zika virus research.[61] The virus's presumed low pathogenicity has contributed to a paucity of basic research on Zika virus. During the initial discovery of Zika virus, animal studies in NHP (*Cercopithecus aethiops centralis*, *M. mulatta*, and *C. ascanius schmidti*), mice and other rodents (for example, cotton rats [*Sigmodon hispidus*]), guinea pigs (*Cavia porcellus*), and rabbits (*Oryctolagus cuniculus*) produced equivocal results, with some animals manifesting clinical signs ranging from mild fever and rash to death.[16] Initial studies of the virus showed that Zika virus is neurotropic, although these studies were confounded in many cases by the use of intracerebral inoculations.[6,16] Much more extensive research has been performed on similar flaviviruses and tropical diseases, for which reviews of animal models exist,[8,11,36,99] and knowledge gained from those studies has been and will continue to serve as a starting point for Zika virus research. Future research should highlight the underlying pathogenesis of Zika virus in both mature and embryonic infections, target potential therapeutics and vaccines, as well as uncover the lineage-associated differences in Zika virus that may have led to the recent outbreaks. Here we summarize the animal models that have been used to study Zika virus during this initial wave of increased research focus.

Small Animal Models

The development of small animal models of Zika virus infection has been and will continue to be an important step in understanding the pathophysiology of this virus. Mouse models, in particular, allow for large sample sizes, the use of spontaneous and induced mutations on defined genetic backgrounds, and the ability to perform detailed and broad prospective research studies. These advantages support in-depth analyses of the immunologic and pathologic characteristics of Zika virus infection as well as quick screening of potential therapeutics and vaccines.

Models of Zika virus infection in adults. After the discovery of Zika virus, initial studies in mice required many serial passages of the virus in mice to produce consistent phenotypes.[16] With the advancement of genetic engineering, mouse models can now be developed more readily, and several mouse models of Zika virus infection have been described recently. Several of these models have altered interferon responses, which are an important component of antiviral defense.[58] These mouse models include single and double knockouts of the receptors for the type I (*Ifnar1*) and type II (*Ifngr1*) interferons on either the 129/Sv or C57BL/6 genetic background.[34,58,87] These inbred strains have been designated A129 and AG129 for the single and double knockouts, respectively, on the 129 genetic background and IFNAR1, for the single knockout on the B6 genetic background.

Mice that lack receptors for type I interferon, IFNα/β—including the inbred strain A129 (129S2 *Ifnar1*^{tm1Agt})[34]—have been previously described as models of disease due to Dengue virus[64,68,94,78] and other viruses.[42,53,72] In a study where 5- to 6-wk-old A129 mice were inoculated with an African lineage of Zika virus (MP1751, *Aedes africanus* isolate) subcutaneously in the leg, high viral loads and criteria for euthanasia were reached within 6 d postinfection (dpi), with pathologic lesions noted primarily in the CNS and spleen.[20] Compared with the A129 mice, 129Sv/Ev normal controls displayed decreased viral loads, with no clinical phenotype observed.[20] This lethal phenotype in A129 mice is similar to that observed in studies with other arboviruses.[5,7,19,67,75] Another study investigated the A129 strain in response to a Zika virus of Asian lineage (FSS13025, Cambodia) after inoculation at 3, 5, and 11 wk of age.[71] All groups displayed signs of illness, such as hunched posture and ruffled fur, but the most severe disease and lethality were observed only in the 3-wk-old group.[71] In contrast, CD1 and C57BL/6J normal controls and sham-infected controls displayed no appreciable phenotype.[71] The investigators of this study[71] also examined the effect of Zika virus on AG129 mice (129/Sv *Ifnar1*^{tm1Agt} *Ifngr1*^{tm1Agt}), which lack both type I (IFNα/β) and type II (IFNγ) interferon receptors.[87] This model was previously used to investigate Dengue[77] and yellow fever[84] viruses, as well as many others. In the study, 3-wk-old AG129 mice infected with Zika virus through either the intraperitoneal or intradermal route displayed neurologic signs including tremors and ataxia, with subsequent humane endpoints reached at 6 dpi.[71] Analysis of the tissues from both strains demonstrated that the highest viral loads were in testes and brain. The presence of virus in the testes is consistent with the reports in human literature of sexual transmission of Zika virus.[27,60] Comparing A129 and AG129 revealed that AG129 mice were more susceptible to Zika virus, demonstrating neurologic signs and hindlimb paralysis (Figure 1), although both models still resulted in a lethal phenotype.[71,76] In a related study, both 3- to 4-wk-old and 8-wk-old AG129 mice were inoculated with various doses of Zika virus (H/PF/2013, French Polynesia, Asian lineage), ranging from 10^5 to 1 pfu.[3] All of the mice, regardless of dose, became moribund and were euthanized. The mice exhibited signs of illness by 5 dpi, typified by weight loss, lethargy, and a hunched posture. Histopathologic lesions were most severe in the muscles and brain.[3] Interestingly, no neurologic signs or paralysis were reported in this study,[3] in contrast to an earlier study.[71] This difference may be attributable to the different Asian lineage viral strains used, Cambodian compared with French Polynesian.

