RESEARCH ARTICLE

Maternal Antibody-Mediated Disease Enhancement in Type I Interferon-Deficient Mice Leads to Lethal Disease Associated with Liver Damage

Julia María Martínez Gómez¹,²*, Li Ching Ong³*, Jian Hang Lam¹,², Siti Amanlina Binte Aman⁴, Eshele Anak Libau¹,², Pei Xuan Lee¹,², Ashley L. St. John⁴, Sylvie Alonso¹,²,³*

¹ Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, ² Immunology programme, Life Sciences Institute, National University of Singapore, Singapore, ³ Infectious Disease programme, Singapore-MIT alliance for Research and Technology (SMART), National University of Singapore, Singapore, ⁴ Emerging Infectious Diseases programme, Duke-NUS, Singapore

☯ These authors contributed equally to this work.

* micas@nus.edu.sg

Abstract

Epidemiological studies have reported that most of the severe dengue cases occur upon a secondary heterologous infection. Furthermore, babies born to dengue immune mothers are at greater risk of developing severe disease upon primary infection with a heterologous or homologous dengue virus (DENV) serotype when maternal antibodies reach sub-neutralizing concentrations. These observations have been explained by the antibody mediated disease enhancement (ADE) phenomenon whereby heterologous antibodies or sub-neutralizing homologous antibodies bind to but fail to neutralize DENV particles, allowing Fc-receptor mediated entry of the virus-antibody complexes into host cells. This eventually results in enhanced viral replication and heightened inflammatory responses. In an attempt to replicate this ADE phenomenon in a mouse model, we previously reported that upon DENV2 infection 5-week old type I and II interferon (IFN) receptors-deficient mice (AG129) born to DENV1-immune mothers displayed enhancement of disease severity characterized by increased virus titers and extensive vascular leakage which eventually led to the animals’ death. However, as dengue occurs in immune competent individuals, we sought to reproduce this mouse model in a less immunocompromised background. Here, we report an ADE model that is mediated by maternal antibodies in type I IFN receptor-deficient A129 mice. We show that 5-week old A129 mice born to DENV1-immune mothers succumbed to a DENV2 infection within 4 days that was sub-lethal in mice born to naïve mothers. Clinical manifestations included extensive hepatocyte vacuolation, moderate vascular leakage, lymphopenia, and thrombocytopenia. Anti-TNFα therapy totally protected the mice and correlated with healthy hepatocytes. In contrast, blocking IL-6 did not impact the virus titers or disease outcome. This A129 mouse model of ADE may help dissecting the mechanisms involved in dengue pathogenesis and evaluate the efficacy of vaccine and therapeutic candidates.
Author Summary
Severe dengue has been linked to secondary heterologous infection or to primary infection in 6–9 month old babies born to dengue immune mothers. The leading hypothesis for such observations is the antibody-dependent enhancement (ADE) phenomenon in which cross-reactive, but non-neutralizing, heterologous antibodies, or sub-neutralizing levels of homotypic antibodies, enhance uptake of dengue virus into host cells. We report here the development of a dengue ADE mouse model that is mediated by maternal antibodies acquired during gestation and breastfeeding. Dengue infection of mice born to mothers immune to a heterologous dengue serotype resulted in rapid death of the mice, accompanied by increased virus titers, severe liver damage and heightened systemic inflammation. Anti-TNFα therapy fully rescued the animals. This novel mouse model may help further understand dengue pathogenesis and provides a platform for testing the efficacy of vaccine and therapeutic candidates.

Introduction
Dengue is the most serious and widespread arthropod borne viral disease worldwide with an estimated 390 million people infected mainly in the tropical and subtropical regions, and 3 billion people at risk of infection in over 100 countries [1]. The etiological agent of dengue, dengue virus (DENV), belongs to the genus Flavivirus within the Flaviviridae family, which also includes Japanese encephalitis, West Nile, and yellow fever viruses. DENV is an enveloped virus with a single-stranded, positive-sense RNA genome. There are four antigenically distinct serotypes of DENV (DENV1-4) that may co-circulate in the same geographical area [1]. The virus is primarily transmitted to humans by the highly urbanized *Aedes aegypti* female mosquito which has spread globally due to increased trade and travel [2]. *Aedes albopictus* has also been reported to effectively transmit DENV to humans and its capacity to survive in cooler weather has allowed the spread of the virus to more temperate regions such as Europe and North America [3].

Human infection with one of the four DENV serotypes is mostly asymptomatic. When symptomatic, the disease presents itself in a wide spectrum of clinical manifestations, ranging from mild acute febrile illness to self-limiting classical dengue fever (DF) to the severe dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS) [4]. The hallmarks of DHF/DSS are haemorrhagic manifestations and increased vascular permeability, respectively, the latter resulting in fluid loss which may progress to life-threatening hypovolemic shock.

While infection with one DENV serotype is believed to confer life-long protection against that particular serotype, secondary infection with a heterologous serotype may lead to severe disease. Epidemiological studies over the last few decades have indeed reported that most of the DHF/DSS cases occur upon secondary infection with a heterologous DENV serotype [5, 6]. Increased risk of DHF/DSS was also reported in infants at 5–9 months of age born to dengue immune mothers when maternally acquired antibodies wane to sub-neutralizing levels [7–10]. These epidemiological observations were explained by the antibody-dependent enhancement (ADE) of infection hypothesis, whereby actively (during primary infection) or passively (through maternal transfer) acquired anti-DENV antibodies cross react but fail to neutralize a heterologous (or homologous) serotype of DENV [5]. Mechanistically, antibody-opsonized DENV gains entry into activating Fc receptor (FcR)-bearing cells such as dendritic cells and monocytes resulting in increased viral replication which in turn triggers the massive release of inflammatory and vasoactive mediators that contribute to the disease severity [11–13].
The lack of suitable animal models for DENV infection has seriously hindered the understanding of dengue pathogenesis and the pre-clinical evaluation of prophylactic and therapeutic candidates. Immunocompetent mice are generally not susceptible to DENV infection partly due to the virus inability to interfere with the murine type I IFN response [14–17]. However, a handful of studies have described that upon intravenous administration of high doses (10⁸ pfu) of DENV to immunocompetent mice, viral load could be detected in the serum and other organs, accompanied with relevant clinical manifestations such as hemorrhage and thrombocytopenia [18, 19]. However, immunocompetent mouse models are seldom used due to the major shortcoming of low/undetectable systemic infective viremia.

Alternatively, a variety of mouse genetic backgrounds lacking various immune components have been explored that displayed increased susceptibility to DENV infection [20–24]. Among these models, AG129 mice, which are deficient in interferon (IFN)-α/β and –γ receptors, were shown by us and others to allow effective replication of a number of DENV strains, mainly from serotype 1 and 2 [23, 25–28]. ADE models have also been reported in these mice whereby administration of cross-reactive, sub- or non-neutralizing antibodies led to enhanced disease severity [29, 30]. We have recently reported that maternally acquired heterotypic dengue antibodies induced ADE in AG129 mice, with earlier death, increased viremia and increased vascular permeability upon DENV2 infection of mice born to DENV1-immune mothers compared to mice born to naïve mothers [31]. While this model is supported by many epidemiological observations, the lack of an intact IFNγ signaling pathway in these mice limits the study of certain aspects of the dynamic interactions between the antibodies, virus particles and immune cells. Indeed, the antibody production in AG129 mice has been reported to be biased towards the IgG1 subclass due to the lack of IFNγ [23]. However in humans, DENV infection results mainly in IgG1 and IgG3 [32] which corresponds in mice to the IgG2a subclass. Consistently, DENV infection of immune competent mice mainly results in the production of IgG2a antibodies [33]. As IgG subclasses display differential affinities for various activating and inhibitory Fc-receptors [11–13], their role and impact on disease severity in the context of ADE is likely to be different. Therefore, we sought to address this aspect by reproducing the maternal ADE model in A129 mice, which are type I IFN-receptor deficient but have an intact IFNγ signaling pathway. We report here that DENV2 infection of young mice born to DENV1-immune mothers resulted in enhanced disease severity characterized by death, increased viremia, and severe liver damage. The detrimental role of TNFα in this model was also demonstrated.

