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Mouse models of dengue virus infection for vaccine testing

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Abstract

Dengue is a mosquito-borne disease caused by four serologically and genetically related viruses termed DENV-1 to DENV-4. With an annual global burden of approximately 390 million infections occurring in the tropics and subtropics worldwide, an effective vaccine to combat dengue is urgently needed. Historically, a major impediment to dengue research has been development of a suitable small animal infection model that mimics the features of human illness in the absence of neurologic disease that was the hallmark of earlier mouse models. Recent advances in immunocompromised murine infection models have resulted in development of lethal DENV-2, DENV-3 and DENV-4 models in AG129 mice that are deficient in both the Interferon-α/β receptor (IFN-α/β R) and the interferon-γ receptor (IFN-γR). These models mimic many hallmark features of dengue disease in humans, such as viremia, thrombocytopenia, vascular leakage, and cytokine storm. Importantly AG129 mice develop lethal, acute, disseminated infection with systemic viral loads, which is characteristic of typical dengue illness. Infected AG129 mice generate an antibody response to DENV, and antibody-dependent enhancement (ADE) models have been established by both passive and maternal transfer of DENV-immune sera. Several steps have been taken to refine DENV mouse models. Viruses generated by peripheral *in vivo* passages incur substitutions that provide a virulent phenotype using smaller inocula. Because IFN signaling has a major role in immunity to DENV, mice that generate a cellular immune response are desired, but striking the balance between susceptibility to DENV and intact immunity is complicated. Great strides have been made using single-deficient IFN-α/βR

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mice for DENV-2 infection, and conditional knockdowns may offer additional approaches to provide a panoramic view that includes viral virulence and host immunity. Ultimately, the DENV AG129 mouse models result in reproducible lethality and offer multiple disease parameters to evaluate protection by candidate vaccines.

Keywords

dengue; vaccine; mouse model; Interferon receptor

1. INTRODUCTION

1.1. Background

Dengue is a mosquito-borne disease caused by four serologically and genetically related viruses termed DENV-1 to DENV-4, each of which is a distinct species in the genus *Flavivirus*, family *Flaviviridae*. The positive-sense RNA genome encodes a single polyprotein that is post-translationally processed into ten distinct proteins: three structural proteins (capsid, premembrane/membrane (prM/M) and envelope (E)) that are present in the mature virion and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) that are only expressed during viral replication[1][2]. The envelope (E) protein comprises the majority of the mature viral surface and contains most of the epitopes eliciting neutralizing antibodies[3][4]. Clinical symptoms of primary infection with one DENV include a self-limiting acute febrile illness with headache, myalgia, or rash. Recovery is accompanied by the development of long-term homotypic immunity. However, cross-protective, heterotypic immunity is short-lived, so the individual remains susceptible to secondary infection by one or more of the other three DENVs[5]. Antibody dependent enhancement (ADE) and T cell antigenic sin are potential complications of secondary infections because the strong homotypic immunity generated against the first DENV is only weakly heterotypic towards a second DENV[4][6]. Presentations of severe dengue include dengue hemorrhagic fever (DHF) characterized by thrombocytopenia, gastrointestinal bleeding, and plasma leakage, and dengue shock syndrome (DSS) which is characterized by DHF symptoms accompanied by hypovolemic shock[7][8]. Despite many advancements in dengue research, a vaccine to combat disease has not been licensed. Significantly, vaccines must protect against the four DENVs simultaneously to avoid the potential complications from immune enhancement.

1.2. Necessity for a suitable model

In nature the only vertebrate hosts for DENVs are primates. Thus, it is no surprise that the inability to appropriately model human dengue disease in animals has been a major obstacle to the development of vaccines and therapeutics. DENV has two natural transmission cycles; the urban cycle which involves *Aedes* mosquitoes and humans, while sylvatic (jungle) transmission occurs between mosquitoes and monkeys[9]. Generally, non-human primates (NHP)have been used to study dengue infection and vaccine candidates because they develop acute viremia and mount a strong neutralizing antibody response after challenge[10][11]. These models continue to improve[12], but the majority of the studies have not resulted

in severe clinical outcomes or lethality [13][14][15]. Additionally, NHP models are not suitable for early stage preclinical testing because of the associated high cost and limited animal availability. Mouse models of severe dengue could provide a stringent platform to evaluate efficacy because a vaccine that can protect from lethal disease should also prevent milder infections. The development of reproducible, lethal DENV infection models that include hallmarks of severe dengue disease provides the opportunity to expand preclinical testing to include a spectrum of clinically relevant parameters that have been impossible to assess in past decades.

2. MOUSE MODELS

2.1. Immunocompetent mice

Initial attempts to establish small animal models of dengue involved the traditional approach of adaptation of human virus isolates by passage in suckling-mouse brain. Virus was inoculated intracranially and harvested from the brains to prepare the suspension for the subsequent passage. This propagation method resulted in a neurological disease phenotype that is unlike the multi-organ involvement typically observed in clinical dengue infections. Additionally, as the viruses became adapted and more virulent in mice, there was a concurrent attenuation in the ability to cause human disease [16][17]. Comparisons between parent and derivative strains have led to the identification of neurovirulence determinants [18][19][20]. Subsequent models have been established by intraperitoneal inoculation of mouse-brain-adapted DENV strains. The regimen used in these studies generated strains that did not cause neurological manifestations of disease, instead the mice developed clinical signs of human disease, such as viremia, thrombocytopenia, systemic cytokine responses, and, in some cases, lethality [21], [22]. DENV-2 strain 16681, a 1967 Thai human isolate passaged in mosquito cell culture, can cause hemorrhage when inoculated at 10^9 PFU into 4–5 week old C57BL/6 mice [23]; however, results were widely varied with some mice developing additional signs of dengue. Although some DENV strains do induce a limited viremia in some mouse strains, the overwhelming majority of immunocompetent mouse models do not result in clinical signs of dengue infection. Their use requires careful interpretation of results because their characteristics may differ greatly from the naturally-acquired strains encountered in the wild.