AG129 mice were used in another study, where they were infected with Zika virus (MR766, African lineage) at 8 to 14 wk of age and displayed a dose-dependent onset of neurologic signs, including paralysis, and subsequent lethality.[98] The mice experienced worsening clinical signs, which started at 10 dpi,[98] a later time point than reported for the same mouse strain in other reports.[3,71] However the mice were older than those in other studies and were infected with a lower dose of an African lineage Zika virus. The study[98] also evaluated the use of an inhibitor of viral RNA-dependent RNA polymerase, 7DMA, in AG129 mice; the drug delayed the course of disease compared with that in controls (23 dpi compared with 14 dpi). Finally, compared with AG129 mice, infection of 8- to 9-week-old SCID mice produced delayed clinical signs and lethality, averaging 40 dpi for SCID mice.[98]

Another Zika virus investigation[39] examined several strains of mice that have alterations in both the type I and type II interferon responses. AG129, *Ifnar1*$^{-/-}$, *Irf3*$^{-/-}$*Irf5*$^{-/-}$*Irf7*$^{-/-}$ interferon regulatory factor triple-knockout mice, and *Irf3*$^{-/-}$ and *Irf5*$^{-/-}$ single-knockout mice (the latter 4 strains on the C57BL/6 genetic background) yielded the most notable findings.[39] These mice were inoculated at 4 to 6 wk of age with Zika virus (both Asian and African lineages). The AG129, *Ifnar1*$^{-/-}$, and triple-knockout mice displayed neurologic signs such as hindlimb weakness and paralysis when injected with Zika virus of an Asian lineage (H/PF/2013), and all mice eventually succumbed to illness. This finding is in agreement with another report, in which 5- to 6-wk-old

Figure 1. Uninfected and Zika-virus–infected AG129 mice. An example of bilateral hindlimb paralysis in AG129 provided by one of the authors (CMN). The affected mouse was inoculated retroorbitally 7 d previously with 1×10^4 pfu of the MR766 Zika virus strain.

triple-knockout mice displayed hindlimb weakness at 6 dpi after retroorbital injection of Asian lineage Zika virus (FSS13025).[44] Interestingly, *Ifnar1*$^{-/-}$ mice inoculated with the original African lineage isolate of Zika virus (MR766) showed marked reduction in clinical signs and lethality in comparison.[39] B6, CD1, and *Irf5*$^{-/-}$ single-knockout mice demonstrated no clinical signs when inoculated at similar ages, but when 1-wk-old B6 mice were infected, 33% succumbed to infection within 24 h, thus suggesting that Zika virus pathology is highly age-dependent.[39] To that effect, *Ifnar1*$^{-/-}$ mice were inoculated with Zika virus (H/PF/2013) at 3, 4, and 6 mo and demonstrated a reduction in lethality at these ages compared with the earlier inoculation time points.[39] Furthermore, B6 WT mice inoculated with Zika virus concurrent with the administration of an IFNAR1-blocking antibody displayed no clinical signs but established an elevated viremia.[39] These experiments with older mice and with blocking antibodies might serve as animal models for vaccine efficacy studies. Analysis of Zika virus RNA in a previously mentioned study[71] showed high viral loads in a variety of tissues, including CNS and testes, consistent with other recent reports. Additional experiments with the following strains reported no adverse outcomes: *Mavs*$^{-/-}$, *Irf3*$^{-/-}$, *Irf5*$^{-/-}$, *Ifnlr1*$^{-/-}$, *Ifit1*$^{-/-}$, *Ifit2*$^{-/-}$, *Ifitm3*$^{-/-}$, *Isg15*$^{-/-}$, *Ube1l*$^{-/-}$, *Mb21d*$^{-/-}$, and *Tmem173*$^{-/-}$(STING).[39] A related study investigated 7-wk-old C57BL/6 mice that were treated with an IFNAR1-blocking antibody and were inoculated with both Asian (H/PF/2013) and African (Senegal 1984) lineages into the footpad.[30] Mice displayed Zika virus RNA within testis, epididymis, and related fluids; alteration of the testicular architectural structure; and decreased testicular size, with more severe lesions noted with the African lineage.[30] The experiment was repeated in *Rag1*$^{-/-}$ mice, and similar lesions were noted, however the damage to Leydig cells was decreased compared with those in C57BL/6 mice, implying that both direct viral infection and adaptive immune responses cause testicular damage after Zika virus infection.[30] When *Ifnar1*$^{-/-}$ and C57BL/6 mice were inoculated intraperitoneally with Asian lineage Zika virus (SMGC1),[89] *Ifnar1*$^{-/-}$ mice displayed lethality (20%), and surviving animals had substantial epididymitis and orchitis but no lesions in the prostate or seminal vesicles.[48] These models may serve in the future to investigate the effects of Zika virus on the male reproductive tract.