Results

Cross-reactive, neutralizing and enhancing properties of sera from DENV1-immune mothers and their offspring

Infection of adult female A129 mice via the intravenous (iv) route with 10⁶ PFU of DENV1 resulted in an asymptomatic infection with barely detectable viremia by plaque assay (S1A Fig). However, detection of anti-NS1 IgG antibodies in the sera of these mice 6 weeks post-infection (p.i.) confirmed that the virus had replicated productively (S1B Fig). High IgG antibody titers against homologous DENV1 and heterologous DENV2 virus were measured by ELISA in the sera from these DENV1-immune female mice (Fig 1A). The same assays were performed with the serum from 5–6 weeks old mice born to these DENV1-immune mothers. The total anti-DENV1 and anti-DENV2 IgG titers measured were 1 log lower than those measured in their mother (Fig 1C). Remarkably, the anti-DENV1 IgG antibody response was mainly (DENV1-immune mothers) or exclusively (mice born to the DENV1-immune mothers) of the IgG2a sub-class (Fig 1B & 1D).
Fig 1. DENV-specific antibody titers in DENV1-immune mothers and their offspring. Five to six weeks old naive female A129 mice were infected iv with 10^6 PFU of DENV1 and bled at 6 weeks post-infection (n = 3–5) (A&B). A129 mice born to naive or DENV1-immune mothers were bled at the age of 5–6 weeks old (n = 5) (C&D). The antibody titers were determined by indirect ELISA against UV-inactivated DENV1/DENV2 particles as indicated. (A, C) Total IgG titers against DENV1 or DENV2 as indicated on the top of the graphs. (B, D) Anti-DENV1 IgG1, IgG2a and IgG2b titers. Titers were determined as the lowest dilution factor that gives an OD value of 3 times the mean blank. Dotted line denotes the lowest dilution of the sera and a nominal titer of 50 or 66.7 was assigned to samples with titers below the lowest dilution.

doi:10.1371/journal.pntd.0004536.g001
Neutralizing capacity of the serum from DENV1-immune mothers and their offspring was also evaluated against DENV1 and DENV2 using classical in vitro plaque reduction neutralization test (PRNT). Results show that the sera from DENV1-immune mothers displayed some neutralizing capacity against both DENV1 and DENV2, with PRNT50 titers of about 140 and 50, respectively (Table 1). Expectedly, the neutralizing capacity of the sera from mice born to DENV1-immune mothers was significantly lower than that seen with their mother, with average PRNT50 values of 78 and 13 against DENV1 and DENV2, respectively. Finally, the enhancing properties against DENV2 of the sera from mice born to DENV1-immune mothers were assayed in vitro. Enhanced DENV2 infection of K562 cells was observed with serum dilutions ranging from 1/20 to 1/160 (Fig 2).

Together these in vitro data demonstrate that serum from mice born to DENV1-immune mothers cross-reacts with but does not neutralize effectively DENV2, which results in enhancement of infection.

Table 1. Neutralizing properties of sera from DENV1-immune mothers and their offspring. Serially diluted (2-fold) heat-inactivated sera from DENV1-immune mothers (6 weeks p.i.), age-matched naïve mothers and 5–6 weeks old mice born to their respective mothers were mixed with either DENV1 or DENV2 prior to infection of BHK cells. The PRNT50 value was determined by nonlinear regression of the serum dilution factor that results in 50% reduction in the number of plaques as compared to the positive control (virus alone). Samples with PRNT50 values less than 1:10 were arbitrarily assigned a value of 5. Sera from DENV1-immune and naïve mothers were pooled whereas sera from mice born to their respective mothers were tested individually (n = 5) and are expressed as average ± SEM.

|                         | DENV1       | DENV2       |
|-------------------------|-------------|-------------|
| Naïve mothers (Age-matched) | 5.00        | 5.00        |
| DENV1-immune mothers (6 weeks post-infection) | 139.12      | 59.20       |
| Mice born to naïve mothers | 10.01 ± 3.02 | 6.33 ± 1.33 |
| Mice born to DENV1 immune mothers | 78.14 ± 10.69 | 13.30 ± 2.38 |

doi:10.1371/journal.pntd.0004536.t001

Fig 2. Enhancing properties of sera from 5–6 weeks old mice born to DENV1-immune mothers. K562 cells were infected with a mixture of DENV2-D2Y98P-PP1 and serially diluted pooled sera from mice born to DENV1-immune mothers (circle) or that from naïve mothers (square). Virus titers in supernatant were determined by plaque assay in BHK cells and expressed as fold enhancement (with respect to the average plaques obtained in the virus alone control).

doi:10.1371/journal.pntd.0004536.g002
Enhanced disease severity upon DENV2 infection of A129 mice born to DENV1-immune mothers

Five to six week old mice born to DENV1-immune females or naïve females were infected iv with $10^6$ PFU of DENV2 clinical isolate D2Y98P-PP1, which we have previously shown to trigger vascular leakage and death in AG129 mice upon primary infection [27, 28]. Here, A129 mice born to DENV1-immune mothers consistently and uniformly died by day 4 post-DENV2 infection (p.i.) whereas the infected age-matched A129 mice born to naïve mothers survived throughout the experimental period (Fig 3A). Both groups started to display clinical symptoms
including hunched back and severe diarrhea at the end of day 3 and at day 4 p.i. However, while the mice born to naïve mothers recovered by day 5 p.i., mice born to DENV1-immune mothers rapidly became lethargic and moribund at which stage they were euthanized (Fig 3B). None of the infected mice displayed signs of paralysis or central nervous system (CNS) involvement.

Comparable viremia titers were measured at day 2 p.i. in both animal groups (Fig 3C). However, viremia was 1 log higher at day 4 p.i. in mice born to DENV1-immune mothers compared to the age-matched control mice (Fig 3C). The viral loads in perfused liver, spleen and brachial/axillary lymph nodes were also significantly higher at day 4 p.i. in mice born to DENV1-immune mothers (Fig 3D). Negligible levels of virus were detected in the brain from the infected mice. Furthermore, as increased vascular leakage was the main feature in the maternal ADE model in AG129 mice [31], we also assessed the vascular permeability in A129 mice. Interestingly, moderate (less than 2-fold increase compared to uninfected controls) and comparable vascular leakage was measured in both groups of DENV2-infected A129 mice born to naïve or DENV1-immune mothers (Fig 3E).

Together, these data indicate that DENV2 infection of young A129 mice born to DENV1-immune mothers led to increased disease severity characterized by lethality and increased viral loads in the blood and several organs. However, these mice did not experience further increase in vascular leakage as compared to their counterparts born to naïve mothers.

Severe liver damage and elevated transaminase levels in DENV2-infected mice born to DENV1-immune mothers

To gain further insights into the pathology displayed by the DENV2-infected mice born to DENV1-immune mothers, histology analysis was conducted. Upon H&E staining, severe liver damage was observed at day 4 p.i. in mice born to DENV1-immune mothers, characterized by widespread cytoplasmic vacuolation of the hepatocytes (Fig 4A). These mice displayed healthy hepatocytes at the earlier time point of day 2 p.i. (S2 Fig). Liver damage observed at day 4 p.i. correlated with elevated systemic levels of aspartate transaminase (AST) (Fig 4B). Instead, mice born to dengue naïve mothers displayed healthy liver (Fig 4A) with normal AST levels (Fig 4B). In addition, no evident damage was observed in the spleen and intestine from DENV2-infected mice born to naïve and DENV1-immune mothers at day 4 p.i. (S2 Fig), thus suggesting that maternal antibody-mediated disease enhancement targets mainly the liver.

A previous study reported the accumulation of DENV particles in liver sinusoidal endothelial cells in an ADE model where AG129 mice were administered enhancing antibodies prior to DENV2 infection [30]. Similarly, we found that DENV NS3 protein mainly co-localized with CD31+ cells, a marker for endothelial cells and to a lesser-extent with CD11b+ cells, a marker primarily expressed on monocytes/macrophages in mice born to DENV1-immune mothers (Fig 5). In mice born to dengue naïve mothers, NS3 staining showed co-localization with similar cell markers, although overall the levels of infection appeared reduced (Fig 5). These observations thus indicate that hepatocytes are not the main target cell population for DENV in both infected groups. This therefore suggests that the extensive cytoplasmic vacuolation of hepatocytes seen in DENV2-infected mice born to DENV1-immune mothers is not due to a direct cytolytic viral effect and correlates with the possible heightened presence of the virus inside the liver sinusoidal endothelial cells.

