Received: 25 August 2020 | Accepted: 1 December 2020

DOI: 10.1002/ame2.12145

ORIGINAL ARTICLE

WILEY

A murine model of dengue virus infection in suckling C57BL/6 and BALB/c mice

Alana B. Byrne[1,2] | Ayelén G. García[1] | Jorge M. Brahamian[1,2] | Aldana Mauri[1] | Adrián Ferretti[1] | Fernando P. Polack[1] | Laura B. Talarico[1,2]

[1]Fundación INFANT, Buenos Aires, Argentina

[2]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

Correspondence
Laura B. Talarico, Laboratorio de Investigaciones Infectológicas y Biología Molecular, Infectología, Departamento de Medicina, Hospital de Niños Ricardo Gutiérrez. Gallo 1330, Buenos Aires (1425), Argentina.
Email: ltalarico@conicet.gov.ar

Present address
Alana B. Byrne and Laura B. Talarico, Laboratorio de Investigaciones Infectológicas y Biología Molecular, Infectología, Departamento de Medicina, Hospital de Niños Ricardo Gutiérrez, Buenos Aires, Argentina
Ayelén G. García, Instituto Nacional de Enfermedades Infecciosas (INEI) - Administración Nacional de Laboratorios e Institutos de Salud (ANLIS) "Dr Carlos Malbrán", Buenos Aires, Argentina
Jorge M. Brahamian, Departamento de Química Biológica-IQUIBICEN (CONICET-UBA), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

Funding information
Consejo Nacional de Investigaciones Científicas y Técnicas, Grant/Award Number: PIP0118; UBS Optimus Foundation, Grant/Award Number: ID 2007-00206; Fundación Bunge y Born; Fondo para la Investigación Científica y Tecnológica, Grant/Award Number: PICT2008-1073

Abstract

Dengue is a significant public health concern across tropical and subtropical regions worldwide, principally causing disease in children. Very young children are at increased risk of severe manifestations of dengue infection. The mechanism of dengue disease in this population is not fully understood. In this study, we present a murine model of dengue virus primary infection in suckling C57BL/6 and BALB/c mice in order to investigate disease pathogenesis. Three-day-old C57BL/6 mice intraperitoneally infected with DENV-2 NGC were more susceptible to infection than BALB/c mice, showing increased liver enzymes, extended viremia, dissemination to organs and histological alterations in liver and small intestine. Furthermore, the immune response in DENV-infected C57BL/6 mice exhibited a marked Th1 bias compared to BALB/c mice. These findings highlight the possibility of establishing an immunocompetent mouse model of DENV-2 infection in suckling mice that reproduces certain signs of disease observed in humans and that could be used to further study age-related mechanisms of dengue pathogenesis.

KEYWORDS

BALB/c, C57BL/6, dengue virus, mouse model, suckling mice

1 | INTRODUCTION

Dengue virus (DENV) is the most prevalent arthropod-transmitted viral infection globally, infecting an estimated 390 million people,[1,2] and causing 50-100 million apparent cases[3] and 10,000 deaths annually.[4] Apparent cases have increased dramatically worldwide; more than doubling every decade since 1990,[5] a trend which may be exacerbated by the influence of climate change and urbanization on vector expansion in coming decades.[3,4] While infection with any of the four dengue serotypes (DENV 1-4) often causes subclinical disease,[6,7] dengue fever is a significant public health concern across tropical and subtropical regions.[5,8] Dengue disease is associated with high fever, myalgia and rash that may lead to potentially life-threatening severe dengue (SD) during defervescence, characterized by thrombocytopenia, capillary leakage, bleeding, increased liver enzymes and complications in multiple organs.[2,9] Furthermore, dengue disease has been associated with long-term disability.[5]

The most important factor for progression to SD is heterotypic secondary infection,[10,11] though severity is ultimately determined by an interplay of many factors, including viral serotype,[12,13] age,[14] host genetic,[15,16] and immune determinants, such as cross-reactive T lymphocytes[17,18] and serostatus.[19,20] This poses a major challenge to vaccine design, since an incomplete or sub-optimal immune response has the potential to prime the individual for enhanced disease upon reinfection.

Dengue affects people of all age groups, but in many parts of the world it is principally a pediatric public health problem.[21] Particularly, over the last 50 years, dengue incidence has increased at a higher rate in infants than in any other group.[22] Infants are at increased risk of dengue hemorrhagic manifestations and shock syndrome. The immaturity of infant's immune system may enhance susceptibility to severe dengue disease, as occurs with other severe infectious diseases that are uncommon at later ages.[23-25] Furthermore, maternally transferred antibodies have been also implicated in dengue disease severity.[26-28] Therefore, the complex pathogenesis of dengue disease during primary infection in infants represents a major challenge to understand age-related mechanisms of disease.

Mouse models of disease are fundamental to understanding viral pathogenesis, disease progression and for preclinical testing of vaccines and pharmaceuticals. Murine models have been a particular challenge for DENV; traditional mouse models of primary and secondary infection utilize mice deficient in or fully lacking interferon (IFN) or STAT receptors,[29] elements whose signaling are key to pathogenesis in humans and vaccine responses.[30,31] Newer models have recapitulated some aspects of disease in non-IFN-based immunodeficiencies or have used humanized mouse models.[29] Some aspects of primary infection, including enzymatic and biochemical alterations and tissue damage have been modeled in immunocompetent C57BL/6 mice infected with mouse adapted-DENV and some clinical isolates.[32-35] Another study documented increased vascular permeability and a platelet reduction in C57BL/6 mice infected with a DENV clinical isolate.[36,37] Because young children are uniquely vulnerable to DENV infections, there has been an effort to establish effective murine models of enhanced disease in young mice, often employing infection schemes that permit the study of maternally acquired antibodies.[38,39]

Here, we present a model establishing a baseline of detectable pathology after DENV primary infection in immunocompetent mice during the phase of exclusive suckling, with special consideration of technical solutions to age-specific issues in animal handling.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The animal protocols used in this study were reviewed and approved by Fundación INFANT Animal Care and Use Committee (IACUC) and were performed according to the guidelines of the INFANT IACUC.

2.2 | Cell lines and virus preparation

Vero (African green monkey kidney) cells were grown in MEM (GIBCO) supplemented with 5% fetal bovine serum (FBS). The C6/36 mosquito cell line from *Aedes albopictus*, adapted to grow at 33°C, was cultured in L-15 Medium (Leibovitz) (GIBCO) supplemented with 0.3% tryptose phosphate broth, 0.02% glutamine, 1% MEM non-essential amino acids solution and 5% FBS. DENV-2 strain NGC was grown in C6/36 cells for 4-6 days. Virus stock was titrated by plaque formation in Vero cells.

UV-inactivation of DENV-2 particles was performed on a Cross Linker XL-1500 using 5 cycles of irradiation at 3600 × 100 μJ/cm^2 dose of UV-light. Plaque assay was performed to ensure that the DENV particles were inactivated.

2.3 | Mouse strains and DENV infection

Five to six-week-old C57BL/6 and BALB/c breeders were obtained from Facultad de Ciencias Veterinarias, Universidad Nacional de la Plata, and housed in Fundación INFANT Animal Facility. Animals were housed and bred under specific pathogen-free conditions in individual ventilated cages, under 12/12 h of light/dark cycles, a temperature of 20-24°C, and a relative humidity of 40-60%. All experimental procedures were approved and were performed according to the guidelines of the INFANT IACUC. In all experiments four to six mice were used per treatment group. Both male and female mice were used in the treatment and control groups. At least two independent experiments were conducted.