In addition, several recent studies have clarified the role of the female reproductive tract in Zika virus pathogenesis. One study using an Asian lineage (FSS13025) inoculated intravaginally showed that AG129 and LysMCre^{+}IFNAR$^{fl/fl}$ (which lack IFNAR in myeloid cells) mice experienced worsening clinical signs and weight loss, with AG129 mice having a poorer outcome.[83] Interestingly, this phenotype was observed only when the mice were inoculated during induced diestrus and not during induced estrus, demonstrating the effect of the estrous cycle on the virus's pathogenic potential.[44] Zika virus was detected until 10 dpi in vaginal washes of AG129 in induced diestrus, indicating that the virus may replicate within the vaginal mucosa.[44] Corroborating this study is another that used the same Zika virus strain (FSS13025) in C57BL/6 mice at 8 to 10 wk of age, demonstrating that viral RNA persisted in the vagina until 4 dpi after intravaginal inoculation of these mice.[95] These results were compared with those from several knockout strains, which showed that *Rag2*$^{-/-}$ mice were no different than WT in regard to vaginal viral RNA load but that *Ifnar1*$^{-/-}$ and *Irf3*$^{-/-}$*Irf7*$^{-/-}$ mice had increased viral RNA levels intravaginally.[95] High-dose (5.2×10^5 pfu) intravaginal inoculation of *Ifnar1*$^{-/-}$ mice resulted in lethality by 9 dpi,[95] consistent with other studies[3,20,39,48,55,71,76,98] that have identified the importance of the interferon pathway in Zika virus pathogenesis. In addition, compared with intraperitoneal administration, intravaginal inoculation of C57BL/6 mice achieved higher Zika virus titers in the spleen.[95]

In another report on *Ifnar1*$^{-/-}$ mice, both WT and *Ifnar1*$^{-/-}$ 4-wk-old mice infected with Asian lineage Zika virus (Paraiba 2015, Brazil) shed viral RNA within tears at 7 dpi.[55] Further analysis at 28 dpi showed that viral RNA persisted in several tissues, including eyes, brain, and spleen, long after Zika virus RNA was undetectable in serum. To determine the infectious capability of Zika virus in tears, ocular secretions and eye tissue homogenates were injected intraperitoneally into AG129 mice. Ocular homogenates harvested at 7 dpi uniformly resulted in lethality in AG129 mice by 10 dpi, but AG129 mice infected with tears collected on 7 dpi or ocular homogenates prepared on 28 dpi did not display clinical signs of Zika virus infection.[55] Despite the lack of clinical evidence of Zika virus infection, intraperitoneal injection of tears from infected mice resulted in serum titers similar to those obtained after direct inoculation of Zika virus,[55] thus providing evidence that tears may serve as a reservoir and source of infection in Zika-infected mammals. Similar concerns were recently brought to light during the Ebola virus outbreak,[88] although whether tear transmission actually contributes to an outbreak scenario is still undetermined.

In an attempt to recapitulate the effects of Zika virus in immunosuppressed humans, including fatal disseminated infection, an immunosuppressed mouse model of Zika virus infection was established recently.[10,65] To this end, 6- to 8-wk-old BALB/C mice were immunosuppressed with dexamethasone and then challenged intraperitoneally with a Puerto Rican clinical isolate (PRVABC59). Compared with controls, the immunosuppressed mice displayed high viral loads at 5 dpi in blood and most tissues, with minimal accompanying inflammation.[10] When the dexamethasone treatment was tapered off, infected mice experienced weight loss and various clinical signs, suggesting that the clinical

deterioration was a combination of the disseminated Zika virus infection and subsequent immune reconstitution after secession of immunosuppressive therapy. Prominent inflammation was noted in various organs on postmortem analysis. Using this model, the investigators then treated infected and immunosuppressed mice with recombinant type I interferons. Treated mice were asymptomatic and had minimal inflammation and detectable Zika virus within tissues, demonstrating that interferon treatment was effective in greatly reducing Zika virus infection this model.[10] This study demonstrates an approach to developing an animal model of Zika virus that uses immunosuppressive therapy.