DENV2-infected mice born to DENV1-immune or naïve mothers display comparable changes in their blood parameters

Blood parameters were assessed at day 2 and 4 p.i in both infected groups. Compared to uninfected mice, both DENV2-infected groups displayed increased neutrophils and reduced
lymphocytes and monocytes counts, which was more evident at day 4 p.i. (S3 Fig). Thrombocytopenia was also seen for both infected animal groups as evidenced by the lower platelet counts measured at both day 2 and 4 p.i. Instead, there was no significant difference in the hematocrit between infected groups and uninfected control, reflecting the moderate vascular leakage measured in the infected mice (Fig 3). The only significant difference between mice born to DEN- 

Fig 4. Liver histology and function in DENV2-infected mice born to DENV1-immune. 5-6-weeks old A129 mice born to either DENV1-immune (black bars) or naïve mothers (open bars) were iv infected with 10^6 PFU of D2Y98P-PP1. (A) Histological analysis of the liver at day 4 p.i. of DENV2-infected mice born to dengue naïve or DENV1-immune mothers and uninfected mice as indicated (n = 3). Images were taken at 20x and 40x magnification. Representative sections from two independent experiments are shown (scale bar – 100μm). (B) Serum levels of aspartate transaminase (AST) were determined at the indicated time points (n = 5 ± SEM per time point), * p<0.05 based on 1-way ANOVA with Bonferroni’s post-test.

doi:10.1371/journal.pntd.0004536.g004

Increased levels of pro-inflammatory cytokines and chemokines in DENV2-infected mice born to DENV1-immune mothers

A hallmark of DHF/DSS is the heightened expression of cytokines that results in various pathological complications [3, 34]. To gain further insights into this A129 ADE model and identify the soluble mediators that could contribute to disease enhancement, the levels of 32 different cytokines and chemokines were measured in the sera from DENV2-infected mice born to naïve or DENV1-immune mothers. At the early infection time point of day 2 p.i., only two
cytokines, namely Granulocyte-colony stimulating factor (G-CSF) and monokine induced by IFN-γ (MIG), were significantly reduced in DENV2-infected mice born to DENV1-immune mothers compared to DENV2-infected mice born to naïve mothers (average values of 2026.72 pg/ml vs 7080.75pg/ml for G-CSF and 128.75pg/ml vs 395.42pg/ml for MIG) at day 4 p.i. when clinical symptoms were observed, a greater number of cytokines became differentially expressed. Higher systemic levels of G-CSF, Eotaxin, LIF, LIX, MIP-1α, MIP-1β, MCP, MIP-2, MIG, RANTES, TNFα, IL-6, IL-10, IL-13 and IL-17 were measured in mice born to DENV1-immune mothers compared to DENV2-infected mice born to naïve mothers (Fig 6). When expressed as fold change, these expression levels range from 1.5 (M-CSF) to 9.37 (LIF) fold-increase (Fig 6). These increased levels of pro-inflammatory cytokines and chemokines measured in DENV2-infected mice born to DENV1-immune mothers therefore support that these mice experienced an exacerbated inflammatory response. 

Key role of TNFα in disease severity

Patients with severe dengue have been reported to display elevated levels of TNFα [35] and in several AG129 ADE models, TNFα blocking antibodies were found to delay the death of DENV2-infected mice [30, 31]. Consistently, increased TNFα levels were measured in DENV2-infected A129 mice born to DENV1-immune mothers at moribund stage (day 4 p.i.) (Fig 6). To assess the role of TNF-α in this A129 ADE model, DENV2-infected mice born to DENV1-immune mothers were administered with a TNF-α blocking antibody or an isotype control at day 2 p.i. Survival and clinical symptoms were monitored and indicated that the anti-TNFα-treated
mice survived throughout the experiment while the isotype control mice were moribund by day 4 p.i. (Fig 7A & 7B). Comparable viremia titers were measured at day 4 p.i. in both anti-TNFα and isotype control treated groups (Fig 7C), thus indicating that the absence of disease enhancement in the anti-TNFα treated group was not due to reduction in virus loads. In contrast, AST level was significantly lower in the anti-TNFα-treated group compared to the isotype control group (Fig 7D), suggesting that the mice had reduced liver damage. This was confirmed by histology analysis whereby mice treated with anti-TNFα antibody displayed no obvious liver damage. In contrast, isotype control mice showed substantial cytoplasmic vacuolation (Fig 7E). Together, these data support that liver damage is the likely cause of death in this ADE model and indicate that TNFα plays a key role in this process.

Role of IL-6 and IFNγ in disease severity

Among the panel of cytokines and chemokines measured, the levels of IL-6 were highly elevated in the DENV2-infected mice born to DENV1-immune mothers compared to mice born to naïve mothers (Fig 6). In addition, elevated levels of this cytokine have been reported in severe dengue patients [36]. In order to investigate the role of IL-6 in this A129 ADE model, DENV2-infected mice born to DENV1-immune mothers were treated with an anti-IL6 blocking antibody at day 2 p.i. In sharp contrast to what was observed when TNFα was neutralized at the same time point, no differences were observed in terms of survival, clinical scores or viremia in the anti-IL6 treated mice compared to the isotype control group (Fig 8A–8C). This thus suggests that increased IL-6 production in mice born to DENV2-immune mothers is not a major contributor to the enhancement of disease severity.

Furthermore, the role of IFNγ in this ADE model was evaluated using a similar strategy. We were particularly interested to test whether the presence of an intact IFNγ signaling pathway in A129 mice plays a role in the limited vascular leakage seen in this ADE model, compared to the ADE model in AG129 mice for which we previously reported extensive vascular permeability.  

Fig 6. Systemic levels of differentially modulated cytokines and chemokines in DENV2-infected A129 mice born to DENV1-immune or naïve mothers at day 4 p.i. Five to six weeks old A129 mice born to either DENV1-immune or naïve mothers were iv infected with 10⁶ PFU of D2Y98P-PP1. At day 2 or 4 p.i., the mice were terminally bled and the systemic levels of cytokines were determined by multiplex ELISA. Average levels of the differentially modulated cytokines (n = 5 each for 2–3 independent experiments) in the sera of DENV2-infected mice born to DENV1-immune mothers (black bars), DENV2-infected mice born to naïve mothers (open bars) and age-matched uninfected mice (stripe bars) were shown and error bar denotes standard deviation between experiments. Fold change were obtained by taking the average cytokine levels of the DENV2-infected mice born to the naïve mothers as reference. Legend: * Fold change is estimated as levels in DENV2-infected mice born to DENV1-immune mothers were above upper detection limit of the multiplex assay. * Levels in DENV2-infected mice born to DENV1-immune mothers were not significantly different from the basal levels in the uninfected control mice.

doi:10.1371/journal.pntd.0004536.g006
as the primary cause of morbidity for the infected mice that have acquired maternally trans-ferred antibodies [31]. Hence, DENV2-infected A129 mice born to DENV1-immune mothers were treated with a neutralizing antibody against IFN-γ at day 2 p.i. There were no significant differences in survival, clinical scores or extent of vascular leakage between the anti-IFN-γ-treated mice and the isotype control mice (Fig 8D–8F). However, increased viremia was

Fig 7. Anti-TNFα treatment of DENV2-infected mice born to DENV1-immune mothers. Five to six-weeks old A129 mice born to DENV1-immune mothers were infected iv with 10^6 PFU of D2Y98P-PP1. At day 2 p.i., the mice were injected iv with 100 μg of anti-TNFα neutralizing antibody (open square) or 100μg of isotype control antibody (black square). (A) Survival rate and (B) mean clinical score of anti-TNFα-treated and isotype control antibody-treated DENV2-infected mice born to DENV1-immune mothers (n = 7–8). (C) Viremia and (D) serum AST levels of anti-TNFα-treated and isotype control at day 4 p.i. (n = 5 ± SEM, * p < 0.05 based on Mann-Whitney test with respect to isotype control). (E) Histological analysis of the liver of anti-TNFα-treated mice, isotype antibody-treated mice harvested at day 4 p.i., and uninfected mice as indicated (n = 3 per group). Images were captured at 20x and 40x magnification. Representative sections from two independent experiments are shown (scale bar= 100μm).

doi:10.1371/journal.pntd.0004536.g007
measured in the anti-IFNγ-treated animals compared to the group treated with the isotype control antibody (Fig 8G). Together, these data support that IFNγ helps limit viral replication in this ADE model but does not seem to play a critical role in the extent of vascular leakage detected in various organs.