2.2. Humanized mice

Humanized mice provide a way to circumvent the limited species tropism of DENV. Initial experiments involved infection of severe combined immunodeficient (SCID) mice (lacking B and T lymphocytes) after reconstitution with immortalized human cell lines or peripheral blood lymphocytes. Generally, the virus was inoculated into the engrafted site (intraperitoneal or intratumor) and outcomes ranged from viremia to paralysis [24][25][26]. Non-obese diabetic (NOD) / SCID mice xenografted with human $CD34^+$ progenitor cells (14% median engraftment) were infected subcutaneously with a 1995 Thai human isolate of DENV-2, strain K0049, and developed clinical signs of human disease such as fever, erythema, and thrombocytopenia, but no animals died following DENV infection. [27]. In a subsequent study the model was improved to 52% median engraftment by using NOD/SCID/IL2rγ^{null} mice, and two of eight mice became seropositive by four weeks post-

infection [28]. These mice experienced a sustained viremia for three weeks post-infection [29], and human cytokine response peaked after eight days [30]. When the route of inoculation was changed to multiple mosquito bites (4–5) per mouse, viremia was sustained for 52 days post-infection, and there was a dramatic increase in the number of mice developing anti-DENV antibodies (14 of 32 mice sampled at days 4–30 post infection)[30].

Another approach used to improve engraftment was based on utilization of reconstituted RAG2$^{-/-}\gamma c^{-/-}$ mice (lacking T-, B-, and NK cells). Infection with DENV-2 16681, a lab-adapted strain, led to an anti-dengue response in 63% (10/16)of mice, and three of the ten mice had neutralizing titers against DENV-2 [31]. Further, NOD/SCID mice implanted with human fetal thymus and liver then irradiated and transplanted with human CD34^{+} cells, or BLT-NOD/SCID, had higher than 50% engraftment[32]. Intravenous inoculation of DENV-2, Colombia 36298, a non-adapted strain resulted in weight loss, clinical signs of illness, as well as detectable viremia, secreted NS1 protein, and human cytokines, but no fever or thrombocytopenia. DENV-specific antibody response was detected in 30% of the mice after 25 days, but only a few had detectable neutralizing titers.

Overall, the DENV-2-infected humanized mice show several clinical signs of human disease: viremia, thrombocytopenia, and a cytokine response, but prolonged periods of mouse viremia are not consistent with human infections, and infections were not lethal. Thus, there are several limitations to the utilization of humanized mice for early stage preclinical testing, including the highly technical process required to generate large numbers of animals with consistently high levels of engraftment, and the variability introduced by differences in mouse strain, the cells used for engraftment, and the virus type[33]. Although the correlates of protection to DENV infection are poorly understood, neutralizing antibody responses are an important measurement in DENV infection models. Humanized mice have generally low seroconversion and neutralizing response, thus they are less suitable for vaccine studies wherein the evaluation of long-term immunity is a priority.

It is worth noting that the BLT-NOD/SCID mice were used as a model to test the ability of an antiviral to reduce viremia[32]. This suggests an increased potential for using humanized mice in antiviral assays, as opposed to testing vaccines in these models for which a robust adaptive response to the virus is required [34].

2.3. Immunocompromised mice

Because wild type (WT) out bred and inbred mice were largely resistant to dengue infection, immunocompromised mice were tested. Athymicnude mice, nu/nu and nu/+ were partially susceptible (60%-40%) to infection by mouse-adapted DENV-1 strain Mochizuki. Some infected animals developed paralysis without detectable viremia, but others remained asymptomatic, although they developed antibody responses [35].

Interferon (IFN) signaling plays a principal role in protection against dengue disease in humans (recently reviewed in [36]) Therefore it was hypothesized that mice without a complete IFN response should be sensitive to DENV infection. A ground-breaking study found that mice deficient in IFN-α/β and IFN-γ receptors (AG129) (Table 1) were indeed susceptible to DENV-2 infection[37]. Adult AG129 mice inoculated intraperitoneally with

10^6 PFU of DENV-2 New Guinea C (NGC), a mouse brain-adapted strain, had systemic infection with detectable viral loads in the serum and spleen that peaked at 3 days post-infection (dpi) then subsided, brain viral titers steadily increased until the mice developed neurological disease at 7–12 dpi. Importantly, immunization with non-lethal DENV-2 strains, 16681 and alive attenuated vaccine derivative, PDK-53, led to the generation of neutralizing antibodies and protected AG129 mice against lethal challenge with NGC. These results were a dramatic improvement on the other contemporaneous DENV infection models with a lethal infection by a DENV-2 strain and the ability of a candidate live attenuated vaccine strain to induce a protective immune response against subsequent DENV-2 challenge [38][39].

2.3.1 DENV-2 models

2.3.1.1 Mouse-adapted DENV-2 models: In order to generate a mouse-adapted DENV-2 that would result in systemic disease, the mouse neurotropic strain PL046 was passaged to generate strain D2S10[40] (Table 2). AG129 mice inoculated intravenously with 10^7 PFU D2S10 developed non-neurotropic lethal dengue infection with hallmarks of dengue disease in humans. In contrast, similar or higher doses of PL046 in AG129 mice resulted in paralysis between 2 to 4 weeks post-infection[41][40]. Lethal D2S10 infection was accompanied by high serum and tissue viral loads, histopathology of the liver, spleen, and intestine, and vascular leakage[40]. Also, tumor necrosis factor (TNF)-α production increased as the infection progressed, and anti-TNF-α antibodies delayed mouse mortality, implicating a major role for the cytokine in the mechanism of D2S10 lethality. At lower doses, D2S10 ($<10^7$ PFU) caused neurological disease in the mice following a prolonged disease course[40]