As seen in studies examining intrauterine effects of Zika virus,[13] viremia and clinical signs have been compared between different immunocompetent strains of mice. One study has shown variable susceptibility in 10- to 12-wk-old mice after challenge with Zika virus (Brazil ZKV2015).[38] In that study, both BALB/c and SJL mice displayed a significantly higher viremia than C57BL/6 mice.[38] In addition, both inactivated virus and plasmid DNA vaccines were protective in all three strains of mice that were investigated,[38] correlating with similar findings in rhesus macaques[1] and offering encouragement for the plausibility of human Zika virus vaccines. Another study showed that both 4- and-8 wk-old 129 Sv/Ev mice exhibited transient but widespread viremia after inoculation with a clinical isolate of Asian lineage Zika virus (GZ01).[96] Swiss mice, which were used in one of the initial Zika virus experiments in 1971,[6] have also been investigated recently. Injection of 1-d-old mice either intracerebrally or subcutaneously with a Brazilian clinical isolate (SPH 2015) led to clinical signs that eventually required euthanasia, including lethargy, ataxia and paralysis, in all mice, with the intracerebral group exhibiting signs first.[26] Extensive inflammation was present in the brains of both infected groups, with the cerebral cortex as the main area affected. Interestingly, 2 of the 4 mice inoculated subcutaneously had atonic urinary bladders, presumed secondary to spinal cord lesions;[26] atonicity of the urinary bladder has also been reported in a human case of Zika virus.[52] Atonic bladders have not been reported in other studies, and because the subcutaneous injection was near the lumbar vertebrae,[26] this lesion cannot be interpreted as evidence of preferred site for Zika virus. Figure 2 provides a summary of the results from all of these experiments in mice.

Models of intrauterine Zika virus infection. The embryologic effects of Zika virus have gained widespread acceptance in the human medical field, thus highlighting the usefulness of mouse models, in that rodents and primates both exhibit hemochorial discoid placentation. Despite their broad similarities in placental classification, unique differences during fetal development do exist between these 2 species.[49] The gestational age between these 2 species is vastly different, complicating the study of specific embryologic inoculation time points in mice.[70] Furthermore, well-established differences in embryologic neural development exist between mice and humans,[28] such that the brain development of a mouse pup at postnatal day 1 is comparable to that of a human brain at midgestation.[74] Studies examining genes related to microcephaly have shown milder phenotypes in mice compared with humans,[47,69] with this finding perhaps attributable to the relatively smaller cerebral cortex in mice. The infection of chicken embryos with Zika virus (Mexican isolate) to study its effect on the developing brain induced a microcephaly-like phenotype in surviving embryos.[29] In another study, the injection of Asian lineage Zika virus (SZ01) into the lateral ventricles of ICR mouse fetuses in utero caused marked deregulation of microcephaly-associated genes, cortical thinning, and induction of an immune response within the brain.[43] In addition, Zika virus titers were highest in neural progenitor cells within the brain.[43] Using the same experimental mouse model, convalescent serum from a Zika virus-infected human was injected intraperitoneally, after which treated animals showed a marked reduction in the number of Zika-virus-infected cells in brain, suggesting that convalescent serum may have therapeutic potential for decreasing the embryologic effects of Zika virus.[90] Intracerebral injection of a Mexican clinical isolate of Zika virus (MEX1-44) into embryonic (E) day 14.5 embryos of either C57BL/6J or 129S1/SvImJ mice led to microcephaly, cortical thinning, and neural progenitor cell infection as seen in other studies, but the authors also reported neuronal death, a leaky blood brain barrier, and astrogliosis in the brains of infected embryos.[77] This study adds to the collective understanding of the widespread effects of Zika virus on the developing brain.

Several recent studies have shown that Zika virus infection can be vertically transmitted in immunocompetent mice.[13,93] However, these 2 studies used different Asian-lineage strains of Zika virus. In one,[93] a clinical isolate of Zika virus that originated in Samoa was injected into pregnant C57BL/6 dams at E13.5 either intraperitoneally or into the lateral ventricle of the embryo and showed that Zika virus has a tropism for radial glial cells, an embryologic neural progenitor cell type. Real-time PCR analysis of the fetal brains demonstrated similar alterations in gene expression as have been noted in human neural cultured cells that were infected with Zika virus.[82,93] The absence of appreciable microcephaly in the mice may be due to the difference in the number of radial glial cells between mice and humans.[37] Of the intraperitoneally injected dams, 5 of 9 placentas had Zika virus RNA at 3 dpi, showing that the virus has the capability of crossing the placental barrier.[93] In a similar experiment, a Brazilian clinical isolate of Zika virus administered intravenously yielded differing results, in that for SJL pregnant mice, Zika virus produced transplacental infection and induced profound intrauterine growth restriction, decreased cortical thickness, and ocular abnormalities in the pups; analysis of the SJL pups demonstrated upregulation of genes associated with autophagy and autolysis.[13] In contrast, C57BL/6 pups showed no appreciable change, and the researchers were unable to detect Zika virus in the embryos by using quantitative PCR analysis.[13] Differences in viral susceptibility between SJL and C57BL/6 occurred in another Zika virus study[38] and was previously described in regard to Theiler murine encephalomyelitis virus,[45] another virus that interacts with interferon pathways.[81] In particular, C57BL/6 mice express higher levels of interferon-stimulated genes than do SJL, and this difference may account for the varied susceptibility to the virus, with SJL mice subject to and C57BL/6 resistant to clinical signs from Theiler murine encephalomyelitis virus.[45] Interstrain differences in susceptibility appear to exist after Zika virus infection as well.