**Discussion**

ADE mouse models involving the passive administration of enhancing antibodies (polyclonal immune serum or purified monoclonal antibodies) prior to DENV infection have been widely reported by several groups [29, 30, 37]. This approach is straightforward and allows controlling the dose and nature of the antibodies that are administered. In comparison, the maternal ADE model may be seen as less “artificial” since it closely mimics a situation that is encountered in humans. Since heterologous secondary infection in mice does not lead to enhanced...
disease severity [38], the maternal ADE model represents the second best option to mimic a secondary heterologous infection. Furthermore, it allows the investigation of specific pathogenesis aspects that cannot be addressed in the passive administration ADE model, such as the role of breast milk antibodies in disease enhancement for example. With the recent advances in dengue vaccine development, the protective or enhancing properties of vaccination-induced maternal antibodies in infants, or the possible interference of maternal antibodies on vaccination efficacy in babies and young children are interesting topics that our maternal ADE model can also help investigate.

The vast majority of studies reported so far have used the immunocompromised mice AG129 mice, which are deficient in type I and type II IFN receptors. AG129 mice allow productive infection upon infection with various DENV strains that may be accompanied by relevant clinical manifestations. However, the lack of an intact and functional type II IFN signaling pathway in these mice precludes or limits the investigation of a number of aspects in particular the host adaptive immune responses to DENV infection. A couple of very recent studies have reported the use of mice in which absence of type I IFN receptor is restricted to myeloid cell subsets (dendritic cells and macrophages) [39, 40]. In this model, mice succumbed to DENV infection thereby offering a novel in vivo platform for pathogenesis studies and therapeutic testing [39, 40].

Here, we have used A129 mice, which are deficient in type I IFN receptor but have an intact and functional type II IFN pathway. Infection of adult A129 female mice with a high dose of DENV1 via the iv route resulted in an asymptomatic barely detectable viremia followed by the production of anti-DENV1 antibody titers including antibodies against both structural and non-structural (NS1) proteins, thus indicating DENV1 productive replication. We then showed that 5-week old A129 mice born to these DENV1-immune mothers, succumbed to DENV2 infection within 4 days whereas age-matched mice born to dengue naïve mothers survived the infection. Increased virus titers in the blood and several organs, and severe liver damage were observed in the moribund animals.

In a recently published work using the AG129 mouse strain, we reported that 5-week old mice born to DENV1-immune mothers displayed disease severity enhancement characterized by earlier death, increased virus titers and extensive vascular leakage compared to age-matched mice born to dengue naïve mothers [31].

Whereas disease enhancement is clearly observed for both mouse models, it is interesting to note that the clinical manifestations differ. Indeed, in the AG129 mouse model extensive vascular leakage was observed with 8–18 fold increases in OD values in mice born to DENV1-immune mothers compared to uninfected controls [31]. However, no significant liver damage was seen in the infected mice [31]; on the other hand enhancement of disease severity in the A129 ADE model correlated with severe liver damage but moderate vascular leakage. Given that AG129 and A129 differ by the presence of a functional type II IFN pathway, one could hypothesize that IFNγ helps limit vascular leakage in A129 mice. However, neutralization of IFNγ with a blocking antibody did not impact the extent of vascular leakage measured in the A129 ADE mouse model. This thus supports a negligible role for IFNγ in vascular leakage observed in the A129 ADE mouse model. Instead, we showed that IFNγ clearly limits viral replication, as previously reported in an AG129 model [41]. The fact that the increased viral titers did not translate into earlier death of the animals may be explained by the acute nature of our model where mice die within 4 days.

When comparing the AG129 and A129 ADE models, we propose that the differential route of DENV2 infection as well as the different infectious doses may impact the disease progression and manifestations. While in AG129 10^3 PFU of DENV2 were administered subcutaneously (sc), A129 were infected via the iv route with 10^6 PFU, due to their known greater resistance to
dengue infection \[37, 42\]. Upon sc infection, dendritic cells are likely to be the first host cells infected by DENV \[42, 43\], which will then migrate to the draining lymph nodes where naïve T cells will be activated followed by systemic dissemination of the virus and interaction with maternally acquired heterotypic enhancing antibodies. Instead, iv administration of DENV2 allows immediate interaction of the virus with maternal enhancing antibodies followed by uptake into FcR-bearing cells (mainly monocytes), thus bypassing or minimizing interactions with and infection of DCs. Thus it is possible that in A129 mice, activation of the adaptive immunity through DCs is minimal and leads to acute death (day 4 p.i.) of the infected mice even before further increase in vascular leakage can be detected. Furthermore, the direct delivery of high amounts of virus particles into the bloodstream is likely to overwhelm the highly vascularized organs such as spleen and liver where the greatest increase in virus titers was measured compared to mice born to naïve dams, leading to organ damage and failure.

Nevertheless, the relevance of liver involvement in dengue disease is supported by several case report studies where dengue infection has been associated with involvement of multiple organs, one of the most common being the liver \[44–46\]. The range of involvement includes asymptomatic elevation of liver aminotransferases to severe manifestations in form of acute liver failure \[44, 47–49\]. Other mouse models of dengue infection have also reported hepatic damage characterized by cellular infiltration and vacuolation of hepatocytes, accompanied by transient increase in the ASL and ALT levels and in some cases substantial vascular leakage \[25, 27, 50–56\].

Furthermore, Zellweger and colleagues previously showed enhanced infection of liver sinusoidal endothelial cells in AG129 mice upon passive transfer of enhancing anti-dengue antibodies \[30\]. In our A129 ADE model, we also observed enhanced DENV infection of liver endothelial cells in mice born to DENV1-immune mothers compared to mice born to naïve mothers. These observations thus suggest that the extensive cytoplasmic vacuolation of hepatocytes observed in these mice is not a consequence of a direct cytopathic viral effect. A study reported that anti-NS1 antibodies in absence of dengue infection can result in liver damage \[57\]. The pathological role of maternal anti-NS1 antibodies in our ADE model remains to be investigated. Alternatively hepatocytes apoptosis may result from deregulated cytokines production, in particular TNFα. However, in a recent study investigating liver damage in 13 autopsy cases of DHF/DSS, DENV proteins were detected in hepatocytes and Kupffer cells but not in endothelial cells \[58\].

Among the cytokines that were up-regulated in the DENV2-infected mice born to DENV1-immune mothers, TNFα was identified as a critical player as its \textit{in vivo} neutralization at day 2 p.i. fully protected the animals from disease enhancement. Importance of TNFα in dengue disease severity has been illustrated in various mouse models. In primary DENV2 infection models using either immunocompetent \[59\] or AG129 \[25\] mice, blocking TNFα using an antibody partially protected the animals from liver damage and vascular leakage, respectively. DENV2 infection of TNFα\(^{-/-}\) mice resulted in significant reduction of hemorrhage compared to wild-type infected mice \[18\]. Such observations are not restricted to DENV2 infection. Indeed, complete protection was obtained in a DENV3 lethal AG129 model upon treatment with anti-TNFα antibodies \[60\]. Furthermore, the pathological role of TNFα has been substantiated in other \textit{in vivo} ADE models where anti-TNFα treatment of infected mice improved their mean survival time \[30, 31\]. Hence, it is clear that TNFα is a key pathogenic cytokine responsible for the different disease manifestations such as liver damage and vascular leakage in various murine dengue models. However, the role of TNFα in human dengue is less clear. While several studies have identified a positive correlation of increased TNFα levels with dengue disease severity \[49, 61\], others have noticed no significant differences in expression levels of this cytokine during severe or mild dengue \[62, 63\]. Inconsistencies in TNFα levels in these
cytokine profiling studies of dengue patients have largely been attributed to possible differences in collection time points and discrepancies in the assessment of disease severities [64]. Evaluation of genetic predisposition to severe dengue also yields conflicting conclusions with regards to TNFα polymorphisms. High producing TNFα allele 308A has been determined to be a risk factor for bleeding manifestations during severe dengue [65, 66]. However, the same allele has also been identified to be protective against severe dengue [67]. A dual role for TNFα may be proposed which could act as both a protective and detrimental cytokine, depending on the stage of infection. This hypothesis is supported by an in vitro study showing that early post-infection, TNFα functions primarily as a pro-survival signal and activates NF-κB [68]. As the infection progresses however, the infected cells become less sensitive to TNFα-mediated NF-κB stimulation and undergo apoptosis.

