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Dengue mouse models for evaluating pathogenesis and countermeasures

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Abstract

Dengue virus (DENV) causes the most prevalent arbovirus illness worldwide and is responsible for many debilitating epidemics. The four circulating DENV serotypes infect humans and can cause asymptomatic, mild, moderate, or severe Dengue. Because of the global morbidity and mortality due to Dengue, deployment of a safe and effective tetravalent vaccine has been a high priority, and to date, a partially realized goal. The study of pathogenesis and development of DENV therapeutics and vaccines has been limited by few animal models that recapitulate features of human disease. Over the past two decades, mouse models of DENV infection have evolved with increasing success. Here, we review the utilization and limitations of mice for studying DENV pathogenesis and evaluating countermeasures.

INTRODUCTION

Dengue virus (DENV) causes an estimated 390 million human infections per year, including approximately 96 million symptomatic cases [1, 2]. The four circulating serotypes of DENV, which differ by ~25% to ~40% at the amino acid level in their envelope (E) proteins, are transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitos [3, 4]. Global prevalence analysis estimates that up to 3.9 billion people are at risk of dengue infection in 128 different countries [5]. DENV is a flavivirus in the *Flaviviridae* family, and its positive-sense 11 kilobase RNA genome encodes three structural proteins (capsid, precursor membrane, and E) and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) [6].

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Conflict of Interests.

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Based on World Health Organization (WHO) criteria, Dengue illness is classified into (a) Dengue with and without warning signs and (b) Severe Dengue. Dengue most commonly presents with a constellation of signs and symptoms including fever, headache, nausea, vomiting, myalgia, arthralgia, rash, and leukopenia [7]. Those experiencing Dengue with warning signs also develop gingival bleeding, lethargy, hepatomegaly, hemoconcentration, thrombocytopenia, and fluid extravasation into body cavities resulting in ascites and/or pleural effusions. Severe Dengue encompasses what historically was termed Dengue Hemorrhagic Fever and Dengue Shock Syndrome (DHF/DSS), whereby patients develop plasma leakage, bleeding, respiratory distress, and visceral organ dysfunction particularly in the liver, heart, and central nervous system [7]. In adolescents and adults, Severe Dengue occurs more frequently during secondary dengue infection with a heterologous DENV serotype and has been hypothesized to be due to antibody-dependent enhancement (ADE), a phenomenon where cross-reactive antibodies from the primary DENV infection bind to but fail to neutralize the secondary DENV strain and instead promote infection in myeloid cells expressing Fc- γ receptors [8–13]. This was illustrated in a study of children in Thailand from 1994 to 2006 in which 43% of Dengue patients with secondary infections progressed to DHF compared to 14% of those primary infections [14]. Infants, in contrast, are at highest risk for Severe Dengue at 6 to 9 months of age in the context of primary DENV infection, as passively transferred maternal antibodies wane to sub-neutralizing titers [15]. Patients who develop Severe Dengue are at risk of death especially if diagnosis and fluid replacement are delayed. While few ($< 0.25\%$) dengue infections result in death, the morbidity remains high with intensive resource utilization in developing countries. In 2013, Dengue cost \$8.9 billion to treat and resulted in 1.14 million disability-adjusted life years globally [16, 17]. A meta-analysis in 2018 projected the burden of Dengue to be 2.1 times higher than the 2013 study [18]. In 2015, Dengvaxia (CYD-TDV), a tetravalent DENV vaccine, was licensed for use in dengue endemic regions. However, three years later, it was found that dengue-seronegative vaccine recipients were at higher hospitalization risk after natural DENV exposure [19]. As the WHO recommends Dengvaxia only for dengue-seropositive individuals, there remains a critical need for development and deployment of additional vaccines and effective therapies.