A study examining the transplacental infection of embryos used B6 *Ifnar1*$^{-/-}$ dams crossed to B6 WT sires, and infection of these dams with Zika virus (H/PF/2013) resulted in fetal demise and intrauterine growth restriction of the embryos.[54] Tissue analysis revealed high viral loads within placenta and fetal brains.[54] In a related study from the same group, a blocking antiIFNAR antibody was administered to Zika-infected B6 WT dams; the resulting embryos exhibited mild intrauterine growth restriction without evidence of fetal demise.[54] In addition, pregnant dams

Mouse model	Virus lineage	Route	Age	Outcome	Reference
A129	MP1751 (African)	SC	5–6 wk	Weight loss, lethargy and death after 6 dpi; viral RNA highest in spleen and brain.	20
	FSS13025 (Asian)	IP, ID	3, 5, and 11 wk	3-wk-old mice displayed tremors, lethargy, anorexia, and lethality by 6 dpi; older mice displayed mild symptoms, with 50% mortality in 5-wk group and 0% in the 11-wk mice.	71
AG129	FSS13025 (Asian)	IP, ID	3 wk	Mice displayed neurologic disease, tremors, loss of balance, and lethality by 6 dpi.	71
	FSS13025 (Asian)	IVG	8–12 wk	Mice inoculated after induced diestrus presented with worsening clinical signs and lethality by 22 dpi. This phenotype was not observed in mice inoculated after induced estrus.	83
	H/PF/2013 (Asian)	FP, IP	3–4 wk and 8 wk	Lethargy, hunched posture, and lethality by 8 dpi; viral titers highest at 2 dpi.	3
	MR766 (African)	IP	8–14 wk	Hunched back, lethargy, hindlimb paralysis, and eventual lethality by 18 dpi on average; disease severity was dose-dependent.	98
Rag1$^{-/-}$	Senegal 1984 (African)	FP	7 wk	Mice treated with IFNAR1 blocking antibody 1 d before inoculation demonstrated the presence of Zika virus in testes and epididymis and testicular involution, although Leydig cells were less damaged than in other experiments by the same group. The results imply that the testicular insult was caused by Zika virus and resulting adaptive immune response.	30
Ifnar1$^{-/-}$	MR766 (African), H/PF/2013 (Asian)	FP, IV	5–6 wk; 3, 4, and 6 mo	Hindlimb weakness, paralysis, and 100% lethality by 10 dpi with Asian lineage. Mice had reduced phenotypic severity and some survived with African lineage. Older mice inoculated with Asian lineage had reduced phenotype, and most survived infection in an age-dependent manner.	39
	H/PF/2013 (Asian), Paraiba 2015 (Asian)	FP	4–8 wk	Zika virus viral shedding was noted in tears until 7 dpi.	55
	ZIKA-SMGC-1 (Asian)	IP	6 wk	Resulted in lethality in 20% of mice; remaining mice had profound orchitis and epididymitis.	48
	FSS13025 (Asian)	IVG	8–10 wk	Higher Zika virus RNA levels in vagina than in WT. High-dose IVG challenge resulted in lethality by 9 dpi.	95
Irf3$^{-/-}$ *Irf7*$^{-/-}$	FSS13025 (Asian)	IVG	8–10wk	Higher Zika virus RNA levels in vagina when compared with WT.	95
Irf3$^{-/-}$*Irf5*$^{-/-}$ *Irf7*$^{-/-}$	MR766 (African), H/PF/2013 (Asian)	FP, IV	4–6 wk	Hindlimb weakness, paralysis, and 100% lethality by 12 dpi with both lineages.	39
	FSS13025 (Asian)	IV	5–6 wk	Mice display worsening clinical signs and hindlimb paralysis by 6 dpi.	44
SCID	MR766 (African)	IP	8–9 wk	Hunched back, lethargy, hindlimb paralysis, and eventual lethality by 40 dpi on average; delayed compared with AG129 on same study	98
129Sv/Ev	GZ01 (Asian)	IP	4–8 wk	No clinical signs reported; viremia was higher in younger animals	96
	MP1751 (African)	SC	5–6 wk	No clinical signs reported.	20
SJL	Brazil ZKV2015 (Asian)	IV	10–12 wk	Virus replicated efficiently; no overt clinical signs.	38
CD1	FSS13025 (Asian)	SC	3 wk	No clinical signs reported; no viremia detected.	71
	41519, 41667, and 41671 (African)	FP	4 wk	No clinical signs reported.	39
C57BL/6	MR766 (African), H/PF/2013 (Asian)	SC, IV	5–6 wk	No clinical signs reported.	39
	FSS13025 (Asian)	SC, FP	6 wk	No clinical signs reported; no viremia detected.	71
	FSS13025 (Asian)	IVG, IP	8–10wk	Zika virus RNA persisted in the vagina until 4 dpi, suggesting local replication. Splenic Zika virus RNA levels were higher after IVG than IP.	95
	ZIKA-SMGC-1 (Asian)	IP	6 wk	No clinical signs reported.	48
	Brazil ZKV2015, Puerto Rico PRVABC59 (Asian)	IV	10–12 wk	Viremia was detectable, but at a much lower level than in SJL and BALB/c mice on the same study.	38
	H/PF/2013 (Asian), Senegal 1984 (African)	FP	7 wk	Mice treated with IFNAR1 blocking antibody 1 d before inoculation demonstrated the presence of Zika virus in testes and epididymis and testicular involution. Lesions were more severe with Senegal 1984 strain.	30
Swiss	SPH 2015 (Asian)	IC, SC	1 d	All mice exhibited neurologic signs and paralysis, with IC-inoculated mice exhibiting signs first. Profound inflammation noted in CNS, primarily within cerebral cortex.	26
BALB/c	Brazil ZKV2015, Puerto Rico PRVABC59 (Asian)	IV	10–12 wk	Virus replicated efficiently; no overt clinical signs.	38
BALB/c, immunosuppressed	PRVABC59 (Asian)	IP	6–8 wk	Viremia widespread in immunosuppressed mice, clinical signs and inflammation increase rapidly as immunosuppressive therapy wanes. Treatment with type I interferons greatly decreases Zika virus infection.	10