In conclusion, we report here a novel mouse model of disease enhancement that is mediated by maternally acquired antibodies in the A129 background. It recapitulates a number of clinical manifestations observed in severe dengue patients including increased vascular permeability, thrombocytopenia, lymphopenia, cytokine storm and liver pathology. Since A129 mice are less immunocompromised than AG129, this model offers a better platform not only to study dengue pathogenesis, but also to evaluate the efficacy of therapeutic and vaccine candidates. Specifically, this liver disease-associated model may indeed prove useful for testing the efficacy of novel dengue therapeutics in alleviating hepatic manifestations during severe dengue. In addition, with recent advances in dengue vaccines clinical development, this maternal ADE model may help investigate the impact of maternal antibodies on vaccination efficacy in infants and provide vaccination strategy guidelines.

Materials and Methods

Ethics statement

All the animal experiments were carried out under the guidelines of the National Advisory Committee for Laboratory Animal Research (NACLAR) in the AAALAC-accredited NUS animal facilities (http://nus.edu.sg/iacuc/). NUS has obtained a license (#VR008) from the governing body Agri-Food & Veterinary Authority of Singapore (AVA) to operate an Animal Research Facility. The animal experiments described in this work were approved by the IACUC from NUS under protocol number 2013–04751.

Virus strains and growth conditions

The DENV2 D2Y98P-PP1 strain was derived from a 2000 Singapore clinical isolate (Genbank accession number #JF327392) [69]. DENV1 [Dengue 1 05K3903DK1 (Genbank accession number #EU081242)] was isolated from a patient during a DEN outbreak in Singapore in 2005 [70]. The Aedes albopictus C6/36 cell line (American Type Culture Collection [ATCC #CRL-1660]) was used for propagation of all the DENV strains as described previously [27]. C6/36 cells were maintained in Leibovitz’s L-15 medium (GIBCO) supplemented with 10% fetal calf serum (FCS). Virus stocks were stored at -80°C.

Measurement of DENV specific total IgG, IgG isotypes and anti-NS1 antibodies. The levels of systemic IgG antibodies against DENV1 or DENV2 were determined by indirect enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates (Corning costar, NY, USA) were coated overnight at 4°C with 150 ng/well of UV-inactivated DENV1 or DENV2 or 10 ng/well of purified NS1 protein [70] in PBS. Serially-diluted serum samples were added to the wells and incubated for 1 h at 37°C. HRP-conjugated goat anti-mouse IgG (H+L) (Bio-rad) at 1:3000, or anti-mouse IgG1, IgG2a and IgG2b (Abcam) secondary antibody was used at a 1:10000 dilution. Detection was performed using O-phenylenediamine dihydrochloride
substrate SigmaFast (Sigma Aldrich) according to the manufacturer’s instructions. The reaction was stopped upon adding 50 μl of 1M sulphuric acid and absorbance was read at 490nm using an ELISA plate reader (Bio-rad model 680). The absorbance values against log dilution factors were then plotted and respective titres were determined by taking the absorbance cutoff at three times the mean background.

**In vitro ADE assay.** Pooled sera from 5–6 weeks old mice born to naïve or DENV1-immune mothers were heat-inactivated at 56°C for 30 minutes. Serially diluted mice sera (2-fold dilutions, starting at 1/20 dilution) were incubated with 10^3 PFU of DENV2 (D2Y98P-PP1) for 1 h at 37°C. K562 cells were then infected with the suspensions for 2 h at 37°C at a multiplicity of infection (MOI) of 0.01 before transferring the infected cells in 48-wells flat bottom tissue culture plates (Falcon) at 37°C, 5% CO₂. Virus titers in the culture supernatant after two days of incubation were determined by plaque assay in BHK cells. Fold enhancement for each dilution was calculated by normalizing the titers at respective sera dilution with the average titers in K562 cells infected with the virus in the absence of serum.

**In vitro plaque reducing neutralizing assay (PRNT).** Serial dilutions (2-fold, starting 1/10 dilution) of heat-inactivated serum from mice born to DENV1-immune or naïve mothers was first prepared with RPMI 1640, 2% FCS (Life Technologies), containing 500 PFU/ml of virus. The suspensions were then incubated at 37°C for 1 h. A positive control with virus alone was also included. Plaque assay was then carried out in BHK-21 cells (in triplicates) as described above. The percentage of neutralization was determined by comparing the number of plaques obtained with each serum dilution to that obtained with the positive control. PRNT<sub>50</sub> was determined by nonlinear regression as the serum dilution factor for which 50% reduction in the number of plaques (with respect to the virus control) was obtained. Samples with PRNT<sub>50</sub> values less than 1:10 were arbitrarily assigned a value of 5.

**Plaque assay**
Plaque assay was carried out in BHK-21 cells as described previously [27]. Briefly, 1x10^5 cells BHK-21 were seeded in 24- well plates (NUNC, NY, USA). BHK-21 monolayers were infected with 10-fold serially diluted viral suspensions or mice sera. After 1h incubation at 37°C and 5% CO₂, 1% (w/v) carboxymethyl cellulose was added to the wells. After incubating 4 days for D2Y98P-PP1 or 5 days for DENV1, the cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet for a minimum of 30 min. Then the plates were thoroughly rinsed with water, before counting the plaques. Results were expressed as log<sub>10</sub> [mean ± SD] of plaque forming units (PFU) / ml of serum measured in 5 mice per group per time point. The limit of detection for the assay was set at 10<sup>2</sup> PFU per ml.

**ADE infection mouse model**
A129 mice (129/Sv deficient in IFNα/β receptor) breeders were obtained from B&K Universal (UK). They were housed and bred under specific pathogen-free conditions in individual ventilated cages. Five to six-week old A129 female mice were infected with 10<sup>6</sup> PFU of DENV1 per mouse intravenously (iv), which led to asymptomatic transient viremia. One week post-infection, after virus clearance, the females were mated with naive 6-week old A129 males and the offspring were weaned at 21 days of age. Uninfected A129 females were used to give birth to naïve controls. At 5–6 weeks of age, mice born to DENV1-immune or naïve mothers were administered with 10<sup>6</sup> PFU of DENV2 (D2Y98P-PP1) via the iv route (0.1 ml in sterile PBS). The infected animals were monitored daily for clinical symptoms. The scoring system used was: 0- Healthy; 1- Ruffled Fur; 2- Hunched back; 3- Severe Diarrhea; 4- Lethargic; 5- Moribund. Survival rate was derived from the number of mice that were
euthanized at moribund stage as evidenced by severe diarrhea and extreme lethargy as described previously [27, 28].

Organ processing for viral titer determination
Infected mice were euthanized and perfused extensively with sterile PBS. Pooled left and right brachial and axillary lymph nodes, brain, intestine, spleen, and liver were harvested and homogenized using a mechanical homogenizer (Omni) for 5 minutes in 1 ml RPMI 1640 at medium speed on ice. Thoroughly homogenized tissues were centrifuged at 14,000 rpm for 10 min at 4°C to pellet debris and then the supernatant was filtered using a 0.22 μm diameter pore size filter. The level of infectious virus within the filtrate is thus considered representative of the total level of infectious virus present in the harvested organ. Ten-fold serial dilutions of each tissue homogenate (from neat to 1: 10^4) were assayed in a standard virus plaque assay on BHK-21 cells as described above. Five mice per time point per group were assessed.

Histology
Mice were euthanized and tissues (intestines, spleen and liver) were harvested and immediately fixed in 4% paraformaldehyde in PBS at the indicated time points. Fixed tissues were paraffin embedded and stained with Hematoxylin and Eosin (H&E). One section was obtained per mouse and 3 livers were examined from each of the 2 independent experiments.