The adaptation protocol utilized to generate D2S10 from PL046 resulted in mutations throughout the genome, and coding changes on a surface-exposed basic patch of the viral E protein were implicated in virulence[40][42]. In an infectious clone system, two substitutions of the PL046 E protein, N124D and K128E, were sufficient to reproduce disseminated disease, including vascular leakage and TNF-α elevation[42]. The negative charge on the E protein decreases binding to heparan sulfate on host cells, which in turn, decreases viral clearance by the host [42]. A major concern of the D2S10 model in AG129 mice is the high inoculum required to achieve lethality and the immune deficiency. Thus, additional DENV-2 strains were generated (Table 2)that increased the virulence phenotype and resulted in a similar disease as D2S10 but using lower doses (Table 3)[43], [44][45]. Interestingly, both D220 and D2S20 (Table 2) encode the K122I substitution, further confirming the importance of this region of the E protein for *in vivo* virulence in mice[46], [42].

Another concern with the D2S10-AG129 model is the immune status of the mice. AG129 mice are the most sensitive to DENV, but mice deficient in only the IFN-α/β receptor (A129 and $IFNAR^{-/-}$) have increased susceptibility to infection when more adapted strains or higher inocula are used (Table 3) [43], [47], [45]. In contrast, G129 mice (IFN-γ receptor deficient) are largely resistant to DENVs[41]. Interestingly, sublethal inoculation of DENV-2 S221 into A129 mice does not cause any disease, but the same dose into AG129 mice led all

mice surviving past 10 dpi to develop neurotropic illness[47]. The putative mechanism for this difference is that A129 mice have intact IFN-γ receptor signaling that reduces viral replication during early infection, then aids in IFN-γ $CD8^+$ T cell clearance of virus from the CNS [47].

The antiviral innate immune response in humans involves intracellular pattern recognition receptors, such as Toll-like receptors (TLR) 3 and 7 and the DExD/H box RNA helicases, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation–associated gene 5 (MDA5) sense viral RNA. RIG-I and MDA5 interact with mitochondrial antiviral signaling protein (MAVS) and stimulator of interferon genes (STING), which ultimately results in nuclear translocation of Interferon regulatory factor (IRF)3 and IRF7to activate IFN-α/β genes[36]. To activate pro-inflammatory gene expression by NFκB, IFN-α/β signals through its receptor, activating the Janus kinase (JAK) / Signal transducer and activator of transcription factor (STAT) pathway.

The contribution of innate immune mediators involved in IFN signaling was examined for DENV-2 infection (Table 1 and Table 3). Infection with DENV-2 S221 showed that neither single-deficient $IRF3^{-/-}$ or $IRF7^{-/-}$ mice, nor double deficient $IRF3^{-/-}7^{-/-}$ mice are succumb to infection with S221. $IRF3^{-/-}7^{-/-}$ mice supported high initial levels of virus replication in serum and tissues, but titers decreased after 2 dpi[48]. $MAVS^{-/-}$ mice also survive infection, yet virus replicates to the same level as it does in $IFNAR^{-/-}$ mice for the initial 18 hours post-infection [44]. Therefore, mice lacking MAVS, and/or IRF3 and IRF7, permitted initial virus replication that was controlled within 2 days. Interestingly, the single deficient $STAT1^{-/-}$, double deficient $STAT1^{-/-}2^{-/-}$, and double deficient $STAT1^{-/-}IFNAR^{-/-}$ mice are all susceptible to infection, but not $STAT2^{-/-}$ or $STAT1^{-/-}$IFN-$\gamma R^{-/-}$ receptor mice [49], underscoring the importance of IFN-α/β protection against DENV infection. These studies showed that absence of STAT2, IFN-γR, IRF3, IRF7, or MAVS was tolerated by mice, and infection was ultimately controlled. Recently, species-specific differences in the ability of DENV-2 to interact with the human and mouse immune systems have been discovered and help to contextualize some of the results obtained with mice deficient in innate immune factors [50][51]. Ultimately, a mouse strain with a single-deficiency is being sought after for modeling infection, and these studies narrow the possibilities to either IFN-$\alpha/\beta^{-/-}$ or $STAT1^{-/-}$.

Cell-mediated immunity studies have been conducted using human leukocyte antigen (HLA) transgenic mice backcrossed to $IFNAR^{-/-}$ mice[52]. One week post-infection with 10^{10}genome equivalents (GE) of S221 or mouse adapted DENV-3 D3S5CX, mice were euthanized, and splenic T cells screened. DENV-2 and DENV-3$CD8^+$T cell epitopes were distributed among all of the DENV proteins but most mapped to the nonstructural proteins (97% and 67%, respectively)[52]. Specifically most DENV-2 responses were for epitopes in the NS3/NS5 proteins, and DENV-3 in the NS1/NS3 proteins. Furthermore, 32 of 42 DENV-2 epitopes and 34 of 50 DENV-3 epitopes in mice were identified as human T cell epitopes, demonstrating that results obtained with the HLA transgenic/$IFNAR^{-/-}$ model are faithful to the human T cell response to DENV. Lastly, homologous (DENV2/2) secondary infection generated DENV-2-specific T cell epitopes. However, heterologous infection (DENV2/3) resulted in a repertoire composed of mostly DENV cross-reactive epitopes, with

decreased DENV-2-specific and increased DENV-3-specific epitopes. The implications of these studies are that heterologous DENV infections result in an increased cross-reactive epitope repertoire instead of serotype-specific T cell responses [53]. This model may help to elucidate why T cell responses are insufficient to combat a secondary (heterologous) infection.