Figure 2. Mouse models of Zika virus pathogenicity. FP, footpad; IC, intracerebral; IVG, intravaginal.

infected with Dengue virus lacked evidence of intrauterine growth restriction, and placentas were virus-free, supporting the idea that the tropism of Zika virus for placental tissue is greater than that of other flaviviruses.[54]

Continuing their work investigating intravaginal inoculation of Zika virus, one group inoculated WT dams at E4.5 and E8.5 and demonstrated that when dams were infected on E4.5, developing embryos at E18.5 had mild but significant overall growth defects.[95] Examination of the embryonic brains demonstrated the presence of Zika virus at both time points, illustrating that intravaginal inoculation can result in embryonic infection, even when the gross appearance of the embryo is unaltered. In a related study, this group inoculated pregnant *Irf3*$^{-/-}$*Irf7*$^{-/-}$ mice and *Ifnar1*$^{-/-}$ dams crossed to WT sires at E4.5 and E8.5. Inoculation at E4.5 resulted in significant fetal weight reduction in *Irf3*$^{+/-}$*Irf7*$^{+/-}$ mice and fetal resorption of *Ifnar1*$^{+/-}$ mice. These data support the notion that early embryonic exposure to Zika virus is deleterious and suggest that the intravaginal route can cause embryonic infection.[95]

In total, the presented studies have produced several animal models of varying phenotypic severity that can be used for studying the effects of Zika virus on developing embryos through vertical transmission, intrauterine, or intraembryo inoculation. Studies like these have the potential to help elucidate the pathophysiologic mechanisms of Zika-virus–associated microcephaly that occurs in humans. The results of the intrauterine experiments are summarized in Figure 3.

Large Animal Models

Previous serologic data has shown that a wide array of large animals have an immunologic response to Zika virus, including water buffalo, goats, NHP, lions, sheep, and wildebeest.[14,31,32] Despite these seroprevalence data in other large animal species, NHP have been an integral component of Zika virus knowledge since the initial isolation of Zika virus from a rhesus macaque in 1947 in Uganda.[18] Seroprevalence data have demonstrated that several species of NHP can seroconvert,[31,32,51,97] and some investigators theorize that Zika virus is maintained in a sylvatic cycle within NHP in the wild,[31] similar to other flaviviruses.[8,36] Similarly, NHP are well established as animal models of disease pathogenesis, vaccine development, and therapeutic research for similar tropical viruses.[8,11,36] Because of their similarity to humans, readily available resources, and known infectivity with Zika virus, NHP have become the large animal model of choice for the current wave of Zika virus research.