DENV research and countermeasure development have been hampered by the lack of animal models that fully recapitulate human disease. Dengue infects non-human primates (NHPs) and causes viremia, but most of the key signs of Severe Dengue are absent [20]. In comparison, mice and other rodents (*e.g.*, guinea pigs, hamsters, and rats), are resistant to DENV infection and disease [21, 22]. A failure of DENV to efficiently infect rodents is likely because the virus has evolved mechanisms to evade the human, but not rodent immune system [23] (Figure 1). For instance, DENV NS5 degrades human STAT2, but not mouse Stat2, which dampens the type I interferon (IFN) signaling response and allows for efficient viral replication in human cells [24]. In addition, the DENV NS2B-NS3 protease can cleave human STING, but not mouse Sting, which contributes to restriction of infection in mouse cells [25]. These barriers have proven difficult to overcome using different strains of inbred or outbred mice. However, over the past two decades, progress has been made in manipulating murine models to study DENV. Here, we review recent advances in mouse

models of DENV infection and focus on their use in studying pathogenesis, testing therapeutics, and evaluating the immunogenicity and efficacy of vaccine candidates.

MOUSE MODELS OF DENGUE INFECTION AND PATHOGENESIS

Wild-type mice.

Most immunocompetent adult mice do not sustain substantial infection or signs of disease with clinical DENV isolates when administered through peripheral inoculation routes. Intravenous inoculation of a non-physiological dose (10^8 plaque forming units [PFU]) of an adapted DENV-2 strain 16681 in C57BL/6 mice causes mild hepatitis, whereas intradermal injection with high (10^7 to 10^9 PFU) doses causes local and systemic hemorrhage, thrombocytopenia, and elevated TNF- α levels [26, 27]. Viral infection and dissemination in these models is restricted, as viremia is inconsistently detected [28]. Moreover, vascular leakage is not observed in these DENV-infected immunocompetent mice. Although one group has reported a model of severe systemic disease in wild-type mice after infection with mouse-adapted DENV-2 and DENV-3 strains and used it to define mechanisms of immune control [29–32], these results have not been confirmed by independent groups. Thus, the utility of immunocompetent mice for studying DENV infection, pathogenesis, and disease remains limited.

A more common use of immunocompetent mice has been to evaluate the immunogenicity of DENV vaccine candidates [33–35]. Challenge of immunized mice can assess vaccine efficacy against morbidity and mortality when DENV is administered directly into the brain via intracranial injection route [36, 37] or through intraperitoneal injection of DENV-infected hematopoietic tumor (*e.g.*, K562) cells [38, 39] (Table 1). Survival from lethal intracranial DENV challenge was established for several vaccines platforms including adjuvanted DENV E ectodomain protein and DNA plasmids encoding DENV prM and E [36, 37, 40]. In challenge studies with DENV-infected K562 cells, immunization with DNA plasmid vaccines encoding DENV-1 prM and E proteins protected outbred ICR mice against lethal infection [38]. Similarly, vaccination of BALB/c mice with an E-DIII subunit vaccine reduced viremia after challenge with DENV-infected K562 cells [39]. Notwithstanding their limitations, challenge models of DENV infection in immunocompetent mice allow cost-effective initial assessment of protection for vaccine candidates.

Wild-type, immunocompetent mice also have been used to study some aspects of DENV pathology such as neuropathogenesis, which occurs relatively rarely in humans [41], and hemorrhage (Table 1). Intracranial inoculation with a neuroadapted DENV-2 strain in BALB/c mice recapitulates some features of DENV encephalitis [42] including reactive gliosis, microglial hypertrophy, and neuronal injury. Based on its ability to bind endothelial cells and possibly platelets [43, 44], DENV NS1 protein has been hypothesized to contribute to thrombocytopenia and bleeding in humans. Indeed, whereas intradermal infection of C3H/HeN and C57BL/6J mice with DENV caused local skin hemorrhage, these phenotypes were absent in mice infected with NS1-depleted DENV [44]. Possibly related to this, exogenous administration of DENV NS1 protein induced vascular leakage in uninfected C57BL/6J mice [43].