Suckling (3-day-old) C57BL/6 and BALB/c mice were inoculated intraperitoneally (IP) with 5 × 10^5 PFU/g of DENV-2 NGC (in a maximum volume of 50 μL), or an equal volume of C6/36 cell supernatant (control group). In order to minimize inoculum volume loss and contaminant entry, an inverse IP administration technique was used. First animal immobilization was performed by

the nuchal fold, followed by thorax and abdomen antisepsis with a cotton swab containing a povidone-iodine solution. Inoculum incision was performed with a high-precision injection syringe with a 30G × 1/2″ needle in the subcutaneous level near the penultimate rib and parallel to sternum, and then moved forward into cranium-caudal direction in order to enter into the peritoneal cavity for inoculum administration. Consecutively, the syringe was slowly withdrawn and haemostasis was achieved with cotton wool. Animals were covered with bed material from original cages in order to mask any possible smell from manipulation before returning them to cages.

Survival, cannibalism, maternal rejection and mouse weight were recorded at different days post-infection (PI) until 8 days PI.

2.4 | Measurement of hematological and biochemical parameters

Suckling mice were euthanized at 8 days PI and blood samples were collected in tubes containing K_2-EDTA (Wiener lab). After centrifugation at 2000× *g* for 10 minutes at room temperature, plasma samples were separated. Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), and lactate dehydrogenase (LDH) were measured by UV kinetics-, kinetics- or enzymatic- method at 8 days PI using an Architect 8100 chemistry analyzer (Abbott Laboratories). Platelet counts were measured by microscopy on a CELLDYN 3700 (Abbott Laboratories) according to the manufacturer´s instructions.

Transaminitis was defined as ALT and/or AST plasma values significantly above the ones obtained for control mice. Plasma values of LDH and CK significantly higher than the values obtained for control mice were considered outcomes of disease. Platelet count significantly lower than the value obtained for control mice was also considered an outcome of disease.

2.5 | Histology

After blood collection at 8 days PI, mice were euthanized and tissues (liver, kidney, spleen, small intestine, and brain) were harvested and immediately fixed in 10% formalin in PBS. Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

2.6 | Quantitation of virus in blood and tissues by real time RT-PCR

Blood was extracted from suckling mice at different times (0, 17, 24, 48, 72, 96 and 168 hours) after DENV-2 infection and collected in tubes containing K_2-EDTA (Wiener lab.). RNA was extracted from plasma samples using QIAamp Viral RNA Minikit (QIAGEN) according to the manufacturer´s instructions.

After blood extraction at the corresponding post-infection times, suckling mice were euthanized and the mesenteric lymph nodes as well as a portion of the tissues (liver, kidney, spleen, small intestine, and brain) were immediately snap-frozen in tubes containing a mixture of dry ice/ethanol and later stored at −80°C. Total RNA was extracted from tissues using TRIzol (Invitrogen) according to the manufacturer´s instructions.

The amount of viral RNA in blood and tissues was quantified by using StepOnePlus Real-Time PCR System (Applied Biosystems) employing TaqMan technology, as described previously.[40] Beta-actin was used as endogenous control. Standard curves were generated using 10-fold serial dilutions of viral RNA obtained from purified DENV-2 suspensions.

2.7 | Isolation and stimulation of spleen mononuclear cells

Suckling mice were infected IP with DENV-2 or inoculated with an equal volume of C6/36 cell supernatant (control group). At 8 days PI, spleens were removed from mice under sterile conditions, following euthanasia, and collected in tubes containing RPMI (GIBCO) with 10% FBS. Spleens were disrupted by grinding between two sterile glass slides and splenocytes were separated using a Ficoll gradient (GE Healthcare). Spleen mononuclear cells (1×10^6 cells/well) were incubated in 200 μL of RPMI 10% FBS in the presence of UV-irradiated DENV-2 (UV-DENV-2) (MOI equivalent of 1.7) or RPMI 10% FBS alone. After 72 hours of incubation at 37°C supernatant fluids were removed and assayed for cytokines. Total RNA was extracted from cell pellets using TRIzol and assayed for transcription factors involved in the immune response by qRT-PCR.

2.8 | Cytokine determinations

Cytokines were determined in the supernatants of stimulated splenocytes using immunoassays for IFN-γ and IL-4 (eBioscience) following the manufacturer´s instructions.

2.9 | Quantification of RNA transcripts

The relative quantification of transcription factors mRNA expression (T-bet and GATA3) that regulate each T-helper (Th) cell differentiation pathway (Th1, Th2, respectively) was performed by real time RT-PCR using TaqMan gene expression assays (Applied Biosystems) and the $2^{-\Delta\Delta C}{}_T$ method.[41] Beta-actin was used as internal control.

2.10 | Statistical analysis

Bar, XY graphs and statistical analyses (mean, standard error, and statistical tests) were generated with GraphPad Prism. The

Student´s unpaired *t* test was used to determine statistical significance between two treatment groups. The one-way ANOVA and Bonferroni´s multiple comparison post-test correction was used to evaluate differences amongst multiple groups. Statistical significance is depicted in figures: $^{*}P < .05$, $^{**}P < .01$.

3 | RESULTS

3.1 | DENV infection in suckling mice

In order to establish a mouse model to study possible mechanisms of dengue disease in infants, we started characterizing DENV primary infection in immunocompetent suckling mice (Figure 1). Three-day-old C57BL/6 and BALB/c mice were infected IP with 5×10^5 PFU/g of DENV-2 NGC. Control mice were inoculated IP with an equal volume of C6/36 cell supernatant. The DENV-2 strain NGC was used because it has shown a higher ability to infect mice compared to other DENV strains.[42] Considering that mice are weaned approximately 21 post-natal days (PND) and that solid feeding starts at 16 PND, viral infection was performed at 3 PND and infection was followed until day 8 PI, ensuring that all samples were collected during the exclusive suckling period. Owing to the small size of 3-day-old mice, the implementation of certain infection techniques was difficult, such as intracerebral (i.c.), intravenous (iv) and subcutaneous (s.c.) inoculation. Therefore, the IP route, implementing the age-specific techniques described in methodology, was selected as the most appropriate technique to infect mice (Figure 2).

First, growth and survival curves were analyzed for DENV-2 infected mice compared to control mice. A slight growth retardation was observed for infected C57BL/6 pups compared to uninfected pups (Figure 3A). Conversely, DENV-2 infection did not affect BALB/c mouse growth (Figure 3B). A 100% survival rate was observed for both strains of infected mice until day 8 PI (Figure 3C,D).

3.2 | Hematological and biochemical determinations

Several studies in DENV patients have reported biochemical alterations, such as transaminitis, increased LDH and CK levels in plasma