ADE of DENV-2 infection occurs readily in AG129, A129, and IFNAR$^{-/-}$ mice[43], [54], [55][56]. Passive administration of DENV cross-reactive monoclonal antibodies or DENV-immune mouse sera prior to infection leads to DHF/DSS-like disease, characterized by increased cytokines, elevated viremia, elevated hematocrit, thrombocytopenia, vascular leakage, gastrointestinal bleeding, and TNF-α involvement[54],[55]. Antibodies with homologous activity exhibit both protective and enhancing properties; depending on the amount administered, which supports *in vitro* studies [4][57], and ADE is not observed in mice pretreated with antibody lacking the Fc region[55].. Importantly, enhanced and non-enhanced infections of AG129 are similar; the major differences between the two models are the increased viremia and decreased inoculum required to achieve lethality during ADE(approximately 100-fold less virus)[55], which indicates that severity of mouse ADE disease is correlated to viral loads, a finding similar to human infections, which have higher viremia and NS1 levels during DHF [58].

2.3.1.2 Non-mouse-adapted DENV-2 models: In addition to the PL046-derived DENV-2 strains, the strainD2Y98Pis virulent in AG129 mice (Table 2), and has a broad range of activity, causing lethal infection with as little as 10^4 PFU, although acute mortality (within 10 days) requires 10^6 PFU (Table 3)[59]. Following intraperitoneal infection mice develop systemic viral loads, increased liver enzymes, vascular leakage, damage to the intestine, liver and spleen antibody responses, and cytokine storm, but no thrombocytopenia. Interestingly, the molecular determinant of virulence for D2Y98P maps to the NS4B protein (This is contrast to the DENV-2 PL046-derived strains, which gain virulence by substitution of the E protein [see above]). A single amino acid (NS4B L52F) is sufficient to encode disease in a non-virulent DENV-2 strain [60]. Furthermore, the mechanism of action for viremia occurs through increased RNA synthesis in mammalian cells, not by IFN-α/β antagonism[60]. Subcutaneous infection of AG129 mice with the double plaque-purified D2Y98P-PP1 clone lowered the acute lethal inoculum to 10^5 PFU [61]. In atherapeutic study of D2Y98-PP1 disease, infected AG129 mice that were administered with hypertonic saline had improved clinical signs. This experiment shows supportive therapy delays mortality by only three days and provides a transient control of vascular leakage [62]. More importantly, it highlights the value of a uniformly reproducible animal model that permits statistically significant measurements of outcomes.

Maternal antibody transfer has been implicated as a cause of enhanced disease of infants[63] [4][64]. Consequently, AG129 mice were used to develop an *in vivo* ADE model wherein the offspring acquire cross-reactive antibodies from their mothers[65]. Female AG129 mice were infected with a non-lethal strain of DENV-1, resulting in asymptomatic infection with the development of anti-DENV-1 antibodies. After viral clearance (7 dpi), the female mice were bred with DENV-naïve male mice, and their 5-week-old offspring were infected with D2Y98P-PP1. D2Y98P-PP1-infected mice born to DENV-1-immune mothers had increased

disease and mortality compared to D2Y98P-PP1-infected mice born to naïve mothers. This model of ADE uses more physiological levels of enhancing antibodies, as they are naturally acquired.

In order to establish a dengue mouse model that could elicit protective immune responses, conditional deletions of the IFN-α/βR in dendritic cells ($CD11^+$) only, macrophages ($LysM^+$) only, both $CD11c^+$ and $LysM^+$, and $CD4^+$ T cells were infected with TSV01, an attenuated DENV-2 strain, or D2Y98P and compared to infection of $IFNAR^{-/-}$ and AG129 mice [66]. DENV-2 infection of WT or CD4-$Cre^{+/-}IFNAR^{fl/fl}$ mice did not result in disease; whereas, the double conditional mutant LysM-$Cre^{+/-}$CD11c-$Cre^{+/-}IFNAR^{fl/fl}$ mice were as sensitive as $IFNAR^{-/-}$ mice, succumbed by day 5, had high tissue virus titers, and elevated cytokines [66]. The single conditional mutants LysM-$Cre^{+/-}$, $IFNAR^{fl/fl}$, and CD11c-$Cre^{+/-}$ $IFNAR^{fl/fl}$ had intermediate phenotypes consisting of 25–75% mortality, severe weight loss that was recovered, systemic disease, systemic viral loads, cytokine induction, and generated IFN-γ^+ $CD8^+$ cells. Therefore the conditional mutant models provide for the study of severe DENV infection in mice that mount a $CD8^+$ response.

Ultimately, D2Y98P and PL046-derived infection models in immunocompromised mice are lethal and lead to similar signs of disease; however, the mechanisms of infection and doses required to attain mortality are different.

2.3.2. DENV-3 model in AG129 mice—We recently described a lethal DENV-3 infection model in AG129 mice using C0360/94, a Thai human isolate from 1994[67][68]. Unlike the models described for DENV-2, and previous DENV-3 mouse models[69], [70] [71][40], C0360/94 was neither adapted nor extensively passaged prior to intraperitoneal inoculation of 6–8 week-old AG129 mice, and C0360/94-infected mice do not display any signs of neurological disease, even after infection with a sublethal inoculum[67]. The animals exhibit thrombocytopenia, hunched posture, limited mobility, and severe weight loss resulting in lethality which generally occurson day 4. The mice have reactive spleen phenotypes, splenomegaly, and high splenic viral titers throughout the course of infection. In the liver, mice display signs of activation, necrosis, and a severe glycogen depletion which may be linked to the significant weight loss. Also, vascular leakage and viral loads in the liver increase during the course of infection. Lastly, vascular leakage and viral loads in the intestine are delayed until 2 dpi. Infected mice undergo cytokine storm and a TNF-α-mediated disease that is completely reversed upon treatment with anti-TNF-α. This differs from DENV-2 models, for which anti-TNF-α treatment only extended the survival times. Additionally, 12- and 18-week old mice retain susceptibility to C0360/94, and any mice (6–18 weeks) that survive infection develop neutralizing antibody titers within four weeks, suggesting that C0360/94 infection of AG129 is suitable for vaccine immunization and subsequent challenge studies that require longer time periods to complete.