***Ifnar1*^{-/-} *Ifngr*^{-/-} DKO mice.**

One approach to overcoming the inherent resistance of immunocompetent mice to DENV infection has been to use animals with deficiencies in innate host defense responses. More than twenty years ago, AG129 mice, which lack receptors for both type I (*Ifnar1*^{-/-}) and II (*Ifngr*^{-/-}) IFNs on a 129 SvEv background, were established as vulnerable to DENV infection [45]. Since then, these mice have been used widely to evaluate vaccines and antiviral agents, and to study pathogenesis (Table 2). AG129 mice inoculated through peripheral routes with strains from all four DENV serotypes succumb to or develop severe disseminated infection [46–50]. DENV infection of AG129 mice can result in vascular leakage and changes in blood cell counts (platelets, lymphocytes, and erythrocytes), which is seen in human disease [51]. In addition, ADE of DENV infection can occur reproducibly in AG129 mice. Passive transfer of heterologous DENV serum or cross-reactive, poorly neutralizing mAbs prior to DENV infection results in enhanced infection and mortality [52, 53]. Administration of immune complexes by pre-incubating mAbs or immune sera with DENV before infection of AG129 mice also achieves ADE and enhances mortality [54, 55] (Table 2).

The permissiveness of DENV infection in AG129 mice has facilitated pathogenesis studies that evaluate the significance of naturally occurring mutations. As an example, the appearance of T164S mutation in NS1 was correlated with enhanced dengue disease in the 1997 Cuban DENV-2 outbreak. AG129 mice inoculated with the T164S mutant DENV-2 sustained greater lethality, viral burden, and cytokine levels, and these phenotypes were associated with higher serum levels of NS1 compared to that seen with the parental strain [56].

Another common use of AG129 mice is for evaluating antiviral agents against DENV. Among the classes of potential inhibitors evaluated as either pre- or post-exposure therapy trials are monoclonal antibodies (mAbs) [57], small molecule direct acting antivirals [58–61], and immunomodulatory agents [62, 63]. Therapeutic administration of mAb m366.6 to DENV-2 infected AG129 mice improved survival [57]. Treatment with AZD6244, a MEK/ERK inhibitor, reduced viral burden and increased survival of DENV-2-infected AG129 mice [58]. Therapy with NITD-008, an adenosine nucleoside inhibitor, reduced DENV infection, delayed mortality in DENV-1 and DENV-4 infected AG129 mice, and prevented lethality in DENV-2 and DENV-3 infected AG129 mice [59]. A combination of immunomodulatory treatment of sunitinib, a vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitor, and an anti-TNF- α mAb reduced vascular permeability and mortality in DENV-2 infected AG129 mice under ADE conditions [62]. Combination of sunitinib with erlotinib, two receptor tyrosine kinase inhibitors, also improved survival, morbidity, and viral burden in *Ifnar1*^{-/-} *Ifngr*^{-/-} DKO C57BL/6 mice [63].

This immunocompromised mouse model of DENV infection nonetheless has limitations: (a) DENV infection of *Ifnar1*^{-/-} *Ifngr*^{-/-} DKO 129 SvEv or C57BL/6 mice typically results in death due to central nervous system infection, which is not the principal cause in humans; (b) the absence of type I and II IFN signaling impacts induction of adaptive B and T cell responses, which affects its utility in vaccine studies; and (c) DENV infection in the absence

of type I and II IFN signaling can skew cytokine responses in ways that differ from human disease.

***Ifnar1*^{-/-} mice.**

Ifnar1^{-/-} C57BL/6 or 129 SvEv (A129) mice are deficient only in the IFN- α/β receptor and are less immunocompromised than *Ifnar1*^{-/-} *Ifngr*^{-/-} DKO mice. Infection of *Ifnar1*^{-/-} mice has been established using mouse-adapted DENV strains under both standard and ADE conditions [52, 64] (Table 3). *Ifnar1*^{-/-} mice typically are more resistant to DENV than *Ifnar1*^{-/-} *Ifngr*^{-/-} DKO mice and sustain less mortality [65, 66]. However, DENV can be detected readily in blood and in peripheral organs including the spleen and liver [66]. By using passaged or adapted strains, high inoculation doses, or ADE conditions, *Ifnar1*^{-/-} mice can succumb to DENV infection [64, 67]. Because of their ability to support DENV infection and retain IFN- γ signaling, *Ifnar1*^{-/-} mice have been used to study DENV pathogenesis [66], including the contributions of NS1 antigenemia and serum sialic acid to DENV vascular leakage [67]. Additionally, *Ifnar1*^{-/-} mice were used to show how the viral prM/M structural protein modulates vascular leakage through an IL-1 β inflammasome signaling axis [68]. Thus, *Ifnar1*^{-/-} mice have utility as a less immunocompromised model in probing mechanisms of DENV pathogenesis.