Rhesus macaques, and to a lesser extent cynomolgus macaques (*M. fascicularis*), are the species of NHP that are predominantly used as Zika virus research models. The majority of the work to date has been to establish Zika virus infections with various strains of the virus and to document the physical and viral characteristics after infection. Because of the outbreak and urgent need for the development and dissemination of knowledge about Zika virus, several primate research institutions have presented their research online prior to formal publication.[63,80,86,97]

Studies out of the Oregon and California National Primate Research Centers using Zika virus isolated from Puerto Rico and Brazil, respectively, have demonstrated that clinical signs in infected adult rhesus macaques are generally mild and consist of erythema, mild fever, and transient lymphadenopathy in some animals.[63,86] Another group has investigated a Chinese clinical isolate of Zika virus (GZ01) and report similar clinical findings and extent of viremia as in other reports, with the presence of Zika virus in lacrimal fluid, saliva, and urine.[46] Necropsy of several macaques in the acute stage of viremia (up to 10 dpi) demonstrated Zika virus RNA in the CNS, gastrointestinal tract, and various other organs.[46] Spleen and lymph nodes contained higher levels of Zika virus RNA at 10 dpi compared with 5 dpi, suggesting that the virus may replicate for a longer time period within these organs.[46] The group at the Southern Research Institute has infected cynomolgus macaques with Puerto Rican (PRVABC59), Cambodian (FSS13025), and Nigerian (IBH0656) isolates of Zika virus and have demonstrated an absence of clinical signs despite viremia lasting as long as 14 dpi.[80] Studies from the Wisconsin National Primate Research Center have shown that infection with Zika virus of both Asian and African lineages (FP and MR766) can cause a viremia that lasts until 21 dpi, and virus is present in various body fluids including saliva, urine, vaginal fluid, and cerebrospinal fluid.[23,97] Their work also has shown that Zika virus can be acquired by macaques through mucosal exposure.[97]

To understand whether Zika virus infection results in acquired immunity, infected macaques were rechallenged 10 wk after initial infection. This reinoculation resulted in an absence of detectable virus in plasma, saliva, or urine, suggesting protective immunity.[23] Another study from the same group has shown that infection with an African lineage Zika virus (MR766) in rhesus macaques confers protection when the animals are subsequently challenged with an Asian lineage (FP), as evidenced by a lack of viremia and clinical signs in the macaques.[4,97] This finding suggests that strain selection for vaccine development may not be critical, given the cross protection between lineages.[4] On a different note, several studies have shown that previous exposure to dengue virus has little effect on Zika virus infectivity and pathogenicity, conferring neither protection nor antibody-dependent enhancement.[66,97]

The immunogenicity of Zika virus has also been tested by using several vaccines in rhesus macaques. One study investigated a DNA vaccine of Asian lineage Zika virus (H/PF/2013) and showed that single-dose vaccination reduced viremia after subcutaneous administration of Zika virus, whereas 2-dose vaccination conferred protection in 94% of rhesus macaques.[21] In another study, vaccines comprising purified inactivated virus, plasmid DNA, and a recombinant adenovirus expressing Zika virus all induced Zika-virus–specific neutralizing antibodies and protected animals from viral challenge with both Brazilian and Puerto Rican isolates.[1] Purified immunoglobulins from these vaccinated rhesus conferred passive protection in other animals when exposed to Zika virus.[1] A vaccine challenge study in mice produced similar results,[38] and together, these studies offer promise for the successful production of a Zika virus vaccine for humans.

Several studies to date have investigated the effects of Zika virus infection in pregnant NHP. In one study, 2 first-trimester pregnant rhesus macaques that were infected with Asian lineage Zika virus (H/PF/2013) had a persistence of their viremia until 29 and 71 dpi, and 1 of 2 rhesus infected in the 3rd trimester had viremia at 36 dpi, which is notably longer than has been observed in nonpregnant rhesus (approximately 10 to 12 dpi).[23] This prolonged viremia during pregnancy has been reported in humans as well,[22] although the underlying mechanism is yet to be fully understood. Another study evaluated a pigtail macaque (*M. nemestrina*) inoculated subcutaneously with an Asian lineage virus (FSS13025) at

Mouse model	Virus lineage	Route	Age	Outcome	Reference
Ifnar1$^{-/-}$	H/PF/2013 (Asian)	FP	E6.5–7.5	Knockout dams crossed to WT to produce *Ifnar1*$^{-/-}$ pups resulted in fetal demise and resorption or IUGR. Zika virus RNA was abundant within placental tissue	54
	FSS13025 (Asian)	IVG	E4.5, E8.5	E4.5 mice underwent fetal resorption by 9 dpi; E8.5 embryos demonstrated IUGR.	95
Irf3$^{-/-}$*Irf7*$^{-/-}$	FSS13025 (Asian)	IVG	E4.5, E8.5	E4.5 embryos demonstrated IUGR; high levels of Zika virus RNA were present in placenta and fetus at both time points.	95
SJL	Clinical isolate from patient in Brazil (Asian)	IV	E10–13	Pups displayed IUGR, cortical malformations similar to microcephaly, and ocular abnormalities	13
C57BL/6	Clinical isolate from patient in Brazil (Asian)	IV	E10–13	Pups displayed no clinical signs; no virus detected in pups, in stark contrast to results from SJL mice in same study.	13
	Clinical isolate from patient in Samoa (Asian)	IP, IC	E13.5	Zika virus infection was able to cross the placenta in some dams and infect neural progenitor cells. Intraventricular injection produced much more profound CNS infection than IP inoculation.	93
	FSS13025 (Asian)	IVG	E4.5, E8.5	E4.5 inoculation resulted in moderate IUGR; both time points showed Zika virus in CNS.	95
	MEX1-44 (Asian)	IC	E14.5	Microcephaly, cortical thinning, neural progenitor cell infection, extensive neuronal death, a leaky blood–brain barrier, and astrogliosis were reported.	77
ICR	SZ01 (Asian)	IC	E13.5	Zika virus replicated efficiently within embryos and infected neural progenitor cells. Treatment with convalescent serum decreased Zika virus viral load in the embryo.	43, 90