2.3.3. DENV-4 models in AG129 mice—Soon after developing the AG129 mouse model of DENV-3 C0360/94, we detailed characterization of a virulent DENV-4 model using AG129 mice. Intraperitoneal inoculation of the non-adapted DENV-4 703–4, 1994 Thai isolate[72][73], produces an acute, disseminated disease in AG129 mice that is lethal beginning within four days[74]. As with C0360/94, AG129 mice infected with 703–4

develop thrombocytopenia, vascular leakage, organ damage in the liver and spleen, and a cytokine storm. Systemic viral loads are high at all days after infection and increase significantly in the liver, spleen, and serum from 1 to 3 dpi; whereas, the brains have low-level infection on all days [74]. Similar to studies with C0360/94 infection, both 6- and 18-week-old mice are highly susceptible to 703–4 demonstrating that this model has utility for evaluation of vaccine candidates. Sublethal infection with 703–4 does not lead to neurological disease, and pre-administration of DENV-2 immune serum leads to ADE with 100% mortality[74].

We have also reported a second DENV-4 strain, TVP-376, that causes a very similar lethal disease to strain 703–4 (in AG129 mice) with systemic viral loads, elevated cytokines, and histopathology[75]. Additionally a side-by-side comparison of infection by DENV-4 TVP-376 and DENV-2 D2S10 using the same route of infection and dose was conducted. IFNα/βR and IFNγR deficient mice in both 129 and C57BL/6 genetic backgrounds (AG129, AGB6, respectively) exhibited comparable susceptibility to both viruses, although the lethal doses in AGB6 were slightly different. $MAVS^{-/-}$ mice survived infection with either virus, and minor differences in viremia loads between the two viruses were detected (Table 1 and Table 3). In AG129 mice, a lethal dose of TVP-376 followed by administration of anti-TNFα resulted in a slight prolonging of survival[75]. Also, a small number of mice in the study that survived infection exhibited signs of neurological disease, which was reported for D2S10 infection [40]. The most striking difference in virulence was observed in A129 mice, which succumbed to D2S10 infection but survived infection with DENV-4 TVP-376; the latter led to short-term weight loss and viremia, but no other physical signs of illness were observed. These results showed that DENV-2 and DENV-4 strains can have different outcomes in the same mouse strain.

2.3.4 Tropism of DENV in AG129 and A129 mice—DENV is often found in human cells of the myeloid lineage in spleen, lymph nodes, and liver samples [76][77][78], but lymphocytes have also been identified in some studies [79]. Although DENV replication can be detected in Kupffer cells, endothelial cells and hepatocytes, the results are not consistent. Autopsy samples of DHF patients in Myanmar, contained DENV in Kupffer cells and hepatocytes; whereas, autopsies from patients in Ecuador had DENV-positive hepatocytes but not Kupffer cells[76], [77]. The differences detected in human tissues and cellular tropism of DENV may be due to the use of different methods and the limited availability of opportunities for examination of autopsy and tissue biopsy samples [80].

DENV-2 tropism has been examined in the AG129 and A129 mouse models. During early infection, following subcutaneous inoculation of 10^6 PFU, D2S10 virus replicates in dendritic cells and macrophages of hematopoietic tissues: spleen, lymph nodes, bone marrow, and peripheral leukocytes [81]. Also, during primary infection with DENV-2, NS3 antigen is detected in dendritic cells and macrophages of spleen and lymph nodes, as well as in hepatocytes near the central vein of AG129 mice [76]. More detailed time course experiments showed a biphasic phenotype of DENV-2 multiplication. The first wave targets the spleen and bone marrow (trailed by lymph nodes). When replication in these lymphoid organs starts to subside, the second wave begins with increased multiplication in the liver,

kidney, and heart [82]. Furthermore, during ADE of DENV-2 in AG129 mice, the liver sinusoidal epithelial cells are a major target for replication [54].

Limited tropism information is available for the other DENVs in mice. In AG129 mice, the NS3 of DENV-3C0360/94 was detected in Kupffer cells in the liver, but not in hepatocytes [67]. Furthermore, DENV-4 TVP-376 NS1 and E protein were detected in Kupffer cells. Evaluation of spleen sections showed that early after infection DENV-4 TVP-376 NS1 was localized to the areas surrounding the follicles but became distributed throughout the entire spleen by 3 dpi[75]. A benefit of the AG129 and A129 mouse models is the ability to investigate virus replication, tropism and kinetics[81], [54][82][76].

2.3.5. Summary of AG129 challenge strains—Currently there is no non-adapted murine infection model for DENV-1. Most of the mouse challenge studies use the DENV-1 strains Mochizuki or Western Pacific 74 [17][83]. Partially due to the dates of their isolation, the passage and adaptation histories of these two strains are long and vary among the different studies. AG129 lethality caused by DENV-2 PL046-derived strains, DENV-2 strain D2Y98P, DENV-3 strain C0360/94, and DENV-4 strains 703–4 and TVP-376 share several features of human-like disease that can serve as relevant outcomes for vaccine testing, such as viremia, vascular leakage, and cytokine response. As new studies are completed, differences in the phenotypes of the various models are emerging. Currently, much is known regarding the virulence phenotype of DENV-2 PL046-derived strains. Their ability to cause paralysis in AG129, but not in A129 mice, is due to the absence of protective $CD8^+$ T cells to clear infection and prevent replication in the spleen [47]. On the other hand the lack of neurologic disease during sublethal infection of AG129 mice by D2Y98P, C0360/94, or 703–4 strains[59][67][74] suggests an additional role of virus-specific neurovirulence that is retained in PL046-derived strains after passaging. Taken together, these data serve as an example that the combination of the specific mouse strain and the specific virus strain used for infection both contribute to the outcome of the mouse model and underscore the importance of additional studies with existing and future models to elucidate mechanisms of disease.