To limit ablation of type I IFN signaling to specific myeloid cells, conditional knockout mice have been evaluated in Dengue infection studies (Table 3). Infection of LysM Cre⁺ *Ifnar1*^{fl/fl} mice with and without ADE conditions leads to weight loss and viral infection of visceral organs [69]. In addition, ADE conditions led to altered blood chemistry and hematology, cytokine upregulation, and vascular permeability [69]. Cd11c-Cre⁺ *Ifnar1*^{fl/fl} and LysM-Cre⁺ *Ifnar1*^{fl/fl} specifically delete *Ifnar1* expression in CD11c⁺ cells (e.g., DCs) and LysM⁺ cells (e.g., monocytes, macrophages, and neutrophils), respectively [70, 71]. Additionally, other genes in the IFN pathway, such as STAT1 [72], have been knocked out or edited to dampen IFN signaling and facilitate DENV infection [73].

Humanized and transgenic mice.

As alternatives to studying DENV infection in immunocompromised mice, mouse-human chimeras have been utilized. Initial studies used severe combined immunodeficient (SCID) mice engrafted with human peripheral blood lymphocytes, which supported low levels of DENV-1 viremia [74]. SCID mice transplanted with human HepG2 hepatocarcinoma cells sustained DENV-2 infection in blood, liver, and the brain [75]. Nonobese diabetic/SCID (NOD/SCID) mice engrafted with human CD34⁺ hematopoietic stem cells and inoculated with DENV showed features of human Dengue such as thrombocytopenia and rash [76]. Other variations of humanized mice using different immunodeficient mouse backgrounds and sources of human cells for reconstitution have been used for DENV infection and reviewed recently [77]. An advantage to using humanized mice is the ability to study human immune cell responses to DENV infection and tropism in human leukocytes. Nonetheless, humanized mice are not widely used in the DENV field because: (a) they are costly and time-consuming to generate, which limits throughput; (b) there are variable levels of human cell engraftment, which results in animal-to-animal variation; and (c) infection in these animals still does not result in the severe manifestations of DENV seen in humans.

Transgenic mice have also been used to study DENV immune system interactions. Transgenic expression of human major histocompatibility complex (MHC) alleles has allowed for study of T cell responses to DENV. Vaccination of HLA-A*0201, HLA-A*2402, and HLA-B*3501 transgenic C57BL/6 mice with a lipid nanoparticle-based mRNA vaccine expressing DENV-1 nonstructural proteins induced robust T cell responses [78]. Administration of anti-Ifnar1 antibody immediately prior to DENV-1 challenge facilitated dengue infection in control-immunized transgenic mice but not in the DENV nonstructural protein mRNA immunized mice [78]. Transgenic mice expressing human TNF- α have been used to study dengue inflammatory disease in the brain [79]. DENV-infected human TNF- α -transgenic mice develop severe encephalitis characterized by seizures, limb weakness, and limb paralysis before death [79]. Although transgenic mice do not recapitulate many aspects of DENV disease in humans, they have provided insight into specific aspects of DENV pathogenesis and immunity.

By applying our understanding of how DENV evades the human immune response, particularly in IFN induction or signaling pathways, it may be possible to generate novel mouse models to study Dengue. As mentioned above, DENV restricts type I IFN and IFN-stimulated gene (ISG) production in human cells by degrading STAT2 and STING (Figure 1) [24, 25]. The use of *Stat2*^{-/-} [55, 80, 81] and human STAT2 knock-in (hSTAT2-KI) [82] mice enabled permissive infection of Zika virus, a closely related flavivirus. However, we and others have found relatively poor DENV infection in hSTAT2-KI mice (R.E.C and M.S.D, unpublished results). This suggests the need for humanization of additional immune restriction factors, possibly STING. As other, human-specific antagonistic interactions between DENV and the host immune system are discovered, replacement of mouse genes (or domains) with human orthologs could allow for efficient DENV replication in immunocompetent, ‘humanized’ mice. Alternatively, screening of Collaborative Cross mice with much larger genetic variation might identify strains of mice that are uniquely susceptible to DENV infection and disease [83–85].