Figure 3. Mouse models of Zika virus pathogenicity in embryos. E, embryonic day; FP, footpad; IC, intracerebral; IUGR, intrauterine growth restriction; IVG, intravaginal.

119 d gestation.[2] The fetus underwent ultrasonographic examination weekly, which revealed that the biparietal diameter (a measure of head size and often gestational age) displayed decreased growth when compared with species-specific published data.[2] In addition, MRI of the fetal brain displayed multiple changes in image intensity throughout pregnancy. At time of delivery, 162 d gestation, both the dam and fetus were necropsied, and Zika virus was present in the placenta, fetal brain and liver, and maternal brain, eyes, spleen, and liver.[2] Alterations in the posterior white matter, gliosis, and axon injury were among lesions noted on histopathologic exam of the fetus.[2] This study is the first to report fetal CNS lesions in a Zika-virus–infected pregnant macaque, and further work on this topic is necessary given its translatability to the lesions observed in human fetuses.

Despite all of the current NHP research being done in several species of macaques, analyzing other NHP models infected with Zika virus may be valuable. Studies have shown that New World monkeys, specifically howler monkeys of the genus *Aloutta*, display more severe clinical signs when infected with yellow fever virus than do Old World monkeys.[15,36,57] Studies of both wild and captive New World monkeys exposed to Zika virus may demonstrate a similar outcome. In a related manner, studies investigating the different outcomes in NHP and other species after exposure to various Zika virus lineages may help to uncover the differences between these lineages and, moreover, help to explain why the current Zika virus outbreak has occurred. The African lineage of Zika virus was initially derived from an NHP source, whereas the Asian lineage has become more adapted to human-to-human transmission over the past several decades.[13] This host adaptation was exquisitely demonstrated in one study that infected brain organoid cell cultures derived from chimpanzee pluripotent stem cells.[13] In the study, the Brazilian lineage did not replicate in the chimpanzee cells, whereas the African lineage replicated well.[13] This result shows the ability of the virus to adapt to different primate hosts over time, and such adaptation is a factor to consider when choosing a Zika virus lineage to use for NHP experiments.

Conclusions

Over the last several years, a myriad of research studies on Zika virus have used both large and small animal species. This research has initially focused on detailing the pathogenesis of Zika virus and establishing an understanding of the tissue tropism of the virus. Animal studies mentioned here have demonstrated that the virus has a strong tropism for neural progenitor cells, among

other cell types, consistent with the human clinical findings of Zika-virus–associated neurologic disease. Furthermore, several studies in mice have shown that Zika virus has a tropism for placental tissue and can infect embryos, results that are also consistent with clinical findings noted in people. Other studies mentioned in this review demonstrate the utility of certain strains of mice, as well as NHP, for Zika virus vaccine and therapeutic development. Antivirals that have shown efficacy against other flaviviruses have also shown efficacy within a mouse model of Zika virus,[23,73,98] and currently available antiviral therapeutics should continue to be tested in Zika virus animal models. Similar to what has been seen in other flaviviruses, the interferon pathways play an important role in Zika virus pathogenesis and immune protection.

Zika virus can be from either the Asian or the African lineage, with specific sublineages depending on the location of initial viral isolation. The high mutation rate of RNA polymerases[33] results in nonhomogeneous populations of RNA viruses. Therefore, given different mosquito vectors, bottlenecks during its spread, and nonstandardized in vitro propagation methods, it is not surprising that Zika virus isolates differ. The first Asian lineage Zika virus was isolated in 1966, and genomic analysis has demonstrated consistent viral evolution over time, leading into the recent outbreaks.[24] Additional evidence shows that Zika virus has experienced recombination events, which are unusual among flaviviruses.[25,79] Some of the studies mentioned in this review have demonstrated a variable phenotype that was dependent on Zika virus lineage. To date, no specific lineage is considered the 'gold standard' for testing, and as such, we recommend careful consideration of the literature when choosing a lineage for investigation. The recent availability of Zika virus infectious cDNA clones will aid in resolving this lineage variability.[76,85,91] Moreover, an array of phenotypes can be observed within different mouse strains, and this variability is dependent on the specific mouse strain, mouse age, and route and dose of viral inoculation. All of these factors should be considered in future research studies of Zika virus.

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