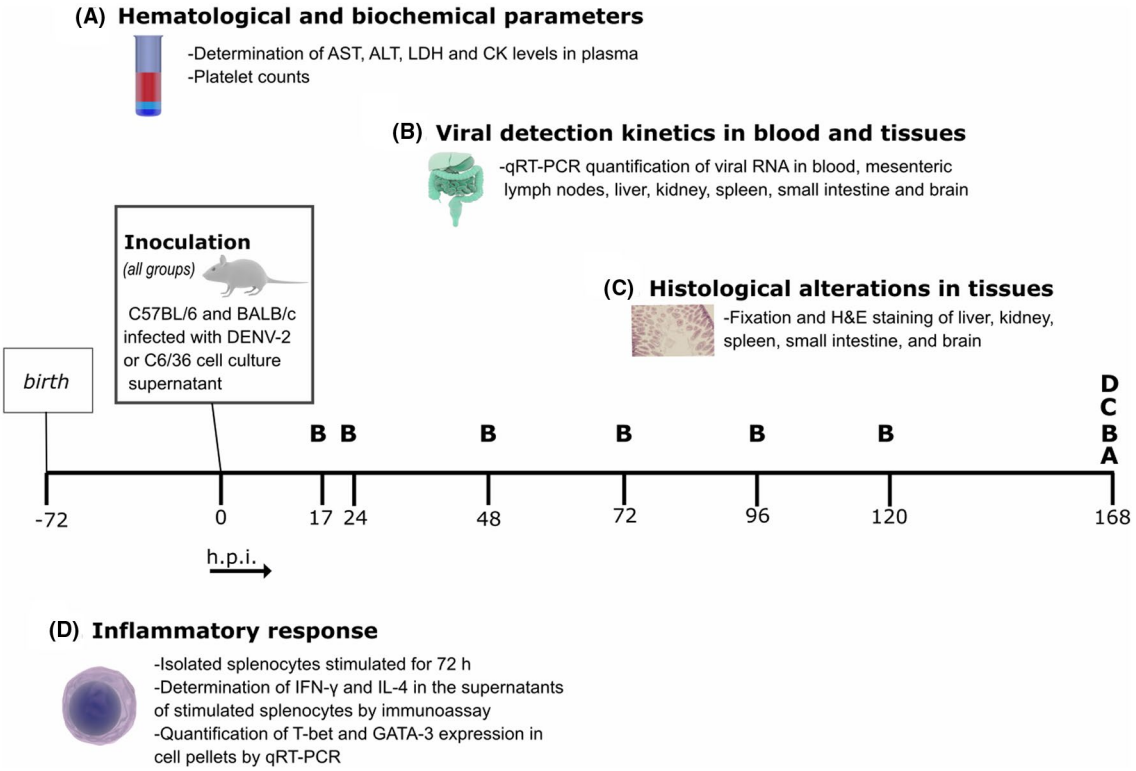

FIGURE 1 Timeline of DENV infection and determinations in suckling mice. C57BL/6 and BALB/c pups were infected IP with 5×10^5 PFU/g body weight of DENV-2 NGC or C6/36 cell culture supernatant (control) at 3 PND. Letters indicate the hours post inoculation (HPI) at which mice were euthanized for the following determinations: A, Plasma levels of aspartate aminotransferase, alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and creatine kinase (CK), and platelet counts; B, Total RNA was extracted from plasma, mesenteric lymph nodes, liver, kidney, spleen, small intestine, and brain and viral RNA was quantified using qRT-PCR; C, Liver, kidney, spleen, small intestine, and brain were fixed, embedded in paraffin and stained with hematoxylin and eosin (H&E); D, Splenocytes were incubated during 72 h with DENV-2 antigen or medium, and after centrifugation, IFN-γ and IL-4 were quantified in cell culture supernatants. In addition, total RNA in cell pellets was used to determine mRNA expression of T-bet and GATA3. In order to reach minimum volumes of plasma for some determinations, blood from 2 mice was pooled

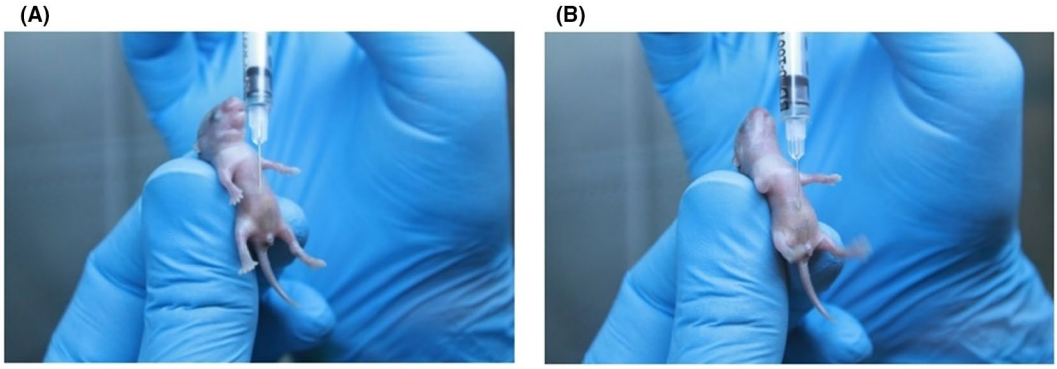

FIGURE 2 Intraperitoneal inoculation of suckling mice. Three-day-old C57BL/6 and BALB/c mice were inoculated with 5×10^5 PFU/g of DENV-2 or cell supernatant by an inverse IP administration technique. A, Mouse immobilization and location of inoculation site. B, Inoculum incision in the subcutaneous level moving forward into cranium-caudal direction and entering the peritoneal cavity for inoculum administration

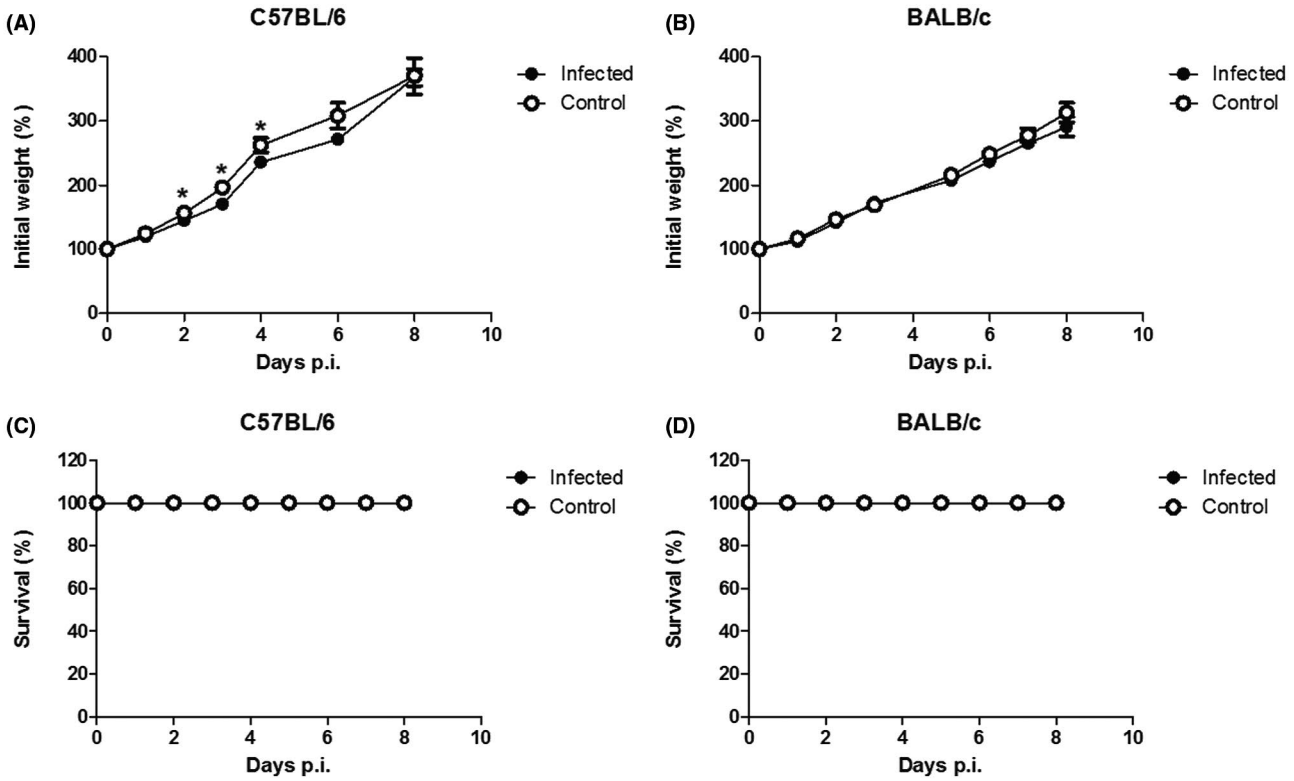

FIGURE 3 Effect of DENV-2 infection on suckling mouse growth and survival. Growth of C57BL/6 (A) and BALB/c (B) infected and control mice until 8 d PI. Survival of C57BL/6 (C) and BALB/c (D) infected and control mice until 8 d PI. Values represent means ± SEM. *$P < .05$, determined by Student's unpaired t test

and thrombocytopenia.[43-47] At day 8 PI, DENV-2 infected C57BL/6 mice showed a significant increase in AST and ALT levels indicating liver damage, and elevated LDH and CK levels in plasma suggesting tissue injury compared to control mice (Figure 4A,C,E,G). BALB/c mice infected with DENV-2 also exhibited a significant increase in CK and a trend towards augmented AST, ALT and LDH levels in plasma compared to control mice (Figure 4B,D,F,H). No significant differences were observed in platelet count in DENV-2 infected mice of either strain compared to control mice.