3. VACCINE TESTING IN DENGUE MOUSE MODELS

Since the discovery that AG129 mice supported systemic DENV infection, vaccines, antivirals, and supportive therapies have been examined in the models [34] [84] [85] [86] [15]. Recent studies with candidate vaccines have shown the strengths and weaknesses of testing vaccines in the AG129 mouse model.

3.1. Immunogenicity and challenge of live-attenuated vaccines

The tetravalent DENV vaccine candidate (TDV) formulation is based on the attenuated DENV-2 PDK-53 strain backbone combined with chimeras into which the prM/E region of DENV-1, −3, and −4 have been substituted. Monovalent formulations for each serotype (TDV-1, TDV-2, TDV-3, and TDV-4) have been tested in AG129 mice[87]. Four weeks after a single immunization with monovalent TDV-1, TDV-2, TDV-3, or TDV-4all mice survived lethal challenge with a mouse-brain adapted DENV-1 strain Mochizuki, but control mice succumbed to infection. When monovalent-immunized mice were challenged with a lethal

dose of neurotropic DENV-2 New Guinea C strain, only monovalent TDV-1 and TDV-2-immunized mice exhibited complete protection, TDV-3 led to 80% protection, and TDV-4 had no protection from lethality, although the survival of animals was prolonged[88]. Immunization with tetravalent TDV formulations also resulted in protection from DENV-1 Mochizuki or DENV-2 New Guinea C.,

TDV was recently tested against lethal challenge with the disseminated DENV-4 703–4 model in AG129 mice [89]. Mice immunized with a single dose of TDV, monovalent TDV-4 or monovalent TDV-2 survived lethal challenge, but all control mice succumbed to infection by day 8. Of note, TDV, monovalent TDV-2, and monovalent TDV-4 -immunized mice had viremia that decreased by 3 days post-challenge, showing that the immunizationprotects AG129 mice against development of dengue disease but not from infection. Additionally, pooled sera from immunized mice collected on day 56 had neutralizing titers to a panel of wild-type DENV-4 strains. Furthermore, splenocytes from TDV-immune mice restimulated *in vitro* and $CD4^+$ T cell responses to the DENV-4 NS3were detected. Together, the results from TDV in AG129 mice indicate that both antibody and cellular responses can be elicited in AG129 mice by a candidate live-attenuated vaccine[89].

Live attenuated mutants in the 2'-O-methyltransferase (2'-O-MTase) of DENV-1 and DENV-2 have also been evaluated as vaccinesin AG129 and IFNAR mice [90]. Infection of AG129 mice with the mutants, based on substitutions in the catalytic tetrad of the 2'-O-MTase, led to reduced viremia when compared to their respective parent virusesDENV-1 WestPac and DENV-2 TSV01. AG129 mice that were immunized with the attenuated 2'-O-MTase DENV-1 or DENV-2 viruses developed neutralizing antibodies after 30 days. Further, mice were protected from homologous infection challenge with parent DENV-1 or DENV-2 viruses. Also, AG129 mice that were administered monovalent or divalent immunization then challenged with heterologous infection did not develop enhanced infection. Furthermore, attenuated mutants protected mice against death and increased induction of TNF-α following highly lethal challenge with 10^7 PFU D2Y98P. Additionally, immunized $IFNAR^{-/-}$ mice were used to study cell-mediated responses, and spleens were restimulated *in vitro* with DENV-2. Attenuated mutant and parent viruses elicited similar $CD8^+$T cell responses, and $CD4^+$ responses were greater following stimulation with parent DENV-2. These results show that mutant 2'-O-MTase is attenuated in AG129 mice, immunization does not lead to enhancement of infection, and that immunization elicits a cellular immune response.

3.2. Immunization with a replicon-based vaccine

A Venezuelan equine encephalitis (VEE)-viral replicon vaccine expressing the E protein ectodomain (E85-VRP) of DENV-1, −2, −3, and −4 has been studied in NHP and was evaluated in AG129 mice against challenge with $10^{8.7}$ GE of DENV-2 S221, a low dose that leads to liver titers and paralysis but can also be used to model ADE (after monoclonal antibody treatment)[91]. Two monovalent immunizations with DENV-2 E85-VRP protected 80% of mice against DENV-2 challenge, significantly reduced liver viral loads, and led to induction of neutralizing antibodies. However, passive transfer of immune serum led to higher liver titers than transfer of naïve sera. DENV-2 E85-VRP-immunized mice depleted

of $CD4^+$ cells had no change in titers after challenge, but $CD8^+$-depleted immunized mice had a significant increase in liver viral loads[91]. Also, lower cytokine levels were present in DENV-2 E85-VRP-immunized mice and $CD4^+$-depleted mice, but cytokine levels of $CD8^+$-depleted mice were similar to those of naïve challenged mice. Changing the immunization dosing schedule from days −14/−5 to days −42/−33 resulted in reduction of liver viral titers post virus challenge, but not the titer of DENV-specific antibodies. It is worth noting that further changes in the immunization schedule to days −61/−33 led to no or lower virus detection following virus challenge[91]. Together, these results show that $CD8^+$ immunity contributes toE85-VRP protection and that lengthening the immunization schedule diminishes the importance of $CD8^+$ immunity.

3.3. Infection with inactivated virus

An aluminum hydroxide-adjuvanted preparation of UV-irradiated S221 (al-UV-DENV2) was used to prime AG129 mice at −14 and −5 days with 10^{11} GE prior to challenge with $10^{8.7}$ GE S221, which leads to paralysis, but is sensitive to ADE [92]. The study was designed so that the priming would lead to generation of a DENV-2 specific antibody response that was non-neutralizing. Indeed, both al-UV-DENV2 primed mice and naïve mice passively transferred with serum from al-UV-DENV2 primed mice had increased mortality and 3 dpi liver titers that mirrored ADE infection[92]. However, transfer of $CD8^+$ cells from S221-infected donors to al-UV-DENV2 primed or non-primed AG129 mice was protective (reduced liver titers and ADE). This study shows that only replicating virus, but not UV-inactivated virus, can induce a protective immune response [92].