Detection of DENV antigen in the liver
Liver tissue was snap-frozen in OCT compound (Tissue-Tek) prior to frozen-sectioning using a cryostat (Leica). Sections (10μm) were fixed onto slides at 4°C in 100% acetone, followed by blocking using 1% BSA in PBS. Tissue sections were then incubated in primary antibodies against CD11b (biotinylated), CD31 (both from eBiosciences), and dengue NS3 (GeneTex) overnight at 4°C. After washing, secondary antibodies including anti-rabbit AlexaFluor-594, streptavidin-conjugated Cy5 (both from Invitrogen), and anti-rat AlexaFlour-488 (Jackson ImmunoResearch) were incubated on the tissue sections in 1% BSA in PBS. Slides were washed and mounted using ProLong Gold Antifade reagent (ThermoFisher Scientific). Confocal images of stained tissue sections were obtained with Leica confocal laser scanning instrument with a channel-series approach to spectral overlap. Images were prepared for publication using ImageJ software.

Hematology
Mouse blood samples were collected in K2EDTA and serum tubes (Greiner bio-one, Alphen a/d Rijn, The Netherlands). Whole blood was immediately analyzed for cell counts using automated hematology analyzer Cell Dyn-3700 (Abbott Laboratories, MediSense Products, MA, USA). Serum aspartate (AST) transaminase levels were quantified using chemistry analyzer COBAS C111 (Roche, Basel, Switzerland).

Assessment of vascular leakage
Vascular leakage was assessed using Evans Blue dye as a marker for albumin extravasation as described previously [27]. Briefly, the mice were injected with 50 μg/g body weight of Evans blue dye (0.5% in sterile phosphate-buffered saline, PBS) via the iv route. After 2 hours, the animals were euthanized and extensively perfused with PBS. The tissues were harvested and weighed prior to dye extraction using 4 ml/g wet tissue of N,N-dimethylformamide (Sigma) at
37°C for 24 h after which absorbance was read at 620 nm. Data were expressed as absolute absorbance at OD_{620} per gram of wet tissue. 5 mice per group per time point were used.

Detection of cytokines and other soluble mediators

A 32-multiplex assay (Merck Millipore, Darmstadt, Germany) was used to measure different cytokine/chemokine in the mouse sera from three independent experiments. The following cytokines and chemokines were measured: Eotaxin, VEGF, TNF-α, RANTES, MIP-2, MIP-1β, MIP-1α, MIG, M-CSF, MCP-1, LIX, LIF, KC-like, IP-10, IL-17, IL-15, IL-13, IL-12 (p70), IL-12 (p40), IL-10, IL-9, IL-7, IL-6, IL-5, IL-4, IL-3, IL-2, IL-1β, IL-1α, IFN-γ, GM-CSF and G-CSF. The assay was performed according to the manufacturer’s instructions. The cytokine concentrations in DENV2-infected mice born to DENV1-immune mothers were then compared to those corresponding in the infected mice born to naïve mothers. Differentially expressed soluble mediators in this ADE context are identified as those (1) with levels in the infected mice born to DENV1-immune mothers that were statistically significant (p < 0.05 based on the Mann-Whitney test) as compared to that in the infected mice born to naïve mothers (for at least two out of three independent experiments) and (2) the levels in infected mice were significantly different from the basal levels in the uninfected controls (p < 0.05 based on Mann-Whitney test).

**In vivo cytokine neutralization**

Five to six-week old A129 mice born to DENV1-immune mothers were infected with 10^6 PFU of D2Y98P-PP1 via the iv route. At day 2 p.i., mice were injected iv with 100 μg of anti-TNFα (eBioscience, Cat. no. 167322-85), anti-IL6 (eBioscience, Cat. no. 16-7322-85), anti-IFNγ (eBioscience, Cat. no. 16-7311-85) or Rat IgG1 isotype control (eBioscience, Cat. no. 16-4301-85) per mouse.

Supporting Information

S1 Fig. Virus replication in 5–6 weeks old DENV1-infected A129 females. Five to six weeks old naïve female A129 mice were infected iv with 10^6 PFU of DENV1 and bled at day 2, 4 and 6 p.i. for determination of viremia by plaque assay in BHK cells (A). At 6 weeks p.i., the mice were also bled and their anti-NS1 titers were determined by indirect ELISA against purified NS1 protein. Sera from age-matched naïve control mice were also included (**p < 0.01 based on Mann-Whitney test) (B).

(TIF)

S2 Fig. Spleen, intestine and liver histology in DENV2-infected mice born to DENV1-immune mothers. 5-6-weeks old A129 mice born to either DENV1-immune or naïve mothers were iv infected with 10^6 PFU of D2Y98P-PP1 (n = 3). Histological analysis of the liver harvested at day 2 p.i. and of the spleen and small intestine harvested at day 4 p.i. was performed. Images were taken at 5x (small intestines and spleen) or 20x (liver) magnification. Representative sections from two independent experiments are shown (scale bar~ 100μm).

(TIF)

S3 Fig. Blood parameters in DENV2-infected mice born to DENV1-immune or naïve mothers. Five to six-weeks old A129 mice born to either DENV1-immune (black bar) or naïve mothers (open bar) were iv infected with 10^6 PFU of D2Y98P-PP1, uninfected controls are depicted with a striped bar. At each of the indicated time points p.i., 5 mice per group were euthanized and blood was collected in EDTA-containing tubes for measurement of various blood parameters including white blood cells (WBC), neutrophils (NEU), lymphocytes (LYM),
monocytes (MONO), hematocrit (HCT), and platelet (PLT) counts. * p<0.05, based on 1-way ANOVA with Bonferroni’s post-test.

(TIF)

Acknowledgments

We are grateful to Prof. S. Vasudevan from Duke-NUS for his kind gift of purified NS1 protein.

Author Contributions

Conceived and designed the experiments: JMMG LCO ALSJ SA. Performed the experiments: JMMG LCO JHL SABA EAL PXL ALSJ. Analyzed the data: JMMG LCO ALSJ SA. Contributed reagents/materials/analysis tools: PXL. Wrote the paper: JMMG LCO ALSJ SA.

References

1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013; 496: 504–507. doi:10.1038/nature12060 PMID: 23563266
2. Simmons CP, Farrar JJ, Nguyen VVC, Wills B. Dengue. N Engl J Med. 2012; 366: 1423–1432. doi: 10.1056/NEJma1110265 PMID: 22494122
3. Guzman MG, Harris E. Dengue. Lancet. 2015; 385: 453–465. doi: 10.1016/S0140-6736(14)60572-9 PMID: 25230594
4. WHO. Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 2nd ed. Geneva; 1997.
5. 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. doi:10.1007/s00705-013-1645-3 PMID: 23471635
6. Halstead SB. Dengue. Lancet. 2007; 370: 1644–1652. doi: 10.1016/S0140-6736(07)61687-0 PMID: 17993365
7. 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(2): 411–419. PMID: 3354774
8. Chau TNB, Quyen NTH, Thuy TT, Tuan NM, Hoang DM, Dung NTP, 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. doi: 10.1086/590117 PMID: 1895189
9. Chau TNB, Hieu NT, Anders KL, Woelbers M, Lien LB, Hieu LTM, et al. Dengue virus infections and maternal antibody decay in a prospective birth cohort study of Vietnamese infants. J Infect Dis. 2009; 200: 1893–1900. doi: 10.1086/648407 PMID: 19911991
10. Simmons CP, Chau TNB, Thuy TT, Tuan NM, Hoang DM, Thien NT, et al. Maternal antibody and viral factors in the pathogenesis of dengue virus in infants. J Infect Dis. 2007; 198: 416–424. doi: 10.1086/519170 PMID: 17597456
11. Chan KR, Zhang SL-X, Tan HC, Chan YK, Chow A, Lim APC, et al. Ligation of Fc gamma receptor IIIB inhibits antibody-dependent enhancement of dengue virus infection. Proc Natl Acad Sci USA. 2011; 108: 12479–12484. doi: 10.1073/pnas.1106681108 PMID: 21746897
12. Filipse J, Wilschut J, Smit JM. Molecular mechanisms involved in antibody-dependent enhancement of dengue virus infection in humans. Traffic. 2012; 14: 25–35. doi: 10.1111/tra.12012 PMID: 22996156
13. Boonnak K, Silke BM, Donofrio GC, Marovich MA. Human Fc RI cytoplasmic domains differentially influence antibody-mediated dengue virus infection. J Immunol. 2013; 190; 5659–5665. doi: 10.4049/jimmunol.1203052 PMID: 23616574
14. Young DF, Chatziandreou N, He B, Goodbourn S, Lamb RA, Randall RE. Single amino acid substitution in the V protein of simian virus 5 differentiates its ability to block interferon signaling in human and murine cells. J Virol. 2001; 75: 3363–3370. doi: 10.1128/JVI.75.7.3363-3370.2001 PMID: 11238662
15. Muñoz-Jordan JL, Sánchez-Burgos GG, Laurent-Rolle M, García-Sastre A. Inhibition of interferon signaling by dengue virus. Proc Natl Acad Sci USA. 2003; 100: 14333–14338. doi: 10.1073/pnas. 2335168100 PMID: 14612562
16. Jones M, Davidson A, Hibbert L, Gruenwald P, Schlaak J, Ball S, et al. Dengue virus inhibits alpha interferon signaling by reducing STAT2 expression. J Virol. 2005; 79: 5414–5420. doi: 10.1128/JVI.79.9.5414-5420.2005 PMID: 15827155