3.3 | Viral detection kinetics in blood and tissues

After DENV-2 IP infection, viremia levels were variable in C57BL/6 and BALB/c pups up to 168 hours PI. In both mouse strains, viral RNA plasma levels peaked at 24 hours PI and again at 72-96 hours PI, decreasing thereafter (Figure 5A,B). In C57BL/6 mice, viral RNA was differentially detected in mesenteric lymph nodes, kidney and liver. DENV-2 RNA levels were low to undetectable up to

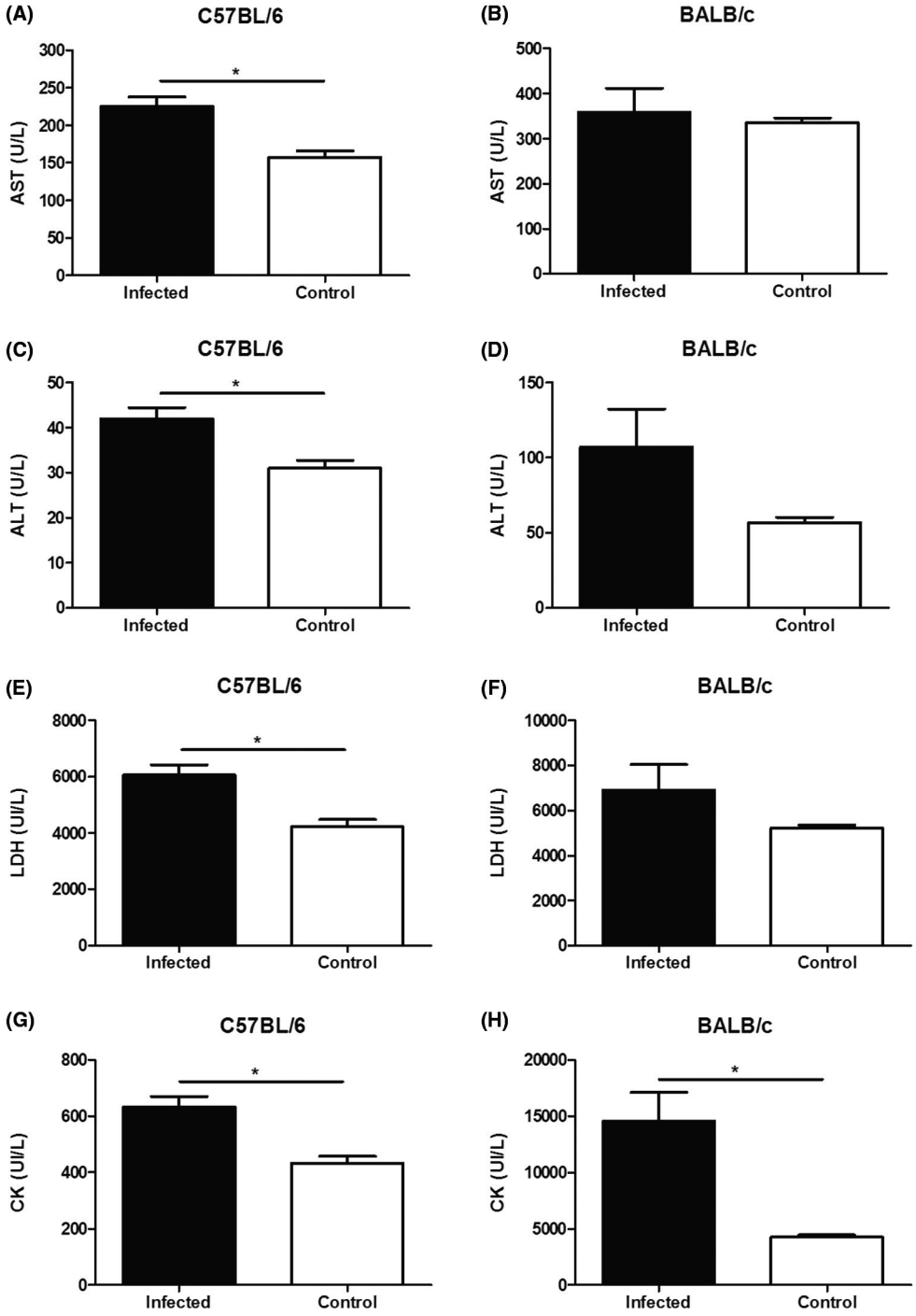

FIGURE 4 Biochemical parameters at 8 d PI in suckling mice infected with DENV-2. Plasma levels of AST (A, B), ALT (C, D), LDH (E, F) and CK (G, H) were determined in C57BL/6 and BALB/c infected and control mice. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; LDH, lactate dehydrogenase. Values represent means ± SEM. *$P < .05$, determined by Student's unpaired *t* test

168 hours PI in mesenteric lymph nodes and kidney, while viral RNA peaked at 17 and 96 hours PI in liver, suggesting transient replication (Figure 5C-E). In contrast, viral RNA levels in spleen, small intestine and brain of C57BL/6 mice were very low to undetectable. Regarding BALB/c mice, DENV-2 RNA was very low to undetectable in solid organs.

3.4 | Histological alterations

In order to analyze possible histological alterations produced by DENV-2 infection of suckling mice, histological sections of several tissues were performed. At day 8 PI, both strains of infected mice showed lymphoid mononuclear infiltrates in liver and intestine, and