Conclusions.

Dengue research has evolved from the first isolation of DENV and passage in mouse brains [86, 87] to the deployment of a licensed live-attenuated tetravalent vaccine with several other candidates in advanced human clinical trials [19, 88]. Early pathogenesis and countermeasure studies were limited due to the lack of susceptible animal models. The introduction of *Ifnar1*^{-/-} *Ifngr*^{-/-} DKO mice into Dengue research allowed for *in vivo* work beyond costly non-human primate studies that fueled our understanding of DENV pathogenesis and expedited discovery and testing of antivirals and vaccines. Variations using *Ifnar1*^{-/-}, humanized, and transgenic mice have supported Dengue research as well by providing models that vary on a spectrum from most to least permissive. *Ifnar1*^{-/-} *Ifngr*^{-/-} DKO mice are used widely because they recapitulate several aspects of Severe Dengue in humans even though they have major deficits in immune responses. An immunocompetent animal model that is susceptible to DENV and develops human disease signs remains a lofty goal. Nonetheless, with the increasing number of mouse models and ability to manipulate expression of specific genes, many questions in Dengue research are being addressed. Thus,

while continued improvement of DENV mouse models is warranted, growth in DENV research no longer depends absolutely on its development.

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Figure 1. DENV restriction of host immune signaling.

DENV antagonism of host proteins involved in IFN induction and ISG expression [89] is denoted by virus symbols. Red virus symbols denote the inhibition of host factors [90–92]. Orange virus symbols denote specific inhibition of human and not mouse proteins [24, 25]. IFN receptors that are knocked out in DENV mouse models are indicated in black.

Table 1.

Immunocompetent mouse models of DENV infection

Mouse	Virus strain	Route of inoculation	Key readout	Reference
BALB/c	DENV1 Hawaii, DENV2 Tr 1751, DENV3 H87, DENV4 H241	Intracranial	Cytokine secretion, neutralization titers, weight loss, lethality	[36, 37, 40, 93]
BALB/c	DENV2 NGC	Intracranial	Morbidity, lethality, neuropathology	[42]
BALB/c	DENV2 NGC	Intracranial	Morbidity, lethality, cytokine secretion	[94]
C3N/HeN and C57BL/6	DENV2 454009 A	Intradermal	Hemorrhage, bleeding time, thrombocytopenia	[44]
C57BL/6J	DENV2 Thailand/16681/84 NS1	Intradermal	Vascular leakage	[43]
C57BL/6	DENV2 Eden2	Intraperitoneal	Thrombocytopenia	[95]
ICR and BALB/c	DENV1 Mochizuki	Intraperitoneal (K562 infected cells)	Viremia, neutralizing titers, lethality	[38]
BALB/c	DENV1–4	Intraperitoneal (K562 infected cells)	Viremia, cytokine expression	[39]
BALB/c	DENV2 NS1	Subcutaneous	Vascular leakage	[96]
C3N/HeN	DENV2 454009A	Intravenous	Viremia, bleeding time	[97]

For readouts, cytokine secretion is measured by ELISPOT assay; morbidity characterization includes mild to severe hind-limb paralysis; vascular leakage is determined using dextran or Evans blue dye; and cytokine expression is measured by mRNA or proteins.

Table 2.Immunocompromised *Ifnar1*^{-/-} *Ifngr*^{-/-} DKO mouse models of DENV infection