17. Shresta S, Kyle JL, Robert Beatty P, Harris E. Early activation of natural killer and B cells in response to primary dengue virus infection in A/J mice. Virology. 2004; 319: 262–273. doi: 10.1016/j.virol.2003.09.048 PMID: 14980486

18. Chen HC, Hofman FM, Kung JT, Lin YD, Wu-Hsieh BA. Both virus and tumor necrosis factor alpha are critical for endothelium damage in a mouse model of dengue virus-induced hemorrhage. J Virol. 2007; 81: 5518–5526. doi: 10.1128/JVI.02575-06 PMID: 17360740

19. John AL St, Rathore AP, Raghavan B, Ng M-L, Abraham SN. Contributions of mast cells and vasoactive products, leukotrienes and chymase, to dengue virus-induced vascular leakage. eLife. 2013; 2. doi: 10.7554/eLife.00481.015

20. Hotta H, Murakami I, Miyasaki K, Takeda Y, Shirane H, Hotta S. Inoculation of dengue virus into nude mice. J Gen Virol. 1981; 52: 71–76. PMID: 7264608

21. Wu SJ, Hayes CG, Dubois DR, Windheuser MG, Kang YH, Watts DM, et al. Evaluation of the severe combined immunodeficient (SCID) mouse as an animal model for dengue viral infection. Am J Trop Med Hyg. 1995; 52: 468–476. PMID: 7771614

22. Lin YL, Liao CL, Chen LK, Yeh CT, Liu CI, Ma SH, et al. Study of dengue virus infection in SCID mice engrafted with human K562 cells. J Virol. 1998; 72: 9729–9737. PMID: 9811707

23. Johnson AJ, Roehrig JT. New mouse model for dengue virus vaccine testing. J Virol. 1999; 73: 783–786. PMID: 9847388

24. Bente DA, Melkus MW, Garcia JV, Rico-Hesse R. Dengue fever in humanized NOD/SCID mice. J Gen Virol. 2005; 79: 13797–13799. doi: 10.1128/JVI.79.21.13797-13799.2005 PMID: 16227299

25. Shresta S, Sharar KL, Prigozhin DM, Beatty PR, Harris E. Murine model for dengue virus-induced lethal disease with increased vascular permeability. J Virol. 2006; 80: 10208–10217. doi: 10.1128/JVI.00062-06 PMID: 17005698

26. Schul W, Liu W, Xu HY, Flaman M, Vasudevan SG. A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs. J Infect Dis. 2007; 195: 665–674. doi: 10.1086/511310 PMID: 17262707

27. Tan GK, Ng JKW, Trasti SL, Schul W, Yip G, Alonso S. A non mouse-adapted dengue virus strain as a new model of severe dengue infection in AG129 mice. PLoS Negl Trop Dis. 2010; 4: e672. doi: 10.1371/journal.pntd.0000672.t001 PMID: 20436920

28. Tan GK, Ng JK, Lim AH, Yeo KP, Angeli V, Alonso S. Subcutaneous infection with non-mouse adapted dengue virus D2Y98P strain induces systemic vascular leakage in AG129 mice. Ann Acad Med Singap. 2011; 40: 523–510. PMID: 22294063

29. Balsitis SJ, Williams KL, Lachica R, Flores D, Kyle JL, Mehlhop E, et al. Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. PLoS Pathog. 2010; 6: e1000790.

30. Zellweger RM, Prestwood TR, Shresta S. Enhanced infection of liver sinusoidal endothelial cells in a mouse model of antibody-induced severe dengue disease. Cell Host Microbe. 2010; 7: 128–139. doi: 10.1016/j.chom.2010.01.004 PMID: 20153282

31. Ng JK, Zhang SL, Tan HC, Yan B, Martínez Gómez JM, Tan WY, et al. First experimental in vivo model of enhanced dengue disease severity through maternally acquired heterotypic dengue antibodies. PLoS Pathog. 2014; 10: e1004031.

32. Wahala WMPB, de Silva AM. The human antibody response to dengue virus infection. Viruses. 2011; 3: 2374–2395. doi: 10.3390/v3122374 PMID: 22355444

33. Smucny JJ, Kelly EP, Macarthy PO, King AD. Murine immunoglobulin G subclass responses following immunization with live dengue virus or a recombinant dengue envelope protein. Am J Trop Med Hyg. 1995; 53: 432–437. PMID: 7485699

34. Fu Y, Yip A, Seah PG, Blasco F, Shi P-Y, Hervé M. Modulation of inflammation and pathology during dengue virus infection by p38 MAPK inhibitor SB203580. Antiviral Res. 2014; 110: 151–157. doi: 10.1016/j.antiviral.2014.08.004 PMID: 25131378

35. Suhartini C, van Gorp ECM, Dolmans WMV, Setiati TE, Hack CE, Djokomoejanto R, et al. Cytokine patterns during dengue shock syndrome. Eur Cytokine Netw. 2003; 14: 172–177. PMID: 14856693

36. Rachman A, Rinaldi I. Coagulopathy in dengue infection and the role of interleukin-6. Acta Med Indones. 2010; 38: 105–108.

37. Zellweger RM, Shresta S. Mouse models to study dengue virus immunology and pathogenesis. Front Immunol. 2014; 5: 151. doi: 10.3389/fimmu.2014.00151 PMID: 24782859
38. Zompi S, Santić BH, Beatty PR, Harris E. Protection from secondary dengue virus infection in a mouse model reveals the role of serotype cross-reactive B and T cells. J Immunol. 2011; 188: 404–416. doi: 10.4049/jimmunol.1102124 PMID: 22131327

39. Zust R, Toh YX, Valdes I, Cerny D, Heinrich J, Hermida L, et al. Type I interferon signals in macrophages and dendritic cells control dengue virus infection: implications for a new mouse model to test dengue vaccines. J Virol. 2012; 88: 7276–7285. doi: 10.1128/JVI.03827-13 PMID: 24741106

40. Pinto AK, Brien JD, Lam C- YK, Johnson S, Chiang C, Hiscott J, et al. Defining new therapeutics using a more immunocompetent mouse model of antibody-enhanced dengue virus infection. mBio. 2015; 6: e01316–15. doi: 10.1128/mBio.01316-15 PMID: 26374123

41. Prestwood TR, Morar MM, Zellweger RM, Miller R, May MM, Yauch LE, et al. Gamma interferon (IFN-γ) receptor restricts systemic dengue virus replication and prevents paralysis in IFN-α/β receptor-deficient mice. J Virol. 2012; 86: 12561–12570. doi: 10.1128/JVI.06743-11 PMID: 22973027

42. Kyle JL, Beatty PR, Harris E. Dengue virus infects macrophages and dendritic cells in a mouse model of infection. J Infect Dis. 2007; 195: 1808–1817. doi: 10.1086/518007 PMID: 17492597

43. Cerny D, Hanifl M, Shin A, Bigliardi P, Tan BK, Lee B, et al. Selective susceptibility of human skin antigen presenting cells to productive dengue virus infection. PLoS Pathog. 2014; 10: e1004548.