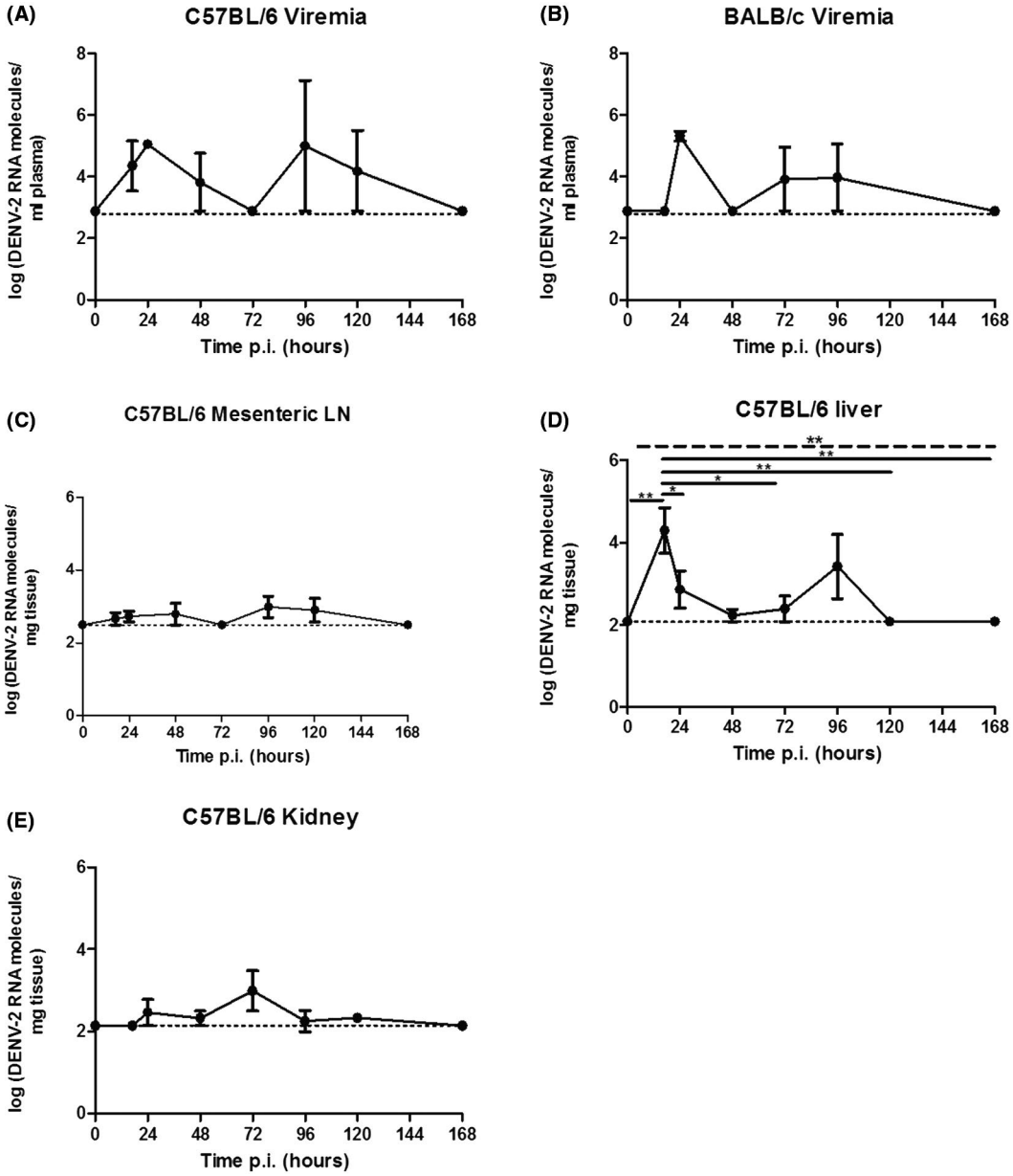

FIGURE 5 Virus detection by qRT-PCR in plasma and tissues after DENV-2 infection in suckling mice. C57BL/6 and BALB/c mice were infected with DENV-2 IP, and at different times PI (0, 17, 24, 48, 72, 96, 120 and 168 h), mice were euthanized and plasma (A, B), mesenteric lymph nodes (C) and organs (D, E) were collected. Total RNA was extracted from tissues and DENV RNA was detected by qRT-PCR. Abbreviations: LN, lymph node. Values represent means ± SEM. $^{*}P < .05$, $^{**}P < .01$ determined by one-way ANOVA (dashed line) and Bonferroni's multiple comparison post-test (solid line). Dotted lines show limits of detection

lytic focuses in the apical region of intestinal villi (Figure 6). Sections of spleen, kidney and brain did not show marked alterations compared to controls. These results suggest that histological damage was similar in C57BL/6 and BALB/c pups.

3.5 | Inflammatory response

Previous studies have reported differential innate and adaptive immune responses to human pathogens in C57BL/6 and BALB/c mice.[48-50] Therefore, we evaluated the immune response to DENV-2 IP infection in suckling C57BL/6 and BALB/c mice by measuring Th1 and Th2 cytokines and associated transcription factors in DENV-stimulated mouse splenocytes (Figure 7). For this purpose, splenocytes were isolated from mice infected with DENV-2 or C6/36 cell supernatant (control group) at 8 days PI and were then exposed to UV-DENV-2 for 72 hours. Splenocytes from infected C57BL/6 mice exhibited a significantly increased production of IFN-γ compared to control mice (159.0 ± 64.5 pg/mL vs 6.0 ± 2.9 pg/mL, for infected and control C57BL/6 mice, respectively, $P < .05$) (Figure 7A). In contrast, splenocytes from infected BABL/c mice showed a low IFN-γ production similar to that of control mice. Additionally, relative T-bet

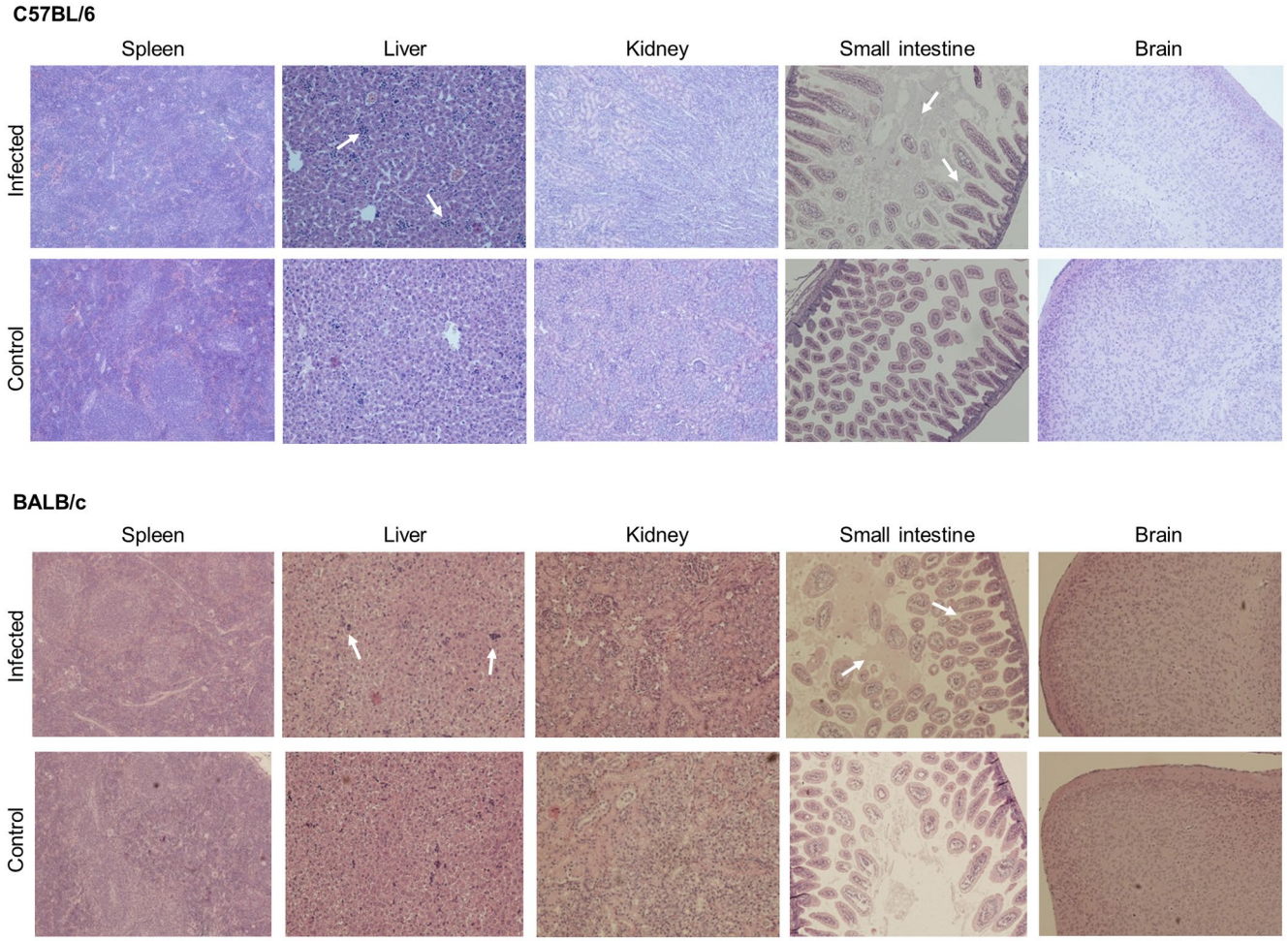

FIGURE 6 Histopathology in suckling mice after DENV-2 infection. Cross sections of various tissues of infected and control C57BL/6 and BALB/c mice at 8 d PI. Arrows indicate lymphoid mononuclear infiltrates in liver and small intestine and lytic focuses in the apical region of intestinal villi in DENV-2 infected mice. Magnification: 200× for liver and kidney, and 100× for spleen, small intestine and brain. H&E staining