3.4. Immunization with a subunit vaccine

Tetravalent dengue EDIIIC is a candidate subunit vaccine composed of recombinant chimeric capsid and E domain III fusion proteins for DENV-1, −2, −3, and −4 and was designed to include both T and B cell epitopes (capsid and E protein, respectively) in the same formulation[93]. Athree-dose immunization regimen of BALB/c and AG129 mice with adjuvanted Tetra DIIIC elicited antibodies specific to the four serotypes. In BALB/c mice, immunization with Tetra DIIIC or infection with DENV-2 results in similar neutralization titers (NT_{50}) (approximately 10-fold higher concentration than the NT_{50} of mice immunized with adjuvant alone). In AG129 mice DENV-2 infection, but not Tetra DIIIC immunization, elicited neutralizing antibody. Tetra DIIIC induced a Th1 response andIgG1 and IgG2a against DENV-1–4. These results show that, because IFN-γ is critical for protection for this vaccine candidate, AG129 did not produce neutralizing antibodies [93].

The EDIIIC-DENV-2 subunit vaccine was tested in the conditional $IFNAR^{-/-}$ mutants. $IFNAR^{-/-}$ mice and the single conditional mutants LysM-$Cre^{+/-}IFNAR^{fl/fl}$ and CD11c-$Cre^{+/-}IFNAR^{fl/fl}$ were immunized with three doses of candidate adjuvanted EDIIIC-DENV-2 subunit vaccine[66]. All three mouse strains produced the same levels of anti-DENV-2 IgG and had similar DENV-2 NT_{50}. Three days after challenge with 10^7 PFU D2Y98P, only the vaccinated CD11c-$Cre^{+/-}$ $IFNAR^{fl/fl}$ mice had a significant reduction in both viremia and mortality. Vaccinated LysM-$Cre^{+/-}IFNAR^{fl/fl}$ mice did not have statistically significantly different viremia, although it reduced approximately 10-fold compared to control; survival was slightly delayed (p=0.051). Vaccinated and placebo-

treated IFNAR$^{-/-}$ mice had similar viremia and although survival was delayed from 4 to 5 days, it was a significant difference[66]. Conditional CD11c-Cre$^{+/-}$ IFNAR$^{fl/fl}$ mice show promise for testing subunit vaccines because they generate a protective response; however, lethality by D2Y98P is not uniform. These results may suggest that the AG129 mouse model is not ideal to evaluate recombinant protein based vaccine candidates, but further work is needed in this area.

4. CONCLUSION

A decades-long deficiency in mouse models of dengue mimicking human disease was substantially met with the development of DENV-2 infection models in AG129 mice. Because vaccines need to protect against all DENV serotypes, the dengue research field has invested in developing mouse models of infection for all of the serotypes. The DENV-2-AG129 mouse models are the most advanced and lead to lethal, acute infection characterized by hallmarks of human illness, and the new DENV-3 and DENV-4 models share similar features. An advantage of these AG129 models is the uniformity of disease outcomes with high lethality, which provide a rigorous challenge of infection that is sensitive to small differences. A major development is the ability to model enhanced infection using passive transfer or naturally-acquired maternal antibodies in offspring mice, which is important because human infections are often severe in the infant population. Also, it is relevant to use low passage clinical isolates because they are genetically related to the endemic viruses causing infections.

There are several challenges posed by studying DENV vaccine candidates in the current mouse models. Most of the models rely on irrelevant routes of infection and high inocula. Also, the use of mouse-passaged strains is appealing because the adaptations can be tracked and the virulence mechanisms can be studied directly but do not mimic disease seen in humans. Lastly, the use of immunocompromised mice is a factor that affects the course of disease and evaluation of vaccines. Specifically, studies with live vaccines have shown that AG129 mice generate protective responses [88][89][90]. However, AG129 mice are lacking aspects of cellular immunity that appear to impact their ability to produce protection following immunization with a non-replicating vaccine [93]. An alternative approach is to use conditional IFNAR$^{-/-}$ knockdowns in dendritic cells or macrophages to study the immune response to a subunit vaccine [66]. Therefore the current evidence points to a combinatorial strategy to study both immunity and disease protection.

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Table 1

Immunocompromised mouse strains used to study DENV.

Mouse strain	Deficiency*	Outcome	References
AG129	IFNα/βR and IFNγR (129 background)	Mortality DENV-2, DENV-3, DENV-4	[40][67][74]
AGB6	IFNα/βR and IFNγR	Mortality DENV-2, DENV-4	[75]
A129	IFNα/βR (129 background)	Mortality DENV-2 Survival DENV-4	[47] [75]
G129	IFNγR in (129 background)	Survival DENV-2	[41]
IFNAR$^{-/-}$	IFNα/βR	Mortality DENV-2	[43]
Mavs$^{-/-}$	MAVS	Survival DENV-2, DENV-4	[44][75]
STAT1$^{-/-}$	STAT1	Mortality DENV-2	[49]
STAT2$^{-/-}$	STAT2	Survival DENV-2	[49]
STAT1$^{-/-}$2$^{-/-}$	STAT1 and STAT2	Mortality DENV-2	[49]
STAT1$^{-/-}$/AR	STAT1 and IFNα/βR	Mortality DENV-2	[49]
STAT1$^{-/-}$/GR	STAT1 and IFNγR	Survival DENV-2	[49]
IRF3$^{-/-}$	IRF3	Survival DENV-2	[48]
IRF7$^{-/-}$	IRF7	Survival DENV-2	[48]
IRF3$^{-/-}$7$^{-/-}$	IRF3 and IRF7	Survival DENV-2	[48]
HLAIIFNAR$^{-/-}$	HLA-A*0201/Kb, A*1101/Kb, A*0101, B*0702, or DRB1*0101 transgenics and IFNα/βR	Not evaluated for mortality#	[52]
LysM-Cre$^{+/-}$IFNAR$^{fl/fl}$	IFNα/βRonly in macrophages	Morbidity, some mortalityDENV-2	[66]
CD11c-Cre$^{+/-}$IFNAR$^{fl/fl}$	IFNα/βRonly in dendritic cells	Morbidity, some mortality DENV-2	[66]
LysM-Cre$^{+/-}$CD11c-Cre$^{+/-}$IFNAR$^{fl/fl}$	IFNα/βRonly in macrophages and dendritic cells	Mortality DENV-2	[66]
CD4-Cre$^{+/-}$IFNAR$^{fl/fl}$	IFNα/βRonly in CD4^{+} cells	Survival DENV-2	[66]