Mouse	Virus strain	Route of inoculation	Enhancing condition	Key readout	Reference
AG129	DENV1 128, DENV2 43, DENV3 80-2, DENV4 B5	Intracranial		Lethality	[57]
AG129	DENV3 D83-144 and DENV3 C0360/94	Intraperitoneal		Viral load, altered blood chemistry, bleeding time, cytokines, liver pathology, neutralization titers. Lethality (C0360/94)	[47]
AG129	DENV1 West Pac 74 DENV2 D2S10, DENV3 C0360/94, DENV4 4703-4, TVP-376	Intraperitoneal		Lethality, morbidity, viremia	[59]
AG129	DENV2 16681	Intraperitoneal		Lethality, viremia, cytokines	[98, 99]
AG129	DENV1 DENV2 DENV3 DENV4	Intraperitoneal	Pre-administration of mAbs and immune complex: preincubation of mAbs with virus	Lethality, viral load	[100]
AG129	DENV2 D2S20	Intravenous		Lethality, viral load, gross pathology, cytokines	[58]
AG129	DENV2 3295 or T164S mutant	Intravenous	Pre-administration of 4G2 (IP)	Lethality, viral load, cytokines	[56]
AG129	DENV2 S221	Intravenous	Pre-administration of 2H2 (IP)	Lethality, viral load, cytokines, vascular leakage	[62]
AG129	DENV2 D2S20	Intravenous	Pre-administration of DENV-immune sera (IP)	Lethality	[101]
AG129	DENV2 S221	Intravenous	Immune complex: preincubation of immune sera or 4G2 with virus	Lethality, cytokines, vascular leakage	[102, 103]
AG129	DENV2 S221	Intravenous	Immune complex: preincubation of immune sera or 4G2 with virus	Lethality	[54, 55]
AG129-hCD46 mice	DENV2 16681	Subcutaneous		Neutralization titers, viremia, cytokine secretion, cytokines	[104]
AG129	DENV2 D220	Not indicated		Neutralization titers, lethality, viremia, morbidity	[105]
AG129	DENV2 S221 and DENV4 703-4	Not indicated	Pre-administration of DENV-immune sera Immune complex: preincubation of immune sera or 4G2 with virus	Lethality, viremia, cytokines	[106]
<i>Ifnar1</i> ^{-/-} <i>Ifngr</i> ^{-/-} C57BL/6	DENV2 TSV01	Intracranial		Mortality, viral load	[107]

For readouts, cytokines are measured in serum, tissue homogenates, or peripheral blood cells by Bioplex, ELISA, or qRT-PCR; morbidity includes weight loss; viral load is determined by titers in spleen, lymph nodes, small and large intestine, liver, and brain; vascular leakage is using an Evans blue dye assay, and cytokine secretion is measured by ELISPOT.

Table 3.

Immunocompromised *Ifnar1*^{-/-} or conditional KO mouse models of DENV infection.

Mouse	Virus strain	Route of infection	Enhancing conditions?	Readout	Reference
<i>Ifnar1</i> ^{-/-} (C57BL/6)	DENV2 05K3295	Intraperitoneal		Viral load, thrombocytopenia, weight loss, cytokines	[108]
<i>Ifnar1</i> ^{-/-} (C57BL/6)	DENV2 S16803	Intraperitoneal		Weight loss, viral load	[109]
<i>Ifnar1</i> ^{-/-} (C57BL/6)	DENV2 Eden	Intraperitoneal		Lethality, thrombocytopenia, platelet activation	[95]
<i>Ifnar1</i> ^{-/-} (C57BL/6)	DENV2 NGC	Intravenous		Weight loss, cytokines, vascular leakage, splenomegaly,	[110]
<i>Ifnar1</i> ^{-/-} (C57BL/6)	DENV2 D220	Intravenous	Pre-administration of 4G2 IP	Morbidity, mortality, sialic acid, NS1; Primary PL046 infection exhibits homologous protection	[67, 111]
Cd11c-Cre ⁺ <i>Ifnar1</i> ^{fl/fl} (C57BL/6)	DENV2 D2Y98P-PP1	Intraperitoneal		Viremia, neutralizing titers, weight loss	[71]
CD11c-Cre ⁺ <i>Ifnar1</i> ^{fl/fl} and LysM-Cre ⁺ <i>Ifnar1</i> ^{fl/fl} (C57BL/6)	DENV2 D2Y98P	Intraperitoneal		Viremia	[70]

For readouts, viral load includes measurements in spleen, liver, brain, kidney, lymph nodes; cytokines are measured in serum and peripheral blood cells using qRT-PCR and ELISA; vascular leakage is determined using an Evans blue dye assay; morbidity scores include lethargy, fur ruffling, and animal hunching.