mRNA expression was higher in C57BL/6 mice compared to that of BALB/c mice (Figure 7B). Interleukin-4 production in splenocyte supernatants from both mouse strains was not significantly different in infected and control groups (Figure 7C). Furthermore, relative GATA3 mRNA expression was similar in C57BL/6 and BALB/c mice (Figure 7D). A 6-fold higher IFN-γ/IL-4 ratio was found in C57BL/6 mice compared to BALB/c mice (30.28 ± 14.83 vs 4.96 ± 2.13, for C57BL/6 and BALB/c mice, respectively), suggesting a trend towards a Th1 bias in C57BL/6 mice.

4 | DISCUSSION

Clinical and experimental evidence have shown that humoral and cellular immunity play a role in both the protection and pathogenesis of dengue illness. Given the immunological immaturity of infants and their vulnerability to severe outcomes during primary infection with DENV, we sought to establish a model that recapitulates important aspects of human DENV illness in immunocompetent neonatal mice in service of better understanding pathogenesis in this uniquely vulnerable population.

C57BL/6 and BALB/c pups were not equally susceptible to infection with DENV-2 NGC. While the C57BL/6 pups had significant increases in AST and ALT, LDH and CK levels, extended viremia and dissemination to organs accompanied by infiltrates in the liver and intestine as well as damage to intestinal villi, BALB/c pups had detectable viremia up to 96 hours PI, and increased CK compared to controls. At 8 days PI infiltrates were detected in the liver and intestine but virus was undetectable in these organs at this point in time. The immune response of DENV-infected C57BL/6 pups had a marked Th1 bias compared to BALB/c pups.

Similarly, adult BALB/c mice infected IP with a non-mouse adapted strain of DENV-2 have been reported to suffer liver injury corresponding with increased serum AST and ALT despite very low viremia.[51] Another group has observed increased ALT and AST, decreased hematocrit and increased platelet count after IP infection of BALB/c adults with brain suspension containing DENV-3 passaged intracerebrally in weaning and then adult BALB/c mice. This adapted strain was also used to infect C57BL/6 adults, resulting in

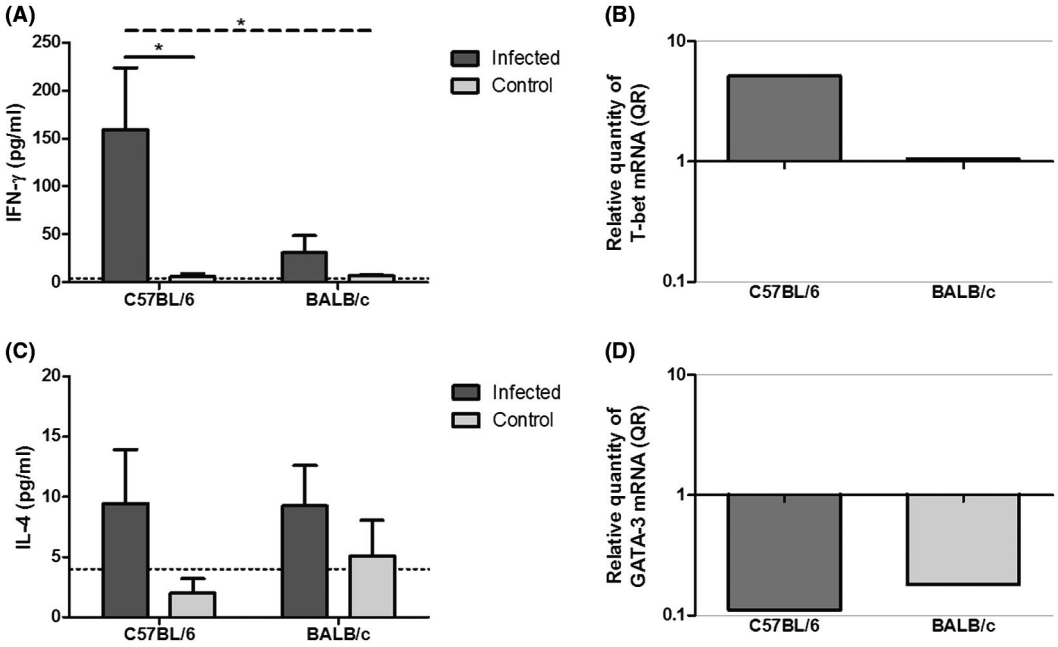

FIGURE 7 Immune response in suckling mice after DENV-2 infection. C57BL/6 and BALB/c mice were infected IP with DENV-2 or C6/36 cell supernatant (control). At 8 d PI, splenocytes were isolated from infected and control mice and stimulated with UV-DENV-2 during 72 h. A, C, Cytokine levels were measured in cell supernatants by immunoassay. B, D, Total RNA was extracted from cell pellets and relative quantity (QR) of transcription factor mRNA corresponding to Th1 and Th2 cell pathways was determined. Values represent means ± SEM. *$P < .05$, determined by one-way ANOVA (dashed line) and Bonferroni's multiple comparison post-test (solid line). Dotted lines show limits of detection

more severe disease, elevated liver enzymes, thrombocytopenia, progressive drop in blood pressure and dose-dependent lethality. In C57BL/6 mice, cytokine levels of IFN-γ, IL-12 and IL-18 were associated with severity.[32] The outcomes of infection described in our study for suckling BALB/c and C57BL/6 mice are consistent with previously published outcomes in adult mice of the same strain.

It has been reported that C57BL/6 adult mice inoculated IP with a neuroadapted strain of DENV-1 had detectable viremia 4-16 days PI, with a peak at 10 days PI, coinciding with a peak in viral replication in the brain. Virus disseminated to spleen, liver and kidneys, all of which fell to undetectable levels by 16 days PI. The peak of viremia coincided with decreased platelet levels. Throughout the infection, mice had increased AST and ALT as well as immune infiltration in liver and increased Th1 associated cytokines IL-12 and IL-18.[33] The present study reports a number of markers of disease in C57BL/6 pups shared by C57BL/6 adults, such as increased liver enzymes accompanied by immune infiltrates and viral dissemination to liver and kidney.

In our study we used a mouse-adapted strain of DENV-2[52] to infect immunocompetent suckling mice by IP inoculation. Even though DENV-2 NGC has been reported to be neurovirulent in mice,[42] viral RNA was neither found in brain nor were alterations observed in histological sections of this tissue by IP inoculation of virus in suckling C57BL/6 and BALB/c mice. In contrast, significant levels of viral RNA were detected in liver of C57BL/6 pups consistent with the finding of lymphoid mononuclear infiltrates in this tissue.