*All mouse strains backcrossed to C57BL/6 unless otherwise noted

#HLA*IFNAR$^{-/-}$ transgenic mice were sublethally infected and sacrificed to study immune responses.

Table 2

DENV-2 strains commonly used in mouse models

Virus	Description	Reference
D2Y98P	Singapore 1998. C6/36 mosquito cells p20	[59]
D2Y98P-PP1	Double plaque-purified clone of D2Y98P	[61]
PL046	Taiwan, neurotropic	[41]
D2S10	From PL046; ten alternate passages between C6/36 mosquito cells and AG129 mice i.v.	[40]
S221	From D2S10; triple plaque-purified clone	[44]
E124/128-IC	PL046 infectious clone with mouse adapted mutations	[42]
D220	From D2S10; ten alternate passages between C6/36 mosquito cells and AG129 mice s.c.	[43]
D2S20	From D2S10; ten alternate passages between C6/36 mosquito cells and AG129 mice i.v.	[45]

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Table 3

Viruses tested for acute, non-neurotropic lethality in immunocompromised mouse strains.

Serotype	Virus	Mouse	Mouse	Route	Outcome	Dose	Reference
DENV-2	D2Y98P	AG129	AG129	i.p.	Mortality	≥10^6 PFU	[59]
	D2Y98P-PP1	AG129	AG129	s.c.	Mortality	≥10^5 PFU	[61]
		AG129	AG129	s.c.	Mortality	ADE + 10^3 PFU	[65]
	D2S10	AG129	AG129	i.v.	Mortality	≥10^7 PFU	[40][55]
		AG129	AG129	i.v.	Mortality	ADE + 10^5–10^6PFU	[55]
		IFNAR$^{-/-}$	IFNAR$^{-/-}$	i.v.	Mortality (60%)	10^7 PFU	[43]
		IFNAR$^{-/-}$	IFNAR$^{-/-}$	i.v.	Mortality	ADE + 10^6–10^7 PFU	[43]
		AGB6	AGB6	i.p	Mortality	$10^{6.4}$ PFU	[75]
		A129	A129	i.p	Mortality	$10^{7.4}$ PFU	[75]
		Mavs$^{-/-}$	Mavs$^{-/-}$	i.p	Survival	$10^{7.5}$ PFU	[75]
	E124/128-IC	AG129	AG129	i.v.	Mortality	$10^{11.2}$ GE	[42]
	D220	IFNAR$^{-/-}$	IFNAR$^{-/-}$	i.v.	Mortality (60%)	10^7 PFU	[43]
		IFNAR$^{-/-}$	IFNAR$^{-/-}$	i.v.	Mortality	ADE + 10^5–10^6PFU	[43]
		A129	A129	i.v.	Mortality	10^7 PFU	[43]
		A129	A129	i.v.	Mortality	ADE + 10^5–10^6PFU	[43]
	S221	AG129	AG129	i.v.	Mortality	≥10^9 GE	[54], [49],[47]
		AG129	AG129	i.v.	Mortality	ADE + $10^{8.7}$ GE	[54]
		IFNAR$^{-/-}$	IFNAR$^{-/-}$	i.v.	Mortality	10^{12}GE	[44]
		A129	A129	i.v.	Mortality	10^{12} GE	[47]
		STAT1$^{-/-}$	STAT1$^{-/-}$	i.v.	Mortality	10^{12} GE	[49]
		STAT2$^{-/-}$	STAT2$^{s-/-}$	i.v.	Survival	≤10^{12} GE	[49]
		STAT1$^{-/-}$2$^{-/-}$	STAT1$^{-/-}$2$^{-/-}$	i.v.	Mortality	10^{10} GE	[49]
		STAT1$^{-/-}$/AR	STAT1$^{-/-}$/AR	i.v.	Mortality	10^{10} GE	[49]
		STAT1$^{-/-}$/GR	STAT1$^{-/-}$/GR	i.v.	Survival	≤10^{10} GE	[49]
		IRF3$^{-/-}$	IRF3$^{-/-}$	i.v.	Survival	≤10^{12}GE	[48]

Table 4

DENV mortality in AG129 mice for frequently used viruses.

Virus	Dose (log_{10} PFU)	*n*	% Mortality[#]	Median survival time (days)	LD_{50}[*] (log_{10} PFU)	Reference
D2S10	7.0	15	93	4.0	6.6	[55]
	6.0	8	0	---		
	5.0	11	0	---		
C0360/94	7.5	15	93	4.0	7.1	[67]
	7.0	2	50	28.5		
	6.5	8	0	---		
703–4	7.3	8	100	4.0	6.8	[74]
	6.3	8	0	---		
TVP-376	7.5	2	100	3.5	6.5	[75]
	7	10	100	5		
	6	8	0	---		
S221	5.6	4 to 7	100	n/a	4.7	[47]
	4.3	4 to 7	100	n/a		

[#]Percentages were calculated from published data in the referenced study.

[*]LD_{50} calculated using Spearman-Karber calculation from data provided in the referenced study, except S221 (published LD_{50}: 2.7×10^9 GE, converted to PFU)

n/a Data not available