44. Nguyen TL, Nguyen TH, Tieu NT. The impact of dengue haemorrhagic fever on liver function. Res Virol. 1997; 148: 273–277. doi: 10.1016/S0923-2516(97)88364-1 PMID: 9272578

45. Póvoa TF, Alves AMB, Oliveira CAB, Nuevo GJ, Chagas VLA, Paes MV. The pathology of severe dengue in multiple organs of human fatal cases: histopathology, ultrastructure and virus replication. PLoS ONE. 2014; 9: e83386. doi: 10.1371/journal.pone.0083386.1001 PMID: 24736395

46. Samanta J. Dengue and its effects on liver. World J Clin Cases. 2015; 3: 125. doi: 10.12998/wjcc.v3.i2.125 PMID: 25685758

47. Kuo CH, Tai DI, Chang-Chien CS, Lan CK, Chioo SS, Liaw YF. Liver biochemical tests and dengue fever. Am J Trop Med Hyg. 1992; 47: 265–270. PMID: 1355950

48. Trung DT, Thao LTT, Hien TT, Hung NT, Vinh NN, Hien PTD, et al. Liver involvement associated with dengue virus type 4 (DENV-4) TVP-376 infection in mice lacking both IFN-α/β and IFN-γ receptors (AG129) and comparison with the DENV-2 AG129 mouse model. J Gen Virol. 2015; 96(10): 3035–3048. doi: 10.1099/jgv.0.000246 PMID: 26296350

49. Kyle JL, Beatty PR, Harris E. Dengue virus infects macrophages and dendritic cells in a mouse model of infection. J Infect Dis. 2007; 195: 1808–1817. doi: 10.1086/518007 PMID: 17492597

50. Sarathy VV, Infante E, Li L, Campbell GA, Wang T, Paessler S et al. Characterization of lethal dengue virus type 4 (DENV-4) TVP-376 infection in mice lacking both IFN-α/β and IFN-γ receptors (AG129) and comparison with the DENV-2 AG129 mouse model. J Gen Virol. 2015; 96(10): 3035–3048. doi: 10.1099/jgv.0.000246 PMID: 26296350

51. Chennarayana D, de Queiroz Prado R, Almeida Xavier E, Cristina de Oliveira N, da Matta Guedes PM, da Silva JS, et al. Immunocompetent Mice Model for Dengue Virus Infection. ScientificWorldJournal. 2012; 2012: 525947. doi:10.1100/2012/525947 PMID: 2266132

52. Costa SM, Paes MV, Barreto DF, Pinhão AT, Barth OM, Queiroz JL, et al. Protection against dengue type 2 virus induced in mice immunized with a DNA plasmid encoding the non-structural 1 (NS1) gene fused to the tissue plasminogen activator signal sequence. Vaccine. 2006; 24(2): 195–205. PMID: 16122850

53. Paes MV, Pinhão AT, Barreto DF, Costa SM, Oliveira MP, Nogueira AC. Liver injury and viremia in mice infected with dengue-2 virus. Virology. 2005; 338(2): 236–246. PMID: 15961136

54. Gonçalves D, de Queiroz Prado R, Almeida Xavier E, Cristina de Oliveira N, da Matta Guedes PM, da Silva JS, et al. Immunocompetent Mice Model for Dengue Virus Infection. ScientificWorldJournal. 2012; 2012: 525947. doi:10.1100/2012/525947 PMID: 2266132

55. Chen HC, Lai SY, Sung JM, Lee SH, Lin YC, Wang WK, et al. Lymphocyte activation and hepatic cellular infiltration in immunocompetent mice infected by dengue virus. J Med Virol. 2004; 73(3): 419–431. PMID: 15170638

56. Sarathy VV, White M, Li L, Gorder SR, Pyles RB, Campbell GA, et al. A lethal murine infection model for dengue virus 3 in AG129 mice deficient in type I and II interferon receptors leads to systemic disease. J Virol. 2015; 89: 12570–1266. doi: 10.1128/JVI.01320-14 PMID: 25392217

57. Lin CF, Wan SW, Chen MC, Lin SC, Cheng CC, Chiu SC, et al. Liver injury caused by antibodies against dengue virus nonstructural protein 1 in a murine model. Lab Invest. 2008; 88(10): 1079–1089. doi: 10.1038/labinvest.2008.70 PMID: 18679379

58. Aye KS, Charmkaw K, Win N, Wai KZ, Moe K, Punyadee N, et al. Pathologic highlights of dengue hemorrhagic fever in 13 autopsy cases from Myanmar. Hum Pathol. 2014; 45: 1221–1233. doi: 10.1016/j.humpath.2014.01.022 PMID: 24767772
59. Atrasheuskaya A, Petzelbauer P, Fredeking TM, Ignatyev G. Anti-TNF antibody treatment reduces mortality in experimental dengue virus infection. FEMS Immunol Med Microbiol. 2003; 35: 33–42. PMID: 12589955

60. Braga EL, Moura P, Pinto LM, Ignácio SR, Oliveira MJ, Cordeiro MT, et al. Detection of circulant tumor necrosis factor-alpha, soluble tumor necrosis factor p75 and interferon-gamma in Brazilian patients with dengue fever and dengue hemorrhagic fever. Mem Inst Oswaldo Cruz. 2001; 96: 229–232. PMID: 11285501

61. Mangione JNA, Huy NT, Lan NTP, Mbanefo EC, Ha TTN, Bao LQ, et al. The association of cytokines with severe dengue in children. Trop Med Health. 2014; 42: 137–144. doi:10.2149/tmh.2014-09 PMID: 25589878

62. Bozza FA, Cruz OG, Zagne SM, Azeredo EL, Nogueira RM, Assis EF, et al. Multiplex cytokine profile from dengue patients: MIP-1beta and IFN-gamma as predictive factors for severity. BMC Infect Dis. 2008; 8: 86. doi: 10.1186/1471-2334-8-86 PMID: 18578883

63. Rothman AL. Cellular immunology of sequential dengue virus infection and its role in disease pathogenesis. Curr Top Microbiol Immunol. 2010; 338: 83–98. doi: 10.1007/978-3-642-02215-9_7 PMID: 19802580

64. Perez AB, Sierra B, Garcia G, Aguirre E, Babel N, Alvarez M, et al. Tumor necrosis factor-alpha, transforming growth factor-β1, and interleukin-10 gene polymorphisms: implication in protection or susceptibility to dengue hemorrhagic fever. Hum Immunol. 2010; 71: 1135–1140. doi: 10.1016/j.humimm.2010.08.004 PMID: 20732366

65. Chuansumrit A, Anantasit N, Sasanakul W, Chaiyaratana W, Tangnararatchakit K, Butthep P, et al. Tumour necrosis factor gene polymorphism in dengue infection: association with risk of bleeding. Pae-diatr Int Child Health. 2013; 33: 97–101. doi: 10.1179/2046905512Y.0000000049 PMID: 23925283

66. Sam S-S, Teoh B-T, Chinna K, AbuBakar S. High producing tumor necrosis factor alpha gene alleles in protection against severe manifestations of dengue. Int J Med Sci. 12: 177–186. doi: 10.7150/ijms.8989 PMID: 25589994

67. Wati S, Rawlinson SM, Ivanov RA, Dorstyn L, Beard MR, Jans DA, et al. Tumour necrosis factor alpha (TNF-) stimulation of cells with established dengue virus type 2 infection induces cell death that is accompanied by a reduced ability of TNF- to activate nuclear factor B and reduced sphingosine kinase-1 activity. J Gen Virol. 2011; 92: 807–818. doi: 10.1099/vir.0.028159-0 PMID: 21148274

68. Grant D, Tan GK, Qing M, Ng JK, Wip A, Zou G, et al. A single amino acid in nonstructural protein NS4B confers virulence to dengue virus in AG129 mice through enhancement of viral RNA synthesis. J Virol. 2011; 85: 7775–7787. doi: 10.1128/JVI.00665-11 PMID: 21632767

69. Schreiber MJ, Holmes EC, Ong SH, Soh HSH, Liu W, Tanner L, et al. Genomic epidemiology of a dengue virus epidemic in urban Singapore. J Virol. 2009; 83: 4163–4173. doi: 10.1128/JVI.02445-08 PMID: 19211734

70. Rozen-Gagnon K, Moreland NJ, Ruedl C, Vasudevan SG. Protein expression and purification. Protein Expr Purif. 2012; 82: 20–25.