Route of infection is a determinant of early cell tropism and the course of infection.[53] For DENV and other vector-borne viruses, using the natural route of infection is often unfeasible and experimental methods may approximate natural infection in humans or select alternative routes that may better reflect other facets of infection. In our model, C57BL/6 and BALB/c pups infected IP with DENV-2 showed 100% survival. Conversely, other mouse models in immunocompetent suckling mice using i.c. inoculation of DENV have shown lower survival rates and have been used to study virulence of DENV strains or antiviral agents.[54-56] Furthermore, immunocompromised suckling mouse models of DENV that used the s.c. or iv routes of infection have exhibited lower survival rates than our immunocompetent mouse model, mainly due to the lack of IFN-I and/or IFN-II signaling pathways or to the enhanced effect of maternal antibodies.[38,39,57]

In previous studies, C57BL/6 and BALB/c mice have shown a distinct immune profile after infection with different human pathogens.[48-50] Concerning our model of DENV primary infection, C57BL/6 pups exhibited a Th1 bias evidenced by a 6-fold higher IFN-γ/IL-4 ratio compared to BALB/c pups. Increased Th1 cytokines have also been detected in adult C57BL/6 mice after DENV primary infection[32,33] and a role for IFN-γ in resistance against infection has been reported.[32]

Interestingly, a number of groups have developed models with young mice, highlighting the importance of immunological immaturity and maternally acquired antibodies in DENV immune response. In one model of neuroinfection using an i.c. passaged DENV-4 strain,

BALB/c pups inoculated up to 7 PND suffered encephalitis and death, while mice infected 14 and 21 PND survived. This strain of DENV-4 was detectable in brain in all mice assayed 6 days PI, but generally did not disseminate to organs.[58]

Work done in weaning and young immunocompromised mice highlights the versatility of experimental designs of a suckling mouse model. In experiments comparing primary infection with clinical DENV isolates in young AG129 mice born to DENV-immune and naive mothers found that mice born to DENV-immune mothers developed vascular leakage and lethal disease, while both groups had transient viremia, increased liver enzymes, cytokine upregulation and viral dissemination to lymph nodes, spleen, intestine and liver.[38] The same group has recapitulated many of these findings in the less immunocompromised A129, which lack type I IFN-signaling pathway compared to the AG129 which lack type I and II IFN-signaling pathways.[39,57]

In conclusion, we present an immunocompetent mouse model of DENV-2 primary infection in suckling mice that reproduces certain aspects of dengue disease observed in humans and that could be used to further study age-related mechanisms of dengue pathogenesis as well as the role of passively transferred anti-DENV antibodies in DENV disease severity. Additionally, the results of this study demonstrate that the technical refinement of IP viral inoculation was safe, effective and minimized harm in C57BL/6 and BALB/c pups; it is an efficient alternative for administration of substances to suckling mice.

ACKNOWLEDGEMENTS

This work was funded by grants from Fundación Bunge y Born, Agencia Nacional de Promoción Científica y Tecnológica (PICT 2008-1073), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (PIP 0118) and UBS Optimus Foundation (ID 2007-00206). ABB is fellow from CONICET, JMB is support professional from CONICET and LBT is member of Research Career from the same institution.

CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

FPP and LBT conceived the study. LBT designed experiments. ABB, AGG, JMB, AM, AF and LBT performed experiments. ABB, AGG, JMB, AM and LBT analyzed the data. FPP and LBT contributed with materials. ABB and LBT wrote the paper.

ORCID

Alana B. Byrne https://orcid.org/0000-0001-9370-1073
Fernando P. Polack https://orcid.org/0000-0002-8432-9158
Laura B. Talarico https://orcid.org/0000-0003-2635-474X

REFERENCES

1. Bhatt S, Gething PW, Brady OJ, et al. The global distribution and burden of dengue. *Nature*. 2013;496:504-507.
2. Wilder-Smith A, Ooi EE, Horstick O, Wills B. Dengue. *Lancet*. 2019;393:350-363.
3. Messina JP, Brady OJ, Golding N, et al. The current and future global distribution and population at risk of dengue. *Nat Microbiol*. 2019;4:1508-1515.
4. Wellekens K, Betrains A, De Munter P, Peetermans W. Dengue: current state one year before WHO 2010–2020 goals. *Acta Clin Belg*. 2020;1-9. https://doi.org/10.1080/17843286.2020.1837576.
5. Stanaway JD, Shepard DS, Undurraga EA, et al. The global burden of dengue: an analysis from the Global Burden of Disease Study 2013. *Lancet Infect Dis*. 2016;16:712-723.
6. Gordon A, Kuan G, Mercado JC, et al. The Nicaraguan pediatric dengue cohort study: incidence of inapparent and symptomatic dengue virus infections, 2004–2010. *PLoS Negl Trop Dis*. 2013;7:e2462.
7. Wu H, Wu C, Lu Q, Ding Z, Xue M, Lin J. Evaluating the effects of control interventions and estimating the inapparent infections for dengue outbreak in Hangzhou, China. *PLoS One*. 2019;14:e0220391.
8. Laserna A, Barahona-Correa J, Baquero L, Castaneda-Cardona C, Rosselli D. Economic impact of dengue fever in Latin America and the Caribbean: a systematic review. *Rev Panam Salud Publica*. 2018;42:e111.
9. Deen J, Lum L, Martinez E, Tan LH. Clinical management and delivery of clinical services. In: *Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control: New Edition*. Geneva, Switzerland: World Health Organization; 2009:25-55.
10. Tsang TK, Ghebremariam SL, Gresh L, et al. Effects of infection history on dengue virus infection and pathogenicity. *Nat Commun*. 2019;10:1246.
11. St John AL, Rathore APS. Adaptive immune responses to primary and secondary dengue virus infections. *Nat Rev Immunol*. 2019;19:218-230.
12. Lovera D, Martinez-Cuellar C, Galeano F, Amarilla S, Vazquez C, Arbo A. Clinical manifestations of primary and secondary dengue in Paraguay and its relation to virus serotype. *J Infect Dev Ctries*. 2019;13:1127-1134.
13. Halsey ES, Marks MA, Gotuzzo E, et al. Correlation of serotype-specific dengue virus infection with clinical manifestations. *PLoS Negl Trop Dis*. 2012;6:e1638.
14. Nunes PC, Sampaio SA, da Costa NR, et al. Dengue severity associated with age and a new lineage of dengue virus-type 2 during an outbreak in Rio De Janeiro, Brazil. *J Med Virol*. 2016;88:1130-1136.
15. Robinson M, Sweeney TE, Barouch-Bentov R, et al. A 20-gene set predictive of progression to severe dengue. *Cell Rep*. 2019;26(1104-1111):e4.
16. Xavier-Carvalho C, Cardoso CC, de Souza KF, Pacheco AG, Moraes MO. Host genetics and dengue fever. *Infect Genet Evol*. 2017;56:99-110.
17. Mongkolsapaya J, Duangchinda T, Dejnirattisai W, et al. T cell responses in dengue hemorrhagic fever: are cross-reactive T cells suboptimal? *J Immunol*. 2006;176:3821-3829.
18. Tian Y, Grifoni A, Sette A, Weiskopf D. Human T cell response to dengue virus infection. *Front Immunol*. 2019;10:2125.
19. Katzelnick LC, Gresh L, Halloran ME, et al. Antibody-dependent enhancement of severe dengue disease in humans. *Science*. 2017;358:929-932.
20. Guzman MG, Alvarez M, Halstead SB. Secondary infection as a risk factor for dengue hemorrhagic fever/dengue shock syndrome: an historical perspective and role of antibody-dependent enhancement of infection. *Arch Virol*. 2013;158:1445-1459.
21. Verhagen LM, de Groot R. Dengue in children. *J Infect*. 2014;69(Suppl 1):S77-86.
22. Elling R, Henneke P, Hatz C, Hufnagel M. Dengue fever in children: where are we now? *Pediatr Infect Dis J*. 2013;32:1020-1022.

23. Fonseca W, Lukacs NW, Ptaschinski C. Factors affecting the immunity to respiratory syncytial virus: from epigenetics to microbiome. *Front Immunol*. 2018;9:226.
24. Principi N, Esposito S. Severe influenza in children: incidence and risk factors. *Expert Rev Anti Infect Ther*. 2016;14:961-968.
25. Talarico LB, Bugna J, Wimmenauer V, et al. T helper type 2 bias and type 17 suppression in primary dengue virus infection in infants and young children. *Trans R Soc Trop Med Hyg*. 2013;107:411-419.
26. Kliks SC, Nimmanitya S, Nisalak A, Burke DS. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. *Am J Trop Med Hyg*. 1988;38:411-419.
27. Chau TN, Quyen NT, Thuy TT, et al. Dengue in Vietnamese infants–results of infection-enhancement assays correlate with age-related disease epidemiology, and cellular immune responses correlate with disease severity. *J Infect Dis*. 2008;198:516-524.
28. Clapham H, Cummings DA, Nisalak A, et al. Epidemiology of infant dengue cases illuminates serotype-specificity in the interaction between immunity and disease, and changes in transmission dynamics. *PLoS Negl Trop Dis*. 2015;9:e0004262.
29. Alves Dos Santos E, Fink K. Animal models for dengue and Zika vaccine development. *Adv Exp Med Biol*. 2018;1062:215-239.
30. Kurane I, Innis BL, Nimmannitya S, Nisalak A, Meager A, Ennis FA. High levels of interferon alpha in the sera of children with dengue virus infection. *Am J Trop Med Hyg*. 1993;48:222-229.
31. Acosta PL, Byrne AB, Hijano DR, Talarico LB. Human type I interferon antiviral effects in respiratory and reemerging viral infections. *J Immunol Res*. 2020;2020:1372494.
32. Costa VV, Fagundes CT, Valadao DF, et al. A model of DENV-3 infection that recapitulates severe disease and highlights the importance of IFN-gamma in host resistance to infection. *PLoS Negl Trop Dis*. 2012;6:e1663.
33. Goncalves D, de Queiroz PR, Almeida Xavier E, et al. Immunocompetent mice model for dengue virus infection. *Sci World J*. 2012;2012:525947.
34. Barros VE, dos Santos-Junior NN, Amarilla AA, et al. Differential replicative ability of clinical dengue virus isolates in an immunocompetent C57BL/6 mouse model. *BMC Microbiol*. 2015;15:189.
35. Marques RE, Besnard AG, Maillet I, et al. Interleukin-33 contributes to disease severity in Dengue virus infection in mice. *Immunology*. 2018;155:477-490.
36. St John AL, Rathore AP, Raghavan B, Ng ML, Abraham SN. Contributions of mast cells and vasoactive products, leukotrienes and chymase, to dengue virus-induced vascular leakage. *Elife*. 2013;2:e00481.
37. Rathore AP, Mantri CK, Aman SA, et al. Dengue virus-elicited tryptase induces endothelial permeability and shock. *J Clin Invest*. 2019;129:4180-4193.
38. Ng JK, Zhang SL, Tan HC, et al. First experimental in vivo model of enhanced dengue disease severity through maternally acquired heterotypic dengue antibodies. *PLoS Pathog*. 2014;10:e1004031.
39. Martinez Gomez JM, Ong LC, Lam JH, et al. Maternal antibody-mediated disease enhancement in type I interferon-deficient mice leads to lethal disease associated with liver damage. *PLoS Negl Trop Dis*. 2016;10:e0004536.
40. Talarico LB, Batalle JP, Byrne AB, et al. The role of heterotypic DENV-specific CD8(+)T lymphocytes in an immunocompetent mouse model of secondary dengue virus infection. *EBioMedicine*. 2017;20:202-216.
41. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402-408.
42. Lee E, Wright PJ, Davidson A, Lobigs M. Virulence attenuation of Dengue virus due to augmented glycosaminoglycan-binding affinity and restriction in extraneural dissemination. *J Gen Virol*. 2006;87:2791-2801.
43. Sam SS, Omar SF, Teoh BT, Abd-Jamil J, AbuBakar S. Review of Dengue hemorrhagic fever fatal cases seen among adults: a retrospective study. *PLoS Negl Trop Dis*. 2013;7:e2194.
44. Padyana M, Karanth S, Vaidya S, Gopaldas JA. Clinical profile and outcome of dengue fever in multidisciplinary intensive care unit of a tertiary level hospital in India. *Indian J Crit Care Med*. 2019;23:270-273.
45. Trojanek M, Maixner J, Sojkova N, et al. Dengue fever in Czech travellers: a 10-year retrospective study in a tertiary care centre. *Travel Med Infect Dis*. 2016;14:32-38.
46. Liao B, Tang Y, Hu F, et al. Serum levels of soluble vascular cell adhesion molecules may correlate with the severity of dengue virus-1 infection in adults. *Emerg Microbes Infect*. 2015;4:e24.
47. Rafi A, Mousumi AN, Ahmed R, Chowdhury RH, Wadood A, Hossain G. Dengue epidemic in a non-endemic zone of Bangladesh: clinical and laboratory profiles of patients. *PLoS Negl Trop Dis*. 2020;14:e0008567.
48. Aoki JI, Muxel SM, Zampieri RA, Muller KE, Nerland AH, Floeter-Winter LM. Author Correction: Differential immune response modulation in early Leishmania amazonensis infection of BALB/c and C57BL/6 macrophages based on transcriptome profiles. *Sci Rep*. 2020;10:4365.
49. Ferreira BL, Ferreira ER, de Brito MV, et al. BALB/c and C57BL/6 mice cytokine responses to Trypanosoma cruzi infection are independent of parasite strain infectivity. *Front Microbiol*. 2018;9:553.
50. Roque S, Nobrega C, Appelberg R, Correia-Neves M. IL-10 underlies distinct susceptibility of BALB/c and C57BL/6 mice to Mycobacterium avium infection and influences efficacy of antibiotic therapy. *J Immunol*. 2007;178:8028-8035.
51. Paes MV, Pinhao AT, Barreto DF, et al. Liver injury and viremia in mice infected with dengue-2 virus. *Virology*. 2005;338:236-246.
52. Gruenberg A, Woo WS, Biedrzycka A, Wright PJ. Partial nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue virus type 2, New Guinea C and PUO-218 strains. *J Gen Virol*. 1988;69(Pt 6):1391-1398.
53. Falzaran D, Bente DA. Animal models for viral haemorrhagic fever. *Clin Microbiol Infect*. 2019;21S:e17-e27.
54. Yu JS, Tseng CK, Lin CK, et al. Celastrol inhibits dengue virus replication via up-regulating type I interferon and downstream interferon-stimulated responses. *Antiviral Res*. 2017;137:49-57.
55. Chen JM, Fan YC, Lin JW, Chen YY, Hsu WL, Chiou SS. Bovine lactoferrin inhibits dengue virus infectivity by interacting with heparan sulfate, low-density lipoprotein receptor, and DC-SIGN. *Int J Mol Sci*. 2017;18:1957.
56. Zou C, Huang C, Zhang J, et al. Virulence difference of five type I dengue viruses and the intrinsic molecular mechanism. *PLoS Negl Trop Dis*. 2019;13:e0007202.
57. Lee PX, Ong LC, Libau EA, Alonso S. Relative contribution of dengue IgG antibodies acquired during gestation or breastfeeding in mediating dengue disease enhancement and protection in type I interferon receptor-deficient mice. *PLoS Negl Trop Dis*. 2016;10:e0004805.
58. Velandia-Romero ML, Acosta-Losada O, Castellanos JE. In vivo infection by a neuroinvasive neurovirulent dengue virus. *J Neurovirol*. 2012;18:374-387.

How to cite this article: Byrne AB, Garcia AG, Brahamian JM, et al. A murine model of dengue virus infection in suckling C57BL/6 and BALB/c mice. *Anim Models Exp Med*. 2020;00:1–11. https://doi.org/10.1002/ame